Accepted Manuscript

Title: Ethanol exposure leads to disorder of blood island formation in early chick embryo

Authors: Guang Wang, Bin-zhen Chen, Chao-jie Wang, Jing Zhang, Lin-rui Gao, Manli Chuai, Yong-ping Bao, Xuesong Yang

PII: S0890-6238(17)30210-1
DOI: http://dx.doi.org/doi:10.1016/j.reprotox.2017.08.003
Reference: RTX 7558

To appear in: *Reproductive Toxicology*

Received date: 21-4-2017
Revised date: 10-7-2017
Accepted date: 3-8-2017

Please cite this article as: Wang Guang, Chen Bin-zhen, Wang Chao-jie, Zhang Jing, Gao Lin-rui, Chuai Manli, Bao Yong-ping, Yang Xuesong.Ethanol exposure leads to disorder of blood island formation in early chick embryo.*Reproductive Toxicology* http://dx.doi.org/10.1016/j.reprotox.2017.08.003

Ethanol exposure leads to disorder of blood island formation in early chick embryo

*Guang Wang[a#], Bin-zhen Chen[a#], Chao-jie Wang[a], Jing Zhang[a], Lin-rui Gao[a], Manli Chuai[b], Yong-ping Bao[c] and Xuesong Yang[a]**

[a]*Division of Histology & Embryology, Key Laboratory for Regenerative Medicine of the Ministry of Education, Medical College, Jinan University, Guangzhou 510632, China*

[b]*Division of Cell and Developmental Biology, University of Dundee, Dundee, DD1 5EH, UK*

[c]*Norwich Medical School, University of East Anglia, Norwich, Norfolk, UK*

Running title: Ethanol affects blood island formation

[#]*Contributed to the work equally.*

[*]*The corresponding author: Xuesong Yang (Email: yang_xuesong@126.com)(Tel: +86-20-85228316)*

Highlights

1. Ethanol affects the hemangioblast migration from the posterior primitive streak to the area opaca.

2. Ethanol enhanced cell differentiation in area pellucida during HH5 and repressed cell differentiation in HH8 chick embryos
3. Ethanol affects vasculogenesis through excess ROS production and altered vascular-associated gene expression

Abstract

Ethanol's effect on embryonic vasculogenesis and its underlying mechanism is obscure. Using VE-cadherin *in situ* hybridization, we found blood islands formation was inhibited in area opaca, but abnormal VE-cadherin^{+} cells were seen in area pellucida. We hypothesise ethanol may affect blood island progenitor cell migration and differentiation. DiI and *in vitro* experiments revealed ethanol inhibited cell migration, Quantitative PCR analysis revealed that ethanol exposure enhanced cell differentiation in area pellucida of HH5 chick embryos and repressed cell differentiation in area pellucida of HH8 chick embryos. By exposing to 2,2′-azobis-amidinopropane dihydrochloride, a ROS inducer, which gave a similar anti-vasculogenesis effect as ethanol and this anti-vasculogenesis effect could be reversed by vitamin C. Overall, exposing early chick embryos to ethanol represses blood island progenitor cell migration but disturbed differentiation at a different stage, so that the disorder of blood island formation occurs through excess ROS production and altered vascular-associated gene expression.

Key words: ethanol; vasculogenesis; chick embryo; blood island; ROS

1. Introduction

Excess alcohol consumption during pregnancy is now a major public concern since ethanol exposure in pregnant women can increase the risk of congenital malformations [1-3]. These congenital malformations include fetal alcohol syndrome (FAS), characterized by cardiac defects, fetal growth restriction, neurodevelopmental delays, and craniofacial malformation [4, 5]. A close correlation between fetal alcohol exposure and impaired vasculogenesis of cortical blood vessels has been demonstrated in humans, rats, and mice [6-8]. These vascular defects may be a contributing factor for abnormal brain development in FAS. This vasculogenesis probably occurs in the early stage that is susceptible to ethanol exposure. The impact of alcohol on vasculogenesis in the developing embryo however remains controversial. Mouse [9], rat [10] and chick [11-15] embryos have been extensively used as models to address the mechanisms underlying the teratogenic effects of ethanol in various systems.

In the developing chick embryo, vasculogenesis involves the migration and differentiation of hemangioblasts from posterior primitive streak derived-mesodermal cells and the formation of primary capillary plexuses in area opaca [16]. Vasculogenesis takes place in the blood islands of area opaca located in the yolk sac. The blood islands not only harbor angioblasts but also hematopoietic cells. Hemangioblasts are the common precursor cells of both angioblasts and hematopoietic cells. Vasculogenesis has been considered as being different from angiogenesis because of the different origins of the endothelial progenitor cells. For

vasculogenesis, the endothelial progenitor cells are derived directly from mesodermal cells whereas in angiogenesis the endothelial progenitor cells are derived from the primary capillary plexuses. Moreover, vasculogenesis is generally considered an embryonic event whereas angiogenesis is regarded as a process that takes place in the adult. It appears now that the concept of vasculogenesis and angiogenesis as being different processes may not be accurate [17-19]. In this context, we revisited the developmental events associated with vasculogenesis in the developing chick embryo.

During blood island formation, a proper cell-cell adhesion is also important for maintaining the integrity of the primary vascular plexus formed by the migrant mesodermal cells. This cell-cell interaction is determined by adhesion molecules, PECAM and VE-Cadherin, expressed by cells located on the lateral borders of the early chick embryo [16]. Moreover, VE-cadherin is expressed in blood island cells-, making it an excellent marker for studying the formation of blood island formation and vasculogenesis. Hemangioblast cell migration is modulated by various genes and pathways such as PDGF, VEGF/VEGFR and ANG-1 pathways [20]. Differentiation is modulated by similar genes and signaling pathways such as PDGF, FGF and VEGFR and likewise [21, 22], blood island fusion process is modulated by various genes and signaling pathways including VEGF, PDGF, FGF and ANG [23, 24].

As a component of oxidative phosphorylation, reactive oxygen species (ROS) plays an important role in the redox control of various signaling pathways[25-27]. However, excessive ROS generation in the body is associated with the pathogenesis of many diseases [28]. Excessive ROS accumulation in the body could interfere with

cellular and physiological functions through the deleterious oxidization of macromolecules, including proteins, lipids, DNA and signal transduction [29]. ROS can act as primary or secondary messengers to promote cell growth or death, and oxidative stress could initiate crucial reactions that either positively or negatively influence embryonic development. Many cells during embryogenesis develop pathological problems due to an imbalance of oxidative stress induced by ethanol exposure [27, 30]. Therefore, a correct balance between ROS production and degradation is crucial to maintain the normal physiological functions of the cell [31, 32].

In this study, the underlying mechanisms of ethanol's effect on the blood island during chick embryogenesis were investigated. In addition, the link between ethanol exposure and excess ROS production during blood island formation was elucidated.

