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REVIEW

The aryl hydrocarbon receptor (AhR) in the regulation of cell–cell contact and tumor growth

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The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor, which is activated by a large group of environmental pollutants including polycyclic aromatic hydrocarbons, dioxins and planar polychlorinated biphenyls. Ligand binding leads to dimerization of the AhR with aryl hydrocarbon receptor nuclear translocator and transcriptional activation of several xenobiotic phase I and phase II metabolizing enzymes, such as cytochrome P4501A1 and glutathione-*S*-transferase, respectively. Since phase I enzymes convert inert carcinogens to active genotoxins, the AhR plays a key role in tumor initiation. Besides this classical route, the AhR mediates tumor promotion and recent evidence suggests that the AhR also plays a role in tumor progression. To date, no mechanistic link could be established between the canonical pathway involving xenobiotic metabolism and AhR-dependent tumor promotion and progression. A hallmark of tumor promotion is unbalanced proliferation, whereas tumor progression is characterized by dedifferentiation, increased motility and metastasis of tumor cells. Tumor progression and presumably also tumor promotion are triggered by loss of cell–cell contact. Cell–cell contact is known to be a critical regulator of proliferation, differentiation and cell motility *in vitro* and *in vivo*. Increasing evidence suggests that activation of the AhR may lead to deregulation of cell–cell contact, thereby inducing unbalanced proliferation, dedifferentiation and enhanced motility. In line with this is the finding of increased AhR expression and malignancy in some animal and human cancers. Here, we summarize our current knowledge on non-canonical AhR-driven pathways being involved in deregulation of cell–cell contact and discuss the data with respect to tumor initiation, promotion and progression.

Introduction

The aryl hydrocarbon receptor (AhR) is a transcription factor belonging to the basic helix-loop-helix/Per/ARNT/Sim (PAS) family (1–3). Among this group of proteins, the AhR is the only one that is activated by a ligand. It was originally discovered due to its stimulation by a variety of planar aromatic hydrocarbons with benzo[*a*]pyrene (B[*a*]P) as prototype (4). To date, >400 exogenous ligands have been identified. In addition to polycyclic aromatic hydrocarbons (PAHs), the AhR is activated by dioxins including dibenzofurans and planar polychlorinated biphenyls (PCBs) (5). One of the most potent ligands known so far is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). It is generally accepted that the toxic responses of these environmental pollutants are the direct consequence of AhR activation. Interestingly, also naturally occurring compounds, such as indoles and several flavonoids, which are present in food may act as AhR agonists. In search for potential endogenous AhR ligands, diverse compounds such as tryptophan derivatives, arachidonic acid metabolites, equilenin, heme metabolites and indigoids have been characterized (6). Furthermore, the AhR is activated by UV photoproducts of tryptophan and is regulated by non-ligand signals such as cyclic adenosine monophosphate (7,8). However, the physiological or toxicological consequences of AhR activation by these ligands are mostly unclear.

Biochemical and genetic studies using prototypic AhR agonists, such as B[*a*]P and TCDD, have led to unravel important AhR-dependent pathways and to understand at least some of the toxic effects of these environmental pollutants. A common response after ligand binding to the AhR is induction of gene expression. In the cytosol, the unliganded receptor forms a complex with two heat shock protein 90 (Hsp90) molecules, at least one immunophilin homologous protein and co-chaperones (Figure 1) (9–11). Binding of the ligand results in nuclear translocation of the AhR, dissociation from the chaperone proteins, heterodimerization with ARNT and subsequent binding of the AhR–ARNT heterodimer to dioxin-responsive elements (DREs) with the consensus core recognition sequence 5′-TNGCGTG-3′, also known as xenobiotic-responsive elements (XREs). This leads to transactivation of several genes encoding phase I and II xenobiotic metabolizing enzymes, such as cytochrome P450s (CYP1A1, CYP1A2 and CYP1B1) and glutathione-*S*-transferase, NAD(P)H: quinone oxidoreductase 1 and aldehyde dehydrogenase 3, respectively (Figure 1) (3,12). Activation of the AhR pathway by PAHs therefore leads to their detoxication and excretion and, at the same time, to their metabolic activation to genotoxic compounds. Studies in CYP1A1 and CYP1B1 knockout mice indicate that CYP1A1 is predominantly important for detoxication, whereas CYP1B1 is required for metabolic activation of B[*a*]P after oral administration (13,14). B[*a*]P is metabolically activated to the ultimate genotoxic B[*a*]P-7,8-diol-9,10-epoxide and finally binds to DNA forming N^2-B[*a*]P-7,8-diol-9,10-epoxide-guanine adducts. Since carcinogenicity of PAHs is lost in AhR knockout mice (15,16), it is generally accepted that this 'canonical' AhR-dependent pathway is required for tumor initiation by PAHs in animals and very likely in humans as well.

In contrast to PAHs, TCDD is metabolically inert and hence does not lead to genotoxic metabolites. However, TCDD is known to be one of the most potent tumor promoters in the liver ever studied in animal models (17). Although still not finally proven in AhR knockout models, it is believed that the tumor-promoting effect of TCDD (and related compounds) is mediated by the AhR. For instance, strains from rats or mice expressing low-affinity AhR show decreased sensitivity in classical tumor initiation/promotion studies (18,19). A role of the AhR in tumor promotion is also suggested by studies in mice expressing a constitutively active AhR (20). However, the canonical AhR pathway evolving xenobiotic metabolism failed to explain the tumor-promoting effects of TCDD, and no mechanistic link between CYP induction and TCDD toxicity could be established so far (21). Moreover, *in vivo* studies in two genetically different rat strains indicate that AhR-driven CYP1A1 induction and tumor promotion can be uncoupled from each other (22). Although it has been shown that TCDD induces suppression of apoptosis *in vitro* upon treatment with UV-C (23) and during tumor promotion in rats (24), the molecular mechanism of AhR-dependent tumor promotion is unknown to date (25). However, the fact that mice expressing a constitutively active AhR show an increase in the development of stomach tumors (26) implies that activation of the AhR leads to deregulation of cell cycle control

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; B[*a*]P, benzo[*a*]pyrene; Cdks, cyclin-dependent kinases; CYP, cytochrome P450; EGCG, epigallocatechin-3 gallate; EMT, epithelial–mesenchymal transition; Hsp90, heat shock protein 90; JNK, c-Jun NH_2-terminal kinase; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T-cells; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; pRB, retinoblastoma protein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element.

