Mechanistic Interrogation of Cell Transformation in Vitro: The Transformics Assay as an Exemplar of Oncotransformation

Gelsomina Pillo

Agency for Prevention, Environment and Energy, Emilia-Romagna

Maria Grazia Mascolo

Agency for Prevention, Environment and Energy, Emilia-Romagna

Cristina Zanzi

Agency for Prevention, Environment and Energy, Emilia-Romagna

Francesca Rotondo

Agency for Prevention, Environment and Energy, Emilia-Romagna

Stefania Serra

Agency for Prevention, Environment and Energy, Emilia-Romagna

Francesco Bortone

Alma Mater Studiorum University of Bologna

Sandro Grilli

Alma Mater Studiorum University of Bologna

Monica Vaccari

Agency for Prevention, Environment and Energy, Emilia-Romagna

Miriam Naomi Jacobs

Department of Toxicology, Centre for Radiation, Chemical and Environmental Hazards Public Health
England

Annamaria Colacci (✉ annamaria.colacci@unibo.it)

Agency for Prevention, Environment and Energy, Emilia-Romagna

Research Article

Keywords: Mechanistic interrogation, cell transformation, vitro, oncotransformation

Posted Date: June 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-559566/v1>

License: (cc) (i) This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

The rodent-based life-time cancer bioassay is still the basic experimental paradigm to assess the carcinogenic potential of chemicals, with relatively little progress regarding the acceptance of in vitro tests.

The Transformics Assay is an in vitro test which combines the BALB/c 3T3 Cell Transformation Assay (CTA) with microarray transcriptomics. It has been shown to improve upon the mechanistic understanding of the CTA, helping to identify mechanisms of action leading to chemical-induced transformation thanks to RNA extractions in specific time points along the process of in vitro transformation.

In this study the lowest transforming concentration of the carcinogenic benzo(a)pyrene (B(a)P) has been tested in order to find molecular signatures of initial events relevant for oncotransformation. Some biological interpretations are presented related to the Enrichment Analysis provided by Metacore.

Results show that after 72 hrs of exposure, the immune and inflammatory responses are the main processes involved in cell response. Furthermore, pathways and processes related to cell cycle regulation, cytoskeletal adhesion and remodeling processes, cell differentiation and transformation were observed.

All these data are interpreted as a consequence of the initial aryl hydrocarbon receptor (AhR) activation leading to a cascade of cellular events and microenvironment modification.

Introduction

Cancer is a consequence of a complex process requiring the alteration and modification of several biological traits that includes changes at the molecular, cellular, tissue and organ level. In humans, this process takes years to become phenotypically evident and the diagnosis is often made when the organ function is already impaired.

Addressing this complexity by using appropriate experimental in vitro models that can resemble the biological process, is still a challenge.

Until relatively recently, the process of carcinogenesis was initially described as a two-step process, initiated by genotoxic events that subsequently orchestrate the cascade of sequential key events, leading to and supporting tumour progression. However in recent years it has become clear that non-genotoxic carcinogenic events play a fundamental role in increasing the risk of human cancer.

The induction of cancer involves the accumulation of genomic alterations, which can be induced directly or indirectly. Carcinogens have conventionally been divided into two categories according to their presumed mode of action, genotoxic carcinogens and non-genotoxic carcinogens. A genotoxic carcinogen has the potential to induce cancer by interacting directly with DNA and/or the cellular apparatus involved in the preservation of the integrity of the genome. Genotoxic compounds were first described in 1980 as chemicals capable of interacting with DNA and inducing tumors in animals (two species, multiple organs), with incidence increasing linearly in a dose dependent fashion, and with no threshold. A non-genotoxic

carcinogen has the potential to induce cancer without interacting directly with either DNA or the cellular apparatus involved in the preservation of the integrity of the genome ^{1,2}

The classificatory distinction between genotoxic and non-genotoxic carcinogenic chemicals is fundamental to current regulatory purposes.

Genotoxicity and mutagenicity can be explored utilising several standardized *in vitro* tests that are accepted in the regulatory context. However, a chemical that is positive in genotoxicity assays, may or may not be carcinogenic ³.

Until relatively recently the rodent carcinogenesis bioassay (RCB) was regarded as the gold standard to study the carcinogenesis process and to identify the carcinogenic properties of both genotoxic and non-genotoxic chemicals. The RCB provides apical endpoints (the tumour in the target organs), some information on the biochemical and morphological alterations accompanying the formation of the tumour, but little or no information on the mechanisms leading to malignancy, or on the key events which mark each step of the process.

The Cell Transformation Assay (CTA) represents an attempt to model the carcinogenesis process in vitro. The assay was developed in the 1960's using different cell lines and protocols, with the intention of replacing the RCB to reduce animal testing, whilst also providing a less expensive and faster tool to test chemicals for carcinogenicity. During the process to develop the Organization for Economic Cooperation and Development (OECD) test guideline, however, some limitations of the CTA were noted, leading to the decision that the assay could not be used as a stand-alone test for predicting carcinogenesis but it could be included in an integrated strategy to contribute to the weight-of-evidence for the chemical classification ^{3,4}.

