Ethanol Induces Enhanced Vascularization Bioactivity of Endothelial Cell-Derived Extracellular Vesicles via Regulation of MicroRNAs and Long Non-Coding RNAs

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Extracellular vesicles (EVs), such as exosomes, have been identified as regulators of vascular remodeling and have promise as therapeutics for vascularization applications. Towards development of EVs as therapeutics, it has been demonstrated that physiological stimuli of angiogenic phenotypes in EV-producing cells can enhance the potency of EVs for vascularization. The goal of this study was to assess whether ethanol, which induces angiogenic phenotypes in endothelial cells, could be employed to enhance endothelial-derived EV vascularization bioactivity. The results indicate that ethanol conditioning of endothelial cells increases the ability of endothelial EVs to induce a pro-vascularization response. This response is due in part to increased CD34 expression in recipient endothelial cells that may result from downregulation of microRNA-106b in EVs isolated from ethanol-conditioned producer endothelial cells. Further, ethanol-induced upregulation of long non-coding RNAs (lncRNAs) HOTAIR and MALAT1 in endothelial EVs was observed to play a significant role in mediating pro-angiogenic effects of these vesicles. Overall, these studies validate ethanol conditioning as a method to enhance the bioactivity of endothelial EVs via regulation of EV-associated microRNAs (miRNAs) and, especially, lncRNAs. Further, the results suggest that alcohol consumption may activate endothelial EVs towards a pro-vascularization phenotype, which could have implications for alcohol-induced tumor angiogenesis.
mediating vascularization bioactivity of EVs\textsuperscript{14,16,17}. Methods to enhance the potency of EVs have been developed, including exogenous loading approaches\textsuperscript{20–26} and cell conditioning via exposure to hypoxia or growth factor stimulation\textsuperscript{17,27,28}. However, these approaches may not be easily adaptable to large-scale biomanufacturing of therapeutic EVs for vascularization applications, thus limiting translational potential.

One substance that may be straightforwardly incorporated into scalable EV production that also induces a pro-vascularization phenotype in endothelial cells is ethanol\textsuperscript{29–31}. Ethanol is already part of large-scale biotechnology production schemes and is relatively cheap and readily available compared to purified growth factors. Ethanol has been shown to induce angiogenic endothelial phenotypes via a variety of pathways\textsuperscript{32–34} and has also been shown to influence the bioactivity and cargo of EVs in other cellular systems\textsuperscript{35,36}. However, there are scarce reports of how ethanol effects on endothelial cells impact bioactivity of EVs derived from these cells.

We hypothesized that ethanol conditioning might increase the vascularization bioactivity of endothelial cell-derived EVs. In these studies, we sought to determine how cellular changes in endothelial cells induced by ethanol are manifest in EVs and to identify specific mechanisms of ethanol-induced regulation of endothelial cell EV activity. We report that ethanol increases the vascularization bioactivity of endothelial cell EVs through at least two distinct mechanisms: downregulation of anti-angiogenic miRNA cargo (miR-106b) and upregulation of pro-angiogenic long non-coding RNA (lncRNA) cargo (MALAT1 and HOTAIR). These findings have implications for generation of EVs for therapeutic vascularization applications and also may shed light on the role of EVs in alcohol-induced angiogenesis in cancer and other physiological settings.

Results

Ethanol stimulates EV production by endothelial cells. As a first step in evaluating the potential of ethanol conditioning as a means to enhance vascularization bioactivity of endothelial cell-derived EVs, the effects of ethanol on EV production were investigated. Concentrations of ethanol beyond 100 mM were found to induce significant cell toxicity in human umbilical vein endothelial cells (HUVECs) (Fig. 1A), thus 100 mM was used as a maximum ethanol level in most experiments. The inclusion of ethanol in the culture medium did not appear to affect the structural integrity of produced EVs, as mean diameters (Fig. 1B) and protein expression levels (Fig. 1C,D) were found to be similar over the range between 0-200 mM ethanol for both HUVEC and human dermal microvascular endothelial cell (HDMEC) EVs (representative blots shown in Supplementary Fig. S1). Notably, up to \textasciitilde 2–3 fold increased EV production by endothelial cells was observed at higher ethanol concentrations (Fig. 1E,F).

