The dioxin receptor has tumor suppressor activity in melanoma growth and metastasis

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Melanoma is a highly metastatic and malignant skin cancer having poor rates of patient survival. Since the incidence of melanoma is steadily increasing in the population, finding prognostic and therapeutic targets are crucial tasks in cancer. The dioxin receptor (AhR) is required for xenobiotic-induced toxicity and carcinogenesis and for cell physiology and organ homeostasis. Yet, the mechanisms by which AhR affects tumor growth and dissemination are largely uncharacterized. We report here that AhR contributes to the tumor–stroma interaction, blocking melanoma growth and metastasis when expressed in the tumor cell but supporting melanoma when expressed in the stroma. B16F10 cells engineered to lack AhR (small hairpin RNA for AhR) exacerbated melanoma primary tumorigenesis and lung metastasis when injected in AhR−/− recipient mice but not when injected in AhR+/+ mice or when co-injected with AhR−/− fibroblasts in an AhR+/+ stroma. Contrary, B16F10 cells expressing a constitutively active AhR had reduced tumorigenicity and invasiveness in either AhR genetic background. The tumor suppressor role of AhR in melanoma cells correlated with reduced migration and invasion, with lower numbers of cancer stem-like cells and with altered levels of β1-integrin and caveolin1. Human melanoma cell lines with highest AhR expression also had lowest migration and invasion. Moreover, AhR expression was reduced in human melanomas with respect to nevi lesions. We conclude that AhR knockdown in melanoma cells requires stromal AhR for maximal tumor progression and metastasis. Thus, AhR can be a molecular marker in melanoma and its activity in both tumor and stromal compartments should be considered.

Introduction

The dioxin receptor (AhR) is a transcription factor with important roles in xenobiotic-induced toxicity and carcinogenesis and in tissue homeostasis (1–3). Notably, AhR positively or negatively influences proliferation and migration depending on the cellular phenotype. AhR increases the growth of transformed cell lines (4) and induces liver (5) and stomach tumors (6) in mice, but it also inhibits the proliferation of breast (7), prostate (8) and liver (9) cancer cells and intestinal carcinogenesis in mice (10). In addition, we have shown that AhR is down-modulated in human acute lymphoblastic leukemia cells (11). These differential effects of AhR on tumor progression could be partially dependent on its cell type-specific roles in cell migration. Although AhR deficiency impairs migration and invasion of fibroblasts (12,13) and endothelial cells (14), it also promotes epithelial cell migration (15). Altogether, these studies indicate that the pro- or antitumoral roles of AhR are tissue specific and suggest that, in order to understand how AhR influences cancer, we must consider both the tumor itself and the surrounding stroma, which, in the skin, contains among others, cells of the mesenchymal (fibroblasts), endothelial and immune lineages.

Here, we have studied the role of AhR in the tumor–stroma interaction in melanoma, a highly aggressive tumor of melanocyte origin that often develops from benign nevi (16). Melanoma is clinically relevant because its high metastatic potential usually results in adverse prognosis and poor patient survival (17,18). Despite that, few clinically relevant molecular markers have been identified for melanoma, being tyrosinase and Melan-A/MART1 (19,20) and B-RAF (21) the most commonly used. Importantly, melanoma dissemination is controlled by the tumor microenvironment, which either restricts or promotes the switch from the radial (localized) to the vertical (metastatic) growth phases (22).

To address the tumor-intrinsic and the stromal-related AhR functions in melanoma, we have used two approaches. First, we have molecularly engineered B16F10 mouse melanoma cell lines to either down-modulate or to constitutively activate AhR in order to investigate how variations in AhR signaling affect melanoma growth and metastasis. Second, we have transplanted those cell lines on AhR+/+ or AhR−/− immunocompetent recipient mice to explore the contribution of stromal AhR expression on melanoma tumorigenesis and metastasis. This scheme allowed us to investigate the interaction between cell-autonomous (melanoma-dependent) and microenvironmental (stromal-dependent) AhR functions in melanoma. We have found that AhR plays antitumorigenic roles in the melanoma cell but pro-tumorigenic roles in the stroma, thus supporting cell type-specific AhR functions in cancer. These results may have clinical relevance because we also observed that AhR expression was frequently reduced in human melanomas relative to benign nevi. Taken together, we suggest that AhR can be a novel molecular target with prognostic or therapeutic value in melanoma.

