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Research paper

Ethanol-induced DNA repair in neural stem cells is transforming growth factor β1-dependent

Steven D. Hicks[a,b,1], Michael W. Miller[a,b,c,d,*]

[a] Department of Neuroscience and Physiology, State University of New York - Upstate Medical University, Syracuse, NY 13210, USA
[b] Developmental Exposure Alcohol Research Center, Binghamton NY 13902, Cortland NY 13045, and Syracuse, NY 13210, USA
[c] Department of Anatomy, Touro College of Osteopathic Medicine, Middletown, NY 10940, USA
[d] Research Service, Veterans Affairs Medical Center, Syracuse, NY 13210, USA

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ABSTRACT

Following neurotoxic damage, cells repair their DNA, and survive or undergo apoptosis. This study tests the hypothesis that ethanol induces a DNA damage response (DDR) in neural stem cells (NSCs) that promotes excision repair (ER) and this repair is influenced by the growth factor environment. Non-immortalized NSCs treated with fibroblast growth factor 2 or transforming growth factor (TGF) β1 were exposed to ethanol. Ethanol increased total DNA damage, reactive oxygen species, and oxidized DNA bases. TGFβ1 potentiated these toxic effects. Transcriptional analyses of cultured NSCs revealed ethanol-induced increases in transcripts related to the DDR (*e.g.*, *Hus1* and *p53*), base ER (*e.g.*, *Mutyh* and *Nthl1*), and nucleotide ER (*e.g.*, *Xpc*), particularly in the presence of TGFβ1. Expression and activity of ER proteins were affected by ethanol. Similar changes occurred in proliferating cells of ethanol-treated mouse fetuses. Ethanol-induced DNA repair in NSCs depends on the ambient growth factors. Gene products for DNA repair in stem cells are among the first biomarkers identifying fetal alcohol-induced damage.

1. Introduction

Neural stem cells (NSCs) are prime targets of ethanol toxicity (Krishnamoorthy et al., 2013; Miller, 2006; Mooney et al., 2013; Nixon et al., 2010; Roitbak et al., 2011; Zawada and Das, 2006). Ethanol-induced damage to NSCs apparently underlies cognitive deficits associated with alcohol use disorder (AUD) and fetal alcohol spectrum disorder (FASD) (Guerri and Pascual, 2010; Heffernan, 2008). Indeed, prenatal exposure to alcohol is the most common cause of developmental disorders (Abel and Sokol, 1992; May et al., 2009; Sampson et al., 1997).

The vitality of cycling NSCs depends on their ability to maintain their DNA integrity (Rehen and Chun, 2006). The delicate balance between continued proliferation and apoptosis relies on the DNA damage response (DDR) (Lakin and Jackson, 1999; Li et al., 2000). Ethanol affects DNA integrity (Brooks, 1997; LaMarche et al., 2003; Pamilla-Castellar et al., 2004); it causes DNA fragmentation in developing cortical neurons (Jacobs and Miller, 2001; Mooney and Miller, 2007; Young et al., 2003).

Ethanol-induced DNA damage occurs through two mechanisms: (1) Ethanol causes the production of reactive oxygen species (ROS) and oxidative damage in immature cortical neurons (Hamby-Mason et al., 1997; Kruman et al., 2012; Ramachandran et al., 2003; Traphagen et al., 2015). (2) Ethanol alters the expression (Cherian et al., 2008; Kuhn and Miller, 1998; Rulten et al., 2008) and activity (Kovota et al., 2013; Miller et al., 2003; Navasumrit et al., 2001; Wilson III et al., 1994; Xu et al., 2005) of DNA repair factors in developing cortical neurons, hepatocytes, and blood cells. One such factor is Trp53 (a.k.a. p53), a key player in the DDR (Lakin and Jackson, 1999; Kuhn and Miller, 1998; Bellamy et al., 1997; Meek, 2009; Morrison et al., 2002; Shieh et al., 2000; Smith and Seo, 2002).

The DDR is initiated through the Rad9-Hus1-Rad1 (9-1-1) complex (Pamilla-Castellar et al., 2004; Bluyssen et al., 1999; Freire et al., 1998; Räschle, 2017; Zhang et al., 2015) which activates p53 (Lieberman et al., 2017; Ljungman, 2000; Roos-Mattjus et al., 2002) and endonucleases such as Apex1 and Nthl1 (Gembka et al., 2007; Zharkov, 2008). Ethanol can alter the expression and phosphorylation of p53 [*e.g.*, Kuhn and Miller, 1998; Cordenonsi et al., 2003; Kannan et al., 2000]. p53-dependent mechanisms of DNA repair in cycling cells are base excision repair (BER) and nucleotide excision repair (NER)

* Corresponding author at: Department of Anatomy, Touro College of Osteopathic Medicine, 60 Prospect Avenue, Middletown, NY 10940, USA.
E-mail address: michael.miller21@touro.edu (M.W. Miller).
[1] Current address: Department of Pediatrics, Penn State Hershey Medical Group, 35 Hope Drive, Suite 102, Hershey PA 17033 USA.

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whereby incorrect bases are removed from a single DNA strand and replaced [*e.g.*, Zharkov, 2008; McCullough et al., 1999; Poletto et al., 2016; Seo et al., 2002]. The DDR, BER, and NER are potential means by which ethanol may alter growth factor-mediated DNA repair (Yan et al., 2010).

The response of proliferating cells to ethanol-induced damage depends on the ambient growth factor. For example, in their own right, fibroblast growth factor (FGF) 2 and transforming growth factor (TGF) β1 affect the proliferation of neural cells (*e.g.*, Davis and Stroobant, 1990; Dobolyi, 2012; Hicks et al., 2010; Luo and Miller, 1997; Luo and Miller, 1999; Siegenthaler and Miller, 2005a). FGF2 is a potent mitogen. It shortens the time a cell takes to pass through the cell cycle, thereby increasing the output from a proliferative population of neural stem and progenitor cells including the NS-5 neural stem cells that are the subject of the present study. In contrast, TGFβ1 has a special role in neural development. It is one of the few growth factors that *inhibits* cell proliferation, primarily by inducing stem cells (*e.g.*, NS-5 cells) to exit the cell cycle. Thus, these two growth factors have opposing effects on the activity of neural stem cells. Ethanol influences the actions of both of these growth factors in forebrain stem cells. This includes their generation (Hicks et al., 2010; Luo and Miller, 1999) and their survival (Hicks and Miller, 2011; Kuhn and Sarkar, 2008). The effects of ethanol on TGFβ1 may be mediated by the effects of ethanol on the recruitment of TGFβ receptors into non-lipid raft domains (Huang et al., 2016).

The present study tests the hypothesis that ethanol-induced DNA damage in NSCs is defined by the growth factor environment of NSCs. This effect results from ethanol targeting of the 9-1-1 complex and excision repair mechanisms (BER and NER). The present study, therefore, examined the effects of ethanol on NSCs treated with one of two different types of agents: an anti-mitogenic (TGFβ1) or pro-mitogenic (FGF2) growth factor.

2. Results

2.1. DNA damage in cultured NSCs

The integrity of the DNA in individual NS-5 cells was evaluated with a comet assay (Fig. 1A). A two-way analyses of variance (ANOVAs) showed that treatment had a significant ($F_{1, 17} = 105.611$; $p < 0.001$) effect on the lengths of the comet tails, a positive indicator of DNA damage, *i.e.*, genomic fragmentation (Fig. 1B). In the presence of either FGF2 or TGFβ1, treatment with moderate amounts of ethanol (200 mg/dl) significantly ($p < 0.05$) increased comet tail lengths compared with cells incubated with a growth factor alone. Higher concentrations of ethanol (400 mg/dl) caused further significant ($p < 0.05$) increases in tail lengths.

In the presence of either growth factor, there was a significant ($F_{1, 17} = 43.052$; $p < 0.001$) effect of ethanol on the tail lengths. A post-*hoc* test showed that in the presence of ethanol at concentrations of 200 or 400 mg/dl, the tail lengths for NS-5 cells cultured with TGFβ1 were significantly ($p < 0.05$) longer than they were for cells cultured with FGF2 and ethanol. Moreover, cells cultured with TGFβ1 and ethanol experienced nearly twice the damage of cells treated with FGF2 and ethanol. This difference was consistent and statistically significant ($p < 0.01$) at both high and low concentrations of ethanol. Thus, ethanol caused DNA damage in a concentration-dependent manner regardless of the growth factor environment.

