Immortalized Mouse Mammary Fibroblasts Lacking Dioxin Receptor Have Impaired Tumorigenicity in a Subcutaneous Mouse Xenograft Model*  


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Although the dioxin receptor, the aryl hydrocarbon receptor (AhR), is considered a major regulator of xenobiotic-induced carcinogenesis, its role in tumor formation in the absence of xenobiotics is still largely unknown. Trying to address this question, we have produced immortalized cell lines from wild-type (T-FGM-AhR+/+) and mutant (T-FGM-AhR−/−) mouse mammary fibroblasts by stable co-transfection with the simian virus 40 (SV-40) large T antigen and proto-oncogenic c-H-Ras. Both cell lines had a myofibroblast phenotype and similar proliferation, doubling time, SV-40 and c-H-Ras expression and activity, and cell cycle distribution. AhR+/+ and AhR+/− cells were also equally able to support growth factor- and anchorage-independent proliferation. However, the ability of T-FGM-AhR−/− to induce subcutaneous tumors (leimyosarcomas) in NOD/SCID-immunodeficient mice was close to 4-fold lower than T-FGM-AhR+/+. In culture, T-FGM-AhR−/− had diminished migration in collagen-I and decreased lamellipodia formation. VEGFR-1/FIT-1, a VEGF receptor that regulates cell migration and blood vessel formation, was also down-regulated in AhR+/− cells. Signaling through the ERK-FAK-PKB/akt-Rac-1 pathway, which contributes to cell motility and invasion, was also significantly inhibited in T-FGM-AhR−/−. Thus, the lower tumorigenic potential of T-FGM-AhR−/− could result from a compromised adaptability of these cells to the in vivo microenvironment, possibly because of an impaired ability to migrate and to respond to angiogenesis.

Many studies over the past decade have characterized the aryl hydrocarbon receptor (AhR)1 as a regulator of the toxic and carcinogenic responses to environmental contaminants such as dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) (1–5). Although very important, this xenobiota-related activity of the AhR does not appear to be its only function in the organism. The large degree of conservation of this receptor among species (6), its constitutive pattern of expression during development and in adult tissues (7), and the many phenotypic alterations found in mice lacking AhR expression (1, 8–13) have provided strong support for the involvement of the AhR in cell physiology independent of xenobiota metabolism.

Novel mechanisms of activation have been described that may associate the AhR to endogenous functions. Thus, this receptor is activated by the natural compounds indirubin and indigo (14), diosmin and diosmetin (15), and by metabolites produced by the aspartate aminotransferase (16). An AhR repressor was identified that regulates AhR activity by binding and sequestering the aryl hydrocarbon receptor nuclear translocator (ARNT) (17). Additional work has also shown that protein kinase C cooperates with ligand binding for receptor activation (18, 19) and that proteasome inhibition activates the AhR in mouse embryo primary fibroblasts in the absence of xenobiota (20, 21).

Among the physiological functions that could require AhR activity, the regulation of the cell cycle and the control of cell proliferation are the best analyzed (reviewed in Ref. 22); yet, the role of this receptor in cell proliferation is still controversial because it can promote or block cell cycle progression.

1 The abbreviations used are: AhR, aryl hydrocarbon receptor; CRIB, Rac binding domain; DAPI, 4,6-diamino-2-phenylindol; ERK, mitogen-activated extracellular signal-regulated kinase; MMP, matrix metalloproteinase; PKB/akt, protein kinase B; RBD, Ras binding domain; SV-40, simian virus 40 large T antigen; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; T-FGM-AhR−/−, transformed mammary gland primary fibroblasts from AhR−/− mice; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; GST, glutathione S-transferase; F32K, phosphatidylinositol 3-kinase; FAK, focal adhesion kinase; ANOVA, analysis of variance; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; Hif-1α, hypoxia-inducible factor 1α gene; Ang-1, angiopeptin-1 gene; Epo, erythropoietin gene.
Several studies support the AhR as an oncoprotein: (i) AhR-defective (AhR-D) mouse hepatoma cells had a prolonged duplication time because of a delayed G1/S transition (23); (ii) mouse embryonic fibroblasts (MEF) lacking AhR entered senescence much earlier than wild-type cells (24) and had a diminished proliferation rate and increased levels of transforming growth β-1 (TGF-β1) (25, 26); (iii) the AhR was relevant in DNA synthesis mediated by p300 and the adenovirus E1A (27); and finally, (iv) constitutive activation of this receptor increased hepatocarcinogenesis in transgenic B6C3F1 mice (28). The AhR also has tumor suppressor activity. It arrests cell proliferation at the G1/S transition in 5L rat hepatoma cells through interaction with hypophosphorylated retinoblastoma (pRb) protein (29). In mouse hepatoma Hepa-1 and in human breast cancer MCF-7 cells, TCDD induced AhR binding and displacement of p300 from E2F-dependent promoters (30). A constitutively active AhR induced apoptosis in Jurkat T cells by arresting cell cycle progression at G1 (31), and AhR activation by ligand binding blocked cell cycle by increasing p27Kip1 expression (32). In this context, a recent report has shown that while the AhR had growth inhibitory activity in epithelial MCF-7 cells, it promoted proliferation in HepG2 hepatoma cells, further suggesting that the contribution of this receptor to proliferation is cell type-dependent (33). Thus, although the contribution of the AhR to cell proliferation is strongly supported, its role in tumor development in the absence of xenobiotics remains largely unknown.

It is increasingly recognized that the stroma plays a major role in determining the rate of tumor growth and invasiveness in vivo (34, 35). Fibroblasts, as one of the most abundant cell types in connective tissue, regulate the synthesis, degradation, and remodeling of the extracellular matrix. Fibroblasts are particularly important in diseases such as breast cancer in which their conversion to smooth muscle α-actin-expressing myofibroblasts is considered a stromal reaction to the invading epithelial tumor cells (desmoplastic reaction) (36, 37). Because secretion of cytokines, proteases, and growth factors by stromal fibroblasts promotes proliferation of tumor cells (38), therapeutic targeting of stromal components constitutes a relevant tool to deprive tumor cells from critical factors needed for their growth, proliferation, and migration. In this context, it is interesting to note that the livers of AhR−/− mice had thickening of the stromal tissue surrounding the portal triads with increased staining for the fibblast markers vimentin and smooth muscle α-actin (39), as well as altered vascular architecture (11).