Materials and methods

Cell lines and mice

B16F10 mouse melanoma cells were from the American Type Culture Collection (Manassas, VA). Human C8161, A375, HBL, DOR and Hmel-1 melanoma cell lines were from the American Type Culture Collection (Manassas, VA). Human FF-C26 fibroblasts were γ-irradiated to halt cell division before use. AhR+/+ and AhR−/− immortalized fibroblasts and aortic endothelial cells were produced as described previously (13,14). Cells were tested to be mycoplasma free by Hoechst 33342 staining. Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamate at 37°C and 5% CO2 atmosphere. AhR+/+ and AhR−/− mice were produced by homologous recombination in embryonic stem cells as described previously (23) and used at 8–10 weeks of age. Mice experiments were approved by the Bioethics and Biosecurity Commission of the University of Extremadura. Mice had free access to water and rodent chow.

Antibodies, vectors and reagents

The following antibodies were used: activated β1-integrin (9EG7), caveolin1 and β-catenin (Becton-Dickinson, Franklin Lakes, NJ); E-cadherin (Millipore, Billerica, MA); AhR (Biomol, Plymouth, PA); total β-integrin-fluorescein isothiocyanate, CD133-PE, CD44-PerCP and CD29-fluorescein isothiocyanate (BioLegend, San Diego, CA); fibronectin (Chemicon, Temecula, CA) and β-actin (Sigma–Aldrich, St Louis, MO). Matrigel solution and matrigel-coated transwells were from Becton-Dickinson. SuperScript II reverse transcriptase and SYBR-Green master mix were from Bio-Rad (Hercules, CA). The constitutively active AhR (CA-AhR) was a generous gift from Dr Fujii-Kuriyama.

Abbreviations: AhR, dioxin receptor; CA-AhR, constitutively active AhR; EMT, epithelial–mesenchymal transition; PBS, phosphate-buffered saline; sh-AhR, small hairpin RNA for AhR; SE, standard error; SMA, smooth muscle actin; TMA, tissue microarray.
Retroviral transduction

B16F10 melanoma cells were transduced with expression vectors containing a small hairpin RNA for AhR (sh-AhR) or a CA-AhR receptor as described previously (24). Mouse aortic endothelial cells were transfected with the sh-AhR expression vector following the same protocol.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting were performed using sh-AhR, wild-type (WT)-AhR and CA-AhR B16F10 total cell extracts as indicated (13).

Clonogenic assays

Clonogenic assays in 2D were done by plating 5 × 10^3 or 10^4 sh-AhR, WT-AhR or CA-AhR cells in plain tissue culture dishes. After 5 days, the medium was removed and clones were washed in phosphate-buffered saline (PBS), stained for 10 min with 0.5% (w/v) crystal violet and counted. For 3D clonogenic assays, cells were grown in matrigel as described previously (14).

Briefly, 5 × 10^3 sh-AhR, WT-AhR or CA-AhR B16F10 cells were seeded on matrigel plugs for 3 days in complete medium. Clones formed were counted and analyzed for cell spreading using the ImageJ software (version 1.45S).

Melanoma cell injection, lung metastasis and extravasation assays

Aliquots of 10^6 exponentially growing cells were injected subcutaneously in both flanks of AhR^+/+ and AhR^-/- recipient mice and tumors were allowed to grow for 15 ± 1 days. For co-injection experiments, 10^5 sh-AhR, WT-AhR or CA-AhR cells were mixed with 4 × 10^5 immortalized AhR^+/+ or AhR^-/- fibroblasts (13) and the cellular mixture was injected subcutaneously in WT mice. In these last set of experiments, each melanoma cell line was injected with AhR^+/+ fibroblasts on one flank and with AhR^-/- fibroblasts on the other flank. Mice were killed and tumors were recovered, weighed and measured with a caliper. Tumor volume was calculated as length × width^2 × 0.4. For lung metastasis, 10^5 cells of each cell line were resuspended in 100 µl of PBS and injected through the tail vein of AhR^+/+ and AhR^-/- recipient mice. After 21 days, mice were killed and the lungs were extracted and observed for the presence of melanoma-derived metastasis. Tumor lesions were recorded and photographed. Lung extravasation was determined after 48 h of cell injection through the tail vein. One hour before killing, mice were injected with 100 µl of rhodamine-conjugated lectin to stain capillaries. Lungs were recovered, fixed in parafomaldehyde, sectioned at 10 µm and the number of melanoma cells found outside the blood vessels were quantified by confocal microscopy.

Cell migration and invasion

Cell migration was analyzed in wound healing experiments as reported in refs 13 and 15 using the Cell-R system (Olympus, Tokyo, Japan). Invasion through matrigel-coated transwells was analyzed using an Olympus FV1000 confocal microscope as described previously (14).

