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Human Primordial Germ Cell Formation Is Diminished by Exposure to Environmental Toxicants Acting through the AHR Signaling Pathway

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Historically, effects of environmental toxicants on human development have been deduced via epidemiological studies because direct experimental analysis has not been possible. However, in recent years, the derivation of human pluripotent stem cells has provided a potential experimental system to directly probe human development. Here, we used human embryonic stem cells (hESCs) to study the effect of environmental toxicants on human germ cell development, with a focus on differentiation of the founding population of primordial germ cells (PGCs), which will go on to form the oocytes of the adult. We demonstrate that human PGC numbers are specifically reduced by exposure to polycyclic aromatic hydrocarbons (PAHs), a group of toxicants common in air pollutants released from gasoline combustion or tobacco smoke. Further, we demonstrate that the adverse effects of PAH exposure are mediated through the aromatic hydrocarbon receptor (*AHR*) and *BAX* pathway. This study demonstrates the utility of hESCs as a model system for direct examination of the molecular and genetic pathways of environmental toxicants on human germ cell development.

Key Words: human embryonic stem cells; germ cell differentiation; reproductive toxicology; polycyclic aromatic hydrocarbons; aromatic hydrocarbon receptor (AHR).

Polycyclic aromatic hydrocarbons (PAHs) are one of the most common components of air pollution and are formed during the incomplete burning of organic substances (coal, oil, tobacco, and meat); they enter the environment mainly as exhaust from automobiles, residential wood burning, forest fires, or secondhand cigarette smoke (Finlayson-Pitts 1997). Numerous studies have linked PAH exposure to tumorigenicity, reproductive failure, and developmental birth defects in laboratory animals (Castro *et al.*, 2008; Detmar *et al.*, 2008; Jacobsen *et al.*, 2008). In parallel, epidemiological studies have linked human PAH exposure to lowered reproductive capacity, pulmonary disease, tumorigenicity, birth defects, and behav-

ioral abnormalities (Fowler *et al.*, 2008; Millman *et al.*, 2008; Perera *et al.*, 2005a,b, 2007). However, although animal and epidemiological studies indicate adverse outcomes in association with PAH exposure, tools to directly assay adverse outcomes of PAH exposure during development of particular human cell lineages have not been available.

A series of studies in mice have demonstrated that oocytes and fetal germ cells in mice are susceptible to exposure to PAHs (Jurisicova *et al.*, 2007; Matikainen *et al.*, 2001, 2002; Pru *et al.*, 2009). Moreover, studies indicate that PAH-mediated oocyte destruction could be prevented by inactivation of the apoptotic gene, *Bax*, and depended on the aromatic hydrocarbon receptor (AHR) to activate Bax expression (Matikainen *et al.*, 2001, 2002). More recently, mouse fetal germ cells were also shown to apoptose in response to incubation with PAHs; however, fetal germ cell toxicity could be prevented by the selective AHR antagonist, α-napthoflavone (ANF) (Coutts *et al.*, 2007; Jurisicova *et al.*, 2007; Matikainen *et al.*, 2001, 2002). Taken together, these studies indicate that PAH exposure can severely reduce the number of developing fetal germ cells and oocytes in mammals (Coutts *et al.*, 2007; Matikainen *et al.*, 2001, 2002). Here, we addressed whether differentiation of human embryonic stem cells (hESCs) to the germ cell lineage, which ultimately gives rise to mature eggs and/or sperm, is altered in the presence of PAHs and/or PAH inhibitors.

MATERIALS AND METHODS

hESC differentiation and treatment. hESC line (H9, XX) was maintained and differentiated as previously described (Kee *et al.*, 2006, 2009). Briefly, adherent differentiation began upon the addition of differentiation media containing 20% fetal bovine serum (Invitrogen, Inc., Carlsbad, CA) and supplemented with Bone Morphogenetic Proteins (BMPs) 4, 7, and 8b (R&D Systems, Minneapolis, MN), reconstituted in 4mM HCl/0.1% bovine serum albumin, and used at 50 ng/ml. 9,10-Dimethylbenz[*a*]anthracene (DMBA;

Sigma, Inc., St Louis, MO), ANF (Sigma), and DMBA-3,4-dihydrodiol (DMBA-DHD; Midwest Research Institute, Kansas City, MO) were dissolved in dimethyl sulfoxide (DMSO) before adding to the media at 1:1000 dilution with indicated final concentrations.