2.2. Reactive oxygen species in cultured NSCs

The presence of the ROS intermediate hydrogen peroxide (H_2O_2) was measured using the fluorescent probe DCFH-DA (2, 7-dichlorofluoresceine diacetate DCFH-DA, Fig. 1C). Ethanol had a significant ($F_{1, 11} = 60.705$; $p < 0.001$) effect on the proportion of NS-5 cells that were 2, 7-dichlorofluoresceine (DCF) positive (Fig. 1D). The growth factor environment also had a significant ($F_{1, 11} = 73.009$; $p < 0.001$)

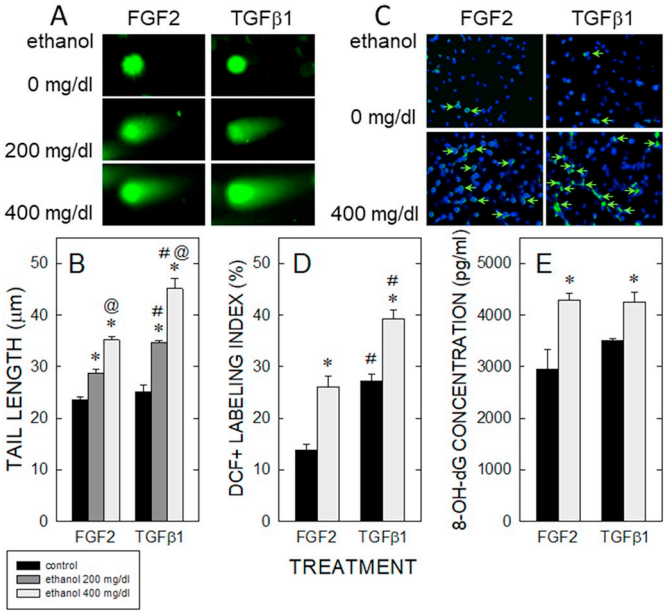

Fig. 1. Effects of ethanol and TGFβ1 on total DNA damage. A. A single cell gel electrophoresis comet assay was used to determine the extent of damaged DNA in NS-5 cells. Cells were cultured in the presence of fibroblast growth factor 2 (FGF2; 10 ng/ml), transforming growth factor β1 (TGFβ1; 10 ng/ml), and ethanol (0, 200, or 400 mg/dl) for 48 h. Following treatment, cells were placed in agarose, lysed, electrophoresed, and labeled with the DNA stain SYBR-green. Fluorescent microscopy was used to measure the distance fragmented DNA migrated: from the head of the cell nucleus to the visible end of the comet tail. B. The mean lengths of comet tails were determined for cells treated with ethanol and FGF2 or TGFβ1. C. NS-5 cells raised with FGF2 or TGFβ1 were challenged with ethanol (0 and 400 mg/dl). Incubation with 2,7-dichlorofluoresceine (DCF) revealed cells generating reactive oxygen species (arrows). All cells were stained with the nuclear label bisbenzimide. D. The labeling index, the proportion of DCF+ cells to bisbenzimide+ cells, was determined for cells treated with a growth factor and ethanol (0 or 400 mg/dl). E. The effects of ethanol and/or TGFβ1 on oxidative DNA damage were measured in NS-5 cells using a modified ELISA for 8-OH-dG. On each graph, bars represent mean values (± the standard errors of the means) for each treatment. $n = 3$. Significant ($p < 0.05$) differences relative to controls without ethanol, cultures treated with 200 mg/dl ethanol, or to FGF2-treated cells are noted by an *, @, or #, respectively.

effect. TGFβ1-alone induced ROS production (14%) than in cells cultured with FGF2 alone. Moreover, ethanol significantly ($p < 0.05$) increased the proportion of ROS+ cells by 12% relative to cells cultured with a growth factor alone. Note that treatment with TGFβ1 and ethanol led to ROS in 13% more cells than did treatment with FGF2 and ethanol.

An 8-hydroxy-2-deoxyguanine (8-OH-dG) assay was used to assess the contribution of ROS to ethanol-induced DNA damage. Ethanol had a significant ($F_{1, 11} = 20.348$; $p = 0.002$) effect on the amount of oxidation-damaged guanosine within NSCs, however, no significant effect of the growth factor was detected (Fig. 1E). Ethanol increased oxidated guanosine in the presence of FGF2 (46%) or TGFβ1 (21%). Both changes were statistically significant ($p < 0.05$). Interestingly, there was no significant difference in oxidative DNA damage among cells treated with ethanol and TGFβ1 *versus* those treated with ethanol and FGF2. This contrasts with measurements of total DNA breaks (see results of comet assay above) which showed a specific increase in ethanol-induced damage in the presence of TGFβ1. Such a discrepancy may result from the effects of TGFβ1 on total DNA repair rather than oxidative stress alone.

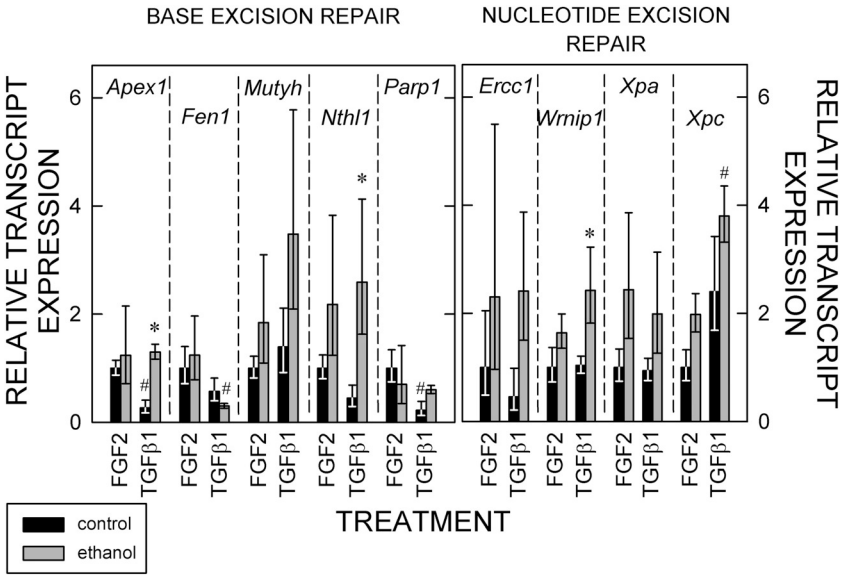

Fig. 2. Effects of ethanol on repair-related transcripts in cultured NSCs. NS-5 cells were treated with ethanol (0 or 400 mg/dl) and a growth factor (FGF2: 10 ng/ml or TGFβ1: 10 ng/ml). The expression of DNA repair related transcripts was determined using quantitative polymerase chain reaction arrays. Transcripts are apportioned into clusters based on their primary function (see www.genecards.org). Each bar represents the mean of three samples and T-bars signify standard errors of the means. Significant differences ($p < 0.05$) caused by ethanol or a growth factor are noted by * and #, respectively.

2.3. DNA repair transcripts in vitro

The effects of ethanol on the expression of 84 transcripts related to DNA repair by cultured NS-5 cells were examined with a quantitative reverse transcriptase polymerase chain reactions (qPCR) array. This included nine transcripts involved in the DDR, eight specific for BER, 13 specific for NER, and five that serve BER and NER. The other 49 transcripts were related to other repair mechanisms (as defined by www.genecards.org). These included transcripts associated with the repair of double strand breaks (DSB), homologous recombination repair (HRR), inter-strand cross-linking (ICL), mismatch repair (MMR), non-homologous end joining (NHEJ), and post-replication repair (PRR). The expression of numerous transcripts was significantly (> 2-fold change; $p < 0.05$) increased by ethanol exposure, particularly those associated with DDR, BER, and NER (Fig. 2). *n.b.* Statistical data for the changes in transcript expression *in vitro* are provided in Supplementary Table 2.

Of the DDR genes, *Hus1* and *Trp53* expression were specifically and significantly affected. *Trp53* also exhibited a significant effect of the growth factor. Other components of the 9-1-1 complex (Rad9 and Rad1) were not significantly affected by ethanol. On the other hand, transcripts for some facilitators of the 9-1-1 complex were also affected by ethanol (*Rad18*) or the growth factors (*Rad17* and *Ube2a*).