Flow cytometry

The analysis of side population cells in sh-AhR, WT-AhR and CA-AhR B16F10 cultures was done in a MoFlo-XDP equipment (Beckman-Coulter, Indianapolis, IN) using published criteria (25). Cells were stained for 90 min

Immunofluorescence

Immunofluorescence was done in an Olympus FV1000 confocal microscope on cultures fixed for 20 min at room temperature in 4% paraformaldehyde (Polysciences Inc, Warrington, PA). Fluorochromes used were Alexa 488 (E-cadherin) and Alexa 633 [β-catenin and α-smooth muscle actin (SMA)]. 4′,6-Diamidino-2-phenylindole was used to stain cell nuclei. Fluorescence distribution analysis was done using the FV10 software (Olympus).

β1-integrin activation measurement

sh-AhR, WT-AhR and CA-AhR B16F10 cells were detached at 4°C in PBS containing 2 mM of ethylenediaminetetraacetic acid, centrifuged, resuspended and incubated at 4°C for 10 min in PBS containing 1% bovine serum albumin to block unspecific antibody binding. The levels of total and active β1-integrin were quantified as described previously (26).

Statistical analyses

Data are shown as mean ± standard error (SE). Statistical comparison between experimental conditions was done using GraphPad Prism 4.0 software (GraphPad, San Diego, CA). The Student’s t-test and the analysis of variance analyses were applied. Experiments were done in duplicate in at least three cultures of each cell line.

Results

AhR down-modulation in the tumor cell increased melanoma growth and metastasis

To analyze the role of AhR in the tumor–stroma interaction in melanoma, we have used B16F10 mouse cells. They were chosen for their ability to grow tumors in non-immunocompromised AhR^+/+ and AhR^-/- mice, which are essential to understand how AhR expression in the stroma affects tumor development. To our knowledge, no equivalent cell line is available from human melanomas. B16F10 cells were transduced with retroviruses encoding either a sh-AhR or a CA-AhR receptor to explore the effects of loss or gain of AhR function, respectively (Supplementary Figure S1A, available at Carcinogenesis Online). B16F10 cells were also transduced with an empty retrovirus as control (Supplementary Figure S1A, available at Carcinogenesis Online). These cell lines will be referred to hereafter as sh-AhR, CA-AhR and WT-AhR, respectively. Immunoblot analysis demonstrated proper knockdown of the endogenous AhR and overexpression of the ectopically expressed receptor in sh-AhR and CA-AhR cells, respectively (Supplementary Figure S1B, available at Carcinogenesis Online). Constitutive AhR activation in CA-AhR cells was demonstrated by the increase in messenger RNA for the Cyp1al target gene (Supplementary Figure S1C, available at Carcinogenesis Online). Silencing or constitutive activation of the AhR pathway did not significantly affect proliferation rates (Supplementary Figure S2A, available at Carcinogenesis Online) or cell cycle distribution of B16F10 cells (Supplementary Figure S2B, available at Carcinogenesis Online).

We then analyzed the effect of the loss- and gain-of-function of AhR in melanoma growth and metastasis. sh-AhR and CA-AhR cells were injected in AhR^+/+ recipient mice and tumor growth was evaluated 15 days later. An inverse correlation between AhR expression and melanoma primary tumorigenesis was found since sh-AhR and CA-AhR cells promoted larger and smaller tumors than control cells, respectively (Figure 1A and Supplementary Figure S3A, available at Carcinogenesis Online). To analyze the effect of AhR in melanoma lung metastasis, the aforementioned cells were injected intravenously in WT mice and the number of metastatic nodules in the lungs counted de visu 21 days later. AhR knocked-down cells induced 2-fold more and larger metastatic nodules than control cells (Figure 1B and C). In this case, however, no statistically significant differences between control and CA-AhR cells were detected (Figure 1B and C), suggesting that, unlike primary tumorigenesis (Figure 1A), endogenous AhR functions appear enough to activate the pro-metastatic program toward the lung. The increased number and larger size of the metastasis induced by sh-AhR cells are probably related to an enhanced fitness to colonize the lung parenchyma

Human melanoma tissue microarrays and immunohistochemistry

Human nevi and melanoma tissues were obtained from the tumor collection of the Infanta Cristina University Clinical Hospital. Each biopsy was diagnosed by two independent pathologists. Human tissue microarrays (TMAs) were assembled from individual biopsies using a 10-mm diameter MTA-I equipment (Sun Prairie, WI). One millimeter cylinders were taken from paraffin-embedded tissues and inserted in triplicate in TMAs containing 17 melanomas and 6 nevi (69 cores). TMAs were sectioned at 3–5 µm and analyzed for AhR and caveolin-1 expression by immunohistochemistry.
AhR expression suppresses melanoma progression because they did not show any significant extravasation advantage when compared with control and CA-AhR cells (Figure 1D).