Ethanol conditioning increases endothelial cell-derived EV vascularization bioactivity. Following from the original hypothesis and given that structurally intact EVs were produced by endothelial cells in the presence of ethanol, EV vascularization bioactivity was directly assessed \textit{in vitro} using an endothelial cell gap closure assay and \textit{in vivo} using a Matrigel plug injection mouse model. EVs isolated from both HUVECs (Fig. 2A) and HDMECs (Fig. 2B) showed increased stimulation of endothelial gap closure based on the ethanol concentration used to condition the EV-producing cells. Additionally, ethanol conditioning (100 mM) endowed endothelial cell-derived EVs with increased ability to recruit host CD31+ cells into injected Matrigel plugs in C57Bl/6 mice (Fig. 2C,D). Together, these data demonstrate increased vascularization bioactivity of EVs derived from endothelial cells cultured in the presence of ethanol compared to those isolated from endothelial cells without ethanol conditioning.

To determine the specific mechanism of the enhanced vascularization bioactivity of endothelial cell-derived EVs induced by ethanol conditioning of producer endothelial cells, we first profiled gene expression in recipient endothelial cells upon stimulation with EVs isolated from producer endothelial cells cultured in the presence or absence of 100 mM ethanol. Among 92 genes examined that are associated with angiogenic activity, a majority (68 of 92) were upregulated upon stimulation by EVs from ethanol-conditioned HUVECs compared...
to unconditioned HUVEC-derived EVs (Fig. 3A, Supplementary Table S1). The mRNA of CD34, a sialomucin protein associated with endothelial cell migration and angiogenesis, was the most significantly differentially regulated (upregulated ~5 fold). This finding was confirmed independently by qPCR using different primers in both HUVECs and HDMECs (Fig. 3B).

**Ethanol conditioning regulates endothelial cell-derived EV miRNA content.** To determine the specific cause of the measured CD34 upregulation, EV-associated protein signaling and miRNA-mediated
ies revealed significant differences in miRNA content between the EV groups (Fig. 5B, Supplementary Table S3),
tions isolated from HUVECs cultured in the presence or absence of 100 mM ethanol was conducted. These stud-
ing in reduced CD34 degradation.
in luciferase activity (Fig. 5A), suggesting that ethanol conditioning of HUVECs alters EV miRNA content result -
such as miR-57338, miR-13739, miR-320e40, and others. Further investigation of miRNAs that have been validated
sequence (Fig. 5D). Thus, specific downregulation of miR-106b partially recapitulated a significant phenotypic
effect of ethanol conditioning on endothelial cell-derived EVs. Overall, these data point to regulation of miR-
expressed in HUVEC-derived EVs (Supplementary Fig. S2), recapitulating a specific effect of ethanol condition-
ing. Assessment of the vascularization bioactivity of these EVs, derived from non-ethanol-conditioned HUVECs
compared to control non-conditioned HUVEC EVs as well as HUVEC EVs from cells transfected with a scrambled antagomir
- untranslated region (UTR) of CD34 was cloned into a luciferase vector that was subsequently
transfected into HUVECs. EVs from HUVECs cultured in the presence or absence of 100 mM ethanol were then applied to recipient HUVECs, and detection of miRNA-specific targeting of CD34 for downregulation was performed based on quantification of luciferase expression. The results of this assay indicated that EVs from non-conditioned producer HUVECs contain miRNAs that specifically target CD34 for downregulation in recipient cells, as these EVs induced a 16.7+/−3.3% reduction in luciferase activity compared to control (Fig. 5A). However, EVs from HUVECs cultured in the presence of 100 mM ethanol induced only a 2.3+/−2.5% reduction in luciferase activity (Fig. 5A), suggesting that ethanol conditioning of HUVECs alters EV miRNA content resulting in reduced CD34 degradation.