Ethanol up-regulated the expression of multiple transcripts involved in BER and/or NER. Of the BER-related transcripts screened, three (*Apex1*, *Mutyh*, and *Nthl1* [*n.b. Nthl1* functions in BER and NER]) were significantly affected by ethanol and three (*Apex1*, *Fen1*, and *Parp1*) were affected by the growth factor. BER-related transcripts that were examined and not significantly affected included *Mare*, *Ogg1*, *Smc1a*, and *Xrcc1*, as well as *Lig1* and *Pole* (enzymes involved in the synthesis of the DNA patch) which also act in NER. Four of the NER-specific mRNAs examined were significantly affected by ethanol (*Ercc1*, *Wrnip1*, *Xpa*, and *Xpc*) and one was affected by a growth factor (*Xpc*). NER-related transcripts that were not significantly affected by ethanol included *Chek1*, *Gadd45a*, *Gtf2h1*, *Gtf2h2*, *Parp2*, and *Rad23a*).

Sixteen transcripts associated with other DNA repair mechanisms were screened. Apparently, none of those involved in DSB, ICL, PRR, or NHEJ (*e.g.*, *Atm*, *Atrx*, *Brca1*, *Brca2*, *Chaf1a*, *Cry2*, *Fancg*, *Mlh3*, *Polh*, *Poli*, and *Tlk1*) was significantly affected by ethanol or a growth factor. One related to HRR (*Rad51c*) and one related to MMR (*Mlh1*) were significantly affected by ethanol and three related to other facets of repair processes were significantly affected by ethanol (*Smc3*) or a growth factor (*Mgmt*, *Smc3*, and *Trpc2*).

Note that more genes were affected and the magnitude of the effect was greater when the NS-5 cells were treated with TGFβ1. Indeed, post-*hoc* tests showed that the effects of ethanol were consistently greater for cells treated with TGFβ1.

2.4. DNA repair transcripts in vivo

Mice were exposed to ethanol *in utero* and the expression of 29 repair-related transcripts was examined in the proliferative compartments of fetal cortex. Statistically significant changes are described in Supplementary Table 3. Six transcripts were significantly ($p < 0.05$) affected by ethanol (Fig. 3). Two DDR (*Hus1* and *Trp53*), four BER (*Apex1*, *Mutyh*, *Nthl1*, and *Parp1*), two NER (*Gadd45a* and *Xpc*), and one HRR (*Xrcc2*) transcripts were altered in NSCs *in vivo*. Transcripts associated with other repair mechanisms were generally not significantly affected by ethanol; the exception being *Mlh3*. Two-way ANOVAs showed significant effects of collection time on the expression of affected transcripts for only *Gadd45a* and *Xpa*.

It is interesting to note that ethanol exposure *in vivo* significantly affected the expression of mRNAs related to growth factor receptors (Fig. 3). This includes the transcripts for the FGF receptor 1 (*Fgfr1*) and the TGFβ1 receptor 1 (*TGFβr1*). No significant differences were detected in the expression of these receptors regarding the timing of the ethanol dosing.

To determine the association between the expression of repair-related transcripts *in vivo* and *in vitro*, negative values for the change of the change in the mean cycles to threshold (ΔΔCp) for the transcripts examined in each condition were plotted (Fig. 4) These data were best fit to linear functions using a regression analysis of the effect of ethanol *in vitro* verses the effects of ethanol *in vivo*. The expression of repair transcripts in NS-5 cells exposed to ethanol and TGFβ1 *in vitro* was positively and significantly ($r = 0.6279$; $p < 0.01$) correlated with the expression of repair transcripts in G13.5 fetuses exposed to ethanol *in vivo*. In contrast, the effect of ethanol with FGF2 *in vitro* did not generate a response that was significantly ($r = -0.1389$; $p = 0.4561$) correlated with the effects of ethanol *in vivo*.

2.5. DNA repair proteins in vitro

The expression of select DNA repair proteins was evaluated with a quantitative immunolabeling method. In general, the expression of repair proteins by NS-5 cells treated with either growth factor varied across treatment groups in patterns similar to those for transcript expression.

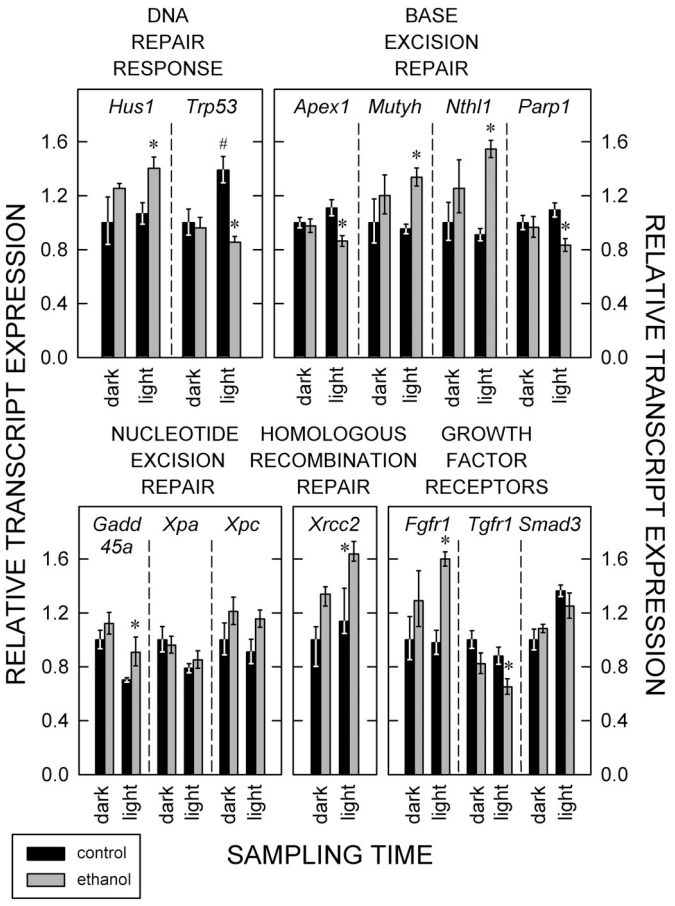

Fig. 3. Effects of ethanol on repair-related transcripts *in vivo*. Pregnant mice were given ethanol-containing liquid diets from G10 to G13 at 5 PM, just before the 12 h dark cycle commenced. The expression of repair-related transcripts in the proliferative zones of fetal cortex was measured at two times (10 PM during the dark cycle on G13 and 1 PM during the light cycle on G13.5) using quantitative real-time polymerase chain reactions. The expression of transcripts associated with facets of the DNA repair mechanisms was calculated for each transcript relative to control animals collected during the dark cycle (set at 1.0). Only transcripts significantly affected by ethanol or the timing of the sampling (based on two-way analyses of variance) are displayed. Significant ($p < 0.05$) differences cause by ethanol (*) or sampling time (#) are noted. Bars represent the mean (± the standard errors of the means) relative transcript expression for each treatment. $n = 4$.

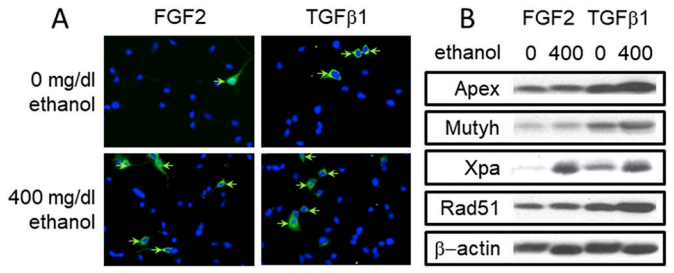

Fig. 5. Protein expression by NS-5 cells. A. The effects of ethanol (0 and 400 mg/dl) and growth factors (FGF2 and TGFβ1) on NS-5 cell expression of p53 was examined immunocytochemically. Arrows identify p53-immunolabeled cells. Bisbenzimide (blue) was a generic stain that identified all cell somata. B. Likewise, the effects of ethanol and the growth factors on NS-5 cell expression of representative DNA repair proteins was determined using immunoblots. Each blot was processed separately with a specific antibody against a specific protein or β-actin.

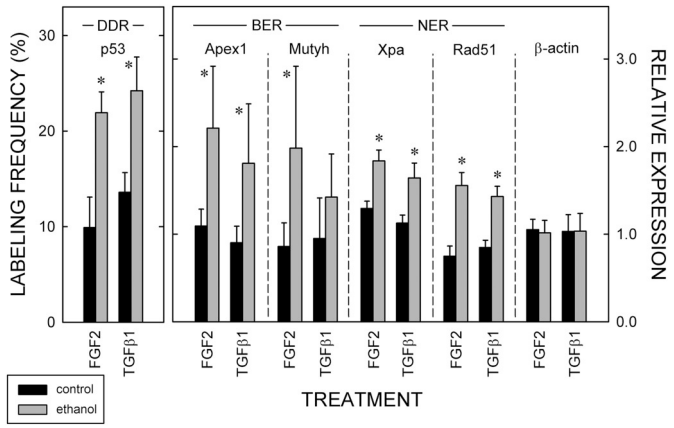

Fig. 6. Effects of ethanol and TGFβ1 on protein expression. Left. The numbers of cells expressing p53 were determined using immunocytochemistry following treatment with ethanol (0 or 400 mg/dl) and/or a growth factor (FGF2 or TGFβ1; 10 ng/ml). Mean cell numbers (bars) and standard errors of the means (T-bars) are shown. Right. The amount of a DNA repair protein expressed by stem cells was quantified with immunoblots. Bars represent the mean net intensity values for each protein (± the standard errors of the means). $n = 3$. Significant ($p < 0.05$) differences induced by ethanol are noted by asterisks.

