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## EGFR may couple moderate alcohol consumption to increased breast cancer risk

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### Abstract

Alcohol consumption is an established risk factor for breast cancer. Nonetheless, the mechanism by which alcohol contributes to breast tumor initiation or progression has yet to be definitively established. Studies using cultured human tumor cell lines have identified signaling molecules that may contribute to the effects of alcohol, including reactive oxygen species and other ethanol metabolites, matrix metalloproteases, the ErbB2/Her2/Neu receptor tyrosine kinase, cytoplasmic protein kinases, adenylate cyclase, E-cadherins, estrogen receptor, and a variety of transcription factors. Emerging data suggest that the epidermal growth factor receptor (EGFR) tyrosine kinase may contribute to breast cancer genesis and progression. Here we integrate these findings and propose three mechanisms by which alcohol contributes to breast cancer. A common feature of these mechanisms is increased EGFR signaling. Finally, we discuss how these mechanisms suggest strategies for addressing the risks associated with alcohol consumption.

### Keywords

alcohol; breast cancer risk factor; EGF receptor; matrix metalloprotease

### Alcohol and other breast cancer risk factors

One in eight American women will be stricken with breast cancer, which is the second leading cause of cancer-related mortality among American women.<sup>1</sup> Approximately 15% of those breast cancer patients who receive surgery and chemotherapy will experience a recurrence of the disease. This is a major contributor to the fact that one-third of those who develop breast cancer will ultimately succumb to the disease.<sup>2</sup> Thus, breast cancer remains an important public health and societal issue and more information is needed concerning the causes of breast cancer and risk factors associated with the disease.

In 50% of breast cancers the only apparent risk factors are gender and age.<sup>1,2</sup> In the other half of breast cancers the risk factors include genetic factors, environmental factors, age of menarche and menopause, and reproductive history.<sup>2</sup> The latter factors underscore the contributions of female reproductive hormones to breast cancer risk.<sup>2</sup>

ErbB family receptor tyrosine kinases and their ligands, members of the epidermal growth factor (EGF) family of peptide growth factors, also play significant roles in breast cancer pathogenesis and progression. The ErbB2 receptor tyrosine kinase, also known as HER2/Neu,

is overexpressed in a significant fraction of breast tumors and this overexpression correlates with the absence of steroid hormone receptor expression and with poor disease prognosis.<sup>3–5</sup> Constitutive ErbB2 signaling causes mammary malignancies in transgenic mouse model systems<sup>6,7</sup> and causes malignant growth transformation in cell culture models of breast cancer.<sup>8,9</sup> Moreover, agents that target ErbB2 are moderately effective at controlling ErbB2-positive breast tumors when these agents are administered as part of a combination chemotherapy regimen.<sup>10</sup>

Although EGF receptor (EGFR) overexpression is observed in breast tumors,<sup>11</sup> the potential roles that EGFR signaling may have in the development or progression of breast cancer are not as well-characterized as those played by ErbB2.<sup>12</sup> Nonetheless, EGFR expression and signaling have been postulated to play roles in the genesis, maintenance, or progression of breast tumors. EGFR appears to be of particular relevance to breast cancer stem cells, triple negative tumors, and basal-type tumors. For example, breast cancer stem cells that exhibit elevated EGFR signaling display resistance to endocrine therapy<sup>13</sup> as well as uncontrolled self-renewal and unlimited proliferation.<sup>14</sup> Approximately 60% of basal-type and triple-negative tumors display elevated EGFR expression. Basal-type and triple-negative breast tumors are typically devoid of estrogen receptor (ER) and HER2 (ErbB2/Neu) expression and are therefore resistant to existing targeted therapies. Thus, EGFR is an attractive target in these types of breast tumors.<sup>15</sup> Indeed, a combination of carboplatin and the EGFR tyrosine kinase inhibitor cetuximab (Erbix) synergistically inhibits proliferation of basal-type breast cancer cell lines.<sup>16</sup>