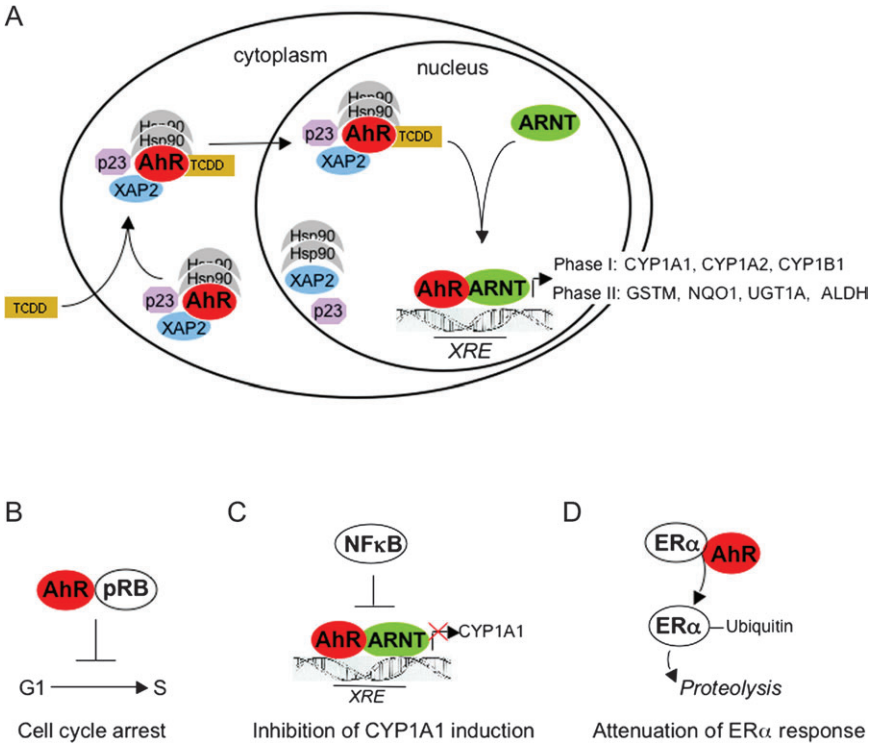

Fig. 1. Canonical and non-canonical signaling pathways of the AhR. (**A**) Schematic representation of canonical AhR signaling pathway. The cytosolic AhR is complexed by two molecules of Hsp90, XAP2 and the co-chaperone p23. Binding of a ligand, e.g. TCDD, leads to a conformational change, thereby allowing nuclear translocation of the AhR complex. In the nucleus, the AhR dissociates from the complex and dimerizes with ARNT. The AhR–ARNT heterodimer then binds to xenobiotic-responsive elements (XREs) in the promoters of genes encoding for several phase I and phase II metabolizing enzymes but also several other genes, e.g. CYP2S1, COX2 or Slug (139). Recruitment of additional co-factors and factors of the basal transcription machinery (not shown) finally allows transcription of these genes. GSTM, glutathione-*S*-transferase M; NQO1, NAD(P)H:quinone oxidoreductase 1; UGT1A, uridine 5′-diphosphate-glucuronosyltransferase 1A; ALDH, aldehyde dehydrogenase (**B**–**D**) Schematic representation of examples of non-canonical AhR signaling. (B) In pRB-proficient cell lines, activation of the AhR by exogenous ligands may lead to direct interaction with pRB and via several mechanisms to inhibition of the transcription factor E2F (99). As a consequence, progression from G_1- to S-phase is blocked. Cell cycle arrest may also be induced by additional mechanisms, such as induction of p27 (36,140). (C) Reciprocal inhibitory effects between the nuclear factor kappa B (NF-κB) and the AhR pathway have been described. One consequence of inhibition of AhR signaling by NF-κB is attenuation of ligand-induced CYP1A1 expression. However, also cooperative effects of the AhR and NF-κB pathways are known (37). (D) The ligand-activated AhR directly associates with estrogen or androgen receptors (ERα or AR) and modulates their function both positively and negatively. Recently, it was shown that the AhR promotes the proteolysis of ERα/AR through assembling a ubiquitin ligase complex, CUL4B(AhR) (38).

in vivo. The role of the tumor-promoting effect of the AhR in humans is still unclear. Although epidemiological data indicate that TCDD is a human carcinogen, which causes hematopoietic, lymphatic and possibly breast cancer (27) and is, therefore, classified as Group 1 carcinogen by the International Agency for Research on Cancer (28), it does not significantly enhance liver tumor formation in humans. However, this observation does not rule out a role of the AhR in tumor promotion in other organs such as lung, which could occur in response to mixtures of PAHs including weak or non-genotoxic AhR ligands.