The expression of p53 was determined immunocytochemically (Fig. 5A). The labeling frequency for cells expressing p53 immunoreactivity was significantly ($F_{1, 15} = 16.267$, $p = 0.002$) greater in ethanol-treated cells (Fig. 6 left). Indeed, the labeling in ethanol-treated cultures was about twice that detected in cells treated with FGF2 alone.

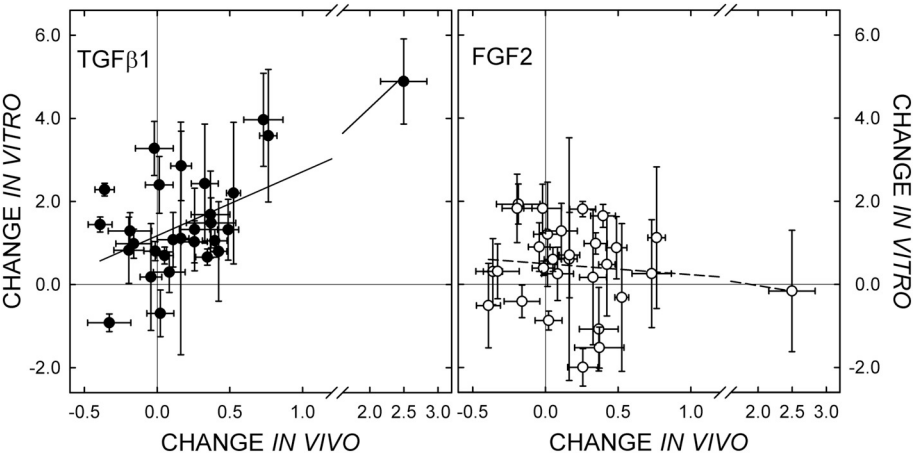

Fig. 4. Correlations between ethanol-induced transcript changes *in vivo* and *in vitro*. Relative gene expression (negative Cp values) for the expression of 29 DNA repair-related transcripts *in vivo* and *in vitro* is plotted. Correlations depict the effects of ethanol *in vivo* (x-axis; sampling time during the dark cycle) *versus* those *in vitro* (y-axis). Data for the effects of ethanol in the presence of TGFβ1 (10 ng/ml; left graph - solid circles) or FGF2 (10 ng/ml; right graph - open circles) *in vitro* were separately plotted. The *in vitro* and *in vivo* data were best-fit by linear regression analyses (TGFβ1: solid line; FGF2: dashed line).

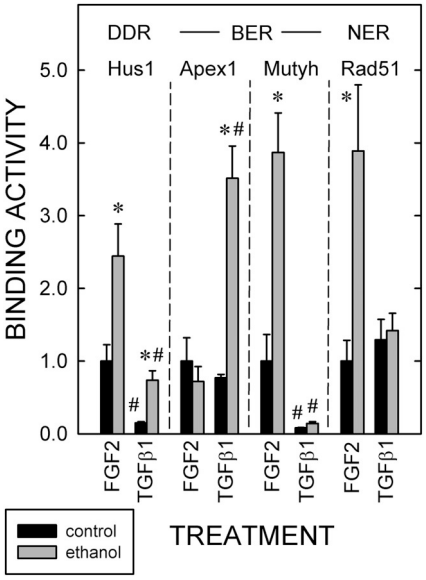

Fig. 7. Effects of ethanol and TGFβ1 on DNA repair activity. DNA repair activity was assessed by quantifying the amount of DNA bound to sentinel repair proteins. DNA bound to individual repair proteins was collected by immunoprecipitation, measured by quantitative polymerase chain reactions, and normalized to unbound DNA content. Bars represent the mean amount of DNA bound to each repair enzyme (± the standard errors of the means) relative to FGF2-treated samples. $n = 3$. Significant ($p < 0.05$) differences caused by ethanol or a growth factor are identified by an * or #, respectively.

Four proteins (Apex1, Mutyh, Rad51, and Xpa) were successfully examined using quantitative immunoblotting (Fig. 5B). Ethanol exposure significantly affected Hus1 ($F_{1, 11} = 8.564$, $p = 0.019$) as well as Apex1 ($F_{1, 23} = 4.572$, $p = 0.045$), Mutyh ($F_{1, 23} = 9.079$, $p = 0.007$), and Xpa ($F_{1, 23} = 20.908$, $p < 0.001$) (Fig. 6 right). Ethanol also increased the proportion of p53-immunopositive cells [see Hicks and Miller, 2011]. The effect of a growth factor was significant for Apex1 ($F_{1, 23} = 36.121$, $p < 0.001$), Xpa ($F_{1, 23} = 45.771$, $p < 0.001$), and Rad51 ($F_{1, 23} = 34.792$, $p < 0.001$; *n.b.* Rad51 is also active in DSB and HRR). In the presence of FGF2, ethanol increased expression of Apex1, Mutyh, Rad51, and Xpa. In the presence of TGFβ1, ethanol exposure affected members of BER and NER pathways, *e.g.*, increasing the expression of Apex1, Xpa, and Rad51.

2.6. DNA repair activity in vitro

A quantitative immunoprecipitation assay of the amount of repair-bound DNA was used to determine whether increases in transcripts and proteins associated with DNA repair produced functional increases in repair activity. qPCR was used to measure the amount of DNA from each treatment group bound to Hus1, Apex1, Mutyh, or Rad51 (Fig. 7). These proteins were chosen as representative members of the DDR, BER, and NER pathways that were affected by ethanol.

Two-way ANOVAs showed that ethanol significantly affected the DNA binding activity of sentinel DDR, BER, and NER proteins. These included Hus1 ($F_{1, 11} = 39.492$, $p < 0.001$), Mutyh ($F_{1, 11} = 13.497$, $p = 0.006$), and Rad51 ($F_{1, 11} = 7.654$, $p = 0.024$). The effect of ethanol on Apex1 binding approached, but did not attain statistical significance ($F_{1, 11} = 4.934$, $p < 0.057$). Nor was there a significant effect of ethanol on p53 binding was detected. Nevertheless, post-*hoc* tests showed that the effect of ethanol on cells treated with TGFβ1 was statistically significant ($p < 0.05$).

Based on two-way ANOVAs, it appeared that growth factor treatment significantly affected the binding activity of DDR and BER proteins: Hus1 ($F_{1, 11} = 61.097$, $p < 0.001$), Apex1 ($F_{1, 11} = 6.176$, $p < 0.038$), Mutyh ($F_{1, 11} = 125.906$, $p < 0.001$). TGFβ1 (relative to FGF2) reduced the DNA binding activity of Hus1 and Mutyh in the presence or absence of ethanol. Interestingly, the activity of Apex1 increased following treatment with TGFβ1 and ethanol compared with FGF2 and ethanol. Ethanol increased DNA binding activity of Hus1, Mutyh, and Rad51 in the presence of FGF2. Ethanol also increased the binding activity of Hus1 and Apex1 in the presence of TGFβ1.

3. Discussion

3.1. DNA damage

Ethanol causes DNA damage in NSCs. The DNA fragmentation in the NSCs results from ethanol-induced oxidative stress; ethanol increases the proportion of ROS+ NSCs and 8-OH-dG production. Ethanol also induces the generation of ROS and oxidative stress in fetal brains (Hamby-Mason et al., 1997; Kruman et al., 2012; Henderson et al., 1995; Miller-Pinsler et al., 2015).

