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Gene expression signatures after ethanol exposure in differentiating embryoid bodies

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ABSTRACT

During the differentiation process, various epigenetic factors regulate the precise expression of important genes and control cellular fate. During this stage, the differentiating cells become vulnerable to external stimuli. Here, we used an early neural differentiation model to observe ethanol-mediated transcriptional alterations. Our objective was to identify important molecular regulators of ethanol-related alterations in the genome during differentiation. A transcriptomic analysis was performed to profile the mRNA expression in differentiating embryoid bodies with or without ethanol treatment. In total, 147 differentially expressed genes were identified in response to 50 mM ethanol. Of these differentially expressed genes, 78 genes were up-regulated and 69 genes were down-regulated. Our analysis revealed a strong association among the transcript signatures of the important modulators which were involved in protein modification, protein synthesis and gene expression. Additionally, ethanol-mediated activation of DNA transcription was observed. We also profiled ethanol-responsive transcription factors (TFs), upstream transcriptional regulators and TF-binding motifs in the differentiating embryoid bodies. In this study, we established a platform that we hope will help other researchers determine the ethanol-mediated changes that occur during cellular differentiation.

1. Introduction

Germ cell tumors are a good source of embryonal carcinoma cells (ECCs), which are used as a valuable tool for investigating embryogenesis and developmental biological processes (Mandal et al., 2016a, 2016b). The main advantages of ECCs are their reproducibility and rapid *in vitro* expansion; thus ECCs are an alternative to embryos in studies investigating the molecular mechanisms of mammalian cell differentiation (Andrews et al., 1994; Guan et al., 1999; Donovan and Gearhart, 2001; Przyborski et al., 2004; Shahhoseini et al., 2010). During differentiation, large-scale sequential changes occur in the expression pattern of genes that are specifically involved in developmental processes. Retinoic acid (RA) is a morphogen derived from retinol (vitamin A) that plays important roles in cell growth, differentiation, and organogenesis. After treatment with RA, ECCs exhibit an induction of different lineages *in vitro*, which is dependent on the RA concentration used and the cell culture conditions (Rohwedel et al., 1999; Soprano et al., 2007; Shahhoseini et al., 2010). The exposure of ECCs to RA under non-adherent culture conditions drives neural specification and is very useful for studying neural differentiation (Cheung et al., 1999; Megiorni et al., 2005).

Different types of factors, such as transcription factors, growth factors, regulators and gene products, are responsible for determining the fate of differentiating cells (Mandal et al., 2015). Neurotoxins, such as ethanol, cocaine and morphine, have the ability to deregulate the differentiation process. Prenatal exposure to alcohol has been reported to have profound effects on many aspects of fetal development. Ethanol disrupts the developing brain, and alcohol consumption during pregnancy can produce a wide range of irreversible cognitive, behavioral, structural, and physical anomalies (Guerri et al., 2009); alcohol consumption during pregnancy is one of the leading preventable causes of birth defects and neurobehavioral disorders (Hagan et al., 2016). Experimental evidence demonstrates that ethanol interferes with many oncogenic phases of brain development and affects crucial processes, such as neuronal migration, neurogenesis, and gliogenesis (Guerri, 2002). Furthermore, the ethanol-induced teratogenesis and

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neurodevelopmental defects occurs during an important critical period in embryogenesis, and heavy ethanol consumption during this period can lead to craniofacial defects and mental disabilities associated with Fetal Alcohol Syndrome (FAS) (Climent et al., 2002; Talens-Visconti et al., 2011; Mandal et al., 2015). However, the exact developmental phases during which alcohol exerts these specific effects on the fetus are not entirely known.

Here, we used an *in vitro* model of early neural differentiation in which ECCs are treated with RA for 48 h with or without ethanol. The main objective of our study is to gain an understanding of the ethanol-mediated changes in transcriptomic expression during differentiation. To the best of our knowledge, our study is the first to show the transcriptomic changes that occur during early differentiation following ethanol exposure. We performed RNA sequencing (RNA-seq) of both control and ethanol-exposed embryoid bodies derived from ECCs. We also carried out an extensive bioinformatics analysis of the gene expression data and selected a group of genes that encode transcription factors (TFs) during early neural differentiation.

2. Materials and methods

2.1. Cell culture and ethanol treatment

Human embryonic carcinoma (NCCIT) cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU ml^{-1} penicillin and 10 µg ml^{-1} streptomycin. The cells (1×10^6 cells) were plated in 90-mm bacterial culture dishes under non-adherent culture conditions in an Opti-MEM growth medium for 24 h to form EBs. The newly formed EBs were then exposed to either RA or RA + EtOH for an additional 48 h. To prevent ethanol evaporation from the culture dishes, the ethanol-treated cells were cultured in a separate CO_2 incubator that was saturated with 50 mM ethanol as previously described by our group (Mandal et al., 2016a, 2016b). A schematic presentation of our experimental procedure is shown in Fig. 1A. To perform the RNA-sequencing, the EBs were treated with 50 mM ethanol, and for the qRT-PCR analysis, an additional 100 and 200 mM ethanol were used.