High-density Real Time-PCR/Quantitative PCR analysis by Fluidigm. Total RNA was extracted using the RNeasy kit (Qiagen, Inc., Valencia, CA) and complementary DNA (cDNA) prepared with SuperScriptIII (Invitrogen) according to the manufacturer's protocols using 1 μg RNA. The cDNA was subjected to a preamplification using 1.25 μl out of 20 μl total cDNA, 1 μl Platinum Taq (Invitrogen), 5 μl CellsDirect 2× Reaction Buffer, and 2.5 μl 0.2× Taqman (Applied Biosystems, Foster City, CA) probe mix. PCR cycle program for preamplification is as follows: 95°C, 10 min; 95°C, 15 s; and 60°C, 4 min for 14 cycles. Assays and samples are prepared according to the Fluidigm protocol and run on a 48 × 48 chip. BioMark (Fluidigm, South San Francisco, CA) program was used to obtain delta C_t value before imported into Excel file sheet to calculate delta-delta C_t value $= 2^{\left(C_{t\text{Gene}} - C_{t\text{Housekeeping genes}}\right)}$. The delta-delta C_t value is then normalized to the control of each experiment to obtain the final normalized expression level. All delta-delta C_t values were calculated using four housekeeping genes (*GAPDH*, *CTNNB1*, *ACTB*, and *CENTRIN*) in high-density RT-PCR/quantitative PCR (qPCR) analysis using the microfluidic Fluidigm system. In contrast, the experiment in Figure 2 only used *GAPDH* as a housekeeping gene. The use of *GAPDH* alone was employed in this case as Figure 2 demonstrates results of standard qPCR reactions in a 7300 Real-Time PCR System (Applied Biosystems) used for screening of short hairpin RNAs (shRNAs) for further analysis.

Western analysis of human AHR. Cells were collected in prechilled PBS with Complete Mini Protease Inhibitor (Roche Applied Science, Inc., Indianapolis, IN) followed by centrifugation for 3 min at 5000 rpm in microcentrifuge at 4°C. Supernatant was removed and pellet resuspended in 200 μl RIPA buffer and stored at −80°C. Samples were thawed and centrifuged again before the supernatant was subjected to bicinchoninic acid protein concentration measurement (Pierce Biotechnology, Inc., Rockford, IL). Thirty-five micrograms of protein was loaded on an 8% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane for 1 h at 100 V in N-cyclohexyl-3-aminopropane-sulfonic acid (CAPS) buffer (10mM CAPS, 20% methanol, pH 11). The membrane was blocked overnight in 5% nonfat milk at 4°C. Mouse monoclonal antibody to AHR (Abcam, Cambridge, MA) was diluted to 1:1000 in 5% nonfat milk followed by goat anti-mouse secondary horse radish peroxidase (Zymed [Invitrogen], Carlsbad, CA) at 1:20,000. Illuminated signal was detected using the ECL Plus System (Amersham, Piscataway, NJ).

shRNA vectors and preparation of lentivirus. shRNA was used to target *AHR* by the BLOCK-iT Inducible H1 Lentiviral RNAi System (Invitrogen). Double-stranded oligos were generated, ligated into the pENTR vector, and transfected into 293FT cells for initial screening. After 24 h, RNA was harvested using the RNeasy kit and cDNA generated using SuperScriptIII with 1 μg total RNA input. The destination lentiviral vector was generated by recombining the pENTR vector with the pLenti4/BLOCK-it-DEST vector via the Gateway technology according to the manufacturer's protocol. Lentiviral supernatant carrying the pLenti4/BLOCK-it-DEST-shAHR vector was generated by cotransfection with 10 μg of each vector with 10 μg Vsvg and 15 μg Δ8.9 into 293FT cells grown on T175. Supernatant was harvested after 3 days and ready for transduction into hESCs or frozen at −80°C until further usage. hESCs prepared for transduction were plated to 50% confluency on matrigel-coated plates. Polybrene was added to the lentivirus supernatant for a final concentration of 8 μg/ml. A 0.5 ml of the mixture was incubated with hESCs in a well of six-well plate for 6 h at 37°C before adding 2.5 ml conditioned media (hESCs media incubated overnight with irradiated MEFs). hESCs were incubated overnight before being washed 2× with PBS and replacing with new conditioned media. The next day, Zeocin was added at 2 μg/ml final concentration to new conditioned media, and the transduced hESCs were selected for 3 days before beginning differentiation as described above.