AhR knockdown increased migration and invasion of mouse melanoma cells
Examination of sh-AhR, WT-AhR and CA-AhR B16F10 cells in 2D and 3D cultures indicated that AhR was linked to significant changes in cell morphology. In 2D cultures, the sh-AhR clones usually grew in spread cell colonies with reduced intercellular contacts (Figure 2A, left top). These cells also formed highly migratory colonies in 3D cultures that were very different in morphology from the rounded colonies produced by control cells (Figure 2A, left bottom) and that were reminiscent of cells undergoing an epithelial–mesenchymal transition (EMT). This phenotype was a direct effect of AhR since the CA-AhR clones showed an exacerbated, ‘epithelial-like’ morphology under both culture conditions (Figure 2A, right panels). Consistent with an EMT-like process, the sh-AhR and CA-AhR cells had increased or reduced migration in wound healing (Figure 2B) and
Fig. 2. AhR knockdown alters melanoma cell morphology, migration and invasion. (A) sh-AhR, WT-AhR and CA-AhR B16F10 cells were grown in 2D (upper left) or 3D (lower left) and clone morphology was analyzed. The number of clones formed in 2D cultures (middle) and the number of spreading cells in 3D matrigel cultures were counted (right). Arrows mark the contour of the clones. n.s., nonsignificant. (B) Cell migration was analyzed in wound healing assays as indicated (15). (C) Invasion of sh-AhR, WT-AhR and CA-AhR B16F10 cell lines was quantified by confocal microscopy in matrigel-coated transwells. (D) sh-AhR, WT-AhR and CA-AhR melanoma cells were analyzed by immunofluorescence for the expression of E-cadherin, β-catenin and α-SMA. 4′,6-Diamidino-2-phenylindole staining was used to locate cell nuclei. (E and F) Protein levels of epithelial and mesenchymal markers (E) and of caveolin1 (F) were determined in each B16F10 cell line by immunoblotting. Numbers under the figure indicate relative protein levels. (G) β1-integrin expression and activation were analyzed by flow cytometry as indicated in Materials and methods. β1t and β1a stand for total and activated β1-integrin, respectively. Data are shown as mean ± SE from triplicate experiments in at least two cultures of each line. Bar 50 µm.
AhR expression suppresses melanoma progression

matrigel-based invasion assays (Figure 2C) with respect to control WT-AhR cells.

Given the observations previously described, we examined whether the morphology of the sh-AhR and CA-AhR cells could correlate with an EMT and mesenchymal–epithelial transition process, respectively. We combined immunofluorescence and immunoblotting to analyze changes in the classical markers E-cadherin, β-catenin, fibronectin and α-SMA, as well as in the EMT inducer Snail. In agreement with an EMT-like process, the more spread and migratory sh-AhR cells partially delocalized E-cadherin and β-catenin from the plasma membrane and increased their α-SMA content by more than 2-fold when compared with both WT-AhR and CA-AhR cells (Figure 2D and E). Additionally, sh-AhR cells had a moderate increase in Snail and in the mesenchymal marker fibronectin with respect to the less tumorigenic CA-AhR cells (Figure 2E).

We have recently observed in murine fibroblasts that AhR exerts a dual opposite role activating caveolin1 and inhibiting β1-integrin (26). Considering these results, and since caveolin1 is involved in melanoma malignancy and its expression is E-cadherin dependent (27), we analyzed caveolin1 and β1-integrin in our B16F10 cell lines. Immunoblot analyses revealed that caveolin1 was heavily upregulated in CA-AhR cells but down-modulated in sh-AhR knockdown cells (Figure 2F). Using flow cytometry, we also found that AhR knockdown upregulated the expression (Figure 2G, left) and the activation (Figure 2G, right) of β1-integrin, a caveolin1-inhibited integrin that regulates melanoma motility and invasion (28,29).

