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Does the Aryl Hydrocarbon Receptor Regulate Pluripotency?

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Abstract

Recent evidence from embryonic stem cells suggests that the aryl hydrocarbon receptor (AHR) plays a central role in the regulation of pluripotency, a short-lived property of cells in the early blastula inner cell mass (ICM). Four key observations support this conclusion. The first is the temporal association between upregulation of AHR expression and the onset of cell differentiation, which argues for the AHR as a determinant of cell fate decisions. The second is the repression of the pluripotency factors OCT4 and NANOG by the AHR, which depresses their function and contributes to the cell's exit from pluripotency. The third is the temporal association between changes in global DNA methylation and stage-dependent AHR expression, which parallel each other during embryonic development, suggesting that AHR helps configure a repressive chromatin structure that controls differentiation. The fourth is the incidence of early developmental aberrations that take place in *Ahr*-null mice and cause the disruption of their embryonic program, which is likely to be a consequence of the loss of pluripotency of the *Ahr*^{-/-} ICM cells.

In this short review, we will focus on the modulation of pluripotency as a novel function of the AHR, and on the potentially detrimental developmental outcomes that may result from exposure to environmental toxicants. This line of enquiry brings us to the tantalizing conclusion that by activating mechanisms that modulate pluripotency, AHR regulates embryonic development. The likelihood that exposure to environmental AHR ligands might disrupt developmental processes is a reasonable corollary to this conclusion.

Keywords

Aryl hydrocarbon receptor; embryonic development; pluripotency; cell cycle regulation; cell fate decisions; epigenetic reprogramming

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1. Introduction

For nearly three decades, the primary function of the AHR, the only member of the bHLH/PAS family of transcription factors activated by known ligands [1], was thought to mediate the toxic responses to environmental contaminants such as PAHs, HAHs, and co-planar PCBs. However, discoveries of endogenous ligands and clues from animal studies during the last 10 - 15 years have uncovered new functions for this receptor in the regulation of normal physiology, cellular differentiation, and embryonic development [2-11]. Due to its functional duality between toxicity and normal physiology (Fig. 1) the AHR is uniquely positioned not only to convert signals from environmental toxicants into alterations of embryonic development, but to trigger a sustained state of cardiovascular insufficiency that increases the risk of adult disease [2, 8, 12-16]. Consonant with the postulates of the Barker Theory of the Developmental Origins of Health and Disease [17], developmental cardiac insufficiency may translate in the adult into congenital heart disease.

Disruption of endogenous AHR signaling, either by gene deletion or by exposure to dioxin or other DLCs, leads to alterations of embryonic development and results in pathogenic phenotypes, pointing at the possibility that one of AHR's endogenous functions might be to regulate aspects of the differentiation program defined by cell type specific epigenomic processes. Several lines of evidence support the view that the AHR safeguards normal embryogenesis by coordinating the choices between proliferation and differentiation, two concerted processes that eventually bring about pluripotency loss [18-20]. Appropriate control of AHR expression appears to be essential to maintain pluripotency, an objective that the blastomeres attain by maintaining the AHR in a state of positive repression mediated by the pluripotency factors themselves in combination with Polycomb Group repressors [18, 21]. AHR repression may provide the ICM cells with the opportunity to reprogram gene expression, implementing epigenetic mechanisms that bring about global changes in DNA methylation [22-27]. In addition, the AHR might also play a significant role in the determination of cell fate decisions, as suggested by the correlation between AHR expression and lineage specification after implantation [9, 28]. The fact that the pattern of AHR expression parallels these changes suggests that the association of the receptor with a repressive chromatin configuration might ultimately play a determinant role in differentiation. In this context, the presence of multiple early embryonic defects in *Ahr*-null mice suggests that the lack of AHR may disrupt embryonic programming [28-35].