Growth factors influence the response of NSCs to toxins such as ethanol. TGFβ1, a potent anti-mitogenic agent, increases the proportion of ROS+ NS-5 cells relative to that detected among cells treated with FGF2. Likewise, TGFβ1 increases the production of ROS in systemic cells/tissues (Herrera et al., 2001; Liu and Desai, 2015; Yao et al., 2007). ROS production can cause metabolic alterations (Arrick et al., 1992; Wu and Cederbaum, 2003) and neuronal DNA damage through depurination, deamination, or double-strand breaks (Brooks, 1997; Mansouri et al., 2001; Singh et al., 1995). In contrast, TGFβ1 can suppress genomic instability in mice keratinocytes (Singh et al., 1995). Differences in DNA integrity following TGFβ1 exposure can be explained by the role of TGFβ1 in apoptosis (Hicks and Miller, 2011; Schuster and Krieglstein, 2002). To maintain genomic stability, TGFβ1 may promote DNA fragmentation and apoptosis in individual NSCs.

TGFβ1 potentiates the toxic effects of ethanol on the integrity of NS-5 cell DNA. Such results are consistent with evidence that ethanol causes oxidative stress and DNA fragmentation *in vitro* (Jacobs and Miller, 2001; Ramachandran et al., 2003; Hicks and Miller, 2011) and *in situ/vivo* (Mooney and Miller, 2007; Young et al., 2003; Hamby-Mason et al., 1997; Henderson et al., 1995). Thus, ethanol causes DNA damage *via* oxidative stress and ethanol-induced ROS generation and this is potentiated by TGFβ1.

3.2. DNA repair

Ethanol promotes growth factor-mediated DNA repair in NSCs by altering their DNA damage responses (Fig. 8). In the presence or absence of ethanol, the expression of transcripts/proteins associated with the DDR and excision repair is increased. The patterns of these increases generally mirror each other, the exception being *Apex1*/Apex1 in NSCs treated with FGF2. Moreover, the genomic response *in vitro* generally parallels that induced by ethanol in cortical proliferative zones *in vivo*, *e.g.*, *Hus1*, *Trp53*, *Apex1*, *Mutyh*, *Nthl1*, and *Xpc*. One notable limitation of this conclusion is the absence of protein-level data *in vivo* that would determine if changes in the translation of mRNA obligatorily relate to protein concentration shifts.

There are many reasons why *in vitro* transcript data may not perfectly parallel changes either of *in vitro* protein expression or of *in vivo* transcript expression. Conversion of mRNA concentration shifts to protein level shifts is regulated by non-coding factors such as micro-ribonucleic acids. These epi-transcriptomic molecules may override up-regulation at the transcript level by binding target mRNAs and preventing protein translation. Such regulation may occur in response to rate-limiting sensors that prevent overproduction of a single component of a DNA repair complex. This would explain why some DNA repair elements display parallel mRNA and protein level changes *in vitro* (*e.g.*, Trp53), though others do not (*e.g.*, Mutyh). Given the influence of growth factor concentrations on the DNA repair response *in vitro*, it is not surprising that *in vivo* mRNA patterns vary slightly in a setting

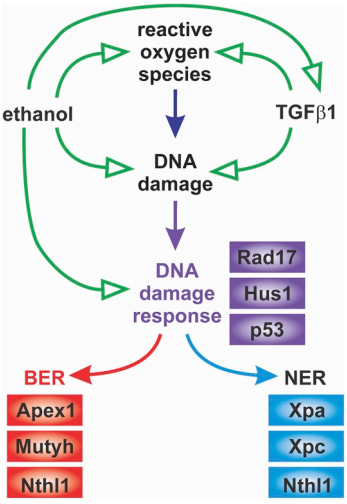

Fig. 8. Effects of ethanol and TGFβ1 on DNA repair. The schematic identifies the cascade of production of reactive oxygen species by ethanol, the generation of DNA damage, and the DNA damage response, primarily through nucleotide and base excision repair.

where growth factor concentrations are constantly fluctuating and cannot be tightly control. The *in vivo* environment is more complex and includes additional growth factors that may impact the DNA repair response of NSCs.

Nevertheless, studies of sentinel, targeted genes show that the binding activity of ethanol-targeted genes is affected by ethanol and growth factors in cultured NSCs. Therefore, the genomic response induced by ethanol in cultured NSCs (a) often presages changes in protein expression, (b) models the *in vivo* transcript response, and (c) results in functional changes that underlie ethanol- and growth factor-induced DNA repair.

The growth factor environment affects ethanol-induced DNA repair in cultured NSCs; notably, ethanol promotes the expression of specific DNA repair transcripts involved in DDR (*Rad17*, *Rad18*, and *Trp53*), BER (*Apex1* and *Nthl1*), NER (*Wrnip1* and *Nthl1*) in the presence of TGFβ1. These culture data are consistent with *in vitro* studies showing TGFβ1-mediated interference of DNA repair (Chen et al., 2016; Tummala and Dokal, 2016), role of TGFβ1 in radiation suppression of NER in cultured human keratinocytes (Qiang et al., 2016), and TGFβ1 modulation of DDR to radiation in TGFβ1-deficient mouse epithelia (Ewan et al., 2002). The implication is that at least in part, ethanol causes its effects on DNA repair through TGFβ1-regulated mechanisms. This is further supported as gestational ethanol exposure causes changes in the *in vivo* expression of the TGFβ and FGF receptors in the cortical proliferative zones [(Miller, 2003), present study] and the effects of ethanol on NSCs treated with TGFβ1 *in vitro* correlate positively and significantly with the response *in vivo* (see Fig. 4). No such correlation is evident for cells treated with FGF2.

Although there are many components of the DNA damage response system, the effects of ethanol on TGFβ1-regulated repair are quite specific. In NS-5 cells, ethanol potentiates TGFβ1-mediated DDR *via* three reinforcing mechanisms.

(1) Ethanol affects the expression of facilitators of repair (*e.g.*, *Rad17* and *Rad18*) in NS-5 cells treated with TGFβ1. Rad17 binds to damaged DNA and recruits the 9-1-1 complex [*e.g.*, Lee and Dunphy, 2010; Niida and Nakanishi, 2006; Sancar et al., 2004; Yang and Zou, 2006].
(2) Ethanol increases the expression of *Hus1*/Hus1 *in vitro* and *in vivo*; the only exception is for protein expression in cells treated by FGF2. As a component of the 9-1-1 complex, Hus1 is critical for stabilizing the DNA during its replication or repair (Sancar et al., 2004; Levitt et al., 2005; Yazinski et al., 2009). Though ethanol affects the 9-1-1 complex, it has no apparent effect on PCNA. This is surprising because PCNA, like the 9-1-1 complex, is a toroidal protein that serves as a sliding clamp anchoring DNA polymerase during DNA replication and repair (Essers et al., 2005; Shivji et al., 1992). Thus, along with the targeted effect of ethanol on Hus1, the lack of an ethanol-induced effect on PCNA shows that ethanol affects DNA repair by specifically interfering with the function of the 9-1-1 complex.
(3) p53 links the DDR system with effectors of repair [*e.g.*, Ljungman, 2000; Seo et al., 2002; Hwang et al., 1999; Zurer et al., 2004]. Ethanol increases *Trp53* expression and the number of p53-immunopositive NS-5 cells in the presence of TGFβ1. Paradoxically, the amount of p53 expression is lower in the cortical proliferative zones. These *in vivo* data are at variance with a previous study of rat cortex showing that ethanol promotes fetal p53 expression (Kuhn and Miller, 1998). Presumably another variable is at play in the present *in vivo* study, *e.g.*, the timing and perseverance in transcript/protein expression.

Ethanol-induced changes in cultured NSCs are generally consistent with changes in fetal and adult rodent brain. They are indicative of ethanol-induced changes in DNA repair and mediation by TGFβ1.

Ethanol up-regulates the expression of BER and NER transcripts/proteins in NSCs including *Mutyh*/Mutyh and *Apex1*/Apex1. Mutyh is a glycosylase that corrects for the inappropriate insertion of guanine instead of cytosine caused by oxidative damage. It has physical and functional interactions with the 9-1-1 complex, particularly Hus1, and facilitates the actions of Apex1 (Balakrishan et al., 2009; Parker et al., 2001; Shi et al., 2006; Hwang et al., 2015). Apex1 is the major endo/exonuclease repairing apurininc/apyrimidinic sites that result from oxidative damage or the action of glycosylases designed to remove bases (*e.g.*, Mutyh) (Balakrishan et al., 2009; Lu et al., 2006; Luncsford et al., 2010). Ethanol also potentiates the expression of *Nthl1*, the mRNA coding for another AP endo/exonuclease that participates in BER and NER. Other targets of ethanol in NER are forms of Xeroderma pigmentosum, *Xpa* and *Xpc*. These proteins are important in the damage recognition initiating NER (Sugasawa, 2008). Thus, ethanol, especially in the presence of TGFβ1, induces the expression of transcripts/proteins required for initial steps of excision repair including the identification of damage and the expression of enzymes that remove damaged and inappropriate bases/nucleotides.