Beside its function in tumor initiation and tumor promotion, recent studies suggest that the AhR also plays a role in tumor progression, i.e. in the transition from a benign to a malignant tumor, in animal models and very likely also in humans. For instance, AhR expression is higher in invasive than in non-invasive tumor cells and tissue (29,30), and the level of expression even correlates with malignancy in lung tumors (31). Down-regulating the AhR function in lung adenocarcinoma cells diminishes anchorage-independent growth *in vitro* (31). Up-regulation of nuclear AhR expression in human urothelial tumors is associated with increased invasion and poor prognosis (32). Importantly, fibroblasts derived from AhR knockout mice show decreased tumorigenicity and migration in a xenograft model due to down-regulation of the proto-oncogene Vav3 leading to a decrease in Rac1 activity (33,34). In support of this, 7,12-dimethylbenz[*a*]anthracene-induced breast tumors in rats are more invasive when the animals are co-exposed to a dioxin-like PCB (35). In summary, there is strong evidence for a pivotal role of the AhR not only in PAH-dependent tumor initiation but also in tumor promotion and progression. The current data indicate that the AhR induces transcription of genes beyond metabolism. Thus, in addition to the canonical signaling cascade shown in Figure 1A, several non-canonical mechanisms of the AhR have been described recently (Figure 1B–D). For instance, activation of the AhR leads to cell cycle arrest in G_1-phase in several cell lines, which is at least partly due to a direct association of the AhR with the hypophosphorylated retinoblastoma protein (pRB), thereby inhibiting progression into S-phase (36). It was also found that the transcription factor nuclear factor kappa B modulates AhR signaling. For example, direct interaction of the AhR with nuclear factor kappa B leads to down-regulation of AhR-dependent induction of CYP1A1 *in vitro* and *in vivo* (37). In addition to its well-known function as a transcription factor, the AhR has been shown to possess E3 ubiquitin ligase activity, e.g. leading to degradation of the estrogen receptor α (38).

Cell–cell contact is known to be a critical regulator of cellular proliferation, differentiation and motility. Inhibition of proliferation by cell–cell contact is generally referred to as contact inhibition or contact-dependent inhibition of growth (39). In adult tissues, contact inhibition is thought to be continuously active, playing a critical role in the repression of somatic cell proliferation. Vice versa, release from contact inhibition *in vivo* and *in vitro* is associated with abnormal cellular proliferation (40). Since tumor promotion is characterized by unbalanced proliferation either due to increased proliferation or decreased level of apoptosis, it is very likely that loss of contact

inhibition is a possible event during tumor promotion. Moreover, loss of proper cell–cell adhesion is the *sine qua non* for tumor progression, which rests on dedifferentiation, migration and invasion of tumor cells.

Recent work from our laboratory and others has shown that the AhR triggers pathways leading to a release from contact inhibition as well as loss of cell–cell adhesion. In this review, we summarize the current knowledge about the action of the AhR on cell–cell contact, thereby inducing loss of contact inhibition, dedifferentiation and migration. (For a recent review on the role of the AhR in cell substratum adhesion, integrins and matrix metabolism, see 41.) In the first section, we will focus on signal transduction pathways of contact inhibition in fibroblasts and in epithelial cells as far it is relevant for understanding the crosstalk between cell–cell contact and the AhR. We will then describe the action of the AhR on contact inhibition. Finally, data will be presented on the involvement of the AhR in disturbing cell–cell adhesion leading to dedifferentiation and migration. The data will be discussed as to tumor promotion and progression.

Contact inhibition and cell–cell adhesion

Contact inhibition

In vitro, contact inhibition becomes apparent by the fact that adherent, non-transformed cells are arrested in G_1-phase at a critical cell density forming a confluent monolayer. In contrast, transformed cells are characterized by loss of contact inhibition manifested by a higher saturation density and the emergence of multi-layered foci. Despite its importance for cell cycle control, knowledge about the molecular mechanisms mediating contact inhibition and its deregulation during tumorigenesis is still scarce (42–46). It is important to note that the growth-inhibitory signal of contact inhibition finally leads to a cell cycle arrest in G_0/G_1-phase. To date, it is still not fully clear how this inhibitory signal is transduced from the cell membrane to the nucleus and how it is integrated into the cell cycle machinery. In order to understand the impact of contact inhibition on cell cycle regulatory proteins, we shortly describe fundamental issues of the eukaryotic cell cycle (for detailed description of cell cycle regulation, see 47–49).

The eukaryotic cell cycle

Cyclin-dependent kinases (Cdks) are known to be master kinases in cell cycle regulation. The activity of Cdks, which belong to the family of serine–threonine kinases, is strictly dependent on association with cyclins. Cyclin protein levels oscillate during the cell cycle in a defined manner, thus—according to the classical view—ensuring proper activation of specific Cdks at the correct time. In G_1-phase, cyclin D/Cdk4 and downstream cyclin E/Cdk2 phosphorylate members of the pRB family, thus allowing G_1/S-phase transition. Rb proteins bind to and modulate the activity of transcription factors, such as E2F family members, histone deacetylases and chromatin remodeling complexes, thereby repressing transcription of S-phase-specific genes, such as cyclin A. When pRB becomes phosphorylated by cyclin D/Cdk4 and cyclin A/Cdk2, it dissociates from E2F, thus allowing E2F to function as a transcriptional activator. In early S-phase, Cdk2 then binds to cyclin A, changing substrate specificity of Cdk2. It is generally believed that the critical function of the cyclin A–Cdk2 complex is phosphorylation of substrates that start DNA replication and coordinating the end of S-phase, such as DNA polymerase α, proliferating cell nuclear antigen, replication protein A or cell division cycle 6. S/G_2- and G_2/M transitions are regulated by Cdk1, which is sequentially activated by cyclin A and then cyclin B. The activity of Cdks is further modulated by activating and/or inhibiting phosphorylations and dephosphorylations. In addition, the activity of Cdks is regulated by association of small inhibitory proteins, known as p15, p16, 18, p19 (INK family) and p21, p27 and p57 (CIP/KIP family).