Towards identifying specific miRNA participants in this effect, analysis of the whole miRNomes of EV populations isolated from HUVECs cultured in the presence or absence of 100 mM ethanol was conducted. These studies revealed significant differences in miRNA content between the EV groups (Fig. 5B, Supplementary Table S3), including substantial downregulation of several miRNAs that are reported to negatively regulate angiogenesis, such as miR-57338, miR-13739, miR-320e29, and others. Further investigation of miRNAs that have been validated to target CD34 for downregulation, including miR-106b41, miR-125a42 and miR-942, showed that miR-106b was downregulated >5 fold in EVs from both HUVECs and HDMECs cultured in the presence of 100 mM ethanol compared to cells cultured without ethanol (Fig. 5C). To confirm the specific role of miR-106b, HUVECs (in the absence of ethanol) were transfected with an antagomir that was validated to significantly decrease miR-106b expression in HUVEC-derived EVs (Supplementary Fig. S2), recapitulating a specific effect of ethanol conditioning. Assessment of the vascularization bioactivity of these EVs, derived from non-ethanol-conditioned HUVECs but with diminished levels of miR-106b, showed increased ability to induce endothelial gap closure compared to control non-conditioned HUVEC EVs as well as HUVEC EVs from cells transfected with a scrambled antagomir sequence (Fig. 5D). Thus, specific downregulation of miR-106b partially recapitulated a significant phenotypic effect of ethanol conditioning on endothelial cell-derived EVs. Overall, these data point to regulation of miR-106b as a crucial component of the mechanism of enhancement of endothelial cell-derived EV vascularization bioactivity by ethanol conditioning.

**Ethanol conditioning regulates endothelial cell-derived EV long non-coding RNA content.** The ethanol-induced downregulation of miRNAs that negatively regulate angiogenesis is not necessarily sufficient to induce pro-vascularization bioactivity in endothelial cell EVs. To assess additional possible molecular players, long non-coding RNA (lncRNA) content in EVs was investigated, as lncRNAs have been reported as EV cargo components43. Specifically, lncRNAs known to induce angiogenesis via regulation of endothelial cell function, including HOTAIR44, MALAT145, and TUG145,46 were assessed by qPCR. Both HOTAIR and MALAT1 were significantly upregulated in EVs from HUVECs cultured in the presence of 100 mM ethanol (Fig. 6A). To assess specific roles of these lncRNAs, an approach similar to that described for miR-106b was used. In this case, HUVECs were transfected with siRNA specific to either HOTAIR (Fig. 6B), MALAT1 (Fig. 6C), both HOTAIR

![Figure 4](image-url) Endothelial cell receptor tyrosine kinase stimulation by EVs. (A) Phosphorylated RTK arrays were incubated for 30 min with lysates from recipient HUVECs stimulated by 100 µg/ml EVs from producer HUVECs cultured in the presence or absence of 100 mM ethanol (EtOH) for 24 h. Ovals indicate phospho-epidermal growth factor receptor (pEGFR) and rectangles indicate phosphor-insulin receptor (pIR). Blots shown are representative of three independent experiments. (B) The same conditions were used to generate separate immunoblots for pEGFR and pIR using different antibodies (n = 3; H1975 cells were used as a positive control for pEGFR (+)).
and MALAT1 (double transfection, Fig. 6D) or a scrambled siRNA sequence. The depletion of HOTAIR and MALAT1 from cellular RNA and EV RNA was confirmed by q-PCR (Supplementary Figs S3 and S4). The cells were then cultured in the presence or absence of 100 mM ethanol and assessment of the vascularization bioactivity of the EVs isolated from these groups of cells was conducted using endothelial gap closure assays. These experiments revealed that downregulating MALAT1 expression alone and MALAT1 and HOTAIR expression together significantly abrogated the increase in vascularization bioactivity in HUVEC EVs associated with
ethanol conditioning (Fig. 6C,D). Overall, these data indicate that IncRNA regulation is a crucial aspect of ethanol-induced effects on endothelial cell EV bioactivity.