We have focused this commentary on the regulation of pluripotency by the AHR and on the potentially deleterious outcomes that result from exposure to environmental toxicants. We will discuss the evidence showing the involvement of AHR in the maintenance of the stem cell pool and the determination of cell fate decisions, including an analysis of phenotypes arising in the *Ahr* knockout mice. In closing, we will discuss the mechanisms by which AHR might regulate pluripotency.

2. AHR and the homeostasis of pluripotency

2.1 AHR expression and DNA methylation during mouse embryonic development

The first few cleavage divisions of embryonic development set in motion the transition from the totipotency of the cells in the morula to the pluripotency of the ICM cells in the blastocyst and reset the epigenomic plasticity of the embryo. Among the biochemical modifications that reconfigure chromatin structure and mark this transition, DNA methylation and epigenomic plasticity appear to proceed in opposite directions: the earlier the embryonic time, the greater the epigenomic plasticity and the lesser the extent of DNA methylation [26]. Later, as the embryo implants, the DNA methylome is established in a cell type specific manner that correlates with its fate specification. Interestingly, AHR expression parallels the global demethylation progress of the maternal DNA (Fig. 2). Fertilized eggs have high levels of *Ahr* mRNA and/or protein and their expression is maintained in the embryos up to the 4-cell stage morula, at which time individual blastomeres can be segregated according to the level of *Ahr* expression [22, 23]. *Ahr* mRNA expression completely disappears during the cleavage divisions and becomes *de novo* detectable in early blastocysts [24, 25, 27]. After implantation, the earliest that AHR expression has been detected is in E7.5 embryos [9], becoming thereafter detectable in almost all developing organs [24, 28]. These observations suggest that the AHR may play a stage-dependent developmental function, possibly behaving as a differentiation-promoting factor that opposes pluripotency. Consistent with this hypothesis, our recent study suggests that *Ahr* expression must be repressed lest the ES cells would slow down proliferation and lose pluripotency [18, 21]. A similar AHR expression pattern and regulatory function is found in the differentiation of hematopoietic stem cells and cancer stem cells [36-39] suggestive of a widespread AHR function in the ontogeny of diverse stem cell lineages.

Recently, TCDD exposure was showed to demethylate the promoter of the hepatic *Cyp1a1* gene and induce persistent gene expression changes through the crosstalk of AHR signaling with the thymine DNA glycosylase TDG and the ten-eleven translocation methyl dioxygenases TET2 and TET3 [40]. The parallel between AHR expression level and the amount of globally methylated DNA suggests that the AHR may be functionally associated with the organization of a repressive chromatin configuration. If this were the case, disruption of AHR signaling would lead to aberrant DNA methylation and ultimately to altered ontogeny. In good agreement with this prediction, maternal exposure to TCDD was shown to lead to DNA hypermethylation of murine CD8⁺ T cells and cause long-lasting effects in response to influenza virus infection later in life [13]. Similarly, rats exposed to TCDD *in utero* showed a good correlation between hypermethylation of the *Brca1* gene promoter in mammary tissues and the incidence of breast cancer in adulthood [41]. Furthermore, TCDD exposure during *in vitro* ES cell differentiation decreased the expression levels of the DNA methyltransferase genes *Dnmt3a* and *Dnmt3b* [9], suggesting that AHR may also regulate the expression of enzymes responsible for *de novo* DNA methylation. In conclusion, the AHR appears to have a stage-dependent developmental function that coordinates the organization of the embryonic epigenome through modulation of the DNA methylome.