Fluorescence-activated cell sorting analysis and Caspase 3/7 assay. Single-cell suspensions were prepared first by incubating differentiated hESCs

in Collagenase Type IV (1 mg/ml) for 10 min followed by 10 min TrypLE (Invitrogen) treatment. Cell pellet was resuspended in 0.5 ml differentiated media and passed through a 40-μm strainer. Cell suspensions were then subjected to fluorescence-activated cell sorting (FACS) analysis with BD FACSAria system (BD Biosciences, San Jose, CA). Cells were sorted for either VASA:GFP+ or VASA:GFP−. One thousand cells of each group were collected in 100 μl PBS and mixed with 100 μl of Caspase-Glo 3/7 reagent according to manufacturer's protocol (Promega, Madison, WI). Luminescence was measured after 1 h of incubation at room temperature with Fluostar Optima (BMG, Offenburg, Germany).

RESULTS

To examine the effect of PAH exposure on human germ cell development, we tested whether the prototypical PAH, DMBA, or its metabolite, DMBA-DHD, affected germ cell differentiation from hESCs. Our previous studies indicated that expression of human germ cell–specific genes, including *VASA* and *DAZL*, is induced by culture with BMP-4, -7, and -8b (Kee *et al.*, 2006). We observed that addition of either DMBA or DMBA-DHD at the concentrations used previously in mouse studies (Matikainen *et al.*, 2001) reduced the expression of the early germ cell–specific genes, *VASA*, *DAZL*, and *PRDM1* (*BLIMP1*) (Fig. 1). Expression of *VASA* and *DAZL* decreased to ~0.2 to 0.02 of control levels, respectively, with a lesser effect observed for *PRDM1* (0.4–0.06 of control). Consistent with previous studies in mice in which DMBA-DHD was more potent than DMBA (Matikainen *et al.*, 2001), in our studies, we observed a similar decrease in germ cell gene expression with 1μM DMBA and 0.1μM DMBA-DHD and observed that 1μM DMBA-DHD was more potent than 10μM DMBA. This indicated that DMBA-DHD is at least one log more potent than DMBA, as measured by the decrease in germ cell–specific gene expression (Fig. 1). Concurrent with analysis of germ cell–specific gene expression, we also analyzed expression of the apoptotic gene, *BAX*, and two somatic cell markers, *NES* and *KDR*. We observed that expression of *BAX* increased 3- to 16-fold with addition of DMBA and DMBA-DHD, respectively. When the antagonist of AHR, ANF, was added with DMBA-DHD, *BAX* expression decreased, whereas that of the germ cell lineage markers *VASA*, *DAZL*, and *PRDM1* increased relative to DMBA-DHD addition alone. Although the rescue by ANF did not restore germ cell expression to the same level as controls, the partial rescue strongly suggested that DMBA-DHD acted through the AHR pathway. In contrast, we noted that both DMBA and DMBA-DHD did not significantly alter expression of the two somatic gene markers *KDR* (mesodermal marker) and *NES* (ectodermal marker), and moreover, no rescue by ANF was observed. Taken together, these observations indicate that PAH addition to differentiating hESCs resulted in a specific decrease in expression of germ cell–specific genes that are diagnostic of primordial germ cells (PGCs).