2. Materials and Methods

2.1 Chick embryos and treatments

Fertilized chick eggs were obtained from the Avian Farm of the South China Agriculture University (Guangzhou, China). The eggs were incubated until the required Hamburger and Hamilton (HH) stage [33] inside a humidified incubator (Yiheng Instrument, Shanghai, China) at 38℃ and 70% humidity. EC (early chick) culture was employed to culture gastrula chick embryos which make them amenable to experimentation. For the whole-mount embryo treatments, the HH0 chick embryos in EC culture were treated with 2% (342.5mM) ethanol [34], 1 μl/ml SU5402

(Sigma-Aldrich, USA), 5μM AAPH (Sigma-Aldrich, USA), 5mg VC (Sigma-Aldrich, USA) + 5μM AAPH or 5mg VC + 2% (342.5mM) ethanol, while the control embryos were exposed to simple saline as a control. For the half-side embryo treatments, 2% (342.5mM) ethanol was directly applied to one side of the gastrula-stage embryos (HH3), while the other side was exposed to simple saline as a control [35]. The treated embryos were incubated for either 18 or 45 hours then fixed in 4% paraformaldehyde for histological, morphological and molecular analysis.

2.2 In situ hybridization

Whole-mount *in situ* hybridization of chick embryos was performed according to standard *in situ* hybridization protocol [36]. Digoxigenin-labeled probes were synthesized to detect VE-cadherin mRNAs [37]. Whole-mount stained embryos were photographed and then frozen sections of thickness of 20 μm were prepared for histological analysis.

2.3 RNA isolation and qPCR analysis

Total RNA was isolated from both HH5 and HH8 chick embryo heads（N>25） using a Trizol kit (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA synthesis and SYBR® Green qPCR assay were performed using a PrimeScript™ RT reagent kit (Takara, Japan). All specific primers used are described in Supplementary Figure S1. Reverse transcription and amplification reactions were performed in Bio-Rad S1000™ (Bio-Rad, USA) and ABI 7000 thermal cyclers,

respectively. Analysis of an invariant endogenous control gene GAPDH was performed in parallel to confirm that equal amounts of RNA were used in each reaction. The ratio between the intensity of the fluorescently stained bands corresponding to genes and GAPDH was calculated to quantify the level of the transcripts for those genes mRNAs. The RT-PCR result was representative of three independent experiments.

2.4 Explant culture

We divided the streak-stage chick embryos (HH3) into 6 equal segments, with the fifth segment from the cranial side treated as the posterior primitive streak. The posterior primitive streak explants were cultured in DMEM-F12 culture medium (Life Technologies) at 37C and 5% CO2.26 The incubation time varied according to experimental requirements. Each treatment was performed in triplicate.

2.5 The trace of cell migration trajectory with DiI.

Carbocyanine dye 1,1V-dioctadecyl-3,3,3V,3V-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, Inc.) was used to label small groups of primitive streak cells. A 2.5% stock solution of DiI was diluted in ethanol, 1:10 in 0.3 M sucrose, and injected into the posterior primitive streak of HH3 chick embryo by air pressure through a micropipette, pulled from a 1 mm glass capillary in a vertical micropipette puller (WD-2, Chengdu Instrument Company). Typically, each labeled tissue in the posterior primitive streak contained approximately 10−30 cells.

2.6 Photography

Following *in situ* hybridization, the whole-mount embryos were photographed using a stereo-fluorescent microscope (Olympus MVX10) with the associated Olympus software package Image-Pro Plus 7.0. The embryos were sectioned into 14 mm-thickness slices using a cryostat microtome (Leica CM1900) and then the sections were photographed using an epi-fluorescent microscope (Olympus LX51, Leica DM 4000B) with the CW4000 FISH Olympus software package.

2.7 Data analysis

All data analyses and graphics were performed using the Graphpad Prism 5 software (Graphpad Software, CA, USA). The results were presented as the mean value (± SE). Statistical significance was determined using paired t test, independent samples t test, or one-way analysis of variance (ANOVA). $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ indicate statistically significance between control and drug-treated groups. P values < 0.05 were considered to be significant.

3. Results

3.1 VE-cadherin expression pattern in gastrula chick embryos

In situ hybridization showed that VE-cadherin was not expressed in area opaca of HH4 staged chick embryos (Fig. 1A-A1). These VE-cadherin^{+} mesodermal cells

could be clearly observed at high magnification in later stages (Figs. 1B1-E1). In HH6 chick embryos, VE-cadherin was highly expressed in the forming blood islands in extra-embryonic area opaca (Figs. 1B-B1) although VE-cadherin expression within the blood islands was still weak. When embryos had developed beyond the HH7-HH8 stage, VE-cadherin expression in the blood islands was much stronger (Figs. 1C-D). In HH10 chick embryos, the blood island in area opaca formed into primary vascular plexus (Fig. 1E). This spatiotemporal expression pattern for VE-cadherin (Fig. 1F) suggested that the gene might be involved in the early stages of vasculogenesis during early embryonic development. The process involves the mesenchymal cells in the primitive streak migrating though the area pellucida and then forming VE-cadherin^{+} blood island in area opaca (Fig. 1F1).

VE-cadherin whole-mount *in situ* hybridization was performed on HH4, 6, 7, 8 and 10 chick embryos.(A) In HH4 chick embryos, VE-cadherin was not expressed in area opaca. (A1) Higher magnification of the area opaca. (B) In HH6 chick embryos, VE-cadherin was mainly higher expressed. (B1) Higher magnification of the area opaca (dotted square outline in B) showed VE-cadherin was expressed more prominently in the blood islands. (C) In HH7 embryo, VE-cadherin was mainly expressed in primitive streak, neural tube, somites, presomitic mesoderm and blood islands of area opaca. (C1) Higher magnification of the area opaca (dotted square outline in C) revealed that VE-cadherin was expressed more strongly in blood islands. (D1) Higher magnification of the area opaca (level indicated by dotted line in D), showing VE-cadherin expression was concentrated in the blood islands and similar to HH7. (E) In HH10 embryo, VE-cadherin was expressed in the blood islands of area opaca. (F-F1) Schematic drawing showing

the process of blood island formation in early chick embryo. Scale bars: 500 mm in A-E1.

3.2 Ethanol exposure inhibited blood island formation

The effects of saline (control) and ethanol (2%) on the developing blood island of chick embryos was investigated (Figs. 2A). On gross examination, we found the blood islands exposed to ethanol appeared less than in the control (Figs. 2B-C). These VE-cadherin^{+} blood islands could be clearly observed in high magnification (Figs. 2B1- C1). As illustrated in the schematic drawing in red, the blood islands were found abnormally aggregated in the area opaca as scatter type (Fig. 2C2) compared to control as gathered type (Fig. 2B2). Closer analysis of the blood island confirmed the morphological observation, i.e., the blood island density in the ethanol-treated group is significantly lower (con: 41.12±2.619, n=14; eth: 16.51±1.010, n=14, $p<0.001$; Fig. 2D) while the isolated blood island number increased more than in the saline treated group (con: 3.8±0.48, n=6; eth: 4.83±0.40, n=6, $p>0.05$; Fig. 2E). Interestingly, abnormal VE-cadherin^{+} cells appeared at the area pellucida in ethanol treated embryos (Fig. 2C1, red arrow, n= 6/14).