Endogenous ligands for ErbB receptors also appear to play important roles in human breast cancers. For example, the EGFR ligand amphiregulin stimulates breast tumor cell motility<sup>17</sup> and is sufficient and necessary for tumor cell expression of parathyroid hormone-related protein (PTHrP), an important contributor to tumor cell invasion and colonization of bone.<sup>18,19</sup> Reduced expression of matrix metalloproteases (MMPs) in SCP20 human breast tumor cells is associated with decreased osteoclastic stimulation by these cells and reduced amphiregulin release by these cells. These data suggest that amphiregulin/EGFR signaling is coupled to bone colonization of breast tumor cells and can be regulated by matrix metalloproteases. Indeed, the EGFR antagonistic antibody cetuximab or the EGFR tyrosine kinase inhibitor gefitinib inhibits bone metastasis by SCP20 cells in mouse xenograft assays.<sup>20</sup> Moreover, the fact that EGFR ligands can stimulate ErbB2 signaling through heterodimerization with EGFR<sup>21</sup> suggests that EGFR and its ligands may play a significant role in at least some ErbB2-dependent breast tumors. Indeed, pharmacological agents that target EGFR or its ligands continue to be investigated as potential breast cancer therapeutics.<sup>22–24</sup>

Several environmental factors appear to contribute to breast cancer risk, including ionizing radiation, pesticides, and diet.<sup>2</sup> A number of studies have identified an association between moderate alcohol consumption and increased breast cancer risk.<sup>25–29</sup> For example, average consumption of 14 or more drinks per week in a five-year period prior to breast cancer diagnosis increased breast cancer risk by 82%.<sup>30</sup>

## Alcohol may exert its effects via several mechanisms

There appear to be multiple mechanisms by which alcohol may contribute to breast malignancies or may modulate the behavior of mammary epithelial and tumor cells *in vivo* and *in vitro*. Alcohol is metabolized in a variety of tissues, including the breast. Alcohol dehydrogenase converts ethanol to acetaldehyde, which can form adducts of DNA and can cause DNA cross-links and G:A transitions at A:T base pairs. Indeed, acetaldehyde displays weak mutagenic and carcinogenic activity.<sup>29,31</sup> Xanthine oxidoreductase and aldehyde oxidase are found in breast tissue and metabolize acetaldehyde to reactive oxygen species (ROS),

including the superoxide anion free radical ( $O_2^{\bullet-}$ ), the neutral hydroxide free radical ( $OH^{\bullet}$ ), and hydrogen peroxide ( $H_2O_2$ ). These ROS can contribute to breast malignancies via a variety of mechanisms, including DNA mutation, base deletion, and single and double strand breaks.<sup>29,31</sup>

Additional mechanisms may underlie the contributions of ethanol to breast malignancies. Alcohol at a concentration as low as 0.06% stimulates the expression of estrogen receptor alpha ( $ER\alpha$ ) and the estradiol biosynthesis enzyme aromatase in human breast cancer cell lines.<sup>32,33</sup> In fact, moderate alcohol consumption of 0.7 g/kg is associated with an elevated estrogen concentration in the plasma of postmenopausal women.<sup>34,35</sup> In cell line model systems alcohol concentrations of 0.06% to 0.6% stimulate transcription from the estrogen response element<sup>36</sup> and increased transcription of the progesterone receptor and pS2, two genes whose transcription is regulated by ER.<sup>37</sup> Alcohol stimulation of ER-dependent gene expression may account for the observation that alcohol stimulates proliferation in ER-positive MCF7 human breast tumor cells but has no effect on the proliferation of ER-negative MDA-MB-231 and BT-20 human breast tumor cell lines.<sup>33</sup> However, it has yet to be demonstrated that changes in ER-dependent gene expression underlie the effects of alcohol on the malignant phenotypes of breast tumor cells.