To test if the decreased germ cell gene expression was mediated through the AHR signaling pathway, we constructed short hairpin RNAs to silence *AHR* expression. By disrupting
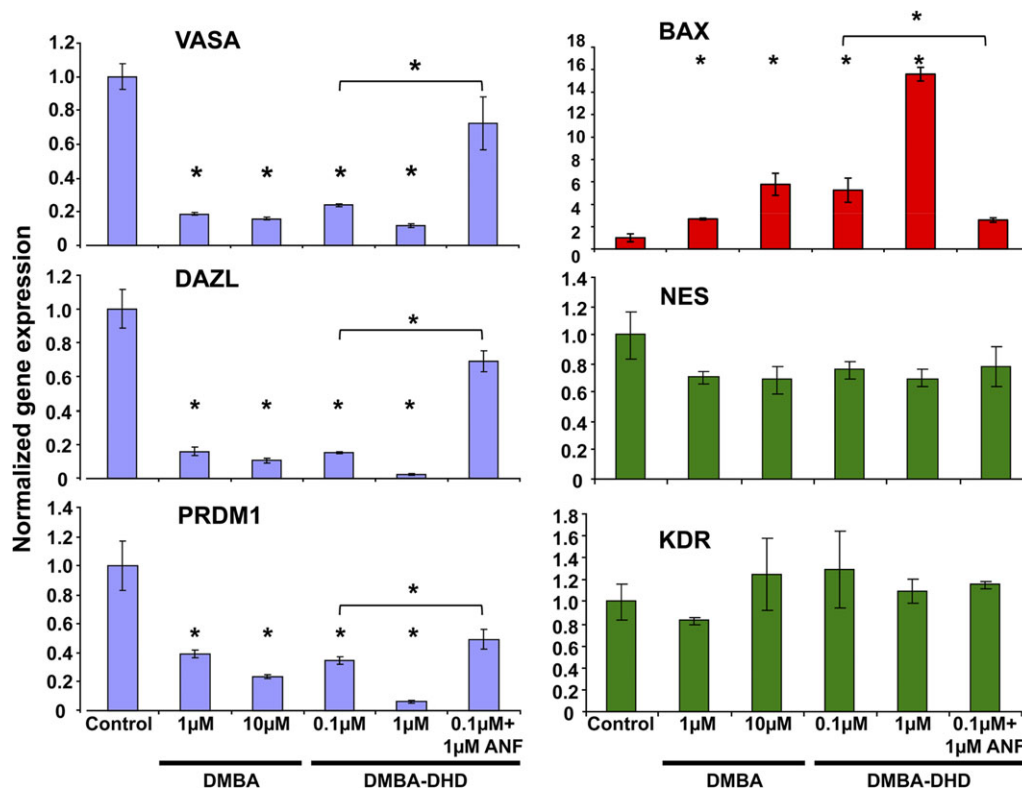
FIG. 1. Expression of early germ cell genes is reduced in the presence of PAHs. Normalized gene expression of *VASA*, *DAZL*, *PRDM1*, *BAX*, *NES*, and *KDR* in differentiated hESCs induced by BMPs to germ cells. Controls are differentiated cells treated only with the solvent, DMSO. DMBA, DMBA-DHD, and ANF are dissolved in DMSO before addition to the differentiating cells. Gene expression is measured by qPCR and normalized first to four housekeeping genes (*GAPDH*, *CTNNB1*, *ACTB*, and *CENTRIN*) followed by normalization to the controls. Error bars are standard deviations from triplicates. Asterisk indicates averages from three independent samples, significantly different from respective controls, by one-way ANOVA analysis; $p < 0.05$.

the essential component of the apoptosis pathway, we expected that the adverse affect of PAH addition to differentiating human germ cell cultures would be alleviated. Five shRNA sequences were chosen to target different regions of human *AHR* (Fig. 2A), subcloned and tested in 293FT cells for their silencing effect on AHR. qPCR measurement of *AHR* transcript levels indicated significant reduction by all short hairpin AHRs (shAHRs) (Fig. 2B), with reductions to approximately 0.8- to 0.3-fold of the control (a silencing vector carrying LacZ targeting sequence). shAHR25 showed the greatest silencing effect followed by shAHR16, shAHR7, shAHR15, and lastly, shAHR10. We recombined shAHR15, 16, and 25 separately into our destination vectors and examined silencing further by Western analysis in both 293FT cells and hESCs (Fig. 3). We noted that Western analysis with lysates of 293FT resulted in detection of two prominent bands that migrated to the expected size of AHR, ~95 kDa, whereas only one protein band was detected in the lysates of hESCs. We observed that a reduction in both the upper and lower bands occurred in 293FT cells and were similarly reduced by all three shAHRs, suggesting that both bands represent legitimate isoforms of AHR, likely to be differentially posttranslationally modified in AHR in 293FT cells. More importantly, shAHR16 and shAHR25 both reduced

AHR protein levels in hESCs more than shAHR15 and the control, shLacZ, consistent with the qPCR analysis of AHR transcript in 293FT cells.