Discussion

This study establishes, for the first time, that ethanol induces enhanced vascularization bioactivity in endothelial cell-derived EVs. Regulation of both EV-associated miRNAs and IncRNAs by ethanol conditioning of producer cells contributes to this effect, which has potential ramifications for therapeutic EV biomanufacturing. These data also suggest that EVs may play an important role in the mechanism of alcohol-induced angiogenesis in cancer.

The finding that miRNAs were regulated in this study is not surprising, as miRNA content has been consistently linked to EV vascularization bioactivity14,16,17 and ethanol exposure has been specifically linked to regulation of EV miRNAs35,36. However, the upregulation of CD34 expression in recipient endothelial cells resulting from ethanol-induced regulation of endothelial cell EV miRNA cargo is interesting and suggests that ethanol exposure may induce transformation of quiescent endothelial cells into angiogenic tip cells via EVs37. Also unexpected was the finding that ethanol conditioning impacts endothelial cell EV bioactivity via regulation of IncRNA cargo, specifically HOTAIR and MALAT1. IncRNAs are reported to constitute a much smaller proportion of total nucleic acid cargo of EVs when compared to miRNAs43. Yet, our results establish that these molecules may play significant roles in defining EV vascularization bioactivity, supporting other studies that have identified bioactive IncRNAs in EVs44,45. Ethanol-induced regulation of pro-angiogenic IncRNA is not unprecedented; MALAT1 has been found to be upregulated in brains of human alcoholics49. However, to our knowledge, this is the first report connecting EV-associated MALAT1 with angiogenic bioactivity. Thus, more extensive study of the effects of ethanol on IncRNA expression in endothelial cells and their EVs may be of interest. Further, therapeutic loading of IncRNAs such as MALAT1 into EVs may be a promising strategy for enhancing vascularization bioactivity.

Finally, as previously stated, the finding that ethanol increases the angiogenic bioactivity of endothelial cell EVs has important implications in cancer. Alcohol consumption has been identified as a risk factor for several cancers, with the links between alcohol and breast and liver cancers being particularly strong. For example, epidemiological studies have consistently shown that breast cancer risk increases with alcohol intake50–52. Among many potential mechanisms, alcohol-induced tumor angiogenesis has been identified as a possible driver of breast cancer progression33,34, with several signaling pathways in endothelial cells being implicated53,54. While activation of these pathways is typically associated with proteins, this study suggests that alcohol exposure to endothelial cells could induce production of pro-angiogenic EVs, which could potentially promote progression of latent tumors (Fig. 7). This possibility is further supported by reports that EVs have been shown to stimulate tumor angiogenesis55,56,57. Thus, the results of this study support further exploration of the potential mechanistic role of endothelial cell-derived EV regulation in alcohol-induced cancer progression.

In summary, this study demonstrates that the pro-angiogenic effects of ethanol on endothelial cells increase the vascularization bioactivity of EVs derived from these cells. This is accomplished in part by regulation of both miRNA and IncRNA components of EVs, with miR-106b downregulation and MALAT1 upregulation having significant effects. These findings could inform future mechanistic studies of the role of EVs in alcohol-induced cancer progression and may also identify new therapeutic cargo or quality control parameters for large-scale biomanufacturing of EVs for therapeutic vascularization applications.