2.2 AHR function in the maintenance of pluripotency

Due to the limitations inherent to the use of ICM cells *in vivo*, the molecular mechanisms involved in maintaining pluripotency have been more often studied with ICM-derived ES cells *in vitro*, in which cells maintenance of pluripotency appears to be the result of the concerted outcome of epigenetic regulation, cell cycle monitored self-renewal, and balanced transcription [42-44]. In these cells, AHR could regulate pluripotency by controlling both self-renewal and gene expression at the transcriptional level, balancing the expression of the pluripotency factors OCT4, NANOG and SOX2 and of differentiation related markers [18]. Under physiological conditions, *Ahr* is repressed in the ES cells, but its untimely derepression causes pluripotency loss and mitotic delay. These observations suggest that AHR may control the pluripotent population by disrupting their self-renewal through lengthening mitosis. The same is true in hematopoietic stem cells, where knockout of *Ahr* or treatment with an AHR antagonist promotes their expansion and proliferation, indicative of the role of AHR in the maintenance of the stem cell pool [38, 39, 45-47]. Unlike in ES cells, however, AHR maintains the hematopoietic stem cell population by restricting their exit from quiescence. Altogether, these observations point at the possibility that AHR, activated by endogenous signals/ligands, may regulate the maintenance of pluripotency by modulating the size of the stem cell pool, a property closely associated with self-renewal ability.

2.3 AHR and cell differentiation

Observations from *in vivo* studies indicate that the premature expression of pioneer transcription factors during embryonic development determines the cell fate and composition of the succeeding cell populations [48, 49]. In fact, it has been proposed that trophoblast and ICM fates could be predicted from the inter-blastomere expression variability of several genes in embryos at the 4-cell-stage [23], the *Ahr* being one of these genes. Thus like other genes, such as *Sox21*, that have a cell-fate determining function, *Ahr* might also be engaged in the determination of preimplantation embryonic fates. However, when the AHR is untimely derepressed in ES cells, this function causes them to restrict their spontaneous differentiation into cardiomyocytes and to initiate a short-lived commitment to neuroglia [18]. Hence, by priming lineage specification, the AHR may function as a pioneer transcription factor. The possibility of segregating blastomeres on the basis of *Ahr* expression suggests that AHR might be associated with the determination of preimplantation embryonic fates. Consistent with this hypothetical AHR function, *Ahr*^{-/-} ES cells, which have higher OCT4 expression levels than wild type, also have a delayed cardiomyocyte differentiation [10]. Furthermore, *in vivo* studies in *Ahr* knockout mice have shown that, although loss of the gene is not lethal, many embryos are unable to survive through *in utero* development [29] and of those that develop to term, the few that survive the perinatal period grow up with multi-organ dysfunction [30-35]. These observations suggest that the developmental program of *Ahr*^{-/-} embryos is abnormal, likely because of alterations of pluripotency in the *Ahr*^{-/-} ICM cells. We surmise that, as a result of unbalanced pluripotency, *Ahr*^{-/-} embryos engage in biased cell fate decisions that either cause them to discontinue embryonic development and die *in utero* or to survive with multi-organ defects.

3. Potential mechanisms through which AHR modulates pluripotency

3.1 Regulation of cell cycle

The anti-proliferative function of the AHR is conserved between somatic and pluripotent stem cells [18, 50, 51] and, by regulating ES cell proliferation, could modulate pluripotency. In fact, we have recently shown that AHR controls the expression and activity of several mitotic regulators in ES cells, including MID1, 14-3-3, PP2A-B55 α , CDK1, γ 15pCDK1, and CDK1 [18]. *Ahr* derepression causes the loss of pluripotency and delayed mitotic progression through the disruption of MID1-PP2A-CDC25B-CDK1 signaling. These results illustrate the significance of mitotic control in the maintenance of pluripotency, highlighting how untimely *Ahr* derepression may perturb it. Appropriate control of mitosis has also been linked to developmental reprogramming, as indicated by the high responsiveness of the cytoplasm of mitotic zygotes to somatic nuclear transfer [52]. Furthermore, at the 4-cell stage morula, a time when embryonic decisions between proliferation and apoptosis are made, blastomeres can be segregated on the basis of *Ahr* expression, suggesting that the AHR may function as a regulator of early embryonic cell cycle [23, 53]. Taking into account the high levels of AHR expression in early embryos (up to the 4-cell stage), it seems reasonable to propose that by regulating mitosis the AHR functions to maintain pluripotency.