Cell cycle and contact inhibition

What happens during contact inhibition? It is now clear that the cell ensures cell cycle arrest by cooperation of several mechanisms (Figure 2). The first evidence that cell cycle regulatory proteins, such as cyclin-dependent kinases, are involved in contact inhibition came from Massagué's group in 1994. They showed in (Mv1Lu mink) epithelial cells that the protein level of the CIP/KIP inhibitor p27 increases at confluence, which inhibits Cdk2 activity (50). We and others then showed that p27 is also up-regulated in confluent human fibroblasts (51), in various epithelial cells and many other cell types (52–57 and our unpublished data). Up-regulation of p27 protein levels in contact-inhibited cells is at least partly dependent on sustained activation of p38α mitogen-activated protein kinase (MAPK), which leads to inhibition of the epidermal growth factor receptor signaling (58,59). Although there is no doubt that p27 is a crucial regulator of cell cycle control, it is at least in fibroblasts dispensable for contact inhibition (60). This indicates further inhibitory mechanisms to be involved such as p16 that was identified as a critical mediator of contact inhibition (61). As a consequence of inhibition of Cdk4 and Cdk2, pRB remains in its hypophosphorylated state, thus inhibiting progression into S-phase (50,51,61). We further provided evidence that upstream of p16, the

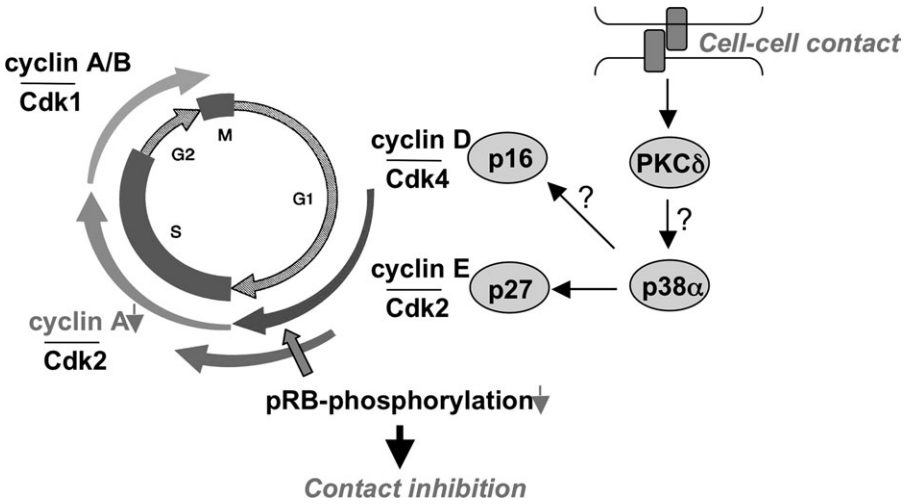

Fig. 2. Signaling cascade of contact inhibition in fibroblasts. Cell–cell contacts lead to a rapid activation of protein kinase Cδ (PKCδ) and a persistent activation of p38α MAPK resulting in accumulation of the KIP inhibitor p27, hence inhibiting cyclin E/Cdk2 activity. Additionally, the INK inhibitor p16 is up-regulated, thereby blocking activity of Cdk4. As a result, pRB remains in its hypophosphorylated state and does not allow transcription of S-phase-specific genes, such as cyclin A. If PKCδ plays a role in activation of p38α MAPK or p38α MAPK is involved in p16 up-regulation remains to be elucidated.

potential tumor suppressor protein kinase Cδ, is involved in contact inhibition (62). Although it is very likely that reorganization of the actin cytoskeleton is one major target of protein kinase Cδ, its precise role in contact inhibition and linkage to the cell cycle machinery still has to be resolved.

Cell membrane proteins

Which cell membrane proteins are responsible for signaling growth inhibition in fibroblasts? Several candidates have been identified, such as contactinhibin and the contactinhibin receptor (63,64), N-cadherin (65), N-CAM (66) and others (42). None of them plays a unique role in contact inhibition and their expression and function entirely depend on the cell type studied.

Cell–cell adhesion in epithelial cells

In epithelial cells, cell–cell adhesion is mediated by Ca^{2+}-dependent homophilic interactions of E-cadherin, which does not only induce contact inhibition, i.e. inhibit proliferation, but also maintain the epithelial phenotype and prevent migration (67–69). Intracellularly, E-cadherin is linked to the actin cytoskeleton by association with α-, β- and/or γ-catenin (plakoglobin). It is generally accepted that one function of E-cadherin is to sequester β-catenin, thereby decreasing the amount of free cytoplasmic β-catenin (70,71). In addition, the cytoplasmic pool of β-catenin is controlled to remain low by proteolytic degradation via the proteasome system (72). Accumulation of cytoplasmic β-catenin, which occurs physiologically during embryonic development and pathologically during tumorigenesis, leads to increased binding of β-catenin to transcription factors of the TCF/LEF family, thereby inducing transcription of not only proliferative but also mesenchymal genes such as *cyclin D1, c-Myc, c-Jun, Id2, Slug or fibronectin* (73,74). Although additional roles of β-catenin in mediating contact inhibition have been identified recently (75), the growth-inhibitory effect of E-cadherin is at least partly mediated by sequestering β-catenin to the plasma membrane, hence preventing transcriptional activation of proliferative genes (76,77 and own unpublished observations). By the same mechanism, E-cadherin prevents transcriptional activation of mesenchymal genes, thereby maintaining the epithelial phenotype.

The role of γ-catenin, a close homologue of β-catenin, seems to be different to that of β-catenin. Obviously, its function in cell adhesion is much more important than in intracellular signaling and transcriptional activity (78). Whereas β-catenin is classified as a proto-oncogene, γ-catenin is considered to be a tumor suppressor. In contrast to β-catenin, γ-catenin is not only associated with E-cadherin but also to desmosomal cadherins (79). Desmosomes are intercellular junctions probably involved in contact inhibition, epithelial differentiation and inhibition of migration similar to the function of and in cooperation with E-cadherin (80).

Epithelial–mesenchymal transition

Disruption of cell–cell adhesion (both E-cadherin-mediated cell adhesion and desmosomes) does not only permit the cells to undergo uncontrolled proliferation but also to dedifferentiate to a mesenchymal phenotype and to migrate, a process which is referred to as epithelial–mesenchymal transition (EMT). EMT is not only a physiological process during embryonic development but also a pathological process whereby primary *in situ* tumors progress toward an invasive or metastatic phenotype. A pivotal initial event in EMT is loss of expression of E-cadherin and γ-catenin. As a consequence, cells lose epithelial markers (i.e. cytokeratine 18), acquire mesenchymal markers such as vimentin, fibronectin and N-cadherin and express proteases that promote cell migration and invasion. Critical mediators of EMT are the zinc finger transcription factors Snail and Slug, which repress transcription of E-cadherin as outlined above (Figure 3) (81).