In an attempt to analyze the contribution of the AhR to tumor development in absence of xenobiotics, we have immortalized primary mammary gland fibroblasts from AhR+/+ and AhR−/− mice by stable co-transfection with the SV-40 large T-antigen and proto-oncogenic c-H-Ras. Although both cell lines had similar proliferation rates, cell cycle distribution and a transformed phenotype in vitro, the ability of T-FGM-AhR−/− to induce subcutaneous tumors in immunodeficient mice was markedly reduced. These results suggest that the absence of AhR could compromise the ability of myofibroblasts to adapt to the in vivo microenvironment and identify this receptor as a potential therapeutic target for the treatment of human cancers of fibroblastic origin.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—TaqDNA polymerase and MMLV reverse transcriptase were from Ecorgen and from Ambion, respectively. Mouse β-actin and secondary rabbit-TRITC antibodies, DAPI, enzymes, and hormones were obtained from Sigma-Aldrich. Collagenase, Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12), OPTI-MEM, Lipofectamine Plus reagent, neomycin sulfate, and trypsin-EDTA were obtained from Invitrogen. Fetal bovine serum (FBS) was from Bio-Whittaker and was heat-inactivated before use. Complete protease inhibitor mixture was purchased from Roche Applied Science. SuperSignal chemiluminescence substrate was obtained from Pierce. [methyl-3H]Thymidine was obtained from PerkinElmer Life Sciences. Protein A/G plus agarose, antibodies against p-Tyr, cyclin D1, cyclin E, p21Cip1, p27Kip1, p-PKB/AKT, PKB/AKT, p-ERK, ERK, H-pan-Ras, and secondary anti-mouse-FITC were from Santa Cruz Biotechnology. Anti-Rac-1 and anti-PAK antibodies were obtained from Transduction Laboratories. Antibodies for SV-40 large T antigen, VEGFR-1, and VEGFR-2 were from NeoMarkers. Smooth muscle α-actin 1A4 and desmin antibodies were from Anacron Diagnosticos. Anti-vimentin was obtained from DAKO and anti-Cd49f/6 integrin from Immunotech.

**Mice and Primary Culture of Mammary Gland Fibroblasts—**AhR-null and wild-type control mice of the same genetic background (C57Bl6/N × 129s/v) were produced as previously described (9). Anti-signal chemicals substrate was obtained from Chemicon. PDGF-R was produced from rat PDGF (C57Bl6/N × 129s/v) as previously described (26). Primary cultures were infected with recombinant adenovirus full-length AhR or AhR null virus (AhRnull) for 48 h (23, 24). pCEFL-AU5-c-H-Ras (proto-oncogenic H-Ras) or a 1:1 mix of both constructs. Proto-oncogenic (cellular) human H-Ras was used rather than the oncogenic, hyperactivated protein, because previous studies had revealed that while AhR activity remained unaffected by expression of the H-Ras proto-oncogene it was inhibited by the oncogenic protein (40). Stable transfectants were obtained after 4–5 weeks of selection in complete medium containing 400 μg/ml neomycin (G418). After isolation and re-plating, clones were considered to be at passage 1.

**Hematoxylin Staining and Immunocytochemistry—**After removing the medium, plates were washed with PBS, fixed in cold methanol for 10 min at −20 °C and air-dried. For hematoxylin staining, cells were dehydrated in PBS and incubated for 3 min in 50% Harris’s hematoxylin. After conventional washes they were mounted with a Nikon El600 microscope. For immunocytochemistry, cells were permeabilized with three 5-min washes in PBS containing 0.05% Triton X-100 (PBS-T). Blocking was performed for 30 min at room temperature in PBS containing 2% bovine serum albumin and 10% goat serum. Cells were incubated for 16 h at 4 °C with primary anti-vimentin, anti-smooth muscle α-actin 1A4, anti-Cd49f/6 integrin, anti-SV-40, anti-AU5, or anti-VEGFR-1 antibodies. After washing in TBS-T, mouse-anti-FITC or rabbit-TRITC secondary antibodies were added, and incubation continued at room temperature for 1 h. Plates were finally washed in PBS as above. Nuclei were stained with DAPI for 5 min and cells observed and photographed using a Nikon El600 fluorescence microscope.

**Cell Proliferation, Cell Number, and Duplication Time—**Cell proliferation was determined by measuring the rate of DNA synthesis. Cultures growing in 24-well plates were incubated with 1 μCi of [methyl-3H]thymidine (specific activity, 7 Ci/mmol) for 2 h. Labeling medium was removed and cells fixed for 2 h at room temperature in 1 ml of methanol/acetic acid (1:1). Fixed cells were washed with 80% ethanol and incubated in 0.05% trypsin-EDTA for 30 min at 37 °C. Cells were then stained with 5 min at room temperature by the addition of 1% (w/v) SDS and incorporated thymidine quantitated in a Beckman LS 3801 liquid scintillation counter. The number of cells attached to the plates was determined in parallel cultures after trypsinization (see above) and cell counting using a hemocytometer. For some experiments, cells were grown in DMEM/F12 medium without FBS. Duplication time was determined from the slope of a semilogarithmic plot representing log of cell number against time.
Flow Cytometry Analysis—Cell cycle distribution and ploidy status of the cultures were determined by flow cytometry DNA analysis. Cells were released from the plates by the addition of 0.25% trypsin, washed in PBS, fixed at 4 °C in 70% cold ethanol, and treated with RNase (10 mg/ml) for 30 min at 37 °C. DNA content per cell was determined in a Cyan flow cytometer (DAKO Cytomation) after staining with propidium iodide (50 μg/ml) for 15 min at room temperature in the dark. For cell cycle analysis, only signals from single cells were considered (10,000 cells/sample).

Cell Lysates and Immunoblotting—Cells were washed with PBS and scraped from the plates at 4 °C under the microscope in lysis buffer (50 mM Tris-HCl, 1% SDS, 2 mM EGTA, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1% Triton X-100, and Complete protease inhibitor mixture). For PKB/AKT and ERK proteins, the following lysis buffer was used: 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate. Lysed cultures were centrifuged 2 h at room temperature in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4). Lysed cultures were centrifuged 2 h at room temperature in TBS-T, the SuperSignal chemiluminescence substrate was added, and another 2 h with the corresponding primary antibodies (H-pan-Ras, pERK, Rac-1, VEGFR-1, and VEGFR-2). After washing in TBS-T, blots were incubated with the horseradish peroxidase-coupled secondary antibody for 1 h at room temperature. Following additional washing in TBS-T, the SuperSignal chemiluminescence substrate was added, and the blots were exposed and developed using the Molecular Imager FX System (Bio-Rad).

Immunoprecipitation—Cells were lysed on ice for 15 min with IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 mM β-glycerophosphate, 1 mM dithiothreitol, and Complete protease inhibitor mixture). Lysates were centrifuged and protein concentration was determined in the supernatants using the Coomassie Plus protein assay reagent (Pierce) and bovine serum albumin as standard. For immunoblotting, 15 μg of protein were denatured, separated on 8% or 12% SDS-PAGE gels, and transferred to nitrocellulose membranes. Membranes were blocked for 2 h at room temperature in TBS-T (50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.5% nonfat milk containing 0.1% Tween 20), incubated with the primary antibody overnight incubation at 4 °C with 2 μg of anti-SV-40 or anti-FAK antibodies, followed by 2-h incubation at 4 °C with protein A/G agarose. Immunoprecipitates were washed four times in IP buffer, denatured, separated on 8% SDS-PAGE gels, and transferred to nitrocellulose membranes. Membranes were blocked as indicated above and overnight incubation at 4 °C with antibodies against p53 (1:1000), Bb (1:300), or pTyr (1:1000). After washing and incubation with the horseradish peroxidase-coupled secondary antibody, the Immobilon blotting membranes were revealed by avidin-biotin complex (ABC) staining.