3.2 Interplay between AHR and pluripotency factors in stem cells

In ES cells, the AHR and the pluripotency factors OCT4 and SOX2 mutually regulate each other's expression. The mouse *Ahr* gene is repressed by the binding of OCT4/SOX2/NANOG complexes on a distal silencer domain of its promoter and is quickly derepressed as the cells differentiate [21]. Untimely AHR derepression downregulates the expression of OCT4 and SOX2 and reduces pluripotency [18]. Hence, although *Ahr* expression in ES cells is under the control of the network of pluripotency factors, an increase of AHR expression is likely to counteract the maintenance of pluripotency and to induce exit from the pluripotent state. Consistent with this idea, recent studies reveal that AHR turns off the expression of OCT4 and NANOG through *Alu*-transposon transcription during differentiation of human embryonic teratocarcinoma cells [37], suggesting that AHR may activate a mechanism that controls the expression of pluripotency genes in both pluripotent and differentiation states.

3.3 Epigenetic regulation through DNA methylation

As mentioned earlier, changes on the embryonic DNA methylome could be a potential mechanism for AHR to modulate pluripotency. Although the molecular mechanisms involved in this AHR function remain to be identified, disruption of endogenous AHR signaling by TCDD exposure or *Ahr* ablation during either embryonic development or ES differentiation attest to the potential involvement of AHR in the regulation of DNA methylome changes associated with lineage specification [9, 13, 41, 54, 55]. These effects of AHR signaling disruption may alter the determination of cell fate decisions during embryonic development by modulating the cell-type specific DNA methylome.

A case in point is the regulation of the imprinted *H19-Igf2* locus, which is targeted by AHR during early embryogenesis. Embryos arising from TCDD-exposed fertilized eggs show

decreased expression of both *H19* and *Igf2*, accompanied by hypermethylation of the locus DMR [56]. Similar results were observed in our studies on TCDD treatment of differentiating ES cells, which resulted in downregulation of *H19* expression and a trend to decrease *Igf2* mRNA levels [9] (Fig. 3A). *In vivo*, TCDD exposure *in utero* or knockout of the *Ahr* gene led to altered expression of *H19* and *Igf2* in embryonic cardiac tissues. Both genes were upregulated in E15.5 and E18.5 sinoatrial node areas of the right atrium, an effect that persisted in both male and female adult mice, in which both genes were downregulated in the right atrium [2, 11] (Fig. 3B). This effect in cardiac tissues, common to *Ahr* knockout and TCDD exposure, suggests that TCDD disrupts endogenous AHR functions in the developing heart. Conceivably, one of the developmental functions of the AHR would be to regulate cardiogenesis by modulating the cardiac DNA methylome and the expression of imprinting genes. TCDD exposure may cause heart dysfunction by disrupting AHR's endogenous signaling. In this regard however, the likelihood that the potential disruptive effect of TCDD and other DLCs may depend on their biopersistence, would suggest that more easily metabolized ligands may be much less toxic to the endogenous AHR functions.

4. Conclusion

Whether and how AHR may regulate pluripotency is an open question. Here, we discuss the evidence and suggest potential mechanisms by which AHR might fulfill this function (Fig. 4). We propose that through activation by endogenous ligands AHR participates in the coordination of multiple biological processes that define pluripotency and embryonic development. One possibility is that AHR might control stem cell self-renewal by regulating the cell cycle. Alternatively, AHR might control the expression of both pluripotency factors and differentiation-related markers and might participate in priming cell fate decisions by regulating the balanced transcription of pluripotency genes. Yet another possibility is that AHR might help coordinate the embryonic epigenome by regulating the DNA methylome and imprinting during embryonic development. The appropriate coordination of these AHR functions would promote the development of embryos with normal phenotypes, while exposure to environmental contaminants could derail endogenous AHR signaling and perturb embryonic ontogeny to cause congenital developmental disease and increased risk of adult disease. Paradoxically, the adult impact of developmental exposure might turn out to be much more significant than the impact of direct exposure to the adult itself.

