A remarkable new target gene for the dioxin receptor
The Vav3 proto-oncogene links AhR to adhesion and migration

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The dioxin receptor (AhR) is possibly the best characterized xenobiotic receptor because of its essential role in mediating the harmful effects of highly toxic environmental pollutants. Despite the fact that AhR-dependent toxicity is a major environmental concern, compelling evidence has recently been produced unveiling novel and remarkable endogenous functions of AhR in cell physiology and tissue homeostasis. Adding to its role in cell proliferation and differentiation, AhR is also involved in the control of cell adhesion and migration, both highly relevant tasks in development and in disease states such as cancer. Interestingly, the effect of AhR on cell migration is cell-type specific because it can sustain or slow down cell motility. Here, I will comment on our recent report showing that AhR is a positive regulator of fibroblast cells migration. Besides characterizing the phenotype of such mesenchymal cells, the most important single finding of our study is that AhR uses the cytoskeleton regulator and oncogen Vav3 to signal through small Rho GTPases, ultimately leading to the physiological control of cell adhesion and migration. These data reveal that AhR activity is required to maintain signaling pathways governing normal cell function and open the question of whether AhR plays a role in cell migration during development and in pathological conditions such as tumor metastasis.

For several decades, the aryl hydrocarbon (dioxin) receptor (AhR) has been studied as a paradigm of xenobiotic-activated intracellular receptor that mediates many of the harmful effects of environmental toxins and carcinogens. These studies attract a great deal of interest because many xenobiotics represent a serious concern to the Biosphere, in general, and to human health, in particular. One of the most important discoveries resulting from all this work is that AhR is a transcription factor that regulates the expression of xenobiotic metabolizing enzymes and, in fact, a quite accurate signaling pathway has been proposed that explains how AhR could mediate toxic responses. Yet, several important observations led to the hypothesis that AhR is not solely intended for xenobiotic metabolism. Its high degree of conservation during evolution and its constitutive expression, its early appearance in metazoans and the physiological roles of closely related proteins are clues about the implication of AhR in physiology. Nevertheless, it has been the production of genetically modified mice and the study of invertebrate models that has provided strong evidence for the involvement of AhR in tissue and organ homeostasis and established the bases to characterize its cellular functions. It should be noted, however, that both activities of AhR (e.g., xenobiotic-related vs. xenobiotic-unrelated) could follow similar molecular pathways since xenobiotics seem to act by exacerbating/deregulating endogenous cellular processes.

AhR shares important functional motifs with proteins that regulate myogenesis, circadian rhythms, organ development and neurophysiology; all members of the basic-helix-loop-helix (bHLH) family of transcriptional regulators. Specifically, AhR belongs to the Class VII of bHLH.
proteins, which have the unique property of containing a PAS (PER-ARNT-SIM) domain for ligand binding. To be functional, AhR needs to dimerize with the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT), a molecular interaction that allows the heterodimer to recognize consensus regulatory binding elements (xenobiotic responsive element, XRE) located in the promoter of target genes; classically cytochromes P450 and phase II enzymes.11 The interest to decipher the endogenous activity of AhR has encouraged the identification of novel AhR target genes through either microarray-based studies12 or by functional analyses of individual candidates (reviewed in refs. 1 and 13). Such ability to regulate the expression of xenobiotoxic unrelated genes gives additional support for the role of AhR in normal cell function.