To address the effect of ethanol in embryonic vasculogenesis in the embryos treated with ethanol at half-side in EC-culture, VE-Cadherin *in situ* hybridization was employed as a blood island marker (Figs. 2G-G2') (Fig. 2F), as described in Materials and Methods and in more detail in a previous publication [33]. Thus, experimental errors from different rates of embryo development could be avoided. The results shows that blood island density at ethanol treated side decreased

significantly (con: 26.55±3.800, n=6; eth: 11.40±2.591, n=6, $p<0.01$; Fig. 2H) in comparison to control side (Fig. 2G-G2', H) while the isolated blood island number increased (con: 2.67±0.67, n=6; eth: 4.33±0.49, n=6, $p>0.05$; Fig. 2I). The increased number of isolated blood islands and decreased blood islands density implies that the blood islands fusion could be affected by ethanol exposure. Above all, we hypothesise that ethanol can affect the processes of blood island progenitor cell migration and differentiation (Fig. 2J).

Schematic drawing shows for early embryo incubation in EC-culture (A). VE-cadherin whole-mount *in situ* hybridization was first performed on HH8 chick embryos both simple saline as control (B) and ethanol (2%) (C). High magnifications and schematic drawing of both the area opaca (dotted square outline in B-C) show the blood island area varies that ethanol treating decreased (B1-C1, B2-C2). (B3-C3) Transverse sections of the area opaca at the levels indicated by dotted line in B1-C1 show the isolated number of blood island differences. (D) The statistics of the blood island density in area opaca described in B1-C1. (E) Showing the isolated number of the blood islands described in B3-C3. (F) Schematic drawing shows for the half-side embryos treatments with ethanol in EC-culture. (G) *In situ* hybridization of embryo treated half side with control and half side with ethanol. (G1-G2) The high magnifications of the area opaca of embryos. (G1'-G2') Transverse section of a representative embryo was shown following ethanol and control side in the area opaca. (H) The statistics of the blood island density in area opaca described in G1-G2. (I) Showing the isolated numbers of the blood island described in G1'-G2'. (J) Schematic drawing showing whether ethanol is involved in cell migration and differentiation during the process of blood island formation in early chick embryo. Scale bars: 500μm in B, C, G and 100μm

in B1-C1,B3-C3,G1-G2'

3.3 VEGF/PDGF/FGF signaling pathways are involved in the ethanol-induced abnormal blood island fusion

It has been well established that VEGF/PDGF/FGF signaling pathways play a very important role in embryonic fusion as they regulate endothelial cell proliferation and migration [38, 39]. Consequently, the disruption of the expression of vascular-related genes was studied using quantitative PCR in the abnormal blood islands that formed as a result of ethanol exposure. The expressions of FGF1 ($p<0.01$), FGF2 ($p>0.05$), PDGFA ($p<0.001$), VEGFA ($p<0.001$), VEGFR1 ($p>0.05$), VEGFR2 ($p>0.05$), VEGFR3 ($p>0.05$), NRP1 ($p>0.05$), NRP2 ($p<0.05$), HIF1 ($p<0.05$), ANG1 ($p<0.01$), and ANG2 ($p>0.05$) show great significance changes. FGF1 expression was increased, while PDGFA and VEGFA expressions were correspondingly repressed in ethanol-treated embryos as compared with control embryos (Fig. 3A). *In situ* hybridization with SU5402 (FGF, VEGF and PDGF receptor-specific tyrosine kinase inhibitor) (Figs. 3B-C, B1-C1) revealed that the blood island density in the SU5402-exposed group was significantly lower in whole-mount (con: 41.12±2.619, n=14; eth: 15.99±0.9606, n=14; $p<0.0001$, Fig.3D) and the isolated blood island number increased in transverse sections (con: 3.83±0.48, n=6; eth: 5.3±0.42, n=6; $p<0.05$, Fig. 3E) compared to control. These findings further validate that FGF/VEGF/PDGF signaling is involved in ethanol-induced the inhibition of the formation of blood islands (Fig. 3F).

(A) *RT*-qPCR analysis showing blood island formation related genes in control and ethanol treated embryos. (B-C1) *In situ* hybridization showing that VE-cadherin is expressed in the lateral mesoderm adjacent to the primitive streak in area opaca in HH8 chick embryos. (B2, C2)The transverse sections were from B1, C1 respectively. (D) The statistics of the blood island density in area opaca described in B1-C1. (E) Showing the isolated numbers of the blood island described in B2-C2. (F) Schematic drawing showing aberrant blood island formation induced by ethanol exposure is associated with PDGF, FGF, et al. Scale bars: 500μm in B-C，200μm in B1-C1 and 100μm in B2-C2.

3.4 Ethanol depresses the migration of mesenchymal cells from the posterior primitive steak

The progenitor cells of blood islands are derived mainly from the posterior primitive steak. In order to determine whether ethanol affects the hemangioblast migration in the formation of blood islands, it was necessary to first study the hemangioblast migration trajectory from the posterior primitive streak to the blood island-forming sites. To follow the cell migration trajectory of hemangioblast cells, DiI dye was injected into posterior primitive streaks in HH3 chick embryos (Figs. 4A-H, A1-H1). This was also achieved by transplanting a piece of the posterior primitive streak into a cell culture (Fig. 4J). We found that exposure to ethanol decreased the migration area of hemangioblasts both in dye-injected embryos (con: 2.103±0.5566, n=8; eth: 0.3116±0.08422, n=8; $p<0.01$, Fig. 4I) and the explants compared with the controls (con: (18.07±3.419) $\times 10^{\times 4}\mu m^2$, n=5; eth: (15.64±4.427)

$\times 10^{\times 4}\mu m^2$, n=5; p<0.05, Figs. 4K-M). Above all, these results indicate that ethanol affects the hemangioblast migration from the posterior primitive streak to the area opaca.

The fluorescent dye was injected into posterior primitive streak and then the mesenchyme cell migration was followed. (A-H) Recording the track on control-control (A-D) and control-ethanol group (E-H). (A1-D1 and E1-H1) are the images merged from fluorescence. (I) The statistics of area spreaded by fluorescence. (J) Posterior primitive streak tissues were also isolated from HH3 chick embryos and maintained for 48 hours. (K-L1) Ethanol-treatment repressed angioblast progenitor cell migration compared (L-L1) with the control (K-K1). (M) Bar chart showing the area of cell migration in 2 groups. (N) Schematic drawing showing ethanol treatment represses precursor cell migration. Scale bars: 1mm in A-H1 and 200μm in K-L1

3.5 Ethanol exposure promotes mesenchymal cells differentiated into VE-cadherin$^+$ cells in area pellucida of HH5 chick embryos

Since ethanol affected the migration of the mesenchymal cells, and there were some VE-cadherin$^+$ cells differentiated in area pellucida (Fig. 2C1) after ethanol treatment, mesenchyme differentiation was also examined to identify any corresponding effects. Whole-mount *in situ* hybridization was carried out in the HH5 embryos. The area pellucida of both ethanol and control are shown in figure 5 (Fig. 5B-B1and Fig. 5A-A1, respectively). The ethanol side blood islands density decreased significantly in comparison to control side (con: 32.18±6.961, n=6; eth: 14.35±1.963, n=6; p<0.05, Fig. 5C) with area opaca decreased (con: 31.60±7.077, n=6; eth:

10.13±1.330, n=6; $p<0.05$, Fig. 5D) while area pellucida increased (con: 0.5843±0.1824, n=6; eth: 4.215±0.8475, n=6; $p<0.01$, Fig. 5E). To further confirm the influence of ethanol on HH5 cell differentiation signaling, the expression of the same genes as HH8 were measured. The expressions of FGF2 ($p<0.01$), VEGFR2 ($p<0.01$), VEGFR3 ($p<0.05$), HIF1 ($p<0.05$) and ANG2 ($p<0.01$) show significant changes. These results show that ethanol treatment promotes mesenchymal cell differentiation via upregulation of FGF2 and VEGFR3 in area pellucida of HH5 chick embryos (Fig. 5G).