Stromal AhR modulates the tumorigenic and metastatic abilities of melanoma cells

To investigate how stromal AhR affects melanoma progression, we inoculated each B16F10 cell line in AhR−/− recipient mice. For primary tumorigenesis, the AhR−/− microenvironment did not significantly affect the growth of either CA-AhR or WT cells (Figure 1A and Supplementary Figure S3A, available at Carcinogenesis Online). In contrast, it severely compromised the growth advantage of sh-AhR cells previously seen in WT mice (Figure 1A). Regarding lung metastasis, the AhR−/− stroma reduced the number of metastatic foci induced by each of the three cell lines under study (Figure 1C). The AhR-expressing stroma likely produces secretable factors that promote melanoma progression since the invasive potential of sh-AhR B16F10 clones was higher in AhR+/+ fibroblast-conditioned medium than in that of AhR−/− fibroblasts (Figure 3A). CA-AhR and WT-AhR cells, on the contrary, did not significantly differ in invasion in either conditioned media (Figure 3A).

Mouse aortic endothelial cells from AhR+/+ mice were transduced with a sh-AhR vector to reduce their basal receptor levels (Supplementary Figure S3B, available at Carcinogenesis Online). The invasion of sh-AhR melanoma clones increased in AhR+/+ endothelium-conditioned medium with respect to that of sh-AhR endothelium

![Fig. 3. Stromal AhR modulates melanoma cell migration. (A and B) sh-AhR, WT-AhR and CA-AhR cells were cultured in medium conditioned by AhR+/+ or AhR−/− mouse fibroblasts (A) or by AhR+/+ or sh-AhR endothelial cells (B) and clone morphology and cell scattering were analyzed by microscopy. (C) sh-AhR, WT-AhR or CA-AhR melanoma cells were co-injected with immortalized fibroblasts from AhR+/+ or AhR−/− mice in WT-AhR mice. Injections were set such as each melanoma cell line was injected with one fibroblast cell line on one flank of the mouse and with the other fibroblast cell line on the other flank. The following number of tumors were analyzed: AhR+/+ fibroblasts—5, 5 and 6 for sh-AhR, WT-AhR and CA-AhR cells, respectively; AhR−/− fibroblasts—6, 6 and 8 for sh-AhR, WT-AhR and CA-AhR cells, respectively. (D) Tumors produced by each cell line in AhR+/+ recipient mice were processed to quantify their blood vessel content (see Supplementary Figure S4A, available at Carcinogenesis Online). Data are shown as mean ± SE from triplicate cultures. Three tumors were analyzed for each experimental condition. Bar 50 µm.](https://academic.oup.com/carcin/article-abstract/34/12/2683/2463949?举行了12位2月19日)
and no significant differences were found for WT-AhR and CA-AhR melanoma cells in either conditioned media (Figure 3B). The sh-AhR endothelium-conditioned medium also reduced the clone formation potential of sh-AhR cells (Supplementary Figure S3C, available at Carcinogenesis Online), again suggesting that AhR is needed to produce stroma-secreted factors required for the growth of melanoma cells depleted of AhR expression. To further sustain this possibility, we decided to co-inject each B16F10 cell line with immortalized AhR+ or AhR− fibroblasts (13) in WT recipient mice. The presence of AhR+ fibroblasts reduced the growth (Figure 3C) and the volume (Supplementary Figure S3D, available at Carcinogenesis Online) of the tumors formed by sh-AhR melanoma cells in WT mice, thus supporting a tumor promoting effect for stromal AhR (see Figure 1A and Supplementary Figure S3A, available at Carcinogenesis Online). AhR+ fibroblasts did not significantly affect the growth of WT-AhR- or CA-AhR-derived tumors with respect to AhR+/− fibroblasts (Figures 3C and Supplementary Figure S3D, available at Carcinogenesis Online). The effect of stromal AhR on the growth of sh-AhR-derived tumors possibly involves an enhanced angiogenic response since the number of blood vessels were higher in sh-AhR than in WT-AhR or CA-AhR tumors (Figures 3D and Supplementary Figure S4A, available at Carcinogenesis Online). Altogether, these results indicate that (i) AhR contributes to the control of melanoma cell migration and invasion, (ii) AhR can repress pro-migratory molecules such as β1-integrin, (iii) AhR hyperactivation leads to the further enhancement of some (caveolin1) but not all (β1-integrin) pro-migratory molecules and (iv) AhR plays intrinsic tumor suppressor-like roles in melanoma cells but pro-tumorigenic roles in the stroma.