We then proceeded to examine the effect of silencing *AHR* on human germ cell differentiation from hESCs in the presence of DMBA-DHD (Fig. 4) and observed that expression of *AHR* was reduced to approximately 50% when shAHR25 was transduced into hESCs and cells were differentiated for 14 days with BMPs. *VASA* and *DAZL* expressions were significantly elevated (rescued) when *AHR* was silenced in the presence of DMBA-DHD. Expression of *PRDM1* was also rescued by shAHR, but to a lesser extent.

To further examine the effects of DMBA-DHD and shAHR at the cellular level in terms of germ cell numbers, we used a VASA:GFP reporter system to harvest human PGCs and quantify differentiation (Kee *et al.*, 2009). For this purpose, hESCs were transduced with the VASA:GFP reporter and selected for stable integration. Silencing vectors against *AHR* or the control, *LacZ*, were introduced into the hESCs, and the cells were differentiated in the presence or absence of DMBA-DHD. In FACS, the majority of differentiated control hESCs reside on the diagonal axis of the FACS plots when the phycoerythrin and fluorescein isothiocyanate (FITC) channels were used to isolate
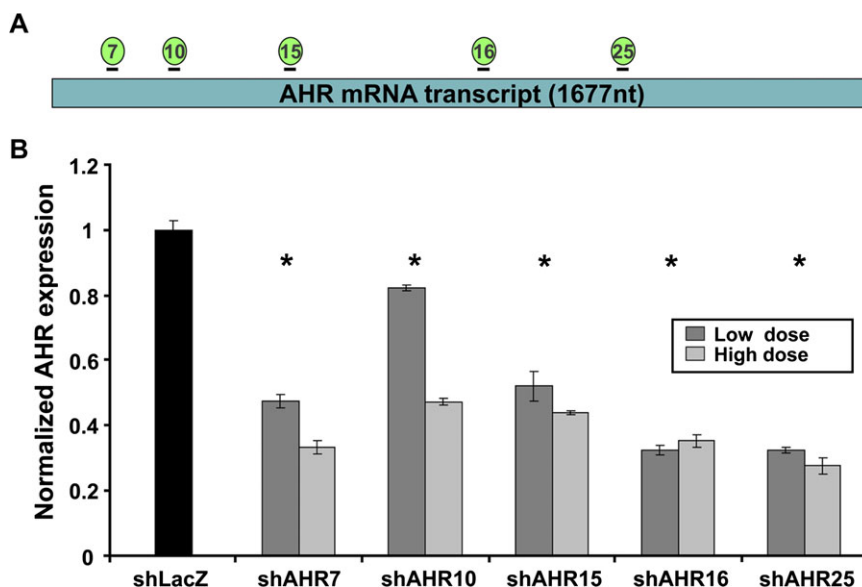
FIG. 2. AHR is silenced in 293FT cells and hESCs. (A) Location of shRNA targeting sequences on the messenger RNA transcript of *AHR*. (B) Normalized *AHR* expressions in 293FT cells with control silencing vector, shLacZ, and five shAHR targeting different regions of *AHR* exons. Two amounts of shAHR, 0.5 and 1 μg, were transfected into 293FT, and qPCR of *AHR* gene expressions were measured after 24 h. One-microgram shLacZ transfection was used as control and for normalization. Asterisk indicates averages from three independent samples, significantly different from respective controls by one-way ANOVA analysis; $p < 0.05$.

VASA:GFP cells (Fig. 5). In hESCs carrying the VASA:GFP reporter, a population of cells also resided on the FITC side of the plot when differentiated and was designated as the VASA:GFP+ cells. Extensive characterization has demonstrated that this population has properties of PGCs, including diagnostic gene expression, methylation status at the imprinted loci and genome-wide, alkaline phosphatase activity, and ability to form embryonic germ cell lines (Kee *et al.*, 2009). We observed that approximately 4.5% of cells were VASA:GFP+ PGCs after 14 days of BMP induction in cells carrying the control vector, shLacZ, without DMBA-DHD treatment (Fig. 5). When hESC cultures were treated with DMBA-DHD, the percentage of VASA:GFP+ PGCs was reduced to 3.1% (the difference in terms of real numbers of PGCs was approximately 908 PGCs out of 20,000 differentiated hESCs in the control vs. 626 PGCs in the DMBA-DHD–treated group). On the other hand, cells carrying shAHR25 showed 7.6 and 7.1% of VASA:GFP+ cells