Soft Agar Assay—Colony formation in soft agar was analyzed in 60-mm cell culture dishes coated with 2 layers of agarose. A 1.5 dilution of cell culture-grade agarose was made in prewarmed culture medium for a final concentration of 1% agar, and 4 ml of this solution added to each culture plate. Agarose was allowed to solidify at room temperature for 10 min and plates placed in the CO2 incubator for pH equilibration. A 1:10 dilution of agarose was also prepared to pre-warm culture medium for a final concentration of 0.5%. T-FGM-AhR+/+ and T-FGM-AhR+/− cells were trypsinized and serially diluted to 105, 5 × 105, 105, or 106 cells/ml. A 100-μl aliquot of each cell dilution was mixed with 900 μl of 0.5% agarose to give final concentrations of 105, 5 × 105, 105, and 106 cells/ml. These cell dilutions were prepared on top of the 1% agarose layer previously solidified in each agar plate. Cells were fed twice per week by the addition of 400 μl of complete culture medium. After 22 days, clones were stained with crystal violet and counted. A total of 7000 cells were seeded into the upper compartment of each well. Complete culture medium was placed in both lower and upper chambers. After 48 h, inserts were removed from the plates, washed in PBS, fixed in 70% ethanol, and incubated for 15 min with 10 ng/ml RNase in PBS. Next, cells were stained with 50 μg/ml propidium iodide at room temperature in the dark and migration-analyzed in a 2100 Radiance confocal microscope (Bio-Rad). For wound closure assays, cells were allowed to reach confluence in serum-containing medium. Wounds were performed in the plates with the aid of a 1-ml pipette tip, and cultures were incubated for 12 h in serum-free medium. Wound closure was monitored by microscopy after staining the cells with hematoxylin.

Matrix Metalloproteinase Activity—MMP activity was determined in conditioned culture medium from T-FGM-AhR+/+ and T-FGM-AhR+/− by gelatin zymography. Cells were grown in OPTI-MEM for 60 h and culture medium recovered and centrifuged at 10,000 × g for 15 min at 4 °C. Cells attached to the plates at the time of the experiment were trypsinized and counted. A volume of conditioned culture medium, the same number of cells was mixed with non-reducing Laemml sample buffer (62.2 mM Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 0.025% bromphenol blue) and applied to 8% SDS-PAGE gels polymerized in the presence of 1% gelatin. After electrophoresis, the gels were washed three times in a solution containing 2.5% Triton X-100 to eliminate the SDS and to allow reconstitution of the proteins. MMP activity was determined by incubating the gels at 37 °C for 16 h (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 5 mM CaCl2, and 0.05% azide). The position of the MMPs was visualized by staining the gels in Coomassie Brilliant Blue G-250 solution.

Rhodamine Phalloidin Staining—Complete medium of cultures at 20–30% confluence was replaced by serum-free DMEM/F12 for 18 h. Cells were then fixed in 4% paraformaldehyde in PBS for 5 min at 4 °C and normalized to 0.5 × 106 cells/100 mm plate for 10 min at room temperature. Cells were blocked in 2% bovine serum albumin for 30 min at room temperature, washed in PBS, and incubated with 4 units of rhodamine phalloidin (Molecular Probes) for 30 min. Fluorescence micrographs were captured on a Nikon E600 microscope.

Reverse Transcription PCR—Total RNA was isolated with the RNeasy Kit from Qiagen following the manufacturer’s instructions. Aliquots of 1 μg of RNA were reverse-transcribed at 42 °C for 60 min using oligo d(T) priming and MMLV reverse transcriptase (Ambion). PCR amplification for Stv40 (340 bp) was performed using the primers forward, 5'-CCTGGGTGTCTTCCATCATC-3' and reverse, 5'-CATGGA-TGACACGACTGTGTGCCC-3'. For Hif-1α (187 bp) the primers were forward, 5'-TGAGGCTCATTAGCAGGATA-3' and reverse, 5'-TACCCTGACTATTCCTCTG-3'. Vegf was amplified with primers that produce fragments (431, 563, and 635 bp) corresponding to the three mRNAs previously described: forward, 5'-ACATCTTCAACGGCGCTTCTGTGTCG-3' and reverse, 5'-AAATGGCAATTCCAGCCACCCG-3'. VEGF-R1 (108 bp) was amplified with the primers forward, 5'-GGAGGTAAGGATGAGGACGTG-3' and reverse, 5'-GTTGATCGACTTCTTGCTTCTC-3'. Tumor growth was determined using the primers forward, 5'-GCCCTGCTGTGTTCTGACTA-3' and reverse, 5'-CAGCAATTGCCCATTGCGT-3'. β-Actin (500 bp) was amplified using the primers forward, 5'-GCTGCAAGAAGACTCTCTGATG-3' and reverse, 5'-TGTTGCTCTGACTAGTGTGAA-3'. Amplification was carried out in a 50-μl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 0.5 μM each primer, 2.5 μCi of [γ-32P]ATP polymerase, and 0.5 μl reverse transcriptase as template. Cycling conditions were as follows: for Stv40 (30 cycles) denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; for Hif-1α (35 cycles) denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min; for Vegf, Vegf-R1, and Vegf-R2 (30 cycles) denaturation 94 °C for 1 min, annealing at 62 °C for 1.5 min, and extension at 72 °C for 1 min. PCR products were visualized in 2% agarose gels stained with ethidium bromide.
Ras and Rac-1 Activation Assays—Plasmids pGEX-RBD and pGEX-PAK-CRIB, which contain the Ras and the Rac-1 binding domains, respectively, fused to glutathione S-transferase (GST), were kindly provided by D. Shalloway and J. G. Collard. All GST fusion proteins were expressed and purified from Escherichia coli BL21 (DE3) as previously described (41) and used within 2–3 days of preparation. Cells were washed twice in ice-cold PBS and lysed in 20 mM Hepes pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA, 25 mM NaF, 1 mM sodium vanadate, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mM benzamidine (for c-H-Ras activation assay), or in 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Nonidet P-40, 100 mM NaCl, 20 mM MgCl₂, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 1 mM dithiothreitol (for the Rac-1 activation assay). Next, cell lysates were incubated with the GST-RBD- or GST-CRIB-containing glutathione-Sepharose beads (15 µl of packed beads corresponding to 15–90 µg of protein) for 30 min at 4 °C with gentle rocking. Bound proteins were eluted by boiling in Laemmli’s sample buffer and resolved in 11% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes and analyzed for active Ras or Rac-1 by immunoblotting (see above) using antibodies against pan-Ras, AU5 (to detect transfected AU5-tagged c-H-Ras), or Rac-1.