During the last few years, our laboratory has been interested in analyzing the endogenous roles of AhR in vitro and in vivo and, in particular, we have focused on its role in cell proliferation and tumor development. A question that is considered of paramount importance in cancer is how the stroma interacts with tumors and by doing so how it modulates their growth and dissemination. In early studies,14 we produced and characterized immortalized fibroblast cell lines from mammary gland stroma of AhR-expressing (AhR+/+) and AhR-null (AhR−/) mice. Subcutaneous xenografts of these cell lines in immunocompromised mice revealed that AhR+/+ fibroblasts had a very limited ability to induce tumors in vivo and, importantly, that they had decreased migration efficiency in a collagen I matrix.14 The fact that this same migratory phenotype was observed in primary fibroblasts isolated from AhR+/+ embryos,13 and since mutation of the Caenorhabditis elegans heterolog AHR-1 also impairs cell and axon migration,15 we decided to further analyze the role of AhR in cell adhesion and migration. A first clue about the potential mechanisms involved come from the differences in cell area and polarization observed between both cell lines. AhR+/+ fibroblasts had larger cell area and were less polarized than AhR−/− fibroblasts. Considering that cell morphology is closely related to F-actin and cytoskeleton dynamics and to the recycling of cell-cell and cell-substratum interactions,16 these results prompted us to investigate actin stress fibers and focal adhesions (FAs) in cells of both genotypes. In agreement, AhR-null cells had a major increase in actin stress fibers and FAs, suggesting the existence of a mechanism connecting AhR to the cytoskeleton.17 Moreover, when we tested the functional significance of these alterations, we found that AhR+/+ fibroblasts had a marked increase in cell adhesion and spreading associated to their impaired migration. A potentially relevant candidate to help explain such effects is the RhoA/Rac1 small GTPase family of proteins. Rho/Rac GTPases are essential regulators of cell adhesion, spreading and migration.18,19 Accordingly, RhoA regulates the formation of F-actin stress fibers and focal adhesions20,21 while Rac1 promotes the formation of lamellipodia and membrane ruffles.19,22 Interestingly enough, in certain cell types, high levels of Rac1 activity are able to inhibit RhoA and to decrease their actin stress fibers content and number of focal adhesions.23,24 In agreement to these observations, highly adherent and less motile AhR+/+ fibroblasts had decreased Rac1 and increased RhoA activities that were relevant for the AhR-null morphology as determined by the use of specific pharmacological inhibitors.

Biochemically, Rho/Rac proteins cycle between inactive (GDP-bound) and active (GTP-bound) states. The GDP to GTP transition is controlled by GDP/GTP exchange factors (GEFs) that favor the rapid transition to the activated state during cell signaling. Remarkably, the proto-oncogene Vav3, a GEF for Rho GTPases with a relevant role in cytoskeleton dynamics, was severely downregulated at the mRNA and protein levels in AhR+/+ fibroblasts. It is particularly relevant that the constitutive expression of Vav3 significantly contributes to the reorganization of the cytoskeleton and to the formation of lamellipodia and membrane ruffles.25,26 Considering these data altogether, we hypothesized that decreased Vav3 expression in AhR-null fibroblasts could alter Rac1 and RhoA activities, cytoskeleton stability and the morphology of the cell, ultimately leading to increased adhesion and reduced migration. In addition to the interest of these observations, these results also identified Vav3 as a novel AhR target gene that expands the number of cellular functions requiring AhR activity. Promoter analyses, chromatin immunoprecipitation (ChIP) assays and site directed mutagenesis led to the observation that Vav3 expression is not inducible by exogenous AhR ligands in neither immortalized nor embryonic fibroblasts. This means that AhR is devoted to the regulation of the constitutive transcription of Vav3. These results add even further strength to our hypothesis stating that AhR signals in a major pathway that governs cell morphology, adhesion and migration. The fact that AhR, a ligand-activated transcription factor, regulates the constitutive expression of Vav3 could appear intriguing. However, Vav3 is not the only gene to be constitutively regulated by AhR, albeit only few have been characterized to date. Former studies on AhR+/− mouse liver revealed that lack of receptor produced a large reduction in constitutive cytochrome P450 1A2 (CYP1A2) mRNA while AhR represses the constitutive expression of the c-Myc proto-oncogene27 and the latent TGFβ binding protein LTBP1.28,29 Taken together, these data support the attractive possibility that AhR regulates a battery of genes controlling cellular homeostasis.

The functional relevance of Vav3/Rac1/RhoA signaling in mediating the AhR-dependent fibroblastic phenotype was demonstrated by down-modulating Vav3 expression through RNA interference (RNAi) in AhR+/+/ fibroblasts. Specific Vav3 small interfering RNAs (siRNA) switched the patterns of actin stress fibers and FAs, as well as the attachment and adhesion efficiencies of AhR+/+ cells to values typical of AhR−/− fibroblasts. Furthermore, the analysis of embryonic fibroblasts from Vav3 knock-out mice30 showed increased cell area and higher actin stress fibers content, a phenotype that resembles that of AhR+/+ fibroblasts. Although the AhR-Vav3 pathway appears functionally relevant for the control of cell adhesion and migration in mesenchymal fibroblast cells, other interesting mechanisms could be also involved. For instance, since AhR has cullin 4B ubiquitin ligase activity,31 the ligand-bound receptor could use the proteasome to adjust the levels of migration-associated
proteins by a mechanism similar to that previously reported for steroid receptors.\(^3\) On the other hand, additional signaling pathways could collaborate and/or converge with Vav3 to finally determine the AhR-dependent cell phenotype. One interesting possibility relies on the dynamics of the cell-substratum interactions because suppression of adherent primary mouse keratinocytes\(^2\) and mouse hepatoma Hepa-1c1 cells\(^3\) induced the nuclear translocation and transcriptional activation of AhR, thus supporting AhR as an intermediate molecule in cell adhesion. Current studies are underway to address those questions and to more precisely define, within the context of AhR expression, the importance of Rac1/RhoA activities in pathologies concerning an altered control of cell migration, as for instance in tumor metastasis.