Inasmuch as understanding the effects of ethanol on specific genes is important, it is also important to appreciate that ethanol has no appreciable effect on most genes. A genome-wide microarray study shows that ethanol affects only 0.43% of the genes expressed by NS-5 cells (Hicks et al., 2010). Among the unaffected genes related to DNA replication and repair are those involved in the patching of the excised bases/nucleotides (*e.g.*, DNA polymerases such as *Polb*, *Pole*, and *Poli* and ligases such as *Lig1*) and genes associated with other repair mechanisms (*e.g.*, ICL, MMR, NHEJ). Moreover, transcripts/proteins associated with HRR (*e.g.*, Rad51 and *Nthl1*) are affected by ethanol which is consistent with a study by Kotova and colleagues (Kovota et al., 2013). This further supports the hypothesis that ethanol specifically targets the early stages of excision repair.

3.3. Clinical ramifications

Generating neurons and glia from NSCs, *i.e.*, neurogenesis, is critical for brain development and neural maintenance/plasticity throughout life. Neurogenesis is the sum of cell proliferation and death, and cell survival depends on DNA integrity, *i.e.*, the success of DNA repair. Ethanol can damage NSC DNA (Brooks, 1997; Hicks and Miller, 2011; Henderson et al., 1995). Such damage can result from oxidative stress and lead to the up-regulation of specific repair mechanisms. Ethanol also differentially regulates the cell cycle of NSCs by altering genomic

expression and inducing hypermethylation (Hicks et al., 2010). Following ethanol-induced DNA damage or hypermethylation, NSCs upregulate repair mechanisms including BER and NER (Lu et al., 1983).

Like ethanol, an ambient growth factor can affect DNA integrity. In contrast to ethanol, TGFβ1 can inhibit the DDR and BER. TGFβ1 downregulates Hus1, a central component of the 9-1-1 damage recognition complex that triggers p53-dependent repair mechanisms such as Apex1 and Mutyh activities (Ljungman, 2000; Roos-Mattjus et al., 2002). In turn, this defines the balance between NSC repair and apoptosis. Thus, TGFβ1 can potentiate ethanol-induced oxidative damage in NSCs by altering DNA repair.

DNA repair mechanisms contribute to cognitive ability (Mattick and Mehler, 2008). This association has been examined in aging (Lu et al., 2004) and chemotherapy (Ahles and Saykin, 2007) patients wherein cognition is reduced. Such people exhibit increased oxidative stress and DNA repair responses. In Alzheimer's patients, similar increases are detected, but these appear to be transient (Lovell and Markesbery, 2007). Interestingly, humans and mice exposed to ochratoxin-A (a common mycotoxin) have compromised cognition. The viability of cultured, proliferating cells from the hippocampi of mice treated with ochratoxin-A is decreased despite a robust DNA repair response (Sava et al., 2007). In contrast, increased expression of DNA repair genes is associated with increased intelligence (Cochran et al., 2006).

An association between neurogenesis and cognition/mental function is compelling (Deng et al., 2010). Neurogenesis is reduced in adults with psychiatric disorders [*e.g.*, Apple et al., 2017; Eisch and Harburg, 2006; Krishnan and Nestler, 2008; Obernier et al., 2002] and increased following environmental and social enrichment (Nithianantharajah and Hannan, 2006; van Praag, 2008). Ethanol exposure, regardless of the timing during the lifespan, targets proliferating NSCs and can lead to mental impairments [*e.g.*, Nixon et al., 2010; Zawada and Das, 2006; Guerri and Pascual, 2010; Heffernan, 2008; Golub et al., 2015; Herrera et al., 2003; Miller and Spear, 2006; Sakharkar et al., 2016]. We posit that ethanol-induced cognitive deficits associated with AUD and FASD result from abnormal DNA repair by NSCs.

Profound damage caused by to ethanol toxicity can occur during the first trimester. This is a time before most organs have developed and when the growing embryo is replete in stem cells and progenitors. Knowing the genes that are richly expressed in an early embryo requires understanding the response of stem cells. Hence, the genes and gene products that are expressed by stem cells and are affected by ethanol *in vivo* offer the promise of a biomarker fingerprint for alcohol-induced damage.

4. Materials and methods

4.1. Cell cultures and treatment conditions

NS-5 cells, non-immortalized NSCs derived from mouse embryonic stem cells, are adhesive, symmetrically expandable NSCs which retain pluripotent potential after prolonged passaging (Conti et al., 2005; Pollard et al., 2006). NS-5 cells were grown at 37 °C and 7.0% CO_2 in 250 ml flasks in serum-free Euromed-N growth medium (Euroclone, Pero, Italy) supplemented with 2.0 mM glutamine, N2 (R&D Systems, Minneapolis MN), 10 ng/ml recombinant human FGF2 (Peprotech, Rocky Hill NJ), and 10 ng/ml recombinant murine epidermal growth factor (EGF; Peprotech). Note that NS-5 cells require a growth factor(s) to maintain their cycling activity and survival (Conti et al., 2005; Pollard et al., 2006). To maintain their stemness, medium was changed regularly (every 1–2 d) and cultures were split upon reaching confluency (every 3–4 d). Thus, at no time during the execution of the study were the stem cells incubated in a growth factor-free medium. This has the disadvantage of making it impossible to assess the effects of a growth factor directly (*i.e.*, through a comparison of the activity of cells treated with a growth factor to the activity of cells raised without a growth factor).

Cells were plated on 100 mm Petri dishes or 24-well plates with glass coverslips for final concentrations of 3.5×10^5 cells/ml. The plates were coated with poly-*l*-ornithine hydrobromide (15 μg/ml; Sigma, St. Louis MO) and laminin (10 μg/ml; Sigma). Cultures were maintained in FGF2/EGF-supplemented Euromed-N medium for 24 h and then switched to growth factor-free conditions for 4 h. This short interval is sufficient to wash out the growth factor, yet not too long to compromise NS-5 cell vitality. Finally, cells were exposed for 48 h to one of the following treatments: FGF2 (10 ng/ml), FGF2 (10 ng/ml) and ethanol (200 or 400 mg/dl; *i.e.*, 43.4 and 86.8 mM, respectively), TGFβ1 (10 ng/ml; R&D Systems), or TGFβ1 (10 ng/ml) and ethanol (200 or 400 mg/dl).

The chosen concentrations of ethanol were necessary to alter the proliferation of neural cells in monolayer cultures [*e.g.*, Hicks et al., 2010; Luo and Miller, 1997; Luo and Miller, 1999] and ventricular zone cells in organotypic cultures (Siegenthaler and Miller, 2005b). The effects of 400 mg/dl ethanol *in vitro* parallel changes caused by ethanol *in vivo* wherein blood ethanol concentrations are ~150 mg/dl (~32.6 mM) (Miller, 1992; Miller and Nowakowski, 1991). To control for ethanol volatility, NS-5 cells were maintained in closed chambers (Luo and Miller, 1997; Adickes et al., 1988).

4.2. Comet assay

A single cell, gel electrophoresis comet assay (R&D Systems, Minneapolis MN) (Rulten et al., 2008; Singh et al., 1988) was performed to quantify the total extent of DNA damage caused by exposure to ethanol (0, 200, or 400 mg/dl). This assay detects single- and double-stranded breaks, as well as apurinic sites and adducts.

Immediately following treatment, adherent cells were trypsinized, pelleted by centrifugation (1500 rpm), and quantified using a hemocytometer. Cells (1×10^5 cells/ml) were suspended in PBS and combined with molten Low Melting Agarose (R&D Systems) at a ratio of 1:10 (*v*/v). This mixture (75 μl) was pipetted onto a two-well comet slide and allowed to solidify at 4 °C for 30 min in the dark. Slides were immersed in pre-chilled lysis buffer under the same conditions for one hr. Excess buffer was removed and the slides were immersed in an alkaline solution (pH > 13) for one hour. After rinsing the slides in Tris/borate/EDTA buffer for 5 min, they were placed in a horizontal electrophoresis chamber and voltage was applied at 1.0 V per cm for 10 min. Finally, slides were fixed in 70% ethanol for 5 min, allowed to air dry, and stained with 50 μl of SYBR Green I per well.