(A-B) *In situ* hybridization showing that VE-cadherin is also expressed in HH5 chick embryos. High magnifications of both the area opaca (dotted square outline in A-B) show the blood island area varies that ethanol treatment decreased (A1-B1). VE-cadherin^{+} cells appear in area pellucida with ethanol treatment (arrows in B1). (C) The statistics of the blood island density in whole-mount embryos. (D) The statistics of the blood island density in area opaca. (E) The statistics of the blood island density in area pellucida. (F) *RT*-qPCR analysis showing blood island formation related genes in control and ethanol treated embryos. (G) Schematic drawing showing ethanol treatment promoted the precursor cell differentiation in area pellucida. Scale bars: 500μm in A-B and 200μm in A1-B1.

3.6 Ethanol exposure causes excess ROS generation during blood island formation

As reported in previous publications, ethanol exposure can induce excess ROS production [34, 40]. 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH, a free radicals generator 25, 40) was used to establish whether excess ROS could also

induce vascular malformation and also in combination with vitamin C (an depressor of ROS production) to see whether the ethanol- or AAPH-induced inhibitive effect can be rescued (Figs. 6A-E2). The results showed that after 45 hours of AAPH exposure, some embryos show a blood island phenotype similar to ethanol treated embryos, while control embryos do not. In AAPH-treated embryos, abnormal VE-cadherin positive cells appeared at the area pellucida as well (Fig. 6B1, red arrow, n= 7/14). The addition of vitamin C was effective in rescuing both the ethanol and the AAPH treated groups. AAPH exposure also reduced the density ($n = 14$, $P < 0.001$; Fig. 6G) but increased the isolated number ($n = 14$, $P < 0.05$ or $P < 0.01$ or $P < 0.001$; Fig. 6H) of blood island in area opaca, while the addition of vitamin C could reverse these effects. The results suggest that the ROS is involved in ethanol-induced abnormal blood island formation in area opaca (Fig. 6I).

(A-E) VE-cadherin whole-mount *in situ* hybridization was implemented in the embryos from control, AAPH, AAPH + VC, ethanol, ethanol + VC treatment groups. (A1-E1) High magnification of area opaca. (A2-E2) Transverse section of area opaca. (F) Bar chart showing the density of blood islands in control, AAPH, AAPH+VC, ethanol, ethanol +VC treated embryos. (G) Bar chart showing the isolated numbers of blood islands in control, AAPH, AAPH+VC, ethanol, ethanol + VC treated embryos. (H) Schematic drawing showing ROS is involved in ethanol-induced abnormal blood island formation in area opaca. Abbreviation: AAPH, 2,2-azobis-amidinopropane dihydrochloride; VC, Vitamin C; ROS, reactive oxygen species; SOD, superoxide dismutase. Scale bars: 500μm in A-E, A1-E1 and 100μm in A2-E2

4. Discussions

Vasculogenesis is the process where de novo blood vessels are formed from migratory mesodermal cells. During gastrulation, the epiblast cells undergo epithelial-mesenchymal transition (EMT) in the caudal region of the primitive streak and emigrate laterally and caudally to the extraembryonic area opaca. At the area opaca, the mesodermal cells give rise to the blood islands. Exposure to harmful environmental factors is known to increase the risk of growth retardation and neural tube defects in the offspring. However, the pregnant woman may be subjected to potential teratogenic factors before she can take precautionary actions. The high risk period for birth defects in the nervous and cardiovascular systems is during the first few weeks of development when these systems are most actively developing. Ethanol is widely consumed in excess and represents a potential teratogen in pregnant women. Therefore, it is important that we comprehend how acute and chronic ethanol exposure disrupts vascular development and the mechanisms involved, so that precautionary measures can be effectively implemented.

Avian and mouse models are often employed to extrapolate the effects of alcohol consumption in human fetus development. In our previously study, excess ethanol exposure was shown to inhibit embryonic angiogenesis through promoting superfluous ROS production during embryo development [40]. A.C. Tufan and N.L. Satiroglu-Tufan found that the exposure of (10, 30 or 50%) ethanol could increase the embryonic mortality rate and inhibit the vascular development in a dose-dependent manner during latter developmental stages of chick embryos [41]. In this study,

gastrula chick embryo (earlier stages) is used as a model to investigate the effects of 2% ethanol exposure on early vascular development. The concentration of the ethanol used in this study is based on our previous study, in which 58% embryos detained at HH4-6 and 10% embryos died after 38-hours ethanol treatment. We have revised it in the revised manuscript [34]. VE-Cadherin was used as a marker to follow the development of the blood islands. In HH8 chick embryos, we revealed that the VE-cadherin was strongly expressed in the blood island. When less developed embryos were treated with ethanol, we could see a distinct decrease in blood island density but number of isolated blood island in section was increased at the corresponding stage. These results suggest that ethanol exposure during the early stages of embryo development impair blood island fusion, which is consistent with previous reports that ethanol could inhibit vascular development in the neonatal brain [6]. The results also indicate that the anti-fusion effect of ethanol can be mediated by VEGF, PDGF and FGF signaling.

These results raise the question as to whether or not ethanol-induced effect involves mesenchyme migration and differentiation and suggest that it is the aberrant expression of mesenchyme cell migration and differentiation, which are partly responsible for the ethanol-induced blood island non fusion. There is growing evidence to indicate that genetic background may determine whether the embryo can adapt to the teratogenic effects of ethanol exposure [42, 43].

In this study, ROS production was increased in chick embryo after treatment with 2％ ethanol and similarly there have been reports in the literature that ethanol

exposure could stimulate excess ROS generation in the human body [44]. The metabolism of alcohol is composed of two steps. First, alcohol dehydrogenase converts alcohol to acetaldehyde and secondly, aldehyde dehydrogenase converts acetaldehyde to acetate. Each of these reactions leads to the formation of one molecule of the reduced form of nicotinamide adenine dinucleotide, enhancing activity of the respiratory chain, including increased oxygen consumption and ROS formation [45]. These excess free radicals may impair normal development of the embryo by inducing apoptosis and necrosis [13, 46]. In this study, the mediation of the anti-angiogenic effect of ethanol by excess free radicals was examined and similar bioactivities using AAPH (a known ROS inducer) instead of ethanol were observed. This suggests that ethanol exposure could induce excess ROS production. Excess ROS is harmful to the cell membrane and organelles so it is likely that excess ROS could correspondingly disrupt blood island formation.