Methods

Cell culture. HUVEC and HDMEC were obtained from Promocell and were generally cultured in EGM2 medium (with growth factors) (Lonza). In gap closure experiments, EBM2 media (without growth factors)

Figure 7. Schematic of potential mechanism of alcohol effects on endothelial cell (EC) EVs. EtOH = ethanol, miRNA = microRNA, IncRNA = long non-coding RNA.
in complete EGM2, a scratch was made in each of the wells using the tip of a 200 μl micropipette. Medium was aspirated and the cells were then washed first with 300 μl of 1X PBS and then with 300 μl of EB2 medium. As positive and negative controls, EGM2 (complete medium) and EB2 (basal medium) were used. Cells were treated with EVs to make the final concentration of EVs 100 μg/ml based on EV protein quantification. Images were taken of the scratches at 0, 9 and 15 h, and the denuded areas were quantified using ImageJ software. Data reported are for the 15 h time point.

To assess bioactivity in vivo, a Matrigel plug assay was performed as described previously. Briefly, 0.4 mL Matrigel (Corning #356231) with or without 100 μg of EVs (in 100 μl PBS) from HUVECs cultured in the presence or absence of 100 mM ethanol was injected subcutaneously into the ventral area of C57Bl/6 mice. After 10 days, mice were euthanized and the Matrigel plugs were excised and fixed in 10% phosphate-buffered formalin. Vascularization bioactivity.

To assess vascularization bioactivity in vitro, an endothelial gap closure assay was used. 30,000 HUVECs were plated in the wells of gelatin-coated 48-well plates. After 24 hours of incubation in complete EGM2, a scratch was made in each of the wells using the tip of a 200 μl micropipette. Medium was aspirated and the cells were then washed first with 300 μl of 1X PBS and then with 300 μl of EB2 medium. As positive and negative controls, EGM2 (complete medium) and EB2 (basal medium) were used. Cells were treated with EVs to make the final concentration of EVs 100 μg/ml based on EV protein quantification. Images were taken of the scratches at 0, 9 and 15 h, and the denuded areas were quantified using ImageJ software. Data reported are for the 15 h time point.

Immunohistochemical analysis. Sections were affixed to glass slides and deparaffinized in xylene, dehydrated in graded alcohol, and finally hydrated in water. Antigen retrieval was performed by boiling the slides in TE buffer (pH 9.0) for 30 min. CD31 primary antibody (Abcam # ab8364) and goat anti-rabbit IgG H&L secondary antibody (Abcam # ab6719) were used in 1:100 and 1:200 dilutions respectively. After incubation with the primary antibodies at 4 °C overnight, the slides were incubated with peroxidase-conjugated anti-rabbit IgG and stained with diaminobenzidine (DAB) chromogen solution (SK-4105) (both from Vector Laboratories), and the primary antibodies at 4 °C overnight, the slides were incubated with peroxidase-conjugated anti-rabbit IgG and stained with diaminobenzidine (DAB) chromogen solution (SK-4105) (both from Vector Laboratories), and then counterstained with hematoxylin. Images were taken with an Olympus BX51 microscope. The percentage of cells with CD31+ was determined by counting the total number of cells and CD31+ cells from each gel section.

RNA profiling and analysis. Human miRNome miScript miRNA arrays (V16.0, 384-well, SA Biosciences, # MIHS-3216ZE) were used to identify the level of miRNAs in EVs from HUVECs cultured in the presence or absence of 100 mM ethanol. EV RNA was isolated by using miRNeasy mini kits (Qiagen #217004) and cDNA was prepared using miScript II RT kits (Qiagen #21860). As recommended in the protocol, ~350 ng of RNA sample was used to make cDNA for one 384-well plate. The miScript miRNA PCR array reaction volume was kept to 10 μl per well for 384-well plate. PCR was performed using an Applied Biosystems Real Time PCR instrument (ABI 7900 Fast HT) according to manufacturer's instructions. Finally, data were analyzed using SA Biosciences software: (http://sabiosciences.com/mirnaArrayDataAnalysis.php).