Alcohol induces other intracellular signaling events. Increased expression of the c-Fos transcription factor is observed in NIH 3T3 cells one hour after treatment with 0.2% alcohol. This increase persists up to 96 hours after treatment with alcohol.<sup>38</sup> Likewise, alcohol concentrations of 1.3% to 5.1% stimulate phosphorylation of the c-Jun NH2-terminal protein kinase (JNK), the p38 mitogen-activated protein kinase (p38 MAPK), and phosphatidylinositol 3-kinase (PI3K).<sup>39,40</sup> Finally, alcohol concentrations as low as 0.12% inhibit expression of the adhesion molecule E-cadherin and cause an increase in cell migration.<sup>41</sup> E-cadherin is a tumor suppressor and loss of E-cadherin expression contributes to tumor progression by enhancing metastatic phenotypes.<sup>42</sup>

Metalloproteases are zinc-dependent endopeptidases. These enzymes are expressed in an inactive, secreted, or transmembrane precursor form. The interaction of a zinc ion with three histidine residues and a cysteine residue (cysteine “switch” residue) hold the metalloprotease in an inactive conformation. Metalloproteases can be activated through cleavage by convertases such as furin. ROS can activate metalloproteases by oxidizing the cysteine switch residue, thereby disrupting its interaction with the zinc ion and allowing the enzyme to adopt an active conformation.<sup>43</sup> Active tumor cell metalloproteases degrade components of the extracellular matrix, such as gelatin and collagens, thereby contributing to tumor cell invasiveness and metastasis.<sup>44,45</sup> In breast cancer cell lines, alcohol concentrations of 1.3% to 5.1% stimulate the expression and secretion of MMPs 2 and 9.<sup>39,46,47</sup> Thus, it is not surprising that alcohol stimulates invasiveness and anchorage-independent proliferation of MCF7 human mammary tumor cells.<sup>32,41</sup> Moreover, small interfering RNAs (siRNAs) specific for MMP2 or small molecule inhibitors of MMP2 reduce the effect of alcohol on anchorage-independent proliferation.<sup>39</sup>

Alcohol appears to stimulate metalloprotease activity via a variety of mechanisms. As discussed earlier, alcohol is metabolized to ROS,<sup>48–50</sup> which then can stimulate metalloprotease activity.<sup>51,52</sup> Thus, it is reasonable to postulate that ROS and their regulation of metalloproteases mediate at least some of the effects of alcohol on the malignant phenotypes of breast tumor cells. Indeed, ROS scavengers inhibit alcohol stimulation of metalloprotease activity and alcohol stimulation of tumor cell invasiveness.<sup>39</sup>

## Alcohol may stimulate tumor cell aggressiveness through increased EGFR signaling

Experiments in fruit flies indicate that increased signaling of EGFR through Erk, but not through p38 or JNK, inhibits the sedative effects of alcohol. Moreover, the sedative effects of alcohol exposure in fruit flies or alcohol consumption by rats appear more rapidly in animals treated with the EGFR tyrosine kinase inhibitor erlotinib. These data suggest that EGFR signaling modulates pathways that couple alcohol to its behavioral effects.<sup>53</sup> In subsequent paragraphs we will discuss numerous other observations that support the hypothesis that EGFR signaling couples alcohol to biological responses, particularly malignant phenotypes of breast tumor cells.