Comet tails were viewed using epifluorescence microscopy (521 nm). The mean length of tails emanating from cells was determined. Accordingly, comet length was measured as the linear distance from the leading edge of the cell membrane to the visible limit of the DNA fragment tail at a fixed illumination of 120 W (100% iris open) on an X-Cite 120 fluorescent lamp (Exfo Lifesciences, Mississauga ON). This approach provided a conservative estimate of comet length by including relatively constant cell widths within the comet measurement. Approximately 25 cells were examined in each of six, randomly selected fields (200 μm × 200 μm) from each of three culture dishes per treatment. The mean comet length for each culture dish was calculated. The grand mean (+/− the standard error of the means) for the three culture dishes per treatment was used in the statistical analyses.

Treatment-induced differences were determined using two-way analyses of variance (ANOVAs) and post-*hoc* Holm-Sidak *t*-tests. The primary comparison in this and other experiments was the pair-wise effects of ethanol on growth factor mediated growth. That is the comparison of the activity of cells in a growth factor (FGF2 or TGFβ1 in the absence or presence of ethanol. Note that the use of two-way ANOVAs allowed for assessments of growth factor effects despite not having cells cultured in the absence of a growth factor due to the requirements of the NS-5 cells.

4.3. Assay for reactive oxygen species

One potential cause of DNA damage is ethanol-induced generation of ROS. ROS include free oxygen radicals produced by the mitochondria during normal metabolism. They accumulate during times of environmental stress and can lead to apoptosis by damaging DNA, proteins, or lipids (Ramachandran et al., 2003; Henderson et al., 1995; Papa and Skulachev, 1997). Cellular accumulations of ROS are a marker for oxidative stress and impending apoptosis.

The effects of growth factors and ethanol on the production of ROS by NS-5 cells were assessed fluorometrically using the oxidation-sensitive fluorescent probe DCFH-DA (Sigma). Intracellular esterases cleaved and ROS oxidized DCFH-DA to form DCF which was detectable with fluorescence microscopy (Gao et al., 2004).

Following treatment in 24-well plates, DCFH-DA (20 mM stock solution) was added directly to the culture medium so that it constituted a final concentration of 1.0 μM. Cells were incubated at 37°C for 15 min, rinsed in 0.100 M phosphate buffered saline (PBS), and fixed by a 30 min wash in 75% ethanol. Cells were stained with the DNA dye bisbenzimide (Hoescht 33,342) for 5 min for the detection of all cells. The mean proportions of cells double-labeled with DCF and bisbenzimide to the total number of bisbenzimide-positive cells were determined. Six randomly selected fields (200 μm × 200 μm) were examined to determine the labeling index for each culture dish. The means (+/− the standard errors of the means) for three independently examined culture dishes per treatment were calculated. Differences among the data for the ethanol and growth factor treatment groups were statistically assessed using ANOVAs.

4.4. Oxidative DNA damage

DNA repair initiated by the 9-1-1 complex results in the activation of 8-hydroxy-2-deoxyguanine (8-OH-dG), a specific product of ROS-induced DNA damage and oxidative stress (Park et al., 2009). Ethanol induces 8-OH-dG activation (Wilson III et al., 1994). Thus, the effects of ethanol and growth factors on the expression of 8-OH-dG were measured by a colorimetric assay (StressMarq Biosciences, Victoria BC). DNA was collected from three samples per treatment group using a DNeasy extraction kit (Qiagen, Valencia CA), denatured and digested using Nuclease P_1 (100 ng/μl; Sigma, St. Louis MO), and incubated at 37°C for 30 min with one unit of alkaline phosphatase (Invitrogen, Camarillo CA).

The assay used a 96-well plate coated with anti-mouse IgG, as well as a tracer consisting of an 8-OH-dG-enzyme conjugate. This format decreased variability and increased sensitivity by recognizing both free and DNA-incorporated 8-OH-dG. Each sample was assayed in duplicate at three dilutions, along with an 8-point standard curve, non-specific and maximum binding wells, and blank and total activity wells. The plates were incubated for 18 h at 4°C before a five step rinse, and development with Ellman's Reagent for 2 h on an orbital shaker at room temperature.

Plates were developed using a Thermo Multiskan Plate Reader (Fisher Scientific) with Ascent Software and absorbance at 405 nm was measured. A standard curve for each plate was generated by plotting the percent amount bound (%B) divided by the maximal amount of binding (B_M) (x-axis) *versus* the log standard concentration (y-axis). The data were examined with a four-parameter log-logit curve fit using a regression analysis. This plot was used to determine the concentration of 8-OH-dG from each sample based on $\%B/B_M$ values. The results were assessed with ANOVAs and Holm-Sidak tests.

4.5. In vitro expression of repair-related transcripts

Changes in the expression of DNA repair-related transcripts were determined using RT^2 Profiler qPCR (SABiosciences, Frederick MD). PCR arrays for the Mouse DNA Damage Signaling Pathway (cat # PAMM-029) were used to assess the expression of 84 genes associated with the *Atm* gene signaling network. Three independent RNA samples from each treatment condition were isolated and reverse transcribed using the RT^2 First Strand Kit (SABiosciences).

Transcript expression was quantified using the Light Cycler 480 System (Roche; Indianapolis, IN) with the SybrGreen Real Time Master Mix (Roche). Mean cycles to threshold (Cp) for each target gene and two reference genes: *Hprt1* and *Hsp90ab1* were recorded. Amplification in the absence of template was used to rule out primer-dimer artifact. End-point melt curve analysis confirmed the presence of single amplicons. As *Hprt1* and *Hsp90ab1* expression were stable across all samples (no statistically significant differences in ethanol- or growth factor-induced transcript expression were detected), the Cp values for the mean of these transcripts was subtracted from Cp values for DNA repair genes and averaged to generate values for the change in the Cp (ΔCp) for each gene in each sample. Mean ΔCp values for each transcript in a sample were compared mean ΔCp values in a second treatment of interest (*e.g.*, FGF2 with ethanol *versus* FGF2-alone). The ΔΔCp values were used to determine relative gene expression across samples. Treatment-induced differences were examined using ANOVAs.

4.6. In vivo expression of repair-related transcripts

The expression of altered gene products was examined in wild-type C57BL6 mice (Charles River Laboratories, Wilmington MA). Animal studies were designed and performed within the guidelines of the Society for Neuroscience. All procedures were approved by the Institutional Animal Care and Use Committees at Upstate Medical University and the Syracuse Veterans Affairs Medical Center.

Animals were maintained in a temperature and humidity controlled environment in which the lights were on between 6:00 AM and 6:00 PM. Pregnant dams were provided ethanol through a pair-feeding paradigm (Hicks et al., 2010; Hicks and Miller, 2011). Mice were mated overnight. Vaginal plug-positive females were identified, and the first morning a plug was observed was described as gestational day (G) 0.5. Females were housed individually throughout pregnancy. They were fed a standard chow and water diet until G10 at which time they were given a PMI Micro-Stabilized Alcohol Rodent Liquid Diet containing 2.5% ethanol (Testdiet, Richmond IN). The ethanol concentration was increased to 4.5% on G11 and maintained until G13.5. The volume of diet consumed was recorded daily. Control mice were pair-fed isocaloric liquid diets. Fresh food was provided daily at 5:00 PM.

Fetuses were taken at two times, once during the dark cycle and once during the light cycle. Accordingly, pregnant dams were sedated with a cocktail (1.0 ml/kg) of ketamine (100 mg/ml) and xylazine (10 mg/ml) at 10:00 PM (during the dark cycle) on G13 or at 1:00 PM (during the following light cycle) on G13.5. Blood was collected from the placental artery at the time of fetal harvesting for measurements of blood ethanol concentration (BEC). BECs were determined with a GM7 Micro-Stat Analyzer (Analox Instruments, London UK). Ethanol-treated mice achieved mean BECs of 135 +/− 26 mg/dl (*i.e.*, 29.2 +/− 5.6 mM) during the dark cycle and 11.2 +/− 2.3 mg/dl (*i.e.*, 2.43 +/− 0.50 mM) during the light cycle.

Fetuses were surgically delivered. Their brains were removed and cut coronally into 400 μm slices using a MacIlwain Tissue Chopper (Mickle Laboratory Engineering, Gomshell UK). Slices were placed in ice cold PBS. A dissecting scope was used to visualize the lateral ventricles, remove the neocortex, and isolate the proliferative regions (the ventricular and subventricular zones). Proliferative zone-enriched samples from fetuses in each litter were pooled into two biological replicates per litter. Tissue samples for each of four litters per treatment group were placed on dry ice for extraction of total RNA and the preparation of cDNA as described above.