AhR knockdown increased the pool of cancer stem-like cells

Next, we explored one potential cause for the tumor suppressor-like role of AhR in melanoma cells. Since EMT can be associated with a stem-like cell phenotype in cancer cells, we analyzed if the sh-AhR B16F10 line contained an increased number of stem-like cells. We monitored by flow cytometry the percentage of ABCB5+/CD29+/CD44− cells (30) that coexpressed CD133 and CD44, two surface markers associated to the stem-like phenotype (31). sh-AhR cells, but not their CA-AhR counterparts, displayed larger numbers of CD133+/CD44−/CD29+ cells when compared with control cells (Figure 4A).

To confirm these results, melanosome formation assays in non-adherent conditions in vitro (32) were performed. We found that the sh-AhR cells generated more and larger melanosomes than either WT-AhR or CA-AhR cells (Figure 4B). The stem-like status of the melanosomes was confirmed by flow cytometry (Figure 4C). These results indicate that one possible mechanism for the tumor suppressor activity of endogenous AhR is to reduce the stem-like cell pool of melanoma cells.

Human melanoma cells and melanomas from human patients had reduced AhR expression

The results above suggest an inverse correlation between melanoma malignancy and the tumor suppressor-like role of AhR. We thus investigated whether AhR could serve as a surrogate marker for melanoma progression and malignancy. We analyzed a battery of five human melanoma cell lines including A375, C8161, HBL, Hmel-1 and DOR. Clone formation assays in 2D showed that C8161 cells produced more spread and scattered clones than did the remaining cell lines analyzed (Figure 5A). C8161 cells also had a more migratory phenotype than A375, Hmel-1 and DOR cells, whereas HBL cells had an intermediate migration potential (Figure 5B). When cultured in matrigel, a similar pattern was obtained being C8161, the most invasive among the cell lines tested (Figure 5C). Interestingly, immunoblot analyses revealed that AhR levels were lower in the highly migratory and invasive C8161 cells than in the less motile A375, Hmel-1 and DOR cells, with HBL cells having an intermediate amount of AhR expression (Figure 6A). Thus, as for the murine B16F10 model, human melanoma cells with reduced AhR expression had increased migration and invasion potentials. In agreement, rescue of AhR expression in C8161 cells (Figure 6B) reduced their migration as compared with the parental cell line (Figure 6C). Moreover, C8161 cells formed more invasive and scattered colonies than A375 cells when grown on FF-C26 human fibroblasts (Figure 6D), further suggesting a negative role for AhR on the invasion of melanoma cells.

We then examined the expression of AhR in human nevi and in melanomas from human patients using tissue arrays and standard immunohistochemistry. Immunofluorescence was also used to confirm AhR expression in human samples (Supplementary Figure S5, available at Carcinogenesis Online). As summarized in Supplementary Table SI, available at Carcinogenesis Online, AhR protein was detected in five out of six nevi lesions analyzed (83%; Figure 6E). In contrast, 14 human melanomas were negative for AhR, whereas 2 biopsies displayed weak immunoreactivity (Supplementary Table SI, available at Carcinogenesis Online, and Figure 6E; n = 16). The negative and weak immunoreactive melanomas correlated with advanced Clark III-IV-V or early Clark <III stages, respectively (Supplementary Table SI, available at Carcinogenesis Online). Since the pro-tumorigenic and metastatic sh-AhR B16F10 cells had reduced caveolin1 expression (Figure 2F), we analyzed caveolin1 levels in the same tissue arrays. Caveolin1 was detected more frequently in nevi lesions (80%) than in malignant melanomas (6%; Supplementary Table SI, available at Carcinogenesis Online, and Figure 6E). AhR and caveolin1 were coexpressed in 67% of all nevi analyzed (Supplementary Table SI, available at Carcinogenesis Online). Some melanomas had detectable AhR expression in the stroma (Supplementary Figure S4B, available at Carcinogenesis Online), which could be suggestive of its effects in that compartment. Thus, AhR and caveolin1 seem inversely correlated with human melanoma progression.

Discussion

AhR promotes or inhibits cell proliferation and migration depending on the phenotype of the target cell (33). However, only few studies have analyzed the role of AhR in tumor development in vivo, and it is mostly unknown if differences in AhR expression contribute to tumor progression and dissemination. Moreover, no studies have yet addressed whether AhR is relevant in the interaction between the tumor cell and the stroma. This encouraged us to investigate the tumorigenic and metastatic response of melanoma cells with varying levels of AhR expression in an AhR-expressing or AhR-lacking microenvironments. Melanoma was studied for its increasing clinical impact and for its metastatic potential that often results in adverse clinical outcomes.