with or without addition of DMBA-DHD. Thus, shAHR not only rescued the reduction of VASA:GFP+ population but also elevated the level of VASA:GFP+ compared with the control cells. These results mirror those that reported an elevated number of primordial oocytes in *Ahr* knockout mice (Robles *et al.*, 2000), again highlighting the AHR pathway as the major apoptotic pathway of mammalian germ cells. As further illustrated, a direct comparison of the number of VASA:GFP+ cells in all four treatment groups demonstrates a significant reduction of VASA:GFP+ at the same GFP+ intensity in the DMBA-DHD–treated shLacZ group but not in the shAHR groups (Fig. 5).

Finally, we sought to determine whether the adverse effect of PAH exposure was specific to human germ cell differentiation by further analysis of apoptosis in the isolated VASA:GFP+ PGC population versus the VASA:GFP− population (Fig. 6). We observed that apoptosis activity, measured by quantitative
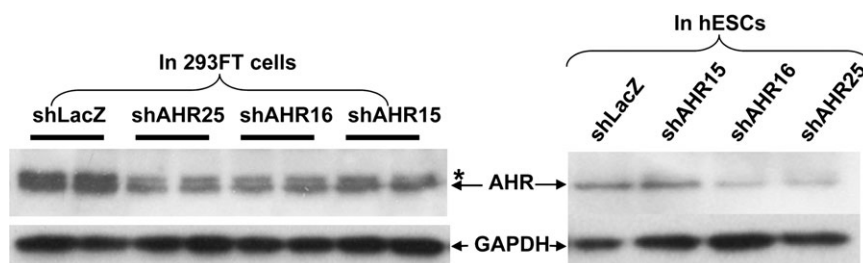

FIG. 3. Western analysis of AHR in 293FT and hESCs. Upper panels are Western blots against AHR in 293FT cells and hESCs, and lower panels are Western blots of GAPDH as loading controls of the same samples. Asterisk indicates second band, which may be a posttranslationally modified form of AHR in 293FT cells.
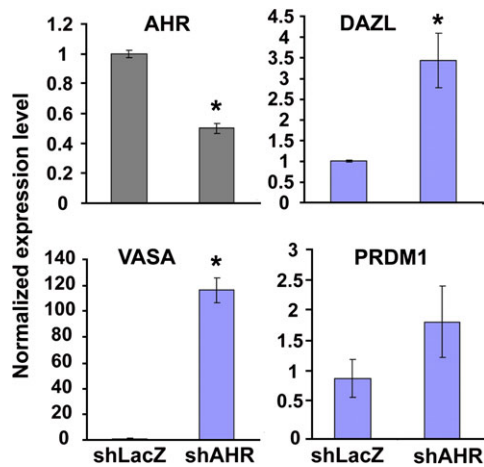
FIG. 4. Gene expressions of *AHR*, *DAZL*, *VASA*, and *PRDM1* in differentiated hESCs treated with 0.1μM DMBA-DHD carrying either control silencing vector shLacZ or shAHR25. Asterisk indicates average from three independent samples, significantly different from respective controls by one-way ANOVA analysis; $p < 0.05$.

Caspase 3/7activity, was significantly increased by the addition of DMBA-DHD in the VASA:GFP+ cells but not in the VASA:GFP− cells. Moreover, this increase of apoptotic activity was reduced by silencing of *AHR* with shAHR16 and shAHR25, confirming that the pathway acted through *AHR* and was specifically altered in the PGC population.

DISCUSSION

Results described here demonstrate that exposure to PAHs, adversely and significantly, affects human PGC differentiation from hESCs. These results also clearly demonstrate that the molecular mechanism underlying reduction in both germ cell–specific gene expression and germ cell numbers is linked to *AHR* and apoptosis of PGCs. Moreover, we noted that increased apoptosis induced by PAHs was specific to PGCs and was not detected in the somatic population. Thus, our analysis demonstrated that the clear detrimental effect of PAHs on hESC differentiation is lineage specific. We note, however, that addition of either DMBA or DMBA-DHD at the concentrations used was based on previous studies in the mouse (Matikainen *et al.*, 2001); data regarding human fetal exposures are lacking.