Overall, this study illustrates and presents a hypothesis of how ethanol was able to inhibit vasculogenesis (Fig. 7A-B). The illustration shows the ethanol-inhibited cell migration and enhanced cell differentiation in area pellucida during HH5, which in turn represses blood island fusion in area opaca during HH8. This process was mediated by VEGF, PDGF and FGF signaling. This explains why ethanol exposure was found to inhibit blood island formation in area opaca, but abnormal VE-cadherin^{+} cells were seen in area pellucida. In addition, ethanol-induced ROS can negatively affect this process. All of these effects combine to allow ethanol to impact negatively on the development of the embryonic vasculature. This study for confirms the need

for further investigation on the precise molecular mechanisms underlying the ethanol-induced impairment of vasculogenesis in embryo development.

(A) Schematic drawing showing formation of chick blood islands was inhibited after exposing to ethanol during chick embryo gastrulation. We can found the abnormal VE-cadherin^{+} cells appeared at the area pellucida in ethanol treated embryos. (B) Schematic drawing showing ethanol-induced ROS negatively affect the blood island formation. In this process, ethanol-inhibited cell migration and –enhanced cell differentiation in area pellucida during HH5, which in turn represses blood island fusion in area opaca during HH8. It mainly mediated by VEGF, PDGF and FGF signaling.

Acknowledgements

The authors also wish to thank Mr Jim Bacon for helpful discussions during the preparation of this manuscript. This study was supported by NSFC grant (81571436, 31401230), Science and Technology Planning Project of Guangdong Province (2014A020213008, 2014A020221091, 2017A020214015), Science and Technology Program of Guangzhou (201710010054, 201510010073), Guangdong Natural Science Foundation (2016A030311044), the Fundamental Research Funds for the Central Universities (21617466) and Research Grant of Key Laboratory of Regenerative Medicine, Ministry of Education, Jinan University (No. ZSYX-M-00001, ZSYX-T-00001 and ZSYX-Y-00002).

References

[1] K.L. Jones, D.W. Smith, The fetal alcohol syndrome, Teratology 12(1) (1975) 1-10.

[2] P.A. May, J.P. Gossage, Estimating the prevalence of fetal alcohol syndrome. A summary, Alcohol Res Health 25(3) (2001) 159-67.

[3] J. McGill, D.K. Meyerholz, M. Edsen-Moore, B. Young, R.A. Coleman, A.J. Schlueter, T.J. Waldschmidt, R.T. Cook, K.L. Legge, Fetal exposure to ethanol has long-term effects on the severity of influenza virus infections, J Immunol 182(12) (2009) 7803-8.

[4] E.L. Abel, Prenatal effects of alcohol, Drug Alcohol Depend 14(1) (1984) 1-10.

[5] K.L. Jones, D.W. Smith, C.N. Ulleland, P. Streissguth, Pattern of malformation in offspring of chronic alcoholic mothers, Lancet 1(7815) (1973) 1267-71.

[6] S. Jegou, F. El Ghazi, P.K. de Lendeu, S. Marret, V. Laudenbach, A. Uguen, P. Marcorelles, V. Roy, A. Laquerriere, B.J. Gonzalez, Prenatal alcohol exposure affects vasculature development in the neonatal brain, Annals of neurology 72(6) (2012) 952-60.

[7] C. Muresan, I. Eremia, Ethanol stimulates the formation of free oxygen radicals in the brain of newborn rats, Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie 43(3-4) (1997) 113-7.

[8] Y. Xu, R. Xiao, Y. Li, Effect of ethanol on the development of visceral yolk sac, Human reproduction (Oxford, England) 20(9) (2005) 2509-16.

[9] Y.Y. Dangata, M.H. Kaufman, Morphometric analysis of the postnatal mouse optic nerve following prenatal exposure to alcohol, J Anat 191 (Pt 1) (1997) 49-56.

[10] K. Sawada, H. Sakata-Haga, S. Komatsu, K. Ohta, Y.G. Jeong, Y. Fukui, A selective loss of small-diameter myelinated optic nerve axons in rats prenatally exposed to ethanol, Congenital anomalies 42(2) (2002) 125-9.

[11] S.R. Bupp Becker, I.A. Shibley, Jr., Teratogenicity of ethanol in different chicken strains, Alcohol Alcohol 33(5) (1998) 457-64.

[12] M.F. Cavieres, S.M. Smith, Genetic and developmental modulation of cardiac deficits in prenatal alcohol exposure, Alcoholism, clinical and experimental research 24(1) (2000) 102-9.

[13] K.A. Debelak, S.M. Smith, Avian genetic background modulates the neural crest apoptosis induced by ethanol exposure, Alcoholism, clinical and experimental research 24(3) (2000) 307-14.

[14] J. Illanes, M. Fuenzalida, S. Romero, P. Gonzalez, D. Lemus, Ethanol effect on the chick embryo ossification: a macroscopic and microscopic study, Biological research 32(2-3) (1999) 77-84.

[15] J.A. Roda-Moreno, M.T. Pascual-Morenilla, O. Roda-Murillo, M. Lopez-Soler, F. Arrebola-Nacle, Action of ethanol on different skull and brain parameters in the chick embryo, Journal of craniofacial genetics and developmental biology 20(1) (2000) 44-8.

[16] W. Risau, I. Flamme, Vasculogenesis, Annu Rev Cell Dev Biol 11 (1995) 73-91.

[17] C.J. Drake, Embryonic and adult vasculogenesis, Birth Defects Res C Embryo

Today 69(1) (2003) 73-82.
[18] A. Eichmann, L. Pardanaud, L. Yuan, D. Moyon, Vasculogenesis and the search for the hemangioblast, Journal of hematotherapy & stem cell research 11(2) (2002) 207-14.
[19] S. Kassmeyer, J. Plendl, P. Custodis, M. Bahramsoltani, New insights in vascular development: vasculogenesis and endothelial progenitor cells, Anatomia, histologia, embryologia 38(1) (2009) 1-11.
[20] M. Shibuya, L. Claesson-Welsh, Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis, Experimental cell research 312(5) (2006) 549-60.
[21] Y. Tanaka, V. Sanchez, N. Takata, T. Yokomizo, Y. Yamanaka, H. Kataoka, P.S. Hoppe, T. Schroeder, S. Nishikawa, Circulation-independent differentiation pathway from extraembryonic mesoderm toward hematopoietic stem cells via hemogenic angioblasts, Cell reports 8(1) (2014) 31-9.
[22] W. Weng, G. Sheng, Five transcription factors and FGF pathway inhibition efficiently induce erythroid differentiation in the epiblast, Stem cell reports 2(3) (2014) 262-70.
[23] P. Devaux, D. Christiansen, S. Plumet, D. Gerlier, Cell surface activation of the alternative complement pathway by the fusion protein of measles virus, The Journal of general virology 85(Pt 6) (2004) 1665-73.
[24] G.Q. Su, Z.H. Huang, Z.F. Liu, A.H. Guo, J.L. Yu, Z. Li, H.J. Song, [Adenovirus-mediated CDglyTK fusion gene system driven by KDR promoter selectively kills MCF-7 breast cancer cells and vascular endothelial cells], Di 1 jun yi da xue xue bao = Academic journal of the first medical college of PLA 24(12) (2004) 1346-9.
[25] F.J. Giordano, Oxygen, oxidative stress, hypoxia, and heart failure, The Journal of clinical investigation 115(3) (2005) 500-8.
[26] C.E. Murdoch, M. Zhang, A.C. Cave, A.M. Shah, NADPH oxidase-dependent redox signalling in cardiac hypertrophy, remodelling and failure, Cardiovascular research 71(2) (2006) 208-15.
[27] D.B. Sawyer, D.A. Siwik, L. Xiao, D.R. Pimentel, K. Singh, W.S. Colucci, Role of oxidative stress in myocardial hypertrophy and failure, Journal of molecular and cellular cardiology 34(4) (2002) 379-88.
[28] (!!! INVALID CITATION !!!).
[29] M. Valko, D. Leibfritz, J. Moncol, M.T. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int J Biochem Cell Biol 39(1) (2007) 44-84.
[30] M.J. Perez, E. Velasco, M.J. Monte, J.M. Gonzalez-Buitrago, J.J. Marin, Maternal ethanol consumption during pregnancy enhances bile acid-induced oxidative stress and apoptosis in fetal rat liver, Toxicology 225(2-3) (2006) 183-94.
[31] M. Karbownik, A. Lewinski, The role of oxidative stress in physiological and pathological processes in the thyroid gland; possible involvement in pineal-thyroid interactions, Neuro Endocrinol Lett 24(5) (2003) 293-303.