Probing of EVs associated with angiogenesis was carried out in recipient HUVECs exposed to EVs isolated from producer HUVECs cultured in the presence or absence of 100 mM ethanol for 24 h. Quantitative PCR using an ABI 7900 Fast HT machine and employing SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used to detect RNA levels in the context of an Angiogenesis PCR array kit (Bio-Rad H384), with settings as recommended for the Supermix reagent. Data were analyzed using the ΔΔCt method. β-actin mRNA and TUG1 IncRNA were selected as controls that were not expected to be affected in control vs. ethanol samples. The sequences of qPCR primers used for this study are available by request.

miRNA activity assay. To evaluate miRNA targeting of CD34, a pMirTarget reporter plasmid (Origene, catalog SC213155) containing the coding sequence for the 3'-UTR of CD34 downstream of firefly luciferase was used. The plasmid was transfected into HUVECs that were plated in 12-well plates 24 h prior to transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After an additional 24 h period, transfected HUVECs were exposed to control solution (PBS) or EVs (100 μg/ml final concentration) from producer HUVECs cultured in the presence of absence of 100 mM ethanol. Cells were harvested after 48 h and lysate was prepared using Luc-Pair™.
Duo-Luciferase Assay Kit 2.0 (Genecopoeia#LPFR-P010) according to the manufacturer’s instructions and normalized luciferase levels were determined using a SpectraMax Gemini plate reader.

**Protein detection.** Receptor tyrosine kinase (RTK) phosphorylation was investigated using Proteome Profiler Human Phospho-RTK Arrays (R&D Systems #ARY001B). HUVECs were incubated with 100 μg/ml EVs isolated from HUVECs cultured in the presence or absence of 100 mM ethanol for 30 min and a total protein lysate was prepared and applied to antibody pre-coated membranes according to the manufacturer’s instructions. The expression levels of pIR and pEGFR were assessed by standard immunoblotting using the following antibodies: pIR (Tyr 1162/1163) (Santa Cruz #sc-25103 P); pEGFR (Tyr1068) (Cell Signaling #2234 S).

**Transfections.** Transfection of HUVECs was performed when cells reached ~50% confluence using HiPerFect reagent (Qiagen #301704). The antagonist of miR-106b-5p (5’-AUC UGC ACU GUC AGC ACU UUA-3’) is a single RNA sequence exactly complementary to miR-106b-5p sequence and was purchased from Integrated DNA Technologies. The concentration of the miR-106b-5p antagonist and negative control was 10 nM. HUVECs mixed with only HiPerFect (mock transfected) and HUVECs transfected with scrambled RNA with HiPerFect were used as controls. After 48 h, cells were harvested and total RNA was prepared to quantiﬁy miR-106b-5p level. To measure miR106b-5p levels in EVs, media was collected after 48 h of transfection from T75 flask. The level of miR106b-5p was measured by q-PCR by using primers specific for miR106b-5p. After conformation of miR106b-5p knockdown in both cells and EVs, a large-scale cell culture was made in T75 flasks and antagonist or scrambled oligo transfections were performed. A similar approach was employed to knockdown HOTAIR and MALAT1 IncRNAs in HUVECs. TriFECTa RNAi kits against both IncRNAs were purchased from Integrated DNA Technologies (IDT). A pool of three siRNAs or control siRNA was transfected by HiPerFect reagent and the final concentrations of transfected RNA oligos were 50 nM. The knockdown of HOTAIR or MALAT1 IncRNAs from cells and EVs was confirmed by q-PCR using primers specific for each IncRNA.

**Statistical analysis.** Parametric statistical tests (one-way analysis of variance (ANOVA) with Bonferroni post-hoc test, 2-sample t-test) were used as appropriate and statistical signiﬁcance level is indicated for each ﬁgure where it was calculated. Data were plotted as mean ± /− standard deviation.

**Data availability.** All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions
T.N.L. and S.M.J. conceived and designed the experiments, analyzed data, and wrote the manuscript. T.N.L., C.A.L. and L.Y.D. performed experiments. All authors reviewed the manuscript.

Additional Information
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