Although the specific decrease of gene expression of germ cell markers was drastic and the apoptosis activity in PGCs (VASA:GFP+ cells) was significantly higher than somatic cells (GFP− cells), the reduction in human PGCs seemed
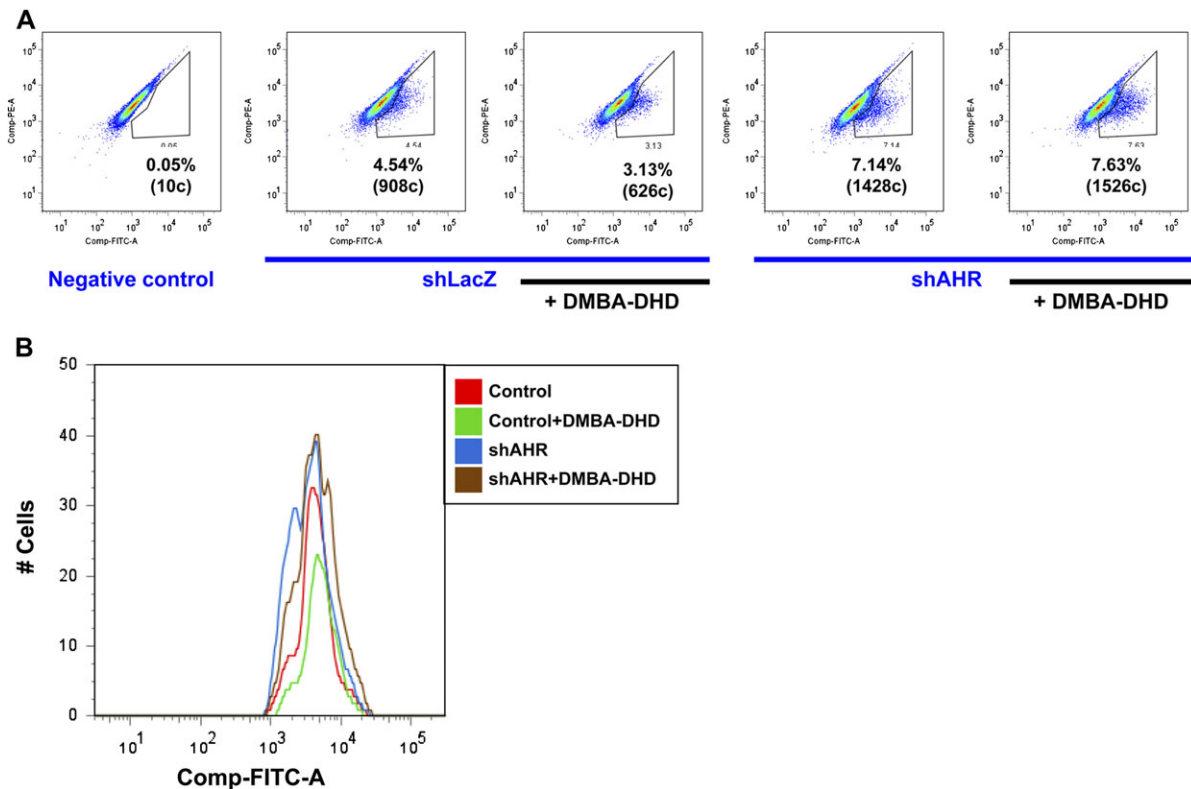