[32] P.K. Singal, A. Bello-Klein, F. Farahmand, V. Sandhawalia, Oxidative stress and functional deficit in diabetic cardiomyopathy, Adv Exp Med Biol 498 (2001) 213-20.
[33] V. Hamburger, H.L. Hamilton, A series of normal stages in the development of the chick embryo. 1951, Dev Dyn 195(4) (1992) 231-72.
[34] S. Li, G. Wang, L.R. Gao, W.H. Lu, X.Y. Wang, M. Chuai, K.K. Lee, L. Cao, X. Yang, Autophagy is involved in ethanol-induced cardia bifida during chick cardiogenesis, Cell Cycle 14(20) (2015) 3306-17.
[35] G. Wang, W.Q. Huang, S.D. Cui, S. Li, X.Y. Wang, Y. Li, M. Chuai, L. Cao, J.C. Li, D.X. Lu, X. Yang, Autophagy is involved in high glucose-induced heart tube malformation, Cell Cycle 14(5) (2015) 772-83.
[36] D. Henrique, J. Adam, A. Myat, A. Chitnis, J. Lewis, D. Ish-Horowicz, Expression of a Delta homologue in prospective neurons in the chick, Nature 375(6534) (1995) 787-90.
[37] N.R. Leslie, X. Yang, C.P. Downes, C.J. Weijer, PtdIns(3,4,5)P(3)-dependent and -independent roles for PTEN in the control of cell migration, Curr Biol 17(2) (2007) 115-25.
[38] J.R. Fu, W.L. Liu, J.F. Zhou, H.Y. Sun, D. Ran, M. Zheng, L. Luo, H. Zhang, Y.F. Zhou, [Role of the sonic hedgehog pathway in regulation of the proliferation, migration and differentiation of hemangioblast derived from AGM], Zhonghua xue ye xue za zhi = Zhonghua xueyexue zazhi 28(11) (2007) 757-61.
[39] H.R. Kim, J.H. Lee, H.R. Heo, S.R. Yang, K.S. Ha, W.S. Park, E.T. Han, H. Song, S.H. Hong, Improved hematopoietic differentiation of human pluripotent stem cells via estrogen receptor signaling pathway, Cell & bioscience 6(1) (2016) 50.
[40] G. Wang, S. Zhong, S.Y. Zhang, Z.L. Ma, J.L. Chen, W.H. Lu, X. Cheng, M. Chuai, K.K. Lee, D.X. Lu, X. Yang, Angiogenesis is repressed by ethanol exposure during chick embryonic development, J Appl Toxicol 36(5) (2016) 692-701.
[41] A.C. Tufan, N.L. Satiroglu-Tufan, The effect of ethanol exposure on extraembryonic vascular development in the chick area vasculosa, Cells, tissues, organs 175(2) (2003) 84-97.
[42] A.E. Adkins, L.M. Hack, T.B. Bigdeli, V.S. Williamson, G.O. McMichael, M. Mamdani, A. Edwards, F. Aliev, R.F. Chan, P. Bhandari, R.C. Raabe, J.T. Alaimo, G.G. Blackwell, A.A. Moscati, R.S. Poland, B. Rood, D.G. Patterson, D. Walsh, C. Collaborative Study of the Genetics of Alcoholism, J.B. Whitfield, G. Zhu, G.W. Montgomery, A.K. Henders, N.G. Martin, A.C. Heath, P.A. Madden, J. Frank, M. Ridinger, N. Wodarz, M. Soyka, P. Zill, M. Ising, M.M. Nothen, F. Kiefer, M. Rietschel, C. German Study of the Genetics of Addiction, J. Gelernter, R. Sherva, R. Koesterer, L. Almasy, H. Zhao, H.R. Kranzler, L.A. Farrer, B.S. Maher, C.A. Prescott, D.M. Dick, S.A. Bacanu, L.D. Mathies, A.G. Davies, V.I. Vladimirov, M. Grotewiel, M.S. Bowers, J.C. Bettinger, B.T. Webb, M.F. Miles, K.S. Kendler, B.P. Riley, Genomewide Association Study of Alcohol Dependence Identifies Risk Loci Altering Ethanol-response Behaviors in Model Organisms, Alcoholism, clinical and experimental research (2017).

[43] Y. Kobayashi, T. Sahara, T. Suzuki, S. Kamachi, A. Matsushika, T. Hoshino, S. Ohgiya, Y. Kamagata, K.E. Fujimori, Genetic improvement of xylose metabolism by enhancing the expression of pentose phosphate pathway genes in Saccharomyces cerevisiae IR-2 for high-temperature ethanol production, J Ind Microbiol Biotechnol (2017).

[44] A. Gonzalez, J.A. Pariente, G.M. Salido, Ethanol stimulates ROS generation by mitochondria through Ca2+ mobilization and increases GFAP content in rat hippocampal astrocytes, Brain research 1178 (2007) 28-37.

[45] B. Halliwell, J.M. Gutteridge, Oxygen toxicity, oxygen radicals, transition metals and disease, The Biochemical journal 219(1) (1984) 1-14.

[46] N.I. Boyadjieva, D.K. Sarkar, Microglia play a role in ethanol-induced oxidative stress and apoptosis in developing hypothalamic neurons, Alcoholism, clinical and experimental research 37(2) (2013) 252-62.