RESULTS

Co-transfection of SV-40 Large T Antigen and Proto-oncogenic c-H-Ras Immortalized Primary Mouse Mammary Gland Fibroblasts—Previous studies have shown that stable co-expression of the SV-40 large T antigen and the activated form of the Ras oncogene induced immortalization of primary cells in culture (42). In this work, primary cultures of stromal fibroblasts were obtained from the mammary gland of AhR+/+ and AhR−/− mice by differential trypsinization and filtration, which allowed separation of fibroblasts from epithelial-derived organoids. Primary fibroblasts were then transfected with pSV3Neo (containing the large T antigen of the SV-40 virus), pCEFL-AU5-c-H-Ras (harboring the coding sequence for the proto-oncogenic human H-Ras) or pSV3Neo+pCEFL-AU5-c-H-Ras and cultures selected for neomycin (G418) resistance for 5–6 weeks. Transfection of AhR+/+ fibroblasts with the constructs pSV3Neo, pCEFL-AU5-c-H-Ras, or pSV3Neo + pCEFL-AU5-c-H-Ras produced 5, 4, and 5 clones, respectively. When using AhR−/− fibroblasts, transfection with the same constructs yielded 3, 1, and 4 clones, respectively. T-FGM-AhR+/+ and T-FGM-AhR−/− clones, transfected with pSV3Neo + pCEFL-AU5-c-H-Ras and propagated up to passage 70, were used for further experiments. Cultures were routinely used at 70–80% confluence, except for xenograft experiments in which cell density was maintained at 50%. A morphological and immunocytochemical characterization of T-FGM-AhR+/+ and T-FGM-AhR−/− clones is shown in Fig. 1. Hematoxylin staining showed that, at cell densities below confluence (e.g. 70–80%), both cell lines had similar fibroblastic phenotype with large nuclei and long cytoplasmic extensions (Fig. 1, panels a and b). At confluence, however, T-FGM-AhR−/− cells adopted a more regular and flattened morphology than T-FGM-AhR+/+; which resulted in cultures with a higher cellular density. 2 Immunocytochemistry for the markers vimentin (Fig. 1, panels c and d) and desmin (not shown) further confirmed the fibroblastic phenotype of these clones. Because T-FGM-AhR+/+ and T-FGM-AhR−/− were also positive for smooth muscle α-actin expression (Fig. 1, panels e and f) and negative for the epithelial markers CD49f/α6 integrin (Fig. 1, panels g and h), cytokeratins AE1/AE3 and E-cadherin (not shown), both cell lines could be phenotyped as myofibroblasts. T-FGM-AhR+/+ and T-FGM-AhR−/− were also analyzed for the level of expression and activity of the transfected proteins SV-40 and AU5-c-H-Ras (Fig. 2). RT-PCR analysis for SV-40 revealed a similar level of mRNA expression in both cell lines (Fig. 2A), a result that was also observed at the protein level by immunocytochemistry (Fig. 2B). The transforming activity of the SV-40 large T antigen takes place, at least in part, through binding and inactivation of the tumor suppressors p53 and retinoblastoma (pRb, p107, and p130) (43, 44). SV-40 activity did not significantly differ between T-FGM-AhR+/+ and T-FGM-AhR−/−, given that the amounts of the target proteins p53 and pRb that could be immunoprecipitated by the viral protein were of similar magnitude in both cell lines (Fig. 2C). Regarding c-H-Ras, T-FGM-AhR+/+ and T-FGM-AhR−/− had similar levels of total (Fig. 2D, H-Ras) and transfected (Fig. 2D, AU5-H-Ras) protein, as determined by Western immunoblot. Immunocytochemistry using an AU5-specific antibody also showed a similar level of transfected protein in both cell lines (Fig. 2E). The activation of basal c-H-Ras (Ras-GTP level) was analyzed by a pull-down assay using antibodies against total c-H-Ras or transfected AU5-c-H-Ras and a GST fusion protein containing the Ras binding domain of Raf (41, 45). As shown in Fig. 2F, the basal amounts of activated c-H-Ras and AU5-c-H-Ras (Ras-GTP) were very similar in T-FGM-AhR+/+ and T-FGM-AhR−/− cells.

T-FGM-AhR+/+ and T-FGM-AhR−/− Cultures Had Similar Proliferation Rates and Cell Cycle Distributions—Proliferation rate, as determined by [3H]thymidine incorporation during DNA synthesis, was very similar between T-FGM-AhR+/+ and T-FGM-AhR−/− (Fig. 3A), even though the mutant cells were slightly more proliferative than the wild-type fibroblasts. This similar rate of cell proliferation was also observed by measuring changes in cell numbers with time in cultures growing in serum-containing medium. As shown in Fig. 3B, wild-type and mutant cells had parallel kinetics of cell accumulation, which revealed a similar proliferation potential. When growing in serum-depleted medium (Fig. 3C), T-FGM-AhR+/+ and T-FGM-AhR−/− also proliferated to a similar extent, thus showing that both cell lines were able to support growth factor-independent proliferation. From the experiments performed in serum-containing medium, we could determine the duplication

2 S. Mulero-Navarro and P. M. Fernández-Salgueiro, unpublished observations.
SV-40 protein was analyzed by immunoprecipitation using a 0.5-mg total cell extract and an SV-40-specific antibody, followed by Western immunoblotting with specific antibodies against p53 and pRb.

A

SV-40 expression in cultures from both genotypes was analyzed by RT-PCR using 1 Ci of [3H]methylthymidine and the radioactivity incorporated measured in a liquid scintillation counter. Cell proliferation in T-FGM-AhR+/− cells using a pull-down assay in which cell-free extracts were incubated with 10 μg of GST-RBD-Raf 1. After washing, cellular proteins bound to the beads were run in SDS-PAGE gels alongside the corresponding cell extracts and immunoblotted against monoclonal anti-pan Ras (c-H-Ras) or AU5 (AU5-c-H-Ras) antibodies. Cell-free extract from 293-T cells, overexpressing the hyperactive AU5-H-Ras V12, was used as positive control and monoclonal AU5 antibody. The bar stands for 5 μm. The experiments were performed in at least two different cell cultures with similar results.