Cell–cell contact as regulator of the AhR

Early work revealed that the AhR is transiently activated by loss of cell substratum adhesion (82,83). Cho *et al.* then demonstrated that the AhR is regulated by cell density, which rests on the observation

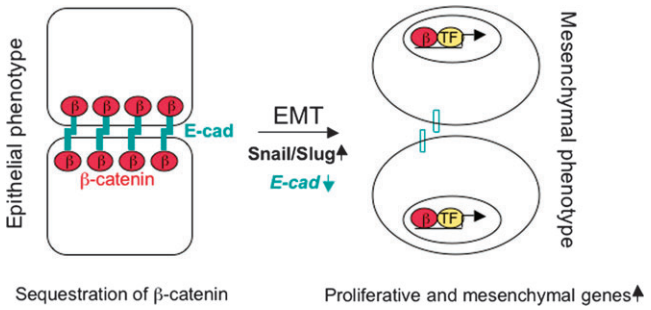

Fig. 3. Schematic illustration of EMT. In epithelial cells, cell–cell adhesion is mediated by homophilic interactions of E-cadherin. One consequence is sequestration of β-catenin, thereby preventing its nuclear translocation. Over-expression of master regulators of EMT, such as Snail and Slug (and others) lead to down-regulation of E-cadherin, hence allowing nuclear translocation of β-catenin. In association with transcription factors (TF) of the TCF/LEF family, β-catenin induces transcription of proliferative and mesenchymal genes (see text for detail). This process is referred to as EMT.

that the AhR is activated by loss of cell–cell contact in the absence of exogenous ligands (84). Thus, in dense cultures of murine C3H10T1/2 fibroblasts, neither nuclear localization of the AhR nor the expression of the AhR target gene CYP1B1 is detectable. However, loss of cell–cell contact either by suspension or seeding the cells at low density provokes nuclear translocation of the AhR with concomitant expression of CYP1B1. In contrast to sustained activation of CYP1B1 by TCDD, transcription of CYP1B1 due to loss of cell–cell contact appears to be transient. Similar findings were obtained with the human keratinocyte cell line HaCaT. Localization of the AhR was found to be predominantly nuclear at sparse cell density and cytoplasmic at confluence (85). Similar data were found upon down-regulating E-cadherin by calcium depletion and in a wound-healing assay. Accordingly, expression of the AhR target gene CYP1A1 is only detectable at low cell density. Furthermore, nuclear export of the AhR is inhibited in sparse cultures by phosphorylation of Ser-68 in the nuclear export signal of the AhR, which is mediated by p38 MAPK (85). In accordance with these *in vitro* findings, the authors demonstrate phosphorylation of the AhR at Ser-68 and nuclear staining of the AhR in the liver of 3-methylcholanthrene-treated mice. Hence, cell–cell contact by E-cadherin provokes a yet unknown signal leading to dephosphorylation of the AhR. It is therefore likely that subcellular distribution of the AhR is regulated by a fine-tuned balance of phosphatases and phosphatase inhibitors in the nucleus, which is dependent on cell density. In contrast to the *in vitro* data described above, expression of the AhR- and TCDD-dependent CYP1A1 induction increases with differentiation of cells in the human epidermis (86). The AhR appears to be localized in the nucleus in proliferating and differentiating murine keratinocytes independent on prior exposure to TCDD (87). Accordingly, exposure of rodent skin to PAHs results in preferential induction of CYP1A1 in differentiating keratinocytes (88).

The AhR is a deregulator of contact inhibition

The first evidence that ligands of the AhR induce proliferation in confluent cell cultures was provided by Milstone and LaVigne more than two decades ago (89). When confluent cultures of newborn human foreskin keratinocytes were treated with nanomolar concentrations of TCDD, cell proliferation was significantly stimulated. The group of Birnbaum then demonstrated that TCDD is able to induce a release from contact inhibition in two human squamous carcinoma cell lines (90). Whereas treatment of exponentially growing cultures with TCDD had no effect on proliferation, treatment of pre-confluent cultures induced a 2-fold increase in saturation density. Later on, similar effects of TCDD on contact inhibition have been observed in the rat liver oval cell line WB-F344 (57,91), Madin-Darby canine kidney and Madin-Darby bovine kidney cells (92: supplementary

data, 93). Interestingly, exposure of WB-F344 cells to other AhR ligands such as PAHs, planar PCBs and some flavonoids also induces a release from contact inhibition, which supports that release from contact inhibition is mediated by the AhR (94–97). Although TCDD-dependent release from contact inhibition was observed in several cell lines and appears to be a common phenomenon, this effect is cell type specific since it is not observed in 5L hepatoma, Hepa, primary murine hepatocytes or HaCaT cells (92 and own unpublished observations). Understanding these cell type-specific responses to TCDD would be instrumental to uncover the basis for the known cell type and organ specificity of TCDD poisoning (98,99).

WB-F344 cells (100) are the best characterized oval cells available so far; when transplanted *in vivo*, they fully differentiate into hepatocytes (101). Oval cells, which are liver stem cells, function as a regenerative reservoir in acute liver damage (102) and may give rise to liver tumors in rodents and humans (103–105). Very recently, Hailey *et al.* (106) published a 2 year exposure study in which rats had been exposed to TCDD or dioxin-like compounds. This study revealed for the first time that oval cells might be targets of TCDD action during liver carcinogenesis. Since transplantation of chemically transformed WB-F344 cells results in the formation of hepatocarcinomas, cholangiocarcinomas and hepatoblastomas (107), we focused on WB-F344 cells as a relevant stem cell culture model for mechanistic studies on TCDD-dependent loss of contact inhibition.