FIG. 5. (a) Silencing of AHR specifically rescues VASA:GFP cells. FACS plots of differentiated hESCs carrying VASA:GFP reporter. Percentages of GFP-positive cells are indicated on each plot of 20,000 cells, with the number of VASA:GFP+ in parenthesis. Negative control is hESCs without VASA:GFP reporter. shLacZ or shAHR25 was transduced into hESCs with or without 1μM DMBA-DHD. (b) Histogram plot of the VASA:GFP–positive populations from (a) comparing intensities and number of cells in 20,000 sorted cells.
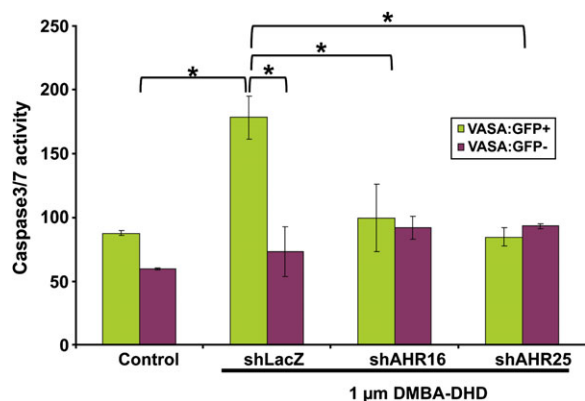
FIG. 6. Caspase 3/7 activity in germ cells is specifically reduced by silencing of AHR. Apoptosis assay measuring Caspase 3/7 activity in FACS cells. Caspase 3/7 activity is triplicate readings of luminescence unit of 1000 sorted cells. Error bars are standard deviations from three measurements. Control is hESCs treated with DMSO and differentiated with BMPs. hESCs carrying shLacZ, shAHR16, or shAHR25 were treated with 1μM DMBA-DHD and differentiated the same way as control. Note that only VASA:GFP+ cells of shLaz had significantly higher Caspase 3/7 activity to all other samples. Asterisk indicates average from three independent samples are significantly different from the respective samples by one-way ANOVA analysis; $p < 0.05$.

minor in the presence of DMBA-DHD. This may be explained if the remaining population of GFP+ cells (3.1% total) includes the population of cells undergoing apoptosis but still intact and viable. In that case, the adverse effect on VASA:GFP+ cell imposed by PAHs could be more severe than the apparent decrease by percentage of VASA:GFP+ cells. We also contrast our results with follicular atresia. Follicular atresia is a process that appears to be regulated in large part via hormonal apoptotic pathways/processes acting through other receptor pathways, such as the tumor necrosis factor-alpha ligand/receptors, Fas ligand/receptors, and others (Kaipia and Hsueh 1997). In contrast, AHR-mediated apoptosis is a chemical-induced apoptosis with ligands of aromatic hydrocarbons and acting through a distinct signaling pathway.

Even though recent studies suggested that pluripotent stem cells might replenish depleted oocyte populations endowed at birth (Johnson et al., 2004, 2005), independent analysis indicated that replenishment of the mammalian germ cell population in females does not occur under normal physiological conditions (Eggan et al., 2006). Hence, the consequences of reducing germ cell numbers by 30–50% in humans in vivo would be expected to strongly and negatively impact fertility because of the limited number of available oocytes at birth and the subsequent decline in numbers until menopause in women. Indeed, in recent studies, we demonstrated that silencing of the germ cell–specific gene, DAZL, results in a twofold reduction in germ cell numbers associated with aberrant expression of germ cell genes in vitro (Kee et al., 2009). This genetic analysis, in vitro, supports results of population-based studies on reproductive status and DAZL polymorphisms (Tung et al., 2006a,b). Thus, it appears that differentiation of hESCs to the germ line can provide an

experimental system for both genetic analysis of germ cell formation as previously shown (Kee et al., 2009) as well as analysis of environmental toxicology as shown here.

Notably in this study, we observed that by silencing AHR, we could directly examine the effect of environmental perturbation and molecular pathway of the adverse effect on germ cell formation, which has never been feasible before on a human genome background. Although gene expression was reduced to 50% (not as complete as the previously reported mouse knockout; Robles et al., 2000), we observed a similar requirement for AHR in hESC differentiation as observed in mouse knockout models. Given the extensive epidemiological data that have been collected retrospectively, in most cases, indicating adverse fetal effects of maternal exposure to PAHs, methods to directly assess lineage-specific human developmental defects are much needed. These studies suggest that at least in regards to PAH exposure, the hESC system allows robust examination of environmental factors or extrinsic factors on human development, especially the germ cell lineage in early development. This opens the door for large-scale toxicological or drug-screening studies for basic or clinical reproductive biology. In the future, investigation of applications to other types of pluripotent stem cells such as induced pluripotent stem cells and analysis of response of individual genetic composition to environmental perturbation is also merited.

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