As discussed above, the physiological activities of AhR could be closer to its xenobiotic-related functions than previously thought. Based on this assumption, it is worthy to consider that AhR could also mediate the effects of toxic xenobiotics on cell adhesion and migration. Interestingly, an initial study revealed that suspension of C3H10T1/2 fibroblasts increased the transcriptional activity of AhR to levels similar to those obtained after treatment with dioxin,\(^1\) suggesting the existence of common mechanisms of regulation. More recently, several relevant studies have defined additional signaling pathways that could mediate the effects of xenobiotics on cell adhesion and migration through AhR. Using human breast cancer MCF-7 cells, Diry and collaborators first reported that dioxin induced a reorganization of the cytoskeleton and an increase in the number of lamellipodia that were associated to higher migration rates of these cells. Mechanistically, such effects on morphology and migration involved reduced expression of the cell-cell interaction protein E-Cadherin and activation of the c-Jun N-terminal kinase (JNK) pathway.\(^3\) In following studies, the same laboratory has further defined the signaling involved in the modulation of adhesion and migration by dioxin. They found that dioxin induces the expression of a member of the Cas family of proteins, Nedd9/Hef1/CasL,\(^3\) which has been postulated as a pro-metastatic gene in the dissemination of several cancers including melanoma\(^7\) and glioblastoma.\(^3\) Moreover, Nedd9/Hef1/CasL appears to have a relevant role in the mechanisms controlling epithelial cell adhesion and migration because it is required for dioxin to modulate E-Cadherin expression and JNK activation through AhR. Thus, altogether, these studies provide strong experimental evidence for a role of AhR in cell adhesion and migration and further support the hypothesis that dioxin acts by mimicking/exacerbating physiological signaling pathways requiring endogenous AhR activity.

The proposed mechanism that could integrate AhR in the Vav3-dependent signaling pathway that controls fibroblast cell adhesion and migration is depicted in Figure 1. We suggest that under physiological cell conditions, endogenous AhR activity maintains constitutive Vav3 expression. A cell status favoring migration will increase Vav3-dependent Rac1 activity which, in turn, will promote the formation of membrane ruffles and stimulate cell migration. In addition, the net balance between the positive modulation through Vav3 and the inhibitory effect exerted by Rac1 will determine RhoA activity and, as a result, the amount of actin stress fibers observed in AhR\(^+/+\) fibroblasts.

In a broad perspective, the involvement of AhR in cell migration adds an important function to its well known implication in the control of cell proliferation, differentiation and apoptosis. Future work will surely provide novel and exciting proofs for the importance of this receptor in developmental processes and in disease states. Particularly relevant are the epithelial-to-mesenchymal transition (EMT) and its reversal, the mesenchymal-to-epithelial transition (MET) since they have major roles in both normal development and in the acquisition of a metastatic phenotype by many tumor cells. The characterization of these new activities will luckily fulfill the mechanistic and functional requirements needed to

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**Figure 1.** Proposed mechanism of AhR-dependent control of cell migration through interaction with Vav3 signaling. Under physiological cell conditions, AhR binds to the Vav3 promoter and regulates its constitute expression. Vav3 protein then serves as GDP/GTP exchange factor (GEF) for the small GTPases Rac1 and RhoA. Once in their GTP-bound form, Rac1 and RhoA modulate cell migration, plasma membrane protrusions and cytoskeleton reorganization. The inhibitory effect of high levels of Rac1 activity on RhoA-dependent control of stress fibers is considered (blunt arrow).
propose AhR as a marker/target molecule susceptible to be modulated in such health threatening illnesses as cancer.

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