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Pluripotency factors and Polycomb Group proteins repress aryl hydrocarbon receptor expression in murine embryonic stem cells

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Abstract The aryl hydrocarbon receptor (AHR) is a transcription factor and environmental sensor that regulates expression of genes involved in drug-metabolism and cell cycle regulation. Chromatin immunoprecipitation analyses, *Ahr* ablation in mice and studies with orthologous genes in invertebrates suggest that AHR may also play a significant role in embryonic development. To address this hypothesis, we studied the regulation of *Ahr* expression in mouse embryonic stem cells and their differentiated progeny. In ES cells, interactions between OCT3/4, NANOG, SOX2 and Polycomb Group proteins at the *Ahr* promoter repress AHR expression, which can also be repressed by ectopic expression of reprogramming factors in hepatoma cells. In ES cells, unproductive RNA polymerase II binds at the *Ahr* transcription start site and drives the synthesis of short abortive transcripts. Activation of *Ahr* expression during differentiation follows from reversal of repressive marks in *Ahr* promoter chromatin, release of pluripotency factors and PcG proteins, binding of Sp factors, establishment of histone marks of open chromatin, and engagement of active RNAPII to drive full-length RNA transcript elongation. Our results suggest that reversible *Ahr* repression in ES cells holds the gene poised for expression and allows for a quick switch to activation during embryonic development.

Abbreviations: AHR, aryl hydrocarbon receptor; AhRE, AHR response element; ARNT, Ah receptor nuclear translocator; bHLH/PAS, basic helix–loop–helix/Per-ARNT-Sim; ChIP, chromatin immunoprecipitation; CTD, carboxyl-terminal repeat domain; EB, embryoid bodies; EMT, epithelial-to-mesenchymal transition; ESC, embryonic stem cells; EZH2, enhancer of zeste homolog 2; H3ac, acetylated histone H3; H3K27ac, acetylated lysine-27 of histone H3; H3K27me3/2/1, tri/di/mono-methylated lysine-27 of histone H3; H3K36me3, tri-methylated lysine-36 of histone H3; H3K4me3/2/1, tri/di/mono-methylated lysine-4 of histone H3; H3K9ac, acetylated lysine-9 of histone H3; H3K9me3/2/1, tri/di/mono-methylated lysine-9 of histone H3; HMT, histone methyltransferase; ICM, inner-cell-mass; iPSC, induced pluripotent stem cells; KDM6A/B, lysine demethylase 6A and 6B; KO, knock out; MET, mesenchymal-to-epithelial transition; MLL, myeloid/lymphoid or mix-lineage leukemia; OSKM, OCT3/4, SOX2, KLF4, MYC; PcG, Polycomb Group proteins; PRC1/2, Polycomb repressive complexes 1 and 2; RING1B, ring finger protein 1B; RNAPII, RNA polymerase II; RNAPII ($S5p^+S2p^-$), RNA polymerase II phosphorylated in CTD serine-5 but not serine-2; RNAPII ($S5p^+S2p^+$), RNA polymerase II hyperphosphorylated in CTD serine-5 and serine-2; SUZ12, suppressor of zeste 12 homolog; TxG, Trithorax Group proteins; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TES, transcription end site; TSS, transcription start site.

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Introduction

AHR is a member of the bHLH/PAS family of transcription factors and the main mediator of teratogenic and carcinogenic toxicities resulting from exposure to planar polycyclic and halogenated aromatic hydrocarbons present in the environment (Hankinson, 1995). Activation by ligand causes AHR to translocate to the nucleus, dissociate from its cytosolic chaperones and heterodimerize with its ARNT partner, also a member of the bHLH/PAS family (Reyes et al., 1992). Binding of AHR–ARNT complexes to AHR response motifs in the promoters of target genes recruits transcription cofactors and associated chromatin remodeling proteins and signals initiation of gene transcription (Hestermann and Brown, 2003; Schnekenburger et al., 2007). Increasing evidence indicates that in addition to the well-known xenobiotic metabolism genes in the Cyp1 family of cytochromes P450, there are other AHR transcriptional targets, including genes involved in cell cycle regulation and morphogenetic processes that may play a vital function during embryonic development (Gasiewicz et al., 2008; Sartor et al., 2009). Such a developmental role may be an evolutionarily conserved primary function of the AHR, a notion supported by the finding that unlike their vertebrate counterparts, AHR orthologs in invertebrates like the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* are not activated by xenobiotic ligands but control expression of homeotic genes involved in neuronal specification during development (Emmons et al., 1999; Hahn, 2002; Qin and Powell-Coffman, 2004; Kim et al., 2006). In mice, *Ahr* ablation leads to impaired vasculature in the kidney, liver sinusoid, and eyes of the neonates (Lahvis et al., 2000) with an ensuing cardiovascular disease that might be directly or indirectly the principal cause of other *Ahr* deficit phenotypes, such as reduced liver size, *patent ductus venosus*, cardiac hypertrophy, hypertension, and fibrosis (Fernandez-Salguero et al., 1995, 1997; Lund et al., 2003, 2008; Lahvis et al., 2005).