Most EGF family peptide growth factors are expressed as transmembrane precursors. Active metalloproteases cleave these precursors, releasing a mature, soluble form of the growth factor that binds ErbB receptors and stimulates their signaling.<sup>43</sup> Agonists for numerous serpentine G protein-coupled receptors (GPCRs) regulate the cleavage of EGF family precursors by metalloproteases, thereby enabling GPCR agonists to stimulate ErbB receptor signaling and coupling to biological responses.<sup>51,54</sup> For example, MMP9 and MMP2 are required for agonists of the G<sub>q</sub>-coupled gonadotropin receptor to stimulate EGFR tyrosine phosphorylation and induction of the downstream transcription factors c-Fos and c-Jun.<sup>51,55</sup> Similarly, the GPCR agonists lysophosphatidic acid (LPA) and carbachol stimulate cleavage and maturation of the precursor form of amphiregulin (AR) by the metalloprotease ADAM17, resulting in enhanced cell migration and increased DNA synthesis.<sup>17,56</sup> Phenylphrine stimulates cleavage of the precursor form of heparin-binding EGF-like growth factor (HB-EGF) by MMP7, leading to arterial vasoconstriction.<sup>51,57</sup> GPCR transactivation of EGFR signaling also stimulates the activity of the c-Jun NH2-terminal protein kinase (JNK), the p38 mitogen-activated protein kinase (p38 MAPK), and phosphatidylinositol 3-kinase (PI3K).<sup>51</sup> It should be reiterated that the activity of JNK, MAPK, PI3K, c-fos, and c-jun increases upon treatment with alcohol,<sup>39, 40,51,55</sup> suggesting that alcohol may activate signaling pathways that are also activated by G<sub>αq</sub>-coupled or G<sub>αi</sub>-coupled GPCRs and that may include EGFR and its effectors. Indeed, angiotensin (AT) stimulation of the G<sub>αq</sub>-coupled AT receptor I leads to ROS production, which in turn stimulates ADAM17-dependent cleavage of HB-EGF.<sup>51</sup> Recall that alcohol is metabolized to ROS. Consequently, we postulate that alcohol metabolism to ROS and subsequent stimulation of metalloprotease activity by ROS could result in increased processing of EGF family precursor proteins and increased EGFR signaling (Figure 1). Indeed, preliminary data from our laboratory indicates that 1% alcohol stimulates MCF7 human breast tumor cells to release AR into the culture medium.

Alcohol concentrations of 0.1% to 0.3% stimulate the transcription of genes whose promoter contains a cyclic AMP (cAMP) response element (CRE).<sup>37</sup> Indeed, alcohol stimulates the activity of some adenylate cyclases (ACs), apparently by stabilizing the enzyme in the active conformation.<sup>58,59</sup> This leads to increased cAMP production, protein kinase A (PKA) activity, phosphorylation of the CRE binding protein (CREB) by PKA, and increased transactivation of CRE promoters by phosphorylated CREB. Because the amphiregulin promoter contains a CRE,<sup>17</sup> it is reasonable to speculate that alcohol may stimulate EGFR signaling through a cAMP-dependent increase in amphiregulin transcription (Figure 2). Indeed, vasoactive intestinal peptide (VIP), which stimulates cAMP production through a G<sub>αs</sub>-coupled GPCR, also stimulates EGFR tyrosine phosphorylation in colonic epithelial cells.<sup>60</sup>

As discussed earlier, alcohol stimulates transcriptional activation by the estrogen receptor. The details of the mechanism by which this occurs remain unclear. Nonetheless, alcohol stimulates ER $\alpha$  and aromatase expression<sup>32,33</sup> and estrogen stimulates ER-dependent TGF $\alpha$  and amphiregulin gene expression.<sup>61–63</sup> Thus, alcohol may stimulate biological responses in breast

tumor cells through ER $\alpha$  stimulation of EGFR ligand expression and EGFR signaling. This hypothesis is consistent with our preliminary observation that 1% alcohol stimulates MCF7 human breast tumor cells to express AR and release it into the culture medium.

Estrogen can also rapidly modulate cellular signaling pathways in the cytosol and at the membrane in the absence of any direct effect of ER on gene expression.<sup>64,65</sup> Some of these nongenomic responses are mediated by a GPCR that stimulates G $_{\alpha i}$ , resulting in activation of matrix metalloproteases and cleavage of the proform of an EGF family growth factor (Figure 1).<sup>64</sup> Other effects appear to be mediated by G $_{\alpha s}$ , resulting in elevated PKA activity and cAMP accumulation (Figure 2).<sup>65</sup> As discussed earlier, this could result in elevated amphiregulin expression via the CRE present in the amphiregulin promoter.

EGFR signaling can modulate ER-dependent signaling.<sup>64</sup> EGFR can couple to the PI3K/Akt pathway, leading to IKK phosphorylation of the estrogen receptor and modulation of ER-dependent gene expression.<sup>66</sup> EGFR signaling can also couple to increased Erk and Jnk activity, resulting in the phosphorylation of various transcription factors, including CREB-binding protein (CBP). Phosphorylated CBP can dimerize with estrogen receptor, leading to ER-dependent gene transcription.<sup>64</sup> Thus, EGFR signaling may lie upstream or downstream of ER in alcohol-induced signaling pathways.