When confluent WB-F344 cultures are treated with nanomolar concentrations of TCDD (or other weak or non-genotoxic AhR ligands such as benzo[*b*]fluoranthene or PCB126), cells cease from G_1 arrest and enter S-phase resulting in a 2-fold increase in saturation density and the emergence of multi-layered foci, which are characteristic features of transformed cells (57,91,94,96,108). That neither exponentially growing cells nor serum-deprived cultures respond to the growth stimulatory effect of TCDD indicates that TCDD does not exert a mitogenic effect *per se* but specifically interferes with the signaling cascade of contact inhibition. The fact that TCDD induces a release from G_1 arrest strongly suggests that cell cycle proteins are targets of TCDD. Indeed, exposure of confluent WB-F344 cells to AhR ligands, such as TCDD, leads to an increase in the S-phase-specific cyclin A and in cyclin A/Cdk2 activity (57,92,109). Interestingly, no increase in the phosphorylation level of pRB was observed (own unpublished observation), indicating that S-phase entry occurs downstream of pRB. In line, ectopic expression of cyclin A in confluent WB-F344 cultures is sufficient to overcome G_1 arrest. Although AhR activation may lead to induction of the activator protein-1-transcription factor c-Jun in 5L hepatoma cells (110), we determined that JunD, but not c-Jun, in association with its partner ATF2 is responsible for cyclin A induction. Activator protein-1 designates a family of dimeric transcription factors consisting of homodimeric Jun family members (c-Jun, JunD and JunB) or heterodimers consisting of Jun with one of the Fos family members (c-Fos, FosB, Fra1 and Fra2) or ATF members (ATF2 and ATFa) (111). Some of them are activated by kinases, such as c-Jun NH_2-terminal kinase (JNK), p38 MAPK (p38) or extracellular signal-regulated kinase (ERK). Interestingly, no increase in the activity of any of the three MAPKs (ERK, p38 and JNK) could be observed in WB-F344 cells in response to TCDD (112 and own unpublished observations). Functional interference with AhR and ARNT revealed that induction of JunD, transcriptional activation of cyclin A and release from contact inhibition in response to TCDD are absolutely dependent on the AhR but very likely independent of ARNT (92,109). This novel signaling cascade triggered by the AhR strongly differs from the classical AhR–ARNT pathway (Figure 4). The functional significance of this pathway has yet to be determined.

In line with a direct effect of TCDD on cell–cell contact, no secretion of the soluble factor transforming growth factor-β was observed (113). This implies that additional targets of TCDD might be cell adhesion molecules. Interestingly, γ-catenin is significantly down-regulated in response to TCDD, whereas no decrease in E-cadherin, α- or β-catenin could be detected (108). Regulation of desmosomal proteins by TCDD has not been analyzed so far. It is not clear if down-regulation of

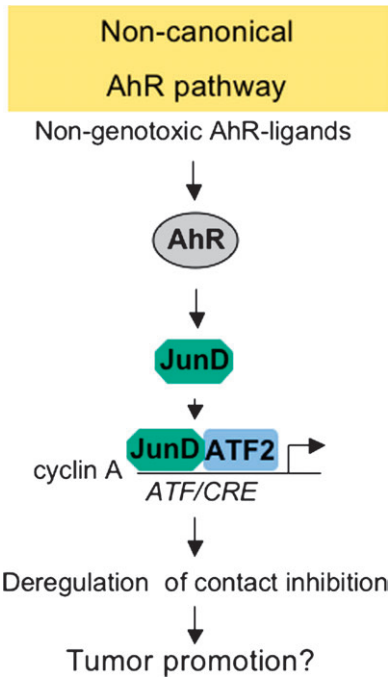

Fig. 4. Proposed novel non-canonical pathway of the AhR. According to the proposed novel non-canonical pathway, non-genotoxic ligands, such as TCDD, lead to activation of the AhR, and very likely independent from ARNT, to induction of JunD, which after heterodimerization with ATF2 results in transcriptional activation of cyclin A finally leading to a release from contact inhibition. If genotoxic ligands may also induce this novel pathway is currently not known.

γ-catenin impairs the function of E-cadherin-mediated cell adhesion and/or of desmosomal cell–cell contacts. The precise mechanism of γ-catenin down-regulation also remains to be elucidated. Since (i) inhibition of γ-catenin degradation by a proteasome inhibitor does not reverse γ-catenin down-regulation and (ii) γ-catenin messenger RNA is down-regulated, it is reasonable to conclude that transcription of γ-catenin is blocked by TCDD, hypothetically by promoter methylation as it has been observed for TCDD-dependent silencing of p16 and p53 (114) or, alternatively, by binding of the AhR to an inhibitory XRE. Such inhibitory XREs have been identified in the promoter region of *c-Fos, COX2 and cathepsin D* gene (115–117). It is supposed that the AhR binds to these DNA sequences, thereby attenuating the activity of other transcription factors such as Sp1 (118). Interestingly, a GC-rich Sp1-binding region is located closely upstream of a putative inhibitory XRE in the human γ-catenin promoter (119).

The AhR is a deregulator of cell–cell adhesion

Increasing evidence is provided that the AhR can stimulate migration and EMT in several cell lines and *in vivo*. Thus, exposure of the human breast cancer epithelial cell line MCF-7 to TCDD or 3-methylcholanthrene leads to down-regulation of E-cadherin, loss of cell–cell adhesion and increased mobility of the cells (120). In contrast to the signaling cascade described in WB-F344 cells, where no increase was observed in the activity of any of the MAPKs in response to TCDD, TCDD leads to a late and persistent activation of JNK in MCF-7 cells. This again indicates cell type and species specificity of AhR function. A gain-of-function analysis revealed that expression of a constitutively active AhR mimicks the effect of TCDD on JNK, i.e. scattering and migration, which supports that the AhR plays a pivotal role in migration of MCF-7 cells. Pharmacological inhibition of JNK blocks the effect of constitutively active AhR and TCDD arguing for JNK as a central mediator of AhR-induced motility. Possible downstream targets of JNK, such as c-Jun, have not been identified in this study. Interestingly, the