2.2. Extraction of total RNA and synthesis of cDNAs

We used RNAiso Plus (Takara BIO, Shiga, Japan) to homogenize the harvested samples for the total RNA extraction according to the manufacturer's instructions. In brief, 200 ml chloroform were mixed and gently inverted for 5 min, followed by centrifugation at 14,000 × *g* at 4 °C for 15 min. In total, 600 µl of isopropanol was added to the collected supernatant and incubated for 1 h on ice. The lysis mixture was centrifuged at 14,000 × *g* for 15 min at 4 °C to decant the supernatant. After washing with ice-cold ethanol (70% v/v), the RNA pellet was centrifuged again for 10 min, and the ethanol was discarded. The RNA pellets were dried by inverting the tube at room temperature, followed by the addition of 20 µl DEPC-treated water. The quality and quantity of the extracted total RNA were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), respectively. The reverse transcription of the extracted RNA was conducted as previously described (Mandal et al., 2016a, 2016b). In brief, the first-strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

2.3. RNA sequencing (RNA-seq)

We used a RiboMinusTM Transcriptome Isolation Kit (Invitrogen) to remove the ribosomal RNAs (rRNAs) from the extracted total RNA. Paired-end transcriptome libraries were constructed using the NEBNext® UltraTM Directional RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA). In total, 100 ng rRNA-

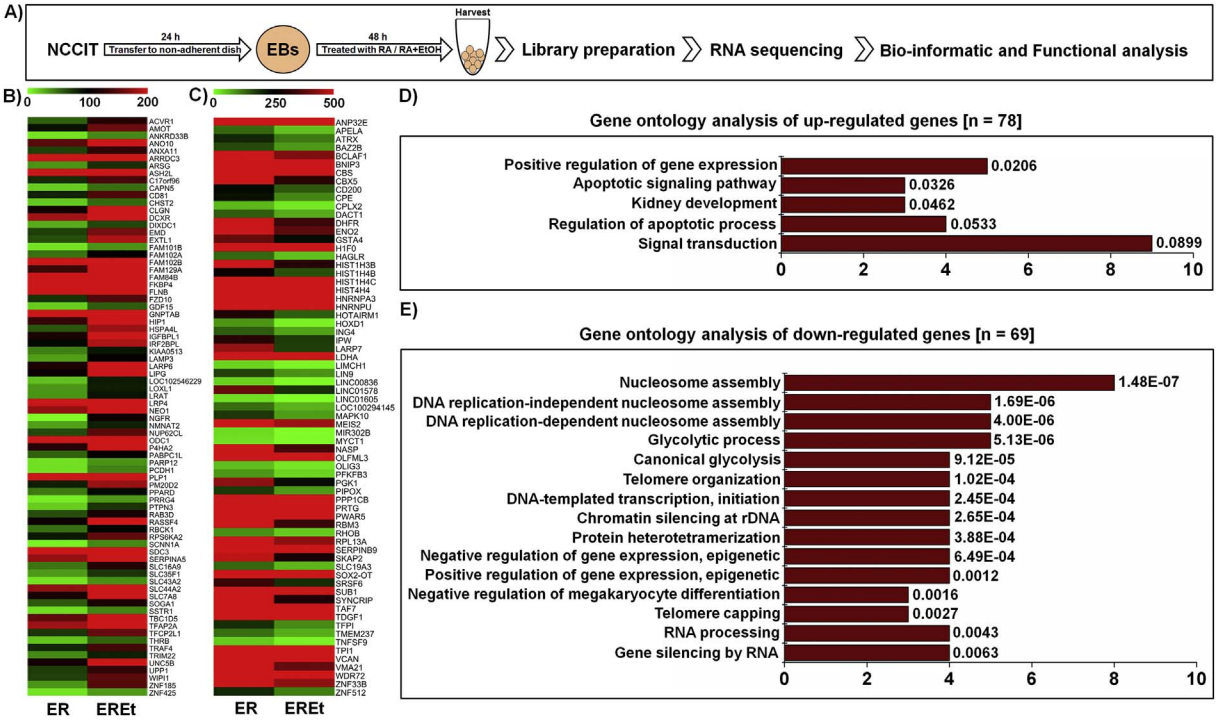

Fig. 1. Genes are differentially expressed in differentiating EBs treated with EtOH. A) Graphical experimental scheme of the differentiation/treatment protocol. NCCIT cells were stabilized and sub-cultured to form EBs. After stabilization, the EBs were treated with or without EtOH for 48 h. The samples were then collected for further analyses. B) and C) represent the heat maps of the up- and down-regulated gene expression levels between the EB + RA (ER) and EB + RA + EtOH (EREt) groups, respectively. The gene expression level of each gene shown in the heat map is scaled and represented as the relative expression change. D) and E) represent the bar graphs of the Gene Ontology analysis (Biological Process) of the up- and down-regulated genes, respectively. Only 5 categories are enriched with up-regulated genes, as mentioned in D. The top 10 significant categories are plotted for the down-regulated genes. Related p-values are indicated in front of each bar. The total number of up- and down-regulated genes is indicated in the titles of the bar graphs in parentheses.