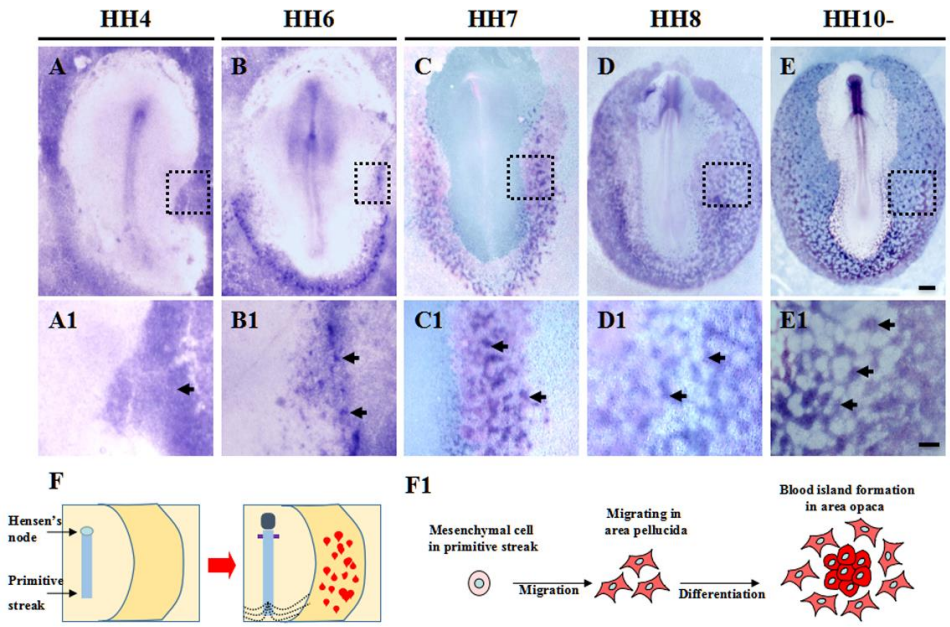

Fig.1. VE-cadherin expression pattern during chick gastrulation.

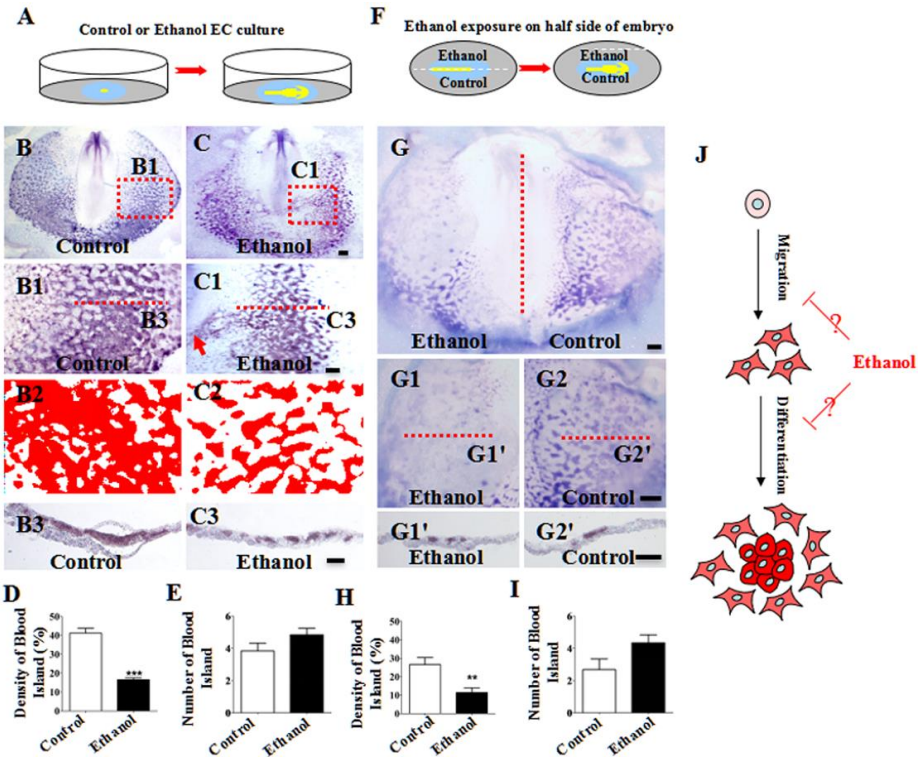

Fig.2. Blood island formation is repressed in ethanol exposure.

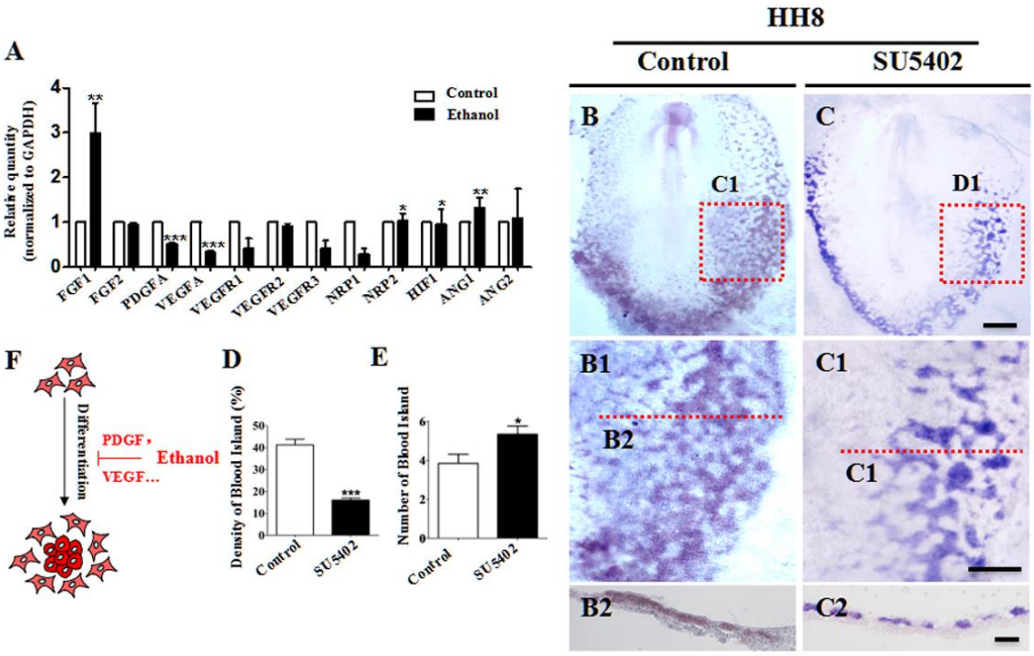

Fig 3. Aberrant blood island formation induced by ethanol exposure is associated with VEGF/PDGF/FGF pathway.