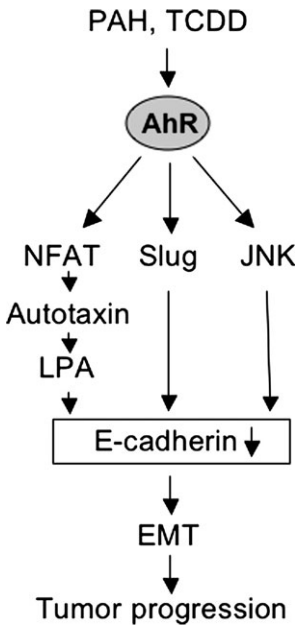

Fig. 5. Potential role of the AhR in EMT and tumor progression. Tumor progression is generally characterized by dedifferentiation of a cell from an epithelial to a mesenchymal phenotype and increased motility. A central process is down-regulation of E-cadherin. Transcriptional inhibition of E-cadherin may either be mediated by AhR-dependent activation of the transcriptional repressor Slug and/or activation of JNK. Breakdown of E-cadherin may also be achieved through activation of the NFAT/autotaxin/LPA pathway (see text for detail).

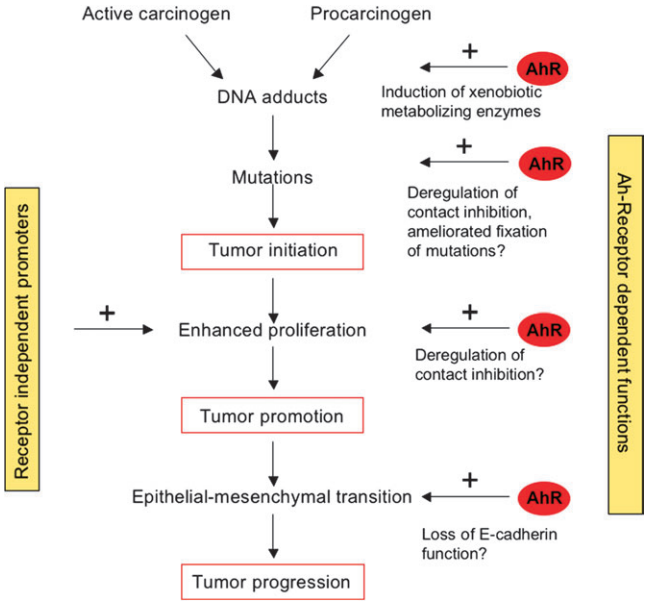

Fig. 6. Hypothetical model of the role of AhR in tumor initiation, promotion and progression. Procarcinogens such as PAHs are known to activate the canonical xenobiotic-responsive element-dependent pathway, thereby leading to their conversion to genotoxic metabolites forming DNA adducts. Mutations are fixed by clonal expansion of initiated cells. Non-genotoxic AhR agonists, such as TCDD, are known to increase cell number, either by inhibition of apoptosis (not included in the figure) (24) or possibly by enhanced proliferation due to loss of contact inhibition providing a mechanistic basis for their tumor-promoting effects. AhR ligands may further lead to breakdown of E-cadherin function by regulating several key players of EMT, thereby driving the process of tumor progression.

JNK/c-Jun pathway has recently been shown to induce DNA methyltransferase 1, thereby leading to promoter methylation on CpG islands and decreased transcription of E-cadherin (121). Whether a similar mechanism is involved in AhR-triggered down-regulation of E-cadherin in MCF-7 cells is not known. In search for an AhR target gene in MCF-7 cells, the authors further identified *Nedd9/Hef1/Cas-L* as the most consistently induced gene in response to 3-methylcholanthrene. They also showed for the human liver hepatoma cell line HepG2 that *Nedd9/Hef1/Cas-L* is a target gene of the AhR, containing two xenobiotic-responsive elements in its promoter, which mediates the effects on JNK activation and E-cadherin down-regulation (122).

Another important AhR target regulating migration might be the nuclear factor of activated T-cells (NFAT) c1/autotaxin signaling pathway (Figure 5). Very recently, Seifert *et al.* (123) discovered that TCDD activates the transcription factor NFAT in MCF-7 cells leading to increased migration. Activation of NFAT resulted in enhanced expression of its target gene *autotaxin*, which is known to exhibit lysophospholipase D activity, thereby generating lysophosphatidic acid (LPA). Although the authors did not investigate the effect of LPA on E-cadherin expression in MCF-7 cells, LPA is known to induce breakdown of E-cadherin-mediated junctions in other cell lines (124).

Ikuta and Kawajiri (125) detected the zinc finger transcription factor Slug as direct target gene of the AhR. Activation of the AhR either by 3-methylcholanthrene or calcium depletion in MCF-7 or HaCaT cells induces transcriptional activation of Slug by binding of the AhR–ARNT complex to an identified XRE in the Slug promoter. Expression of Slug results in down-regulation of the epithelial marker cytokeratine-18 and up-regulation of the mesenchymal marker vimentin. Whether there is a mechanistic link between JNK and Slug, as suggested by observations in lens epithelial cells (126), remains to be determined.

Further evidence for a pivotal role of the AhR in EMT in mammary tumors comes from studies in mouse mammary tumor virus—c-Rel × CK 2a bitransgenic (127) mice. Mammary tumors and cell lines derived from these bitransgenic mice show a highly invasive phenotype with loss of E-cadherin and γ-catenin expression but expression of fibronectin and vimentin. Interestingly, the level of AhR expression seems to correlate with malignancy (128). Human mammary tumors also show aberrant expression of the AhR (129). Co-expression of c-Rel and CK 2 in non-transformed mammary epithelial cells induces the expression of the AhR and Slug and promotes EMT (128). The c-Rel subunit belongs to the family of nuclear factor kappa B transcription factors (130), and CK 2 is a ubiquitously expressed serine–threonine kinase (131). Similar results were obtained in 7,12-dimethylbenz[*a*]anthracene-treated, c-Rel-transformed mammary epithelial cells. Interestingly, the malignant phenotype can be prevented and reversed *in vitro* by co-treatment with the polyphenol epigallocatechin-3 gallate (EGCG) as—even more importantly—EGCG prevents the invasive phenotype of rat mammary cancer induced by 7,12-dimethylbenz[*a*]anthracene *in vivo* (128). The precise mechanism of this protective effect of EGCG is not known so far. However, EGCG has been shown to block AhR-dependent transcription indirectly by binding to Hsp90 (132). It was found that EGCG binds at or near to a C-terminal ATP-binding site in Hsp90 leading to stabilization of the AhR–Hsp90–XAP2 complex, which still translocates to the nucleus but is unable to dimerize with ARNT (133). Thereby, AhR-dependent gene transcription cannot occur (133).