As discussed earlier, alcohol inhibits expression of the cell adhesion tumor suppressor protein E-cadherin, leading to an increase in cell migration.<sup>41</sup> EGFR signaling is coupled to inactivation of the E-cadherin/ $\beta$ -catenin complex in tumor cell lines, thereby contributing to metastatic phenotypes.<sup>67–70</sup> In fact, treatment of lung cancer cell lines with EGFR monoclonal antibodies results in increased E-cadherin expression.<sup>71</sup> Thus, increased EGFR signaling may be responsible for the decrease in E-cadherin expression observed following alcohol treatment.

A hallmark of EGFR signaling is phosphorylation of EGFR on cytoplasmic tyrosine residues. This phosphorylation creates docking sites for effector binding and coupling. Thus, dephosphorylation of these tyrosine residues by phosphatases negatively regulates EGFR coupling to effector proteins and biological responses.<sup>72–74</sup> There is emerging evidence suggesting that alcohol enhances EGFR signaling by inactivating phosphatases that catalyze EGFR dephosphorylation (Figure 3). Recall that alcohol is metabolized to ROS in breast tissue. These ROS can oxidize a conserved, essential cysteine residue in the catalytic domain of phosphatases, thereby disrupting the catalytic activity of the phosphatases.<sup>49,75,76</sup> Therefore, it is plausible to postulate that alcohol may stimulate increased EGFR signaling through ROS-mediated inactivation of the phosphatases that dephosphorylate EGFR tyrosine residues. Indeed, it has been suggested that ultraviolet light and other cellular stresses that contribute to the production of intracellular ROS cause increased EGFR signaling via this mechanism.<sup>77</sup>

Agonist binding to EGFR can cause EGFR heterodimerization with ErbB2, leading to phosphorylation of both receptor molecules and coupling of both receptors to downstream signaling events. Because ErbB2 does not possess soluble agonists, this is an important mechanism by which ErbB2 signaling can be regulated.<sup>78</sup> This heterodimerization of ErbB2 with EGFR may contribute to the effect alcohol has on breast tumor cell behavior. Indeed, this model is supported by the observation that ErbB2 overexpression potentiates the effect of alcohol on invasiveness and other malignant phenotypes of breast cancer cell lines.<sup>39–41,79</sup> ErbB2 overexpression is also associated with increased transcription of MMP2 and MMP9<sup>39,47</sup> and siRNA knockdown of endogenous ErbB2 overexpression is associated with reduced MMP activity.<sup>39</sup>

Finally, amphiregulin induces EGFR coupling to increased expression of MMP2 and MMP9 in breast tumor cell lines; antisense knockdown of amphiregulin expression in the NS2T2A1 breast tumor cell line results in decreased expression of MMP2.<sup>17</sup> Thus, alcohol stimulation

of MMP expression and activity as described in this review may establish a feed forward mechanism that features increased amphiregulin stimulation of EGFR/ErbB2 signaling. This signaling is then coupled to increased expression of MMP2 and MMP9, which contributes to further increases in signaling by the amphiregulin/EGFR/ErbB2 axis and deregulated cellular proliferation, motility, and invasiveness.

Here we postulate three mechanisms by which alcohol may contribute to breast tumor genesis, progression, or aggressiveness. Additional experimentation is necessary to decipher which, if any, of these mechanisms are relevant. These experiments should proceed with an understanding that these mechanisms are not likely to be mutually exclusive. Indeed, at different stages during breast cancer progression distinct mechanisms may be relevant.

The mechanisms discussed here may have important implications in understanding breast cancer progression and potential therapies. Given that alcohol may stimulate estrogen receptor, EGFR, and ErbB2/Her2/Neu signaling, even moderate alcohol consumption may affect the outcome of breast cancer patients whose tumors express these proteins. Moreover, given the central role that ROS and estrogen may have in mediating the effects of alcohol on breast tumor genesis, progression, or aggressiveness, antioxidants and antiestrogens may hold value in blocking the negative effects of alcohol on breast cancer.