To control for consistency of stem cell content in the enriched samples, the expression of the NSC marker nestin was measured at the transcript level using RT-PCR. Briefly, 1.0 μl of cDNA from each sample

was loaded onto a 364-well plate in a solution of 3.5 μl deionized water, 5.0 μl SybrGreen Real Time Master Mix (SA Biosciences), and 1.0 μl of nestin primer pair (R&D Systems; Minneapolis MN). Cp values for nestin in each sample were normalized to the corresponding expression for the reference gene TATA-binding protein (TBP). A ΔΔCp value relative to mean nestin expression was generated for all samples. Only samples comprising nestin contents within one standard deviation relative to the mean were included in the analysis.

To examine the expression of cell death transcripts *in vivo,* a customized PCR array plate (SABiosciences) was designed containing primer sets for 19 cell death-related mRNAs. As previously described, Cp values for each target transcript and reference transcripts (*Gusb*, *Gapdh*, and *Hsp90ab1*) were used to generate ΔCp values. End-point melt curve analyses confirmed the presence of single amplicons. The expression of each transcript was quantified relative to that in control animals as the difference in ΔCp values (*i.e.*, the ΔΔCp) between treatments.

Two-way ANOVAs were used to determine the effect of ethanol exposure and collection time on the expression of death-related transcripts. Statistically significant changes in gene expression ($p < 0.05$) were verified independently by Benjamini-Hochberg post-*hoc* analyses.

The effect of ethanol on the expression of each death-related transcript *in vivo* was compared to the change that occurred *in vitro* be it in the presence of FGF2 or TGFβ1. These data were correlated by best-fitting negative ΔΔCp values for each transcript using a linear regression model. Significant changes in gene expression were determined using ANOVAs followed by post-*hoc* Holm-Sidak tests.

4.7. Immunocytochemistry

NS-5 cells were grown on coverslips and treated with a growth factor (FGF2 or TGFβ1; 10 ng/ml) and ethanol (0 or 400 mg/dl) for 48 h. The NSCs were washed in PBS for 5 min and fixed in 4% paraformaldehyde for 30 min. After a quick rinse in PBS, cells were permeabilized with a 5 min wash in 1.0 N HCl and rinsed three times in PBS. To block non-specific binding, cells were incubated in 1.0% bovine serum albumin with 2.0% donkey serum in PBS (serum-PBS) for 1 h. Following PBS rinses, the cells were incubated with a primary antibody (Supplementary Table 1) for 2 h. After washing in PBS, cells were incubated for 1 h with a donkey anti-rabbit or anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (diluted1:200 in serum-PBS; Jackson, West Grove PA). All samples were labeled with the DNA stain bisbenzimide (Hoescht 33,342; 1:400 in serum-PBS, Sigma) for 5 min before a final PBS wash. Non-specific immunoreactivity was assessed in preparations in which the primary antibody incubation was omitted.

Immunolabeled cells were identified using a Zeiss AxioSkop epifluorescence microscope fitted with an X-Cite lamp (Exfo Lifesciences, Mississauga ON). Counts were taken in two random, non-overlapping fields (200 μm × 200 μm fields in three independent culture dishes per treatment. As a result, ~300 cells were examined in each field. The mean labeling indices for the two replicates were taken as representative of each preparation and used in the statistical comparisons wherein the sample size was three. The effects of growth factor and ethanol were assessed using two way ANOVAs and post-*hoc t*-tests.

4.8. Immunoblotting

Quantitative immunoblotting techniques were used to determine changes in protein expression of cultured NS-5 cells treated with ethanol. Cells were subjected to one of the four treatments for 48 h, removed from the culture dish with a Costar 3010 cell scraper, and lysed in cold RIPA buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1.0% Nonidet P-40, 0.50% sodium deoxycholate, 1.0 mM EDTA, and 0.10% sodium dodecylsulfate) supplemented with a protease inhibitor cocktail (Sigma P-8340). Samples were homogenized and centrifuged at 10,000 rpm for 10 min. The supernatants were removed and stored at −80°C prior to immunoblotting.

The supernatants were thawed and protein concentrations were quantified using the Protein Assay Kit II (Bio-Rad, Hercules CA). Protein fractions (30 μg per sample) were combined with sample buffer (300 mM Tris-hydrochloric acid, 50% glycerol, 5.0% sodium dodecyl sulfate (SDS), 0.025% bromophenol blue, and 250 mM β-mercaptoethanol). Three biological replicates of each sample, along with rainbow molecular weight markers (Amersham Biosciences, Piscataway NJ) were loaded on a 10% SDS-polyacrylamide gel and separated by electrophoresis (40 mA for 3.5 h). Sample proteins were transferred to nitrocellulose membranes overnight, before immunolabeling in a three-step procedure.

Non-specific immunoreactivity was blocked by washing in 5% non-fat dehydrated milk (NFDM) for 1 h at room temperature on an orbital shaker. The membranes were incubated at room temperature for 2 h with primary antibody diluted in 2.5% NFDM (Supplementary Table 1). Membranes were then rinsed (100 mM phosphate-buffered saline and 1.0% Tween-20) and incubated with a horseradish peroxidase linked anti-mouse or anti-rabbit secondary antibody (1:3000 dilution). Tagged proteins were visualized with a chemiluminescent detection agent (Amersham Biosciences) exposed to Kodak Biomax XAR film and developed.

Membranes were stripped by washing in a solution of 62.5 mM Tris-HCl, 2.0% SDS, and 10 μM β-mercaptoethanol at 50°C for 30 min. The stripped membranes were re-probed with an anti-mouse β-actin antibody (1:2000, Sigma). The stable expression patterns of this protein allowed for its use as a reference standard (for the comparison among blots) and loading control. Blot density was measured using a Kodak Image Station and net intensity for proteins of interest were normalized to β-actin intensity for their respective sample.

Statistical significance for differences in protein expression measured in immunoblots was examined using ANOVAs. Post-*hoc* tests for the effects of treatment were performed with Holm-Sidak *t*-tests.

4.9. DNA repair activity assay

DNA repair activity was quantified through the immunoprecipitation of DNA bound to repair proteins followed by qPCR. This method is a novel modification of a technique used for the identification and quantification of transcription factor binding activity (Gossett and Lieb, 2008; Weinmann and Farnham, 2002). After exposure to one of four treatments, NS-5 cells were collected and DNA repair proteins were extracted from three independent samples of 4.0×10^6 cells. Chromatin-bound Hus1, p53, Apex1, Mutyh, and Rad51 were obtained using a ChampionChIP immunoprecipitation kit (SABiosciences; Supplementary Table 1) according to manufacturer instructions. Addition of non-immune serum in place of primary antibody served as a negative control.

Briefly, samples were fixed in phosphate-buffered 4.0% paraformaldehyde, harvested with 0.025% trypsin, and lysed in the presence of a protease inhibitor cocktail (SABiosciences). Chromatin was sheared by sonication into 1000–3000 bp fragments using a Branson Microtip probe. DNA repair proteins of interest (along with bound DNA) were immunoprecipitated using an antibody-protein bead system (SABiosciences). Crosslinks between the repair proteins and DNA were reversed using proteinase K and DNA was isolated with DNA extraction beads (SABiosciences). Total DNA content was quantified for each sample in fractions that had not undergone immunoprecipitation (the pre-immunoprecipitation fraction) and those that had been immunoprecipitated (the post-immunoprecipitation fraction) with Hus1, Apex1, Mutyh, and Rad51.

DNA repair activity was measured as the amount of DNA bound by each repair protein (post-immunoprecipitation fraction) relative to the total unbound DNA in each sample (pre-immunoprecipitation fraction). The TBP gene was quantified in post- and pre-immunoprecipitation

samples from each treatment using a Light Cycler 480 System (Roche). Mean cycles to threshold (Cp) for each sample were recorded and amplification in the absence of DNA template was used to rule out primer-dimer artifact. End-point melt curve analysis confirmed the presence of single amplicons. Post-immunoprecipitation Cp values were normalized to pre-immunoprecipitation Cp values (which were relatively stable across all samples), generating a ΔCp. ΔΔCp values were then generated by normalization of each ΔCp against TBP expression in FGF2 control samples. ΔΔCp values were used to calculate fold-changes in the amount of TBP bound to repair proteins in ethanol-treated samples compared with FGF2-treated samples. Statistical significance was assessed using two-way ANOVAs and Benjamini-Hochberg analyses.

Author contributions

Both authors were involved in the formulation of the project, generation of the experimental design, and data analyses. Dr. Hicks was the primary person responsible for generating the data and Dr. Miller drafted the manuscript.

Competing financial interests

Neither author has any competing interests that may be perceived as influencing the results or discussion of the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2019.02.003.

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