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Abbreviations

AHR	Aryl Hydrocarbon Receptor
DLC	Dioxin-like Compounds
DMR	Differentially Methylated Region

ES	Embryonic Stem
HAH	Halogenated Aryl Hydrocarbon
HSC	Hematopoietic Stem Cells
ICM	Inner Cell Mass
PAH	Polycyclic Halogenated Hydrocarbon
PCB	Polychlorinated Biphenyl
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
TDG	Thymine DNA glycosylase
TET	Ten-Eleven Translocation

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Highlights

The Ah receptor slows down stem cell mitotic progression, playing a central role in the regulation of pluripotency

Early developmental aberrations in *Ahr*-null mice cause the disruption of their embryonic program.

By activating mechanisms that modulate pluripotency, the Ah receptor regulates embryonic development.

Fig. 1.
AHR dual functions in toxicity and normal physiology.

Fig. 2. Schematic illustration of *Ahr* expression and genome-wide DNA methylation throughout the mouse life cycle

(A): High levels of *Ahr* can be detected in early-fertilized eggs until the 4-cell stage morula. The expression completely disappears during cleavage cycles and becomes *de novo* detectable in the early blastocysts. After implantation, *AHR* expression can be detected starting in E7.5 embryos and thereafter can be found in almost all developing-organs. **(B):** Global changes of DNA methylation during epigenetic reprogramming in embryos (from reference [26]). After fertilization, the paternal genome undergoes active demethylation, whereas the demethylation of the maternal genome is slower and depends on DNA replication (by passive demethylation). These post-fertilization demethylation events do not include the differentially methylated regions of genomic imprinting loci. As blastocysts implant and the lineages start to be specified, new DNA methylation profiles are acquired during the specification of embryonic cells. Abbreviations: Z - zygote, 2C - 2-cell stage embryo, 4C - 4-cell stage embryo, 8C - 8-cell stage embryo, 8C-M - 8-cell stage morula, 16C-M - 16-cell stage morula, 32C-M - 32-cell stage morula, EB - early blastocyst, LB - late blastocyst, DMR - differentially methylated region, PGC - primordial germ cell.

Fig. 3. *H19* and *Igf2* expression during *in vitro* differentiation of ES cells and in mouse embryonic and adult heart tissues

(A): Expression levels of *H19* and *Igf2* at 1, 2, 3, and 4 days of ES differentiation with and without TCDD exposure. **(B):** Expression of *H19* and *Igf2* in cardiac sinoatrial node of *Ahr*^{-/-} mice and *Ahr*^{+/+} mice exposed *in utero* to TCDD or corn oil isolated from E15.5 and E18.5 embryonic hearts and in male and female right atria from adult heart. Abbreviations: Diff. - differentiation, Dev. - developmental, E - embryonic day, A - adult, M - male, and F - female.

Fig. 4. Summary of potential mechanisms by which AHR may regulate embryonic development by modulating pluripotency

AHR could regulate embryogenesis by modulating the essential mechanisms that define pluripotency. The involvement of AHR in cell cycle regulation, notably mitotic progression, in early embryonic cells could have a major impact in the maintenance of the stem cell pool. The regulatory role of AHR in the expression levels of pluripotency factors could interfere with the determination of cell fate decisions as stem cells differentiate. AHR could also participate in the acquisition of epigenome plasticity by modulating the cell type specific DNA methylome and genomic imprinting. Collectively, normal AHR functions result in the development of embryos with normal phenotypes; to the contrary, ablation of *Ahr* or derailed endogenous signaling caused by exposure to environmental toxicants would lead to disease and/or increased disease susceptibility.