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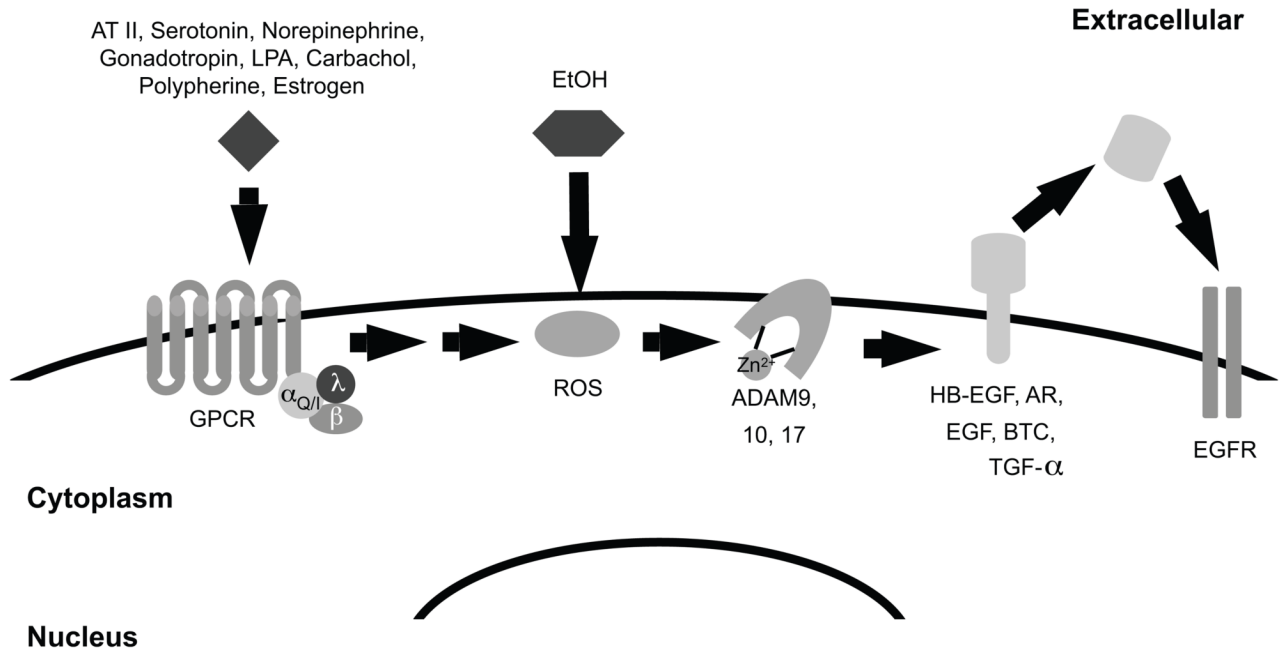
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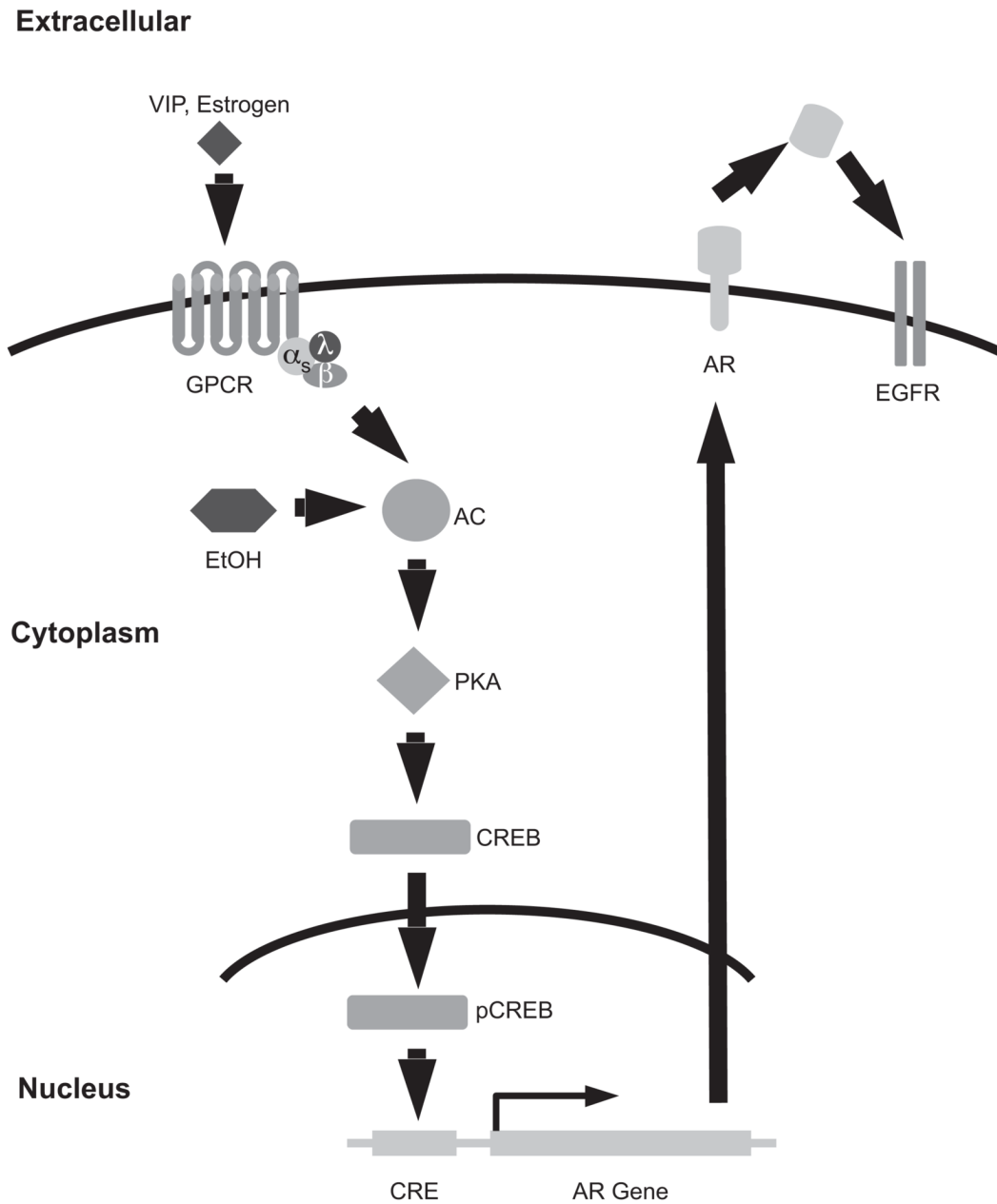
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**Figure 1.**