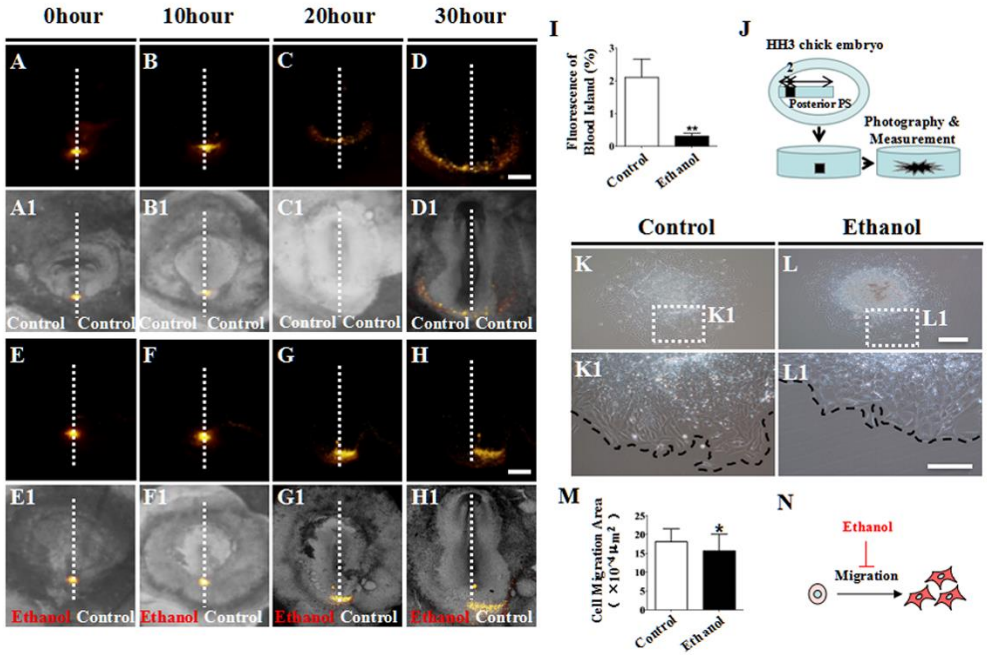

Fig 4. Ethanol treatment represses precursor cell migration.

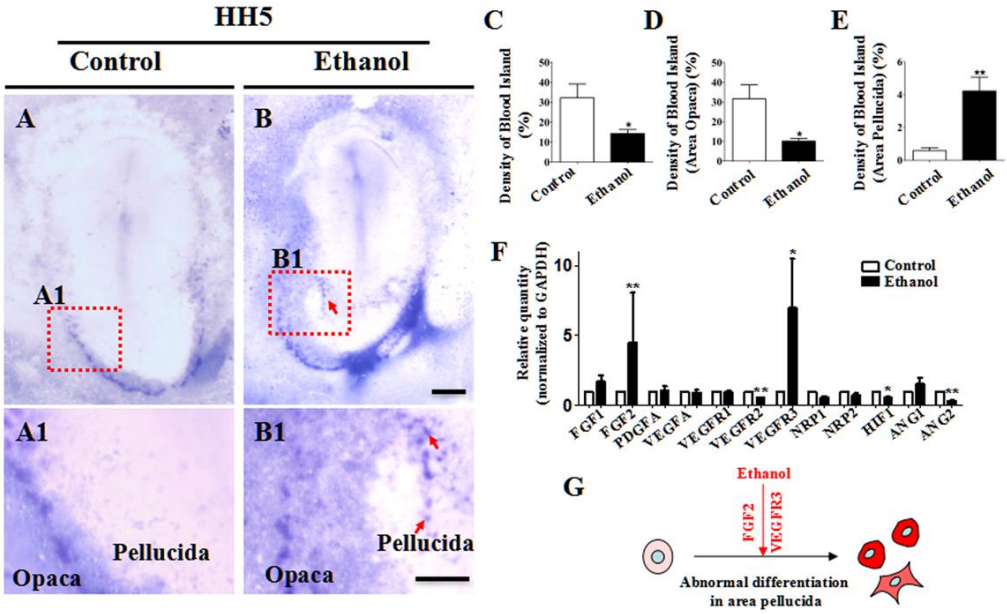

Fig.5. Ethanol treatment disturbance precursor cell differentiation in HH5 chick embryos.

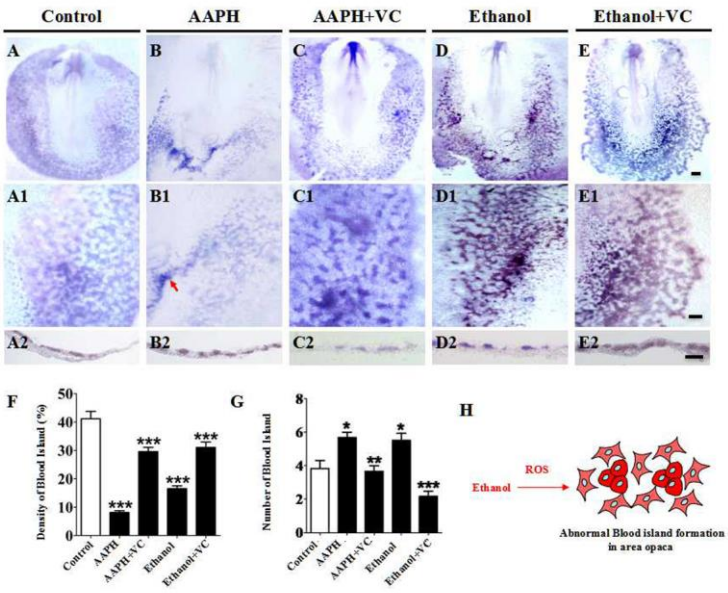

Fig 6. Ethanol treatment induces excess ROS production in early chick embryo.

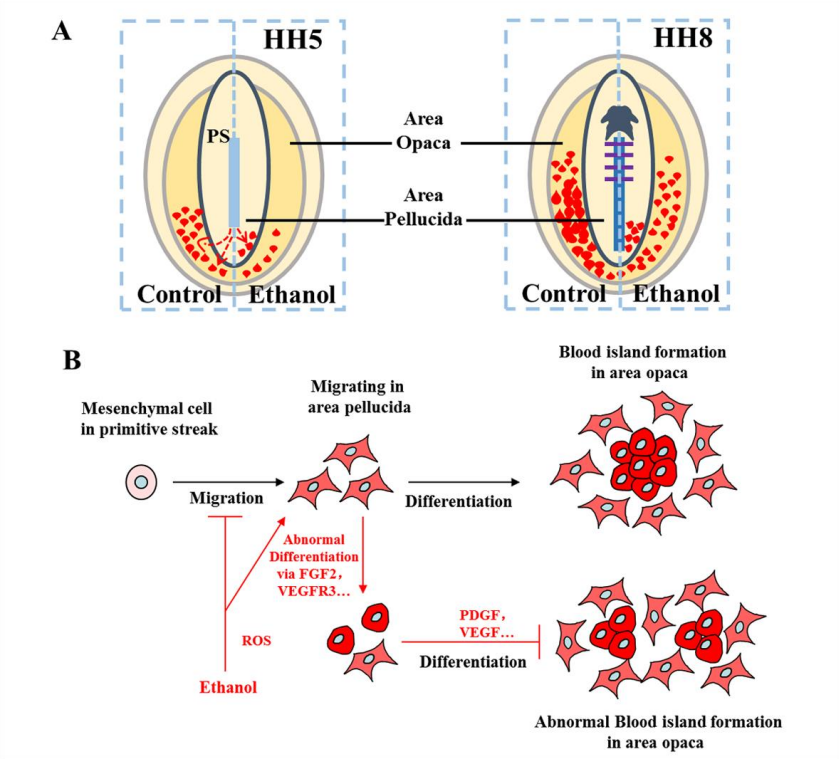

Fig. 7. Schematic of extra-embryonic vasculogenesis in the setting of ethanol.