An important finding of this study is that AhR expression/activation decreases in the tumor cell, melanoma tumorigenesis and metastasis became markedly stimulated. The intrinsic suppressive role of AhR in melanoma cells may be related to its ability to modulate cell migration and invasion, thus strengthening the existence of cell-autonomous AhR functions in cancer (12,14,15). Accordingly, although AhR knockdown favored a mesenchymal-like phenotype with increased migration and tumorigenicity, constitutive AhR activation reduced cell migration and impaired melanoma growth and dissemination. The correlation found in the murine melanoma model (e.g. increased migratory and invasive potentials are associated to reduced AhR levels) was also observed in human melanoma cells, which not only sustains the tumor suppressor function of AhR but also validates the murine model. The change from an anti-invasive (epithelial) to a pro-invasive (mesenchymal) phenotype caused by AhR depletion may reflect an EMT-like process involving E-cadherin and β-catenin, as their cellular localization and expression are altered in metastatic and late-stage human melanomas (34,35). In fact, AhR down-modulation in mouse primary keratinocytes and NMuMG cells and in human HaCaT cells induces an EMT-like process involving changes in the expression and localization of E-cadherin and β-catenin (24). Additionally, the tumor suppressor-like role of AhR in melanoma correlated with the upregulation of caveolin1, which, as AhR, has cell type-dependent tumor suppressor activity (36–38) and...
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is E-cadherin regulated (27). Moreover, AhR and caveolin1 expression positively correlated with a reduced activation of \( \beta_1 \)-integrin, a known promoter of melanoma invasion and dissemination (28,29).

EMT has been associated to stem cell-ness (39) and whereas AhR knockdown enriched, AhR over-activation depleted the subpopulation of B16F10 stem-like cells. Because stem cells are generally considered drivers of tumorigenesis and metastasis (31,32), the antitumoral activity of AhR in melanoma could act by maintaining a reduced pool of undifferentiated stem-like cells. Indeed, AhR activation by xenobiotics inhibited invasiveness (7) and repressed mammosphere formation of breast tumor cells (40), whereas AhR inhibition by StemRegenin increased the number of human hematopoietic stem cells and their

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**Fig. 4.** AhR down-modulation increases undifferentiation markers of B16F10 cells. (A) sh-AhR, WT-AhR and CA-AhR cultures were sorted out to collect the side population cells expressing the adenosine triphosphate-binding cassette transporter ABCB5. These cell fractions were then analyzed by flow cytometry for CD133\(^+\) (left) or CD44\(^+\)/CD29\(^+\) (right). (B) The number of melanospheres formed by each B16F10 cell line under non-adherent culturing conditions (left) and the average number of cells in each melanosphere (right) were determined. (C) Melanospheres were dissociated and the resulting cell suspensions were analyzed for CD133\(^+\) expression by flow cytometry. The experiments were performed by duplicate in three independent cultures. Data are shown as mean ± SE. Bar 50 µm.
We, therefore, propose that AhR suppresses melanoma by a mechanism reducing the number of stem-like cells while inhibiting migration and invasion.

The stroma is an additional compartment that controls tumor progression. Contrary to the tumor suppressor-like activity of AhR in melanoma cells, stromal AhR is needed for malignancy since AhR−/− mice could not sustain tumorigenesis and metastasis of AhR knocked-down cells. Tissue-/stroma-specific factors must be involved in the process because even though AhR-expressing lungs allowed increased pro-metastatic activity at any AhR expression level, primary tumorigenesis was enhanced only for AhR-knockdown melanoma cells. These AhR-dependent stromal effects likely involve yet unidentified diffusible factors because AhR−/− fibroblasts could not sustain sh-AhR tumor growth in WT recipient mice and the AhR-null conditioned medium failed to induce invasion of sh-AhR melanoma cells. Possible candidates are cytokines such as transforming growth factor β because of

**Fig. 5.** Human melanoma cell lines differ in their spreading, migration and invasion potentials. (A) The clonogenic potential of C8161, HBL, A375 and DOR human melanoma cells was determined in 2D cultures. (B) Cell migration was quantified in the indicated cell lines plus Hmel-1 using wound healing assays 24 h after plating. (C) Cell invasion was also analyzed in the five cell lines under study by confocal microscopy using matrigel-coated transwells. Duplicate experiments were done in three cultures of each line. Data are shown as mean ± SE. Bar 50 µm. Note different magnification in A375 and DOR cell lines in panel A.
AhR expression suppresses melanoma progression

AhR expression suppresses melanoma progression since it confers resistance to melanoma chemotherapy when produced in the stroma (43). The fact that the AhR-expressing stroma reduced blood vessel formation in WT-AhR and CA-AhR but not in sh-AhR melanoma suggests that melanoma cells with reduced AhR expression may not respond to stromal inhibitory signals. Actually, stromal AhR seems to contribute to tumor angiogenesis because AhR−/− mice cannot develop a proper angiogenic response (14). Stromal AhR may also modulate melanoma growth and dissemination by promoting the generation of cancer stem-like cells. Diffusible factors secreted by the AhR-expressing microenvironment could synergistically act with melanoma cell-autonomous AhR depletion to sustain an undifferentiated phenotype. The cytokines mentioned previously could in fact act in a paracrine manner on the melanoma cell to sustain stem cell-ness. Such possibility is currently under investigation.