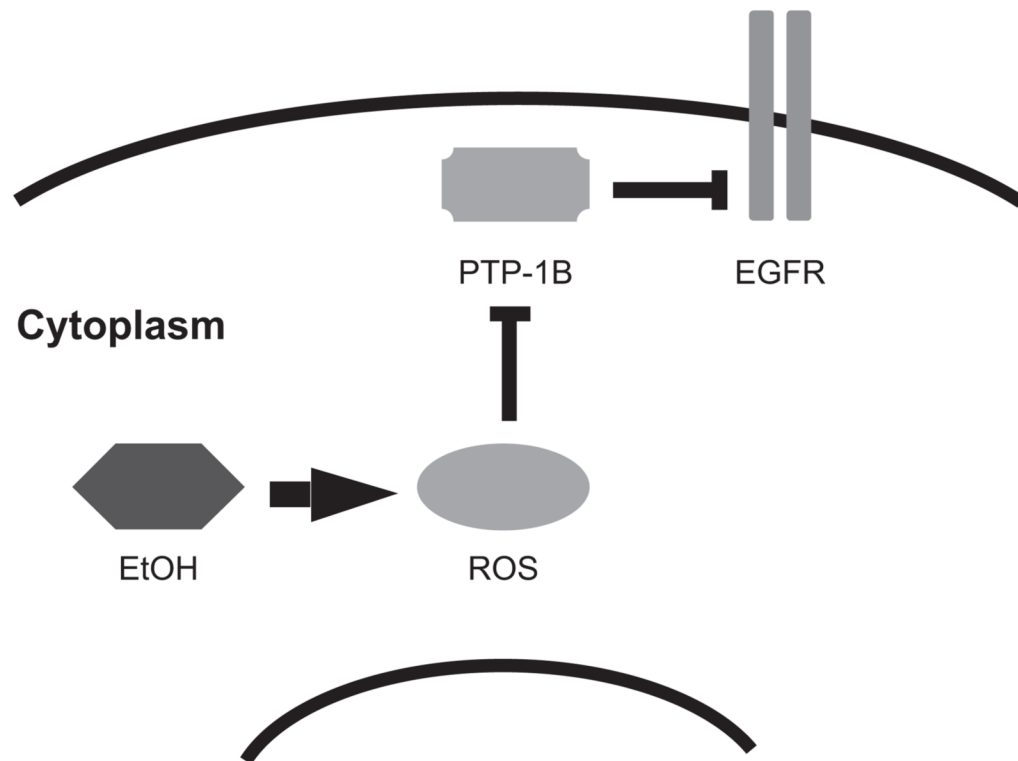
Alcohol may mimic G-protein coupled receptor transactivation of EGFR. Signaling by  $G_{q/i}$ -coupled receptors contributes to the production of reactive oxygen species (ROS). These ROS oxidize a cysteine residue in the catalytic cleft of the ADAM, thereby disrupting the interaction between the zinc ion and the ADAM protein. Disruption of this interaction causes the ADAM protein to adopt an active conformation. Active ADAM proteins can then cleave the precursor form of EGF family growth factors. The cleaved factors bind EGFR and stimulate its signaling activity. Because alcohol causes intracellular accumulation of ROS, it is predicted to stimulate EGFR signaling.