Fig. 2. Transfected SV-40 large T antigen and c-H-Ras have similar levels of activity in T-FGM-AhR+/+ and T-FGM-AhR−/−. A, SV-40 expression in cultures from both genotypes was analyzed by RT-PCR using 1 μg of total RNA and gene-specific oligonucleotides. The expression of β-actin was used to check for RNA integrity and quantitation. B, SV-40 protein expression was determined by immunocytochemistry in T-FGM-AhR+/+ and T-FGM-AhR−/− cultures using a specific antibody coupled to a FITC-conjugated secondary antibody. C, activity of the SV-40 protein was analyzed by immunoprecipitation using a 0.5-mg total cell extract and an SV-40-specific antibody, followed by Western immunoblotting with specific antibodies against p53 and pRb. D, total and transfected c-H-Ras protein expression in T-FGM-AhR+/+ and T-FGM-AhR−/− was determined by Western immunoblotting using 10 μg of total protein and specific antibodies for c-H-Ras and the AU5 tag. E, the level of transfected AU5-c-H-Ras protein was also determined by immunocytochemistry using an AU5-specific antibody and a FITC-conjugated secondary antibody. Nuclei were stained with the DNA binding dye DAPI. F, Ras activity was analyzed in T-FGM-AhR+/+ and T-FGM-AhR−/− cells using a pull-down assay in which cell-free extracts were incubated with 10 μg of GST-RBD-Raf 1. After washing, cellular proteins bound to the beads were run in SDS-PAGE gels alongside the corresponding cell extracts and immunoblotted against monoclonal anti-pan Ras (c-H-Ras) or AU5 (AU5-c-H-Ras) antibodies. Cell-free extract from 293-T cells, overexpressing the hyperactive AU5-H-Ras V12, was used as positive control and immunoblotted against monoclonal AU5 antibody. The bar stands for 5 μm. The experiments were performed in at least two different cell cultures with similar results.

Fig. 3. T-FGM-AhR+/+ and T-FGM-AhR−/− have similar proliferation rates and cell cycle distributions. A, cell proliferation in T-FGM-AhR+/+ (open circles) and T-FGM-AhR−/− (closed circles) was estimated from the rate of [3H]thymidine incorporation during DNA synthesis. Both cell lines were plated in 24-well culture dishes, labeled at different times with 0.5 μCi of [3H]methylthymidine, and the radioactivity incorporated measured in a liquid scintillation counter. Cell proliferation in T-FGM-AhR+/+ (open circles) and T-FGM-AhR−/− (closed circles) was also determined as the increase in cell numbers with time in cultures grown in complete medium (B) or in serum-depleted medium (C). D, duplication times for T-FGM-AhR+/+ (open circles) and T-FGM-AhR−/− (closed circles) were calculated from semilogarithmic plots constructed with data such as those shown in B. Data correspond to mean ± S.D. for triplicate measurements. The experiments are representative from at least two different cultures.

Time for each cell line (Fig. 3D). In agreement with the results indicated above, duplication time was also very similar between T-FGM-AhR+/+ and T-FGM-AhR−/−, reaching values of 11 and 10 h, respectively.

Both cell lines were also analyzed for the expression of regulators of the G1/S transition of the cell cycle such as cyclin D1, cyclin E, p21Cip1, and p27Kip1 (Fig. 4A). Cyclin D1 protein levels appeared to be decreased in T-FGM-AhR−/− with respect to T-FGM-AhR+/+ cells; cyclin E protein, on the contrary, was present at similar amounts in both genotypes. With respect to the expression of negative regulators of the G1/S transition, whereas p27Kip1 protein was undetectable, p21Cip1 protein was present at very similar levels in both cell lines. The analysis of cell cycle distribution and apoptosis was performed by flow cytometry in asynchronous T-FGM-AhR+/+ and T-FGM-AhR−/− cultures (Fig. 4B). The percentage of cells along the different phases of the cycle did not significantly differ between both cell lines, thus indicating that the lower level of cyclin D1 present in T-FGM-AhR−/− did not compromise completion of the G1/S transition and entry into S phase. In addition, apoptotic cell death remained below 5–10% in either T-FGM-AhR+/+ or T-FGM-AhR−/− cultures, further supporting a...
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similar proliferative potential for both cell lines. Protein expression levels for the AhR partner ARNT were similar between T-FGM-AhR+/+ and T-FGM-AhR−/− (not shown).

Immortalized T-FGM-AhR−/− Had a Reduced Potential to Induce Tumors in a Subcutaneous NOD-SCID Mouse Xenograft Model—As shown above, T-FGM-AhR+/+ and T-FGM-AhR−/− had a similar morphological phenotype and SV-40 and c-H-Ras activities and very close proliferation rates and cell cycle distribution. Because co-expression of SV-40 and activated c-H-Ras can induce cellular transformation (42), we next addressed the ability of T-FGM-AhR+/+ and T-FGM-AhR−/− myofibroblasts to induce subcutaneous tumors in immunodeficient mice (Table I). A total of 13 or 12 NOD-SCID mice were injected subcutaneously in both flanks with a cellular suspension of T-FGM-AhR+/+ or T-FGM-AhR−/− myofibroblasts, respectively, and tumors collected after 7–8 weeks. Interestingly, whereas 12 of 13 mice injected with T-FGM-AhR+/+ cells produced tumors (92% efficiency), only 3 of 12 of those injected with T-FGM-AhR−/− cells did (25% efficiency) (Table I). In terms of total number of subcutaneous tumors, 16 were recovered from NOD-SCID mice after 26 injections with FGM-AhR+/+ (61% recovery) and only 5 after 24 injections with FGM-AhR−/− cells (21% recovery). Tumor size was slightly smaller in immunodeficient mice injected with T-FGM-AhR+/+ than in those injected with T-FGM-AhR−/− cells. Therefore, the ability of FGM-AhR−/− cells to induce subcutaneous tumors in this mouse model decreased by 4-fold with respect to the number of animals affected and by 3-fold regarding the total number of tumors produced. Tumors produced by mice injected with FGM-AhR+/+ or FGM-AhR−/− cells were fixed in formalin, sectioned, and analyzed by hematoxylin staining and immunohistochemistry (Fig. 5). Pathology analysis revealed that tumors produced by FGM-AhR+/+ myofibroblasts were similar to those formed by FGM-AhR−/− cells. These high cellularity tumors were organized in dense arrays that infiltrated the surrounding adipose and striated muscle tissues. In either case, tumors were formed by highly elongated cells with a relatively undefined cytoplasm and with long, irregular, and hyperchromatic nuclei containing prominent nucleoli. A large number of mitosis, some of them with atypical features, could also be observed by hematoxylin staining (Fig. 5, panels a–c). Immunohistochemistry for the muscle diagnostic marker desmin revealed growth of the tumor into striated muscle tissue (Fig. 5, panel d). These tumors were also positive for molecular markers previously found to be expressed by T-FGM-AhR+/+ and FGM-AhR−/− cells, such as smooth muscle α-actin (Fig. 5, panel e) and vimentin (Fig. 5, panel f). Thus, from the pathology analysis performed, the subcutaneous tumors induced by T-FGM-AhR+/+ and FGM-AhR−/− in NOD-SCID mice could be classified as leiomyosarcomas, an aggressive malignant tumor of the smooth muscle.