The AhR is involved in the Treg/Th17 differentiation program that regulates immune cell activation through the production of Treg cells (44,45). An interesting possibility is that since Treg suppress the activation of reactive lymphocytes, their depletion in the AhR-lacking stroma could induce an augmented immunological response leading to reduced tumor growth and metastasis. The Treg/Th17 balance is regulated by kynurenine, a high-affinity AhR ligand and a product of the indoleamine 2,3-dioxygenase and the tryptophan 2,3-dioxygenase enzymes (46). Tryptophan 2,3-dioxygenase-dependent synthesis of kynurenine from tryptophan was suggested to suppress antitumor immunity and to support glioblastoma growth, likely by increasing Treg (47). In this context, kynurenine could impair the immune response against melanoma cells by promoting AhR-dependent Treg differentiation, an effect that would increase tumor growth in AhR-expressing but not in AhR-lacking stroma. An additional aspect is how 6-formylindolo[3,2-b]carbazole, an AhR ligand produced from tryptophan by ultraviolet exposure (48), can affect melanoma progression. Exposure of AhR-null mice to ultraviolet could help answer this question.

AHR protein levels were lower in human melanomas with respect to non-malignant nevi, suggesting that the malignant phenotype correlates with reduced AHR expression and that AHR could have a tumor suppressor-like role in melanoma. Importantly, AHR shares with caveolin1 cancer-related properties such as its down-modulation in advanced human melanomas (29) and its implication in cell adhesion and migration (26). Consequently, AHR and caveolin1 could act in concert during melanoma progression and dissemination. Melanoma

Fig. 6. AHR expression correlates with the migration and invasion of human melanoma cell lines and its levels are reduced in human melanoma. (A) C8161, A375, HBL, Hmel-1 and DOR cells were analyzed for AHR protein expression by immunoblotting. The expression of β-actin was used as loading control. (B) AHR expression was rescued in low-expressing C8161 cells (C8161 + AHR) and the amount of cellular receptor was quantified by immunoblotting. (C) The migration ability of the C8161 + AHR cell line was determined in wound healing assays and quantified with respect to control C8161 cells. (D) Low-expressing C8161 and high-expressing A375 human melanoma cell lines were cultured on a monolayer of γ-irradiated FF-C26 human fibroblasts to analyze their clone formation potential under a homogeneous fibroblasts background. Duplicate experiments were done in three cultures of each line. Data are shown as mean ± SE. (E) Immunohistochemistry for AHR and caveolin1 in human nevi and melanomas. Nevi and melanoma were arranged in a TMA as indicated in Materials and methods. AHR and caveolin1 protein were detected by immunohistochemistry using specific antibodies. Micrographs from representative biopsies included in Supplementary Table S1, available at Carcinogenesis Online, are shown. Bar 50 µm.
thus increases the group of AhR-inhibited cancers that also includes acute lymphoblastic leukemia (11), hepatocarcinoma (9), prostate (8) and mammary cancers (49). In summary, AhR activation has tumor suppressor-like activity in melanoma cells because its down-modulation promotes tumorigenesis and metastasis. Oppositely, stromal AhR favors, rather than inhibits, primary melanoma growth and metastasis to the lungs. Therefore, AhR expression has to be accounted for in both, tumor mass and surrounding stroma. One mechanism for the antitumoral activity of AhR could involve the maintenance of a non-migratory/non- invasive phenotype that is converted to a pro-migratory/pro-invasive mesenchymal phenotype by AhR knockdown. AhR may also negatively control the pool of stem-like cancer cells eventually driving melanoma progression. Finally, AhR expression appears to be reduced in invasive human melanoma cells and in melanomas from human patients, which supports its tumor suppressor-like role. Altogether, AhR and caveolin1 could be potential molecular markers in melanoma.

Supplementary material
Supplementary Table S1 and Figures S1–S5 can be found at http://carcin.oxfordjournals.org/
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