**Abbreviations:** AT II, angiotensin II; LPA, lysophosphatidic acid; EtOH, ethanol; GPCR, G-protein coupled receptor; ROS, reactive oxygen species; HB-EGF, heparin-binding, EGF-like growth factor; AR, amphiregulin; EGF, epidermal growth factor; BTC, betacellulin; TGFalpha, transforming growth factor alpha; EGFR, EGF receptor.



**Figure 2.** Alcohol may mimic G-protein coupled receptor stimulated transcription of amphiregulin, an EGF family growth factor. Signaling by G<sub>s</sub>-coupled receptors stimulates adenylate cyclase (AC) production of cAMP. This increases phosphorylation of the transcription factor CREB (cAMP response element binding protein) by protein kinase A (PKA). CREB promotes the transcription of the EGF family growth factor amphiregulin (AR), resulting in increasing EGFR signaling. Alcohol stabilizes AC in the active conformation, resulting in increased PKA activity. Thus, alcohol is predicted to stimulate AR transcription and EGFR signaling. **Abbreviations:** VIP, vasoactive intestinal peptide; GPCR, G-protein coupled receptor; EtOH, ethanol; AC, adenylate cyclase; PKA, protein kinase A; CRE, cyclic AMP response element; CREB, CRE-binding protein; pCREB, phosphorylated CREB; AR, amphiregulin; EGFR, EGF receptor.

## Extracellular



## Nucleus

### Figure 3.

Alcohol may inhibit phosphatases that negatively regulate EGFR. The protein tyrosine phosphatase PTP-1B inhibits EGFR signaling by catalyzing the dephosphorylation of EGFR tyrosine residues. ROS can oxidize a catalytic cysteine residue of protein phosphatases, thereby inactivating them and leading to increased EGFR signaling. Because alcohol causes intracellular accumulation of ROS, it is predicted to stimulate EGFR signaling.

**Abbreviations:** EtOH, ethanol; ROS, reactive oxygen species; PTP-1B, protein tyrosine phosphatase 1B; EGFR, EGF receptor.