The decreased ability of T-FGM-AhR−/− myofibroblasts to induce subcutaneous tumors in this mouse model could be explained by at least two reasons. (i) Those cells had not been fully transformed, a condition that could diminish their ability to support anchorage-independent growth in the recipient mice. (ii) They could be deficient in their ability to establish and adapt to the in vivo microenvironment. To address the transformed status of T-FGM-AhR−/− cells, they were cultured in soft agar and their potential to form colonies compared with that of T-FGM-AhR+/+ myofibroblasts. As shown in Fig. 6, both cell lines formed colonies in soft agar with similar efficiency at different cell dilutions, a result indicative that T-FGM-AhR+/+ as T-FGM-AhR−/−, had the intrinsic potential to induce tumors in vivo.

Cellular Migration, Lamellipodia Formation, and VEGFR-1 Expression Were Impaired in T-FGM-AhR−/− Myofibroblasts—We next analyzed whether T-FGM-AhR−/− cells had an altered response to processes relevant in tumor formation such as cell migration and/or expression of angiogenesis-related genes. T-FGM-AhR+/+ and T-FGM-AhR−/− were seeded in culture transwells coated with either collagen I or matrigel and migration determined by confocal microscopy

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![Fig. 4. Expression of cell cycle regulators and cell cycle distribution in T-FGM-AhR+/+ and T-FGM-AhR−/−](image)

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**TABLE I**

Incidence of subcutaneous tumor formation in NOD-SCID immunodeficient mice injected with immortalized T-FGM-AhR−/− and T-FGM-AhR+/+ cells

<table>
<thead>
<tr>
<th>Number of mice injected</th>
<th>Mice with tumors</th>
<th>Total number of injections</th>
<th>Number of tumors</th>
<th>Tumor volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR+/+</td>
<td>13</td>
<td>26</td>
<td>16</td>
<td>0.92 ± 0.95a</td>
</tr>
<tr>
<td>AhR+/−</td>
<td>12</td>
<td>24</td>
<td>5</td>
<td>0.70 ± 0.71a</td>
</tr>
</tbody>
</table>

* Tumor volume was calculated using the formula π(x² y)/2 where x and y correspond to the width and thickness of the tumor. In NOD-SCID mice injected with T-FGM-AhR+/+ myofibroblasts, the largest tumor obtained had a volume of 3.5 cm³ and the smallest, 0.009 cm³. In NOD-SCID mice injected with T-FGM-AhR−/− cells, the largest tumor reached a volume of 1.7 cm³ and the smallest, 0.009 cm³.

**a** The difference between T-FGM-AhR+/+ and T-FGM-AhR−/− is statistically significant at p < 0.001 (ANOVA).
hematoxylin and eosin. Days of incubation at 37 °C in a CO2 atmosphere, clones were stained with 0.5% agar and placed over a layer of higher density 1% agar. After 22 days of incubation, soft agar support anchorage-independent growth and clone formation in vitro. Clones were stained with crystal violet and counted. Quantitation corresponds to T-FGM-AhR+/+ and T-FGM-AhR−/− cells. The bar corresponds to S.D. from triplicate measurements in at least two different transwells.

In their migratory potential in collagen I matrix. In matrigel conditioned media from T-FGM-AhR+/+ and T-FGM-AhR−/− cultures were collected and MMP activity determined by gelatin zymography. The positions of the MMP-2 and MMP-9 were determined by gelatin zymography. The positions of the MMP-2 and MMP-9 were determined by gelatin zymography.

**Fig. 7.** T-FGM-AhR−/− cells have diminished cell migration and impaired lamellipodia formation. A, T-FGM-AhR+/+ and T-FGM-AhR−/− were seeded in culture transwells coated with either collagen I or matrigel and cell migration monitored by confocal microscopy 36 h later. Migrating cells were located within each matrix by staining with propidium iodide. The results correspond to the mean ± S.D. from triplicate measurements in at least two different transwells. The difference between T-FGM-AhR+/+ and T-FGM-AhR−/− was statistically significant (ANOVA) at p < 0.01. B, cell migration was also estimated from their ability to move in response to a tissue culture wound. T-FGM-AhR+/+ and T-FGM-AhR−/− were grown at confluence and a straight wound made with a pipette tip. Cultures were changed to a serum-free medium for 18 h, stained with hematoxylin, and photographed under light microscopy. The square brackets indicate the extent of wound closure in each cell line. The bar corresponds to 10 μm. C, conditioned media from T-FGM-AhR+/+ and T-FGM-AhR−/− cultures were collected and MMP activity determined by gelatin zymography. The positions of the MMP-2 and MMP-9 were determined by comparison with the HT-1080 human osteosarcoma cell line used as a positive control. MMP-2 activity has been saturated to show MMP-9. D, T-FGM-AhR+/+ and T-FGM-AhR−/− cultures were stressed by serum deprivation over 18 h and lamellipodia formation analyzed by cell staining with rhodamine phalloidin. Lammellipodia are indicated by arrows. Cell nuclei were also stained with the DNA binding dye DAPI. The experiments were performed in at least three independent cultures.

(Fig. 7A). T-FGM-AhR−/− cells showed a significant decrease in their migratory potential in collagen I matrix. In matrigel coating, however, the difference in migration between T-FGM-AhR+/+ and T-FGM-AhR−/− became less pronounced. Wound repair in culture was used as an additional experimental approach to further analyze a deficient migratory potential in T-FGM-AhR−/− cells (Fig. 7B). Eighteen hours after wounding, T-FGM-AhR+/+ readily moved to the center of the wound and started to establish in the available dish space. T-FGM-AhR+/+ on the contrary, mainly remained at the edges of the wound without significantly moving to cover the cell-free space. MMPs are proenzymes that participate in the control of cell migration. T-FGM-AhR+/+ secreted MMP-2 and MMP-9 activities to the medium, although, in comparison, MMP-2 had higher activity than MMP-9 (Fig. 7C). In T-FGM-AhR−/− myofibroblasts, MMP-2 activity was similar, and MMP-9 activity was lower than those present in T-FGM-AhR+/+ cells. Cell migration is also related to reorganization of the α-actin cytoskeleton and to the formation of lammellipodia and phalloidin at the plasma membrane. To determine whether T-FGM-AhR−/− myofibroblasts could reorganize their α-actin fibers in response to stress, both cell lines were grown under serum-free conditions for 18 h and their cytoskeleton analyzed by rhodamine phalloidin staining (Fig. 7D). Whereas serum deprivation induced the formation of lammellipodia in T-FGM-AhR+/+ (left panel, arrows), these structures were rarely present in T-FGM-AhR−/−. Thus, an altered reorganization of the α-actin fibers could contribute to decreased cell migration in T-FGM-AhR−/−.