Several studies have shown a complex pattern of *Ahr* expression during early mouse embryogenesis. Fertilized eggs at the 1-cell stage show detectable levels of *Ahr* mRNA (Dey and Nebert, 1998; Wu et al., 2002) and high levels of AHR activity, as determined by an elevated constitutive mRNA level of the AHR target gene *Cyp1a1* (Dey and Nebert, 1998). Thereafter, *Ahr* mRNA expression is completely silenced between the 2- and 8-cell stages and afterwards increases to a detectable level by late pre-implantation blastocysts (Peters and Wiley, 1995; Dey and Nebert, 1998; Wu et al., 2002). In the post-implantation embryo, *Ahr* mRNA can be demonstrated as early as gestational day 9.5, followed by widespread expansion into almost all developing organs (Abbott et al., 1995; Jain et al., 1998).

Correct reprogramming of the epigenome during embryonic preimplantation stages is essential for the acquisition of pluripotency to ensure the concerted completion of development. The above findings suggest that, concurrent with the time of reprogramming of the embryonic epigenome and establishment of pluripotency in the inner cell mass blastocysts, embryos show low or undetectable levels of *Ahr* expression. It is reasonable to hypothesize that, although needed for post-implantation developmental stages, a functional AHR might be detrimental to the preimplantation process and needs to be silenced during this period.

In ES cells, the pluripotency factors OCT3/4, NANOG and SOX2 form a transcriptional network that controls the expression of several hundred target genes, either by activating the promoters of self-renewal genes or by silencing the promoters of differentiation associated genes (Christophersen and Helin, 2010). The specificity of this silencing resides in the quick regulatory reversibility requiring the interplay between core pluripotency factors, numerous chromatin remodeling complexes, and paused RNAPII molecules, that primes target genes and allows them to be ready for fast activation when required by morphogenetic signals (Medvedev et al., 2012). The promoters of these transcription factors are simultaneously marked by active and repressive histone modifications (i.e., H3K4me3 and H3K27me3, respectively) (Mikkelsen et al., 2007) and are repressed by Polycomb Group-mediated mechanisms, including recognition by Polycomb repressive complexes PRC-1 and -2 which further block transcript elongation by RNAPII (Stock et al., 2007; Endoh et al., 2012). In this study, we examine *Ahr* expression during in vitro non-directed differentiation of mouse ES cells. We find that *Ahr* is silent in these cells, but its expression is quickly restored upon differentiation. ChIP analyses indicate that expression is silenced by the binding of core pluripotency factors and PcG proteins as well as pausing of RNAPII on the *Ahr* promoter. These results are consistent with the concept that *Ahr* silencing is required in ES cells and its expression needed for the completion of subsequent morphogenetic events during differentiation.

Materials and methods

Antibodies and primers

Lists of primary antibodies and primers used in this work are shown in Supplemental Tables S1 and S2.

Culture of embryonic stem cells and in vitro differentiation

C57BL/6N-C2 mouse ES cells (Gertsenstein et al., 2010) bearing the Ahr^{b-1} allele coding for the high ligand-affinity Ah receptor were used throughout this study. Cells were cultured in Dulbecco's Modified Essential Medium (Gibco, Grand Island, NY) supplied with 15% (vol/vol) Knock-Out Serum Replacement (KO-SR, Invitrogen, Grand Island, NY), 1000 units/ml ESGRO Leukemia Inhibitory Factor (LIF, Millipore, Billlerica, MA), 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM MEM non-essential amino acids (NEAA, Invitrogen), and 1 mM sodium pyruvate in a 5% CO_2 humidified incubator at 37 °C. Tissue culture plates used for ES cells were coated with 0.1% gelatin at room temperature for 15 min. Plates with feeder cells were prepared with mouse embryonic fibroblasts pre-treated with 10 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, MO) plated at density of 5×10^4 cells/cm^2. ES cells were cultured either on gelatin coated plates or feeder plates and passaged every second or third day. In vitro non-directed differentiation (hereafter referred to as simply differentiation) was initiated by forming embryoid bodies (EB) in differentiation media either in 25-μl hanging drops (6×10^4 cells/ml) or in