The findings show that ligand-dependent activation of the AhR may induce EMT, a key process during tumor progression. A central mediator of EMT is down-regulation of the cell adhesion molecule E-cadherin. Down-regulation of E-cadherin allows β-catenin to translocate to the nucleus, thereby, in association with transcription factors of the TCF/LEF family, inducing transcription of proliferative and mesenchymal genes. Transcriptional inhibition of E-cadherin may therefore be the result of AhR-dependent activation of the transcriptional repressor Slug and/or activation of JNK. As outlined above, down-regulation of E-cadherin may also be mediated by activation of the transcription factor NFAT, which in turn induces transcription of autotaxin, an enzyme generating LPA. Taken together, at least three

pathways seem to become triggered in response to AhR activation (Figure 5) causing E-cadherin breakdown or lack of its expression, thus stimulating EMT.

The AhR: canonical and non-canonical pathways in concert trigger tumor formation and growth

The critical role of the AhR in tumor initiation rests on the canonical pathway in which activation of the AhR results in the expression of phase I and II enzymes that catalyze detoxication but at the same time 'activation' of procarcinogens causing DNA adduct formation (Figure 1). The factors regulating the balance between activation of procarcinogens into DNA-reactive metabolites and their detoxication have not been clearly determined yet. However, it is suggested that CYP1A1 is predominantly involved in detoxication, whereas CYP1B1 is required for metabolic activation (13,14). Irrespective of this, it is clear that metabolic activation mediated by AhR-regulated functions is a *conditio sine qua non* for tumor initiation (15,16). Since tumor initiation is based on fixation of DNA damage by mutations in critical target genes (oncogenes and tumor suppressor genes) and since mutagenesis by base mispairing and translesion synthesis is bound on DNA replication (134), it is reasonable to posit that any proliferation stimulus during and immediately after the induction of DNA damage and prior to its repair drives the initiation process. Therefore, AhR-triggered functions that end up in stimulation of DNA replication may support DNA damage fixation and, thus, tumor initiation.

An important process counteracting tumor initiation is DNA repair. Data have been reported indicating that ARNT interacts with breast cancer 1 (135) and TCDD may stimulate homologous recombination (136,137), which suggests a possible involvement of the AhR in modulation of DNA repair processes. It would be highly important to elucidate in more detail the role of the AhR in the regulation and the enzymology of DNA repair processes together with their impact on cell cycle regulation. We should note that an important aspect in genotoxin-driven processes is cell death that may counteract mutagenesis by elimination of heavily damaged cells. Early work *in vivo* and *in vitro* (23,24) and the recent finding that the AhR binds to E2F1 and thus is able to inhibit E2F1-mediated apoptosis (138) may be taken to indicate the complex network of AhR-driven functions that in concert support tumor formation and promotion.

Given the role of proliferation in DNA damage fixation and tumor promotion, it is tempting to speculate that loss of contact inhibition in the initiation and post-initiated stage may support pre-initiated cells to expand. Although still not shown *in vivo*, a possible mechanism involved might be AhR-dependent up-regulation of the expression of proto-oncogenes, such as cyclin A by a non-canonical pathway. Breakdown of cell–cell adhesion notably by down-regulation of E-cadherin will lead to EMT with induction of proliferative, mesenchymal and invasive genes, thus stimulating the process of tumor progression, in which at the same time further mutations will be accumulating from fixation of spontaneous DNA damage. AhR agonists affect several key players in EMT, such as JNK, Slug and NFAT, thereby leading to loss of E-cadherin function, which supports a role of the AhR in tumor progression. Based on this hypothetical model (Figure 6), AhR-dependent tumor promoters might be powerful tumorigenic agents because they have the capability to enhance fixation of any DNA damage, expansion of initiated cells, i.e. tumor promotion and finally to drive progression. We should note that in the non-experimental situation, individuals are exposed to mixtures of deleterious compounds such as PAHs that may act both as initiator and promoter, which may at the same time activate both the canonical and the non-canonical functions of the AhR.

Outlook

Both *in vitro* and *in vivo* studies suggest that the AhR deregulates cell–cell contact, thereby inducing release from contact inhibition as well as disruption of cell–cell adhesion, hence leading to EMT. Although some important signaling pathways and key players could be identified so far, it is clear that we are still at the beginning of understanding the role of the AhR in these processes. Therefore, intensive research is still needed to unravel the complex signaling cascade of the AhR. Moreover, *in vivo* studies using appropriate genetic mouse models are needed to finally prove the functional significance of the findings. The fact that EGCG, a component of green tea and inhibitor of the AhR signaling, was able to prevent EMT and thereby the invasive phenotype of breast cancer in an *in vivo* model is at least promising and might help to better understand the process of invasion and metastasis. Importantly, a number of dietary AhR ligands are present in food, some of them known as agonists and some of them acting as antagonists. To date, very little is known about their adverse or beneficial properties and their potential role in tumor promotion, progression or prevention. The novel findings of the non-canonical pathways triggered by the AhR provide a solid basis of new hypotheses that may serve as a ground for further elucidating the molecular events driven by AhR agonists. They will hopefully also help to understand better clinical observations showing that the AhR expression correlates with malignancy in some human cancers.

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