An increasing number of genes have been found to participate in the control of cell migration and angiogenesis. Among them, could be considered relevant those coding for Hif-1α,
Vegf, and its receptors R1 (VegfR-1) and R2 (VegfR-2), Ang-1 and its type 1 receptor (Ang-R1), and Epo. RT-PCR analysis was performed for this battery of genes. We found detectable levels of mRNA expression for Hif-1α, Vegf, VegfR-1, and VegfR-2 in T-FGM-AhR+/+ and T-FGM-AhR−/− cells, whereas expression for Ang-1, Ang-R1, and Epo could not be detected (Fig. 8A). Steady-state mRNA levels for Hif-1α, Vegf, and VegfR-2 were similar in both genotypes. VegfR-1 mRNA, on the contrary, was significantly down-regulated in T-FGM-AhR−/− myofibroblasts (Fig. 8, A and B). Western immunoblotting revealed that the difference in VegfR-1 mRNA between T-FGM-AhR+/+ and T-FGM-AhR−/− was also present at the protein level (Fig. 8C). Further, immunocytochemistry for VEGFR-1 in these cultures also showed a lower level of protein in T-FGM-AhR−/− cells (Fig. 8D). The possible role of AhR in maintaining VegfR-1 levels was also suggested by our unpublished observations showing that TCDD treatment induced VegfR-1 mRNA in a time-dependent manner.

Decreased Migration in T-FGM-AhR−/− Could Be Related to Down-regulation of the ERK-FAK-Rac-1 Pathway—Cell migration is regulated by complex signaling pathways that involve activation of MAPK, FAK, PI3K, and the small GTPase Rac-1. We have analyzed if decreased migration in T-FGM-AhR−/− cells could be related to alterations in this signaling pathway. FAK is a regulator of cell migration that has been suggested to conduct signaling from MAPKs (e.g. ERK) to PI3K and to the small GTPase Rac-1 (46). The activation of this pathway in cells such as myofibroblasts leads to disassembly of α-smooth muscle actin, lamellipodia extension, and migration. The analysis of activated FAK (p-FAK) in T-FGM-AhR+/+ and T-FGM-AhR−/− by immunoprecipitation and Western immunoblotting showed that AhR-deficient cells had lower levels of active kinase than AhR-expressing myofibroblasts (Fig. 9A). FAK phosphorylation and activation is regulated by ERK through the scaffold protein paxillin. Consistent with the observed decrease in p-FAK levels, ERK activation (p42 and p44) was also lower in T-FGM-AhR−/− cells (Fig. 9, right). On the other hand, FAK signals to downstream targets such as PI3K and protein kinase B/AKT (PKB/AKT). Again consistent with the central role of FAK in this pathway, T-FGM-AhR−/− had decreased activation of PKB/AKT (p-PKB) as compared with T-FGM-AhR+/+ cells (Fig. 9B, left). Activated PI3K, from the synthesis of PI(3,4,5)P3, contributes to the activation of the small GTPase Rac-1. In serum-containing medium, the analy-
sis of Rac-GTP levels by pull-down assay revealed that T-FGM-AhR/−− myofibroblasts had a marked reduction in active Rac-1 with respect to T-FGM-AhR+/+ cells (Fig. 9C). Lower levels of active Rac-1 were also found in T-FGM-AhR/−− in serum-free medium (results not shown). Taken together, these data suggest that the ERK-FAK-PI3K-Rac-1 pathway is altered and could be involved in the lower migration ability of T-FGM-AhR/−− cells.

**DISCUSSION**

Recent studies analyzing the implication of the AhR in normal cell physiology, development, and in signaling pathways that regulate cell growth and proliferation have provided a solid experimental support for the involvement of this receptor in cellular functions unrelated to xenobiotic metabolism (reviewed in Refs. 22, 47, 48). The role of the AhR in cell cycle control, cell proliferation, and apoptosis is particularly interesting because its study could lead to more precise knowledge about how this receptor interacts with signals controlling tumor development. Although *in vivo* work has shown that mice lacking AhR are resistant to dioxin-induced toxicity and carcinogenesis (1, 2), and that mice expressing a constitutively active AhR are more susceptible to carcinogen-induced hepatocarcinomas (28), the role of this receptor in cancer in the absence of xenobiotics is largely unknown. In this study, we have produced immortalized mammary gland fibroblast cell lines from wild-type and AhR-null mice and used them as a tool to analyze how the absence of AhR could affect tumor induction *in vivo* and to identify potential pathways that could be involved. Additionally, since stromal fibroblasts are known to modulate growth and spreading of solid tumors (49), these cell lines could also be a tool to study the mechanisms of epithelial-stromal interactions in cancer. As an experimental approach to immortalize mouse mammary fibroblasts, we have used stable co-transfection with the coding sequences for the SV-40 large T antigen and the proto-oncogenic c-H-Ras oncogene, a method previously shown to be effective in promoting transformation of different primary cell types (42–44). Co-transfection with SV-40 and c-H-Ras had similar efficiency in AhR+/+ and AhR/−− fibroblasts because a comparable number of clones were obtained for each genotype. We could select representative T-FGM-AhR+/+ and T-FGM-AhR/−− clones that did not significantly differ in terms of morphology and cell type (myo-
fibroblasts), expression, and activity of SV-40 and c-H-ras, cell proliferation, duplication time, cell cycle distribution, and apoptosis rates. Interestingly, T-FGM-AhR−/− myofibroblasts had lower cyclin D1 levels that did not negatively affect proliferation rates in this cell line, possibly because cyclin E levels were enough to efficiently complete the G1/S transition. In agreement with this possibility, it is known that cyclin D1 and cyclin E sequentially phosphorylate pRb during the G1 and S phases of the cell cycle (50, 51). In addition, since cyclin D1 could be regulated through ERK and Rac-1 (52, 53), decreased ERK and Rac-1 activities in T-FGM-AhR−/− could account for their lower cyclin D1 protein content. Previous studies have shown that primary cells lacking AhR, such as hepatocytes (54), mammary gland fibroblasts1, and embryonic fibroblasts (25), had lower proliferation rates and increased apoptosis, as well as premature senescence (24). T-FGM-AhR−/− cells, on the contrary, showed similar proliferation rates as T-FGM-AhR+/+. Thus, if differences in tumor induction would be expected be- tween T-FGM-AhR−/− and T-FGM-AhR+/+, suggesting that SV-40-c-H-Ras overexpression could overcome the effect of the absence of AhR on cell proliferation. Therefore, if differences in tumor induction would be expected between T-FGM-AhR−/− and T-FGM-AhR+/+, suggesting that SV-40-c-H-Ras overexpression could overcome the effect of the absence of AhR on cell proliferation.

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When T-FGM-AhR−/− myofibroblasts were injected into NOD-SCID-immunodeficient mice using a subcutaneous xenograft model, their capacity to induce tumors was reduced by 3–4-fold with respect to T-FGM-AhR+/+ cells. Tumors that grew from T-FGM-AhR−/− myofibroblasts had an average es- timated volume similar to that produced by wild-type cells, thus suggesting that the absence of AhR was mostly related to decreased tumor incidence. Consistent with the phenotype of the cells, a pathology analysis of the tumors isolated from NOD-SCID mice injected with either T-FGM-AhR−/− or T-FGM-AhR+/+, revealed that they were leiomyosarcomas, a malignant infiltrating soft tissue tumor derived from myofibroblastic cells. Because tumors composed of myofibroblasts are among the less known human malignancies (55), the T-FGM-AhR−/− and T-FGM-AhR+/+ cell lines pro- duced here could be valuable models to study molecular events (e.g., changes in gene expression) associated with the development of leiomyosarcomas. In this context, recent efforts have tried to determine genetic signatures that could underscore the progression of soft tissue sarcomas (56). Furthermore, the R554K genetic polymorphism in the human AhR gene, which in vitro assays have associated to increased induction of AhR target genes (57), appeared to be linked to poor survival in soft tissue sarcoma patients (58).

Colonies formation in soft agar is a classic criterion for the cellular conversion to a transformed phenotype (59, 60). The impaired ability of T-FGM-AhR−/− cells to induce subcutane- ous tumors in NOD-SCID mice could not be attributed to a deficiency in its transformation state because they were able to support anchorage-independent growth and colony formation in soft agar at a level similar to that of T-FGM-AhR+/+ myo- fibroblasts. At this point, the fact that T-FGM-AhR−/− and T-FGM-AhR+/+ had very similar phenotypes led us to suggest that the lower potential for tumor induction of T-FGM-AhR−/− could be mainly related to their adaptability to the in vivo microenvironment. Previous studies have suggested the in- volvement of the AhR in signaling pathways controlling cell migration and angiogenesis. Thus, this receptor is inhibited by the MEK antagonist PD98059 (61), AhR ligands inhibit T-cadherin expression in vascular endothelial cells (62) and disrup- tion of cell-cell interactions activate the AhR in primary keratinocytes and 10T1/2 fibroblasts (63, 64). With respect to migration, T-FGM-AhR−/− cells had decreased migration in a collagen I matrix and in a tissue culture wound assay. Because migration is stimulated by the degradation of the extracellular matrix by metalloproteinases, such as MMP-2 and MMP-9 (65), and because primary AhR−/− fibroblasts had decreased MMP-2 activity (26), we analyzed MMP-2 and MMP-9 in T-FGM-AhR+/+ and T-FGM-AhR−/−. MMP-9 activity was reduced in T-FGM-AhR−/− cells; MMP-2, on the contrary, had similar levels and was in excess with respect to MMP-9 in both cell lines. Although these data could disregard the contribution of MMP-9 to T-FGM-AhR−/− migration, it is interesting to note that MMP-9 specifically induces lung metastasis in mice via VEGFR-1/Flt-1 (66). Interestingly, T-FGM-AhR−/− cells had a marked decrease in VEGFR-1/Flt-1 expression at the mRNA and protein levels; an observation that provides addi- tional support for the role of VEGFR-1/Flt-1 in myofibroblasts migration and in the activation of MMP-9. Additional work analyzing the contribution of VEGFR-1/Flt-1 to cell migration has shown that this receptor modulates reorganization of the actin cytoskeleton (67) and that expression of VEGFR-1 mutants inhibits solid tumor formation (68). Further support for a functional interaction between the AhR and genes involved in vasculogenesis was provided by studies showing that AhR−/− mice had portosystemic shunting and altered liver vasculature (11), a developmental defect that could be rescued by gesta- tional exposure to dioxins (69).

Actin fibers were evident in T-FGM-AhR−/− myofibro- blasts under stress, but these cells failed to develop mem- brane-associated structures involved in cell migration such as membrane ruffles and lamellipodia. A signaling pathway has been proposed that links membrane receptors to lamel- lipodia extension and cell migration. Tyrosine kinase recep- tors (e.g., epidermal growth factor receptor, hepatocyte growth factor receptor, cMET), once activated, signal to in- termediate proteins (e.g., c-Src kinase and/or Ras) that will in turn activate kinases of the MAPK family (MEK and ERK) (46). A central role in this pathway is played by the focal adhesion kinase, FAK, which became activated by phospho- rylation through its association with the scaffold protein paxillin, a target of ERK and MEK (46, 70, 71). One of the downstream targets of FAK is PI3K, which generates PI(3,4,5)P3 and contributes to the activation of the small GTPase Rac-1, a major regulator of focal complexes forma- tion, actin bundling, and elongation of lamellipodia in myo- fibroblasts and endothelial cells (72–74). Consistent with this pathway, the decrease in migration observed in T-FGM- AhR−/− myofibroblasts was coincident with lower levels of activated ERK (p42/p44), decreased phosphorylation (activation) of FAK, reduced PI3K activity determined by the levels of phospho-PKB/AKT, significantly lower Rac-1 activity, and diminished lamellipodia formation. Thus, these data suggest that the AhR could participate in signaling pathways regu- lating cell migration. Previous studies in liver cells and NIH 3T3 fibroblasts have shown that an upstream regulator of that pathway, the c-Src kinase, remains in the cytosol as an inactive complex bound to the AhR and Hsp-90 and that receptor activation is required for release and activation of the kinase (75, 76). Therefore, considering the positive role of AhR in c-Src activation, it is possible that in T-FGM-AhR−/− cells, the absence of AhR could weaken c-Src activation, an effect that could result in decreased Rac-1 activity and lower cell migration.

In summary, the results obtained suggest that the AhR has a role in tumor development in the absence of xenobiotics, possibly by modulating the cellular response to the microenviron- ment. At the molecular level, the AhR could act upstream
on the Src-FAK-Pi3K-Rac-1 pathway that controls cell motility. In addition to this hypothesis, the AhR could be also involved in regulating the expression of VEGFR-1, a major tyrosine kinase receptor involved in cell migration and blood vessel formation. On the other hand, the T-FGM-AhR+/+ and T-FGM-AhR−/− myofibroblast cell lines produced in this work represent a valuable tool to analyze the development of leiomyosarcomas, a soft tissue sarcoma poorly characterized at the molecular level. Therefore, we hypothesize that the absence of AhR could impair tumor development in vivo. From the prognostic point of view, it is tempting to speculate that high levels of AhR expression could be associated with increased tumor susceptibility. These results open new perspectives in AhR research, to determine the molecular mechanisms through which this receptor participates in signaling pathways regulating cell migration and angiogenesis.

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REFERENCES

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