At the same time, it was recognized that there is a need to develop an integrated approach for the testing and assessment (IATA) of non-genotoxic carcinogenesis (NGTxC), and a specific programme of work was established at the OECD Test Guideline Programme ³.

To support the development of the NGTxC-IATA, the mechanistic understanding of the CTA was improved using transcriptomics analysis to facilitate the identification of the key events marking the in vitro cell driven process leading to cell oncotransformation. By using this Transcriptomics assay approach, we were able to discriminate the adaptive and adverse response of BALB/c 3T3 cells exposed to 3-methylcholantrene (3-MCA), suggesting that for this genotoxic chemical, a threshold may exist ⁵.

To better understand the events that mark the turning point from the adaptive to the adverse response, here we report on the testing of the more toxic and carcinogenic compound, B(a)P. B(a)P can act via both genotoxic and non-genotoxic mechanism ^{6,7}, and is considered the reference chemical for the chemical class of polycyclic aromatic hydrocarbons (PAHs) and as such it is used to identify the carcinogenic potency of other PAHs and of complex mixtures containing PAHs via the AhR ⁸, one of the receptor mediated molecular initiating events identified for the integrated approach for the testing and assessment of non genotoxic carcinogen of carcinogens ².

B(a)P was tested in the Transformics assay at the lowest in vitro concentration capable of sustaining the process of oncotransformation.

The Transformics Assay is proposed as a suitable method for inclusion within an IATA for NGTxC, as it provides an in vitro adverse outcome represented and measurable by the "focus", whose association with particular expressed gene profiles can help to identify mechanisms of action leading to chemical-induced transformation. In this way, the "focus" or 'foci' end-point(s), fundamental for the evaluation of the chemical carcinogenic potential, can be supported by direct evidence linking the phenotypic end-point to the molecular events ⁵.

Materials And Methods

Experimental design

The Transformics Assay experimental design is shown in Fig. 1.

A preliminary range finding CTA was performed to select the treatment concentrations to be tested in the Transformics Assay.

Cell culture experiments were conducted in parallel. A set of plates for each treatment (NT, 0.1% DMSO, 0.02 µg/ml B(a)P, 0.2 µg/ml B(a)P, 4 µg/ml 3-MCA) were maintained in culture for the CTA (A-B). The foci formation served as the phenotypic anchoring for the microarray results. **A)** Experimental protocol of the Cytotoxicity assay to evaluate the cell survival after the chemical treatment. **B)** Experimental protocol of the Transformation Assay, with medium changes at specified time points **C)** Integrated experimental protocol: RNA was extracted from cells treated with the Lowest Transforming Concentration (**LTC**) 0.02 µg/ml B(a)P and cells treated with 0.1% Dimethyl Sulfoxide (DMSO), as control. The microarray experiment was conducted with a glass slide containing 8x60K formatted arrays, four of which were hybridised with the treated cells lysate, and four with the control lysate. The GeneSpring GX software (Agilent Technologies) was employed for the statistical analysis of microarray experiments, whereas MetaCore (Thomson Reuters, <https://portal.genego.com/>) was used for the biological analysis. Figure created with BioRender.com

Cells

The original stock of BALB/c 3T3 cells, clone A31-1-1, was obtained from the Health Science Research Resource Bank (Osaka, Japan). Cells were tested, characterized and authenticated by the cell bank for the species origin.

Working cultures were expanded from the original cryopreserved stock.

Cells were grown in M10F, Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL). Only subconfluent cells were used, and the target cells were not maintained beyond the third passage after thawing. Cultures were maintained in a humidified incubator with an atmosphere of 5% CO₂ in air at 37°C⁵.

Chemicals and solutions

3-Methylcholanthrene (3-MCA, 56-49-5, purity 98,9%), Benzo(a)pyrene (B(a)P, 50-32-8, purity 96%) and Dimethyl Sulfoxide (DMSO, 67-68-5, purity 100%) were obtained from Sigma–Aldrich.

DMSO was used at a 0.1% concentration as the vehicle for the two chemicals and as the negative control.

The stock solution of 3-MCA (4 mg/ml) was delivered to the culture medium to reach the final working concentration of 4 µg/ml.

The B(a)P working solutions were prepared by diluting sequential stocks solutions in the culture media to obtain concentrations ranging from 0.0025 µg/ml to 0.04 µg/ml, within the preliminary CTA, and the concentration of 0.02 µg/ml in the subsequent Transformics Assay.

CTA and cytotoxicity assessment

The test was performed according to the EURL-ECVAM (EU Reference Laboratory European Centre for the Validation of Alternative Methods) validated protocol ^{9–11}. This protocol includes cytotoxicity testing within the CTA. Details are reported in Supplementary Information, available Online.

A preliminary CTA was performed in order to identify the lowest transforming concentration (LTC), by testing B(a)P at concentrations of 0.0025µg/ml, 0.005 µg/ml, 0.01µg/ml, 0.02µg/ml and 0.04 µg/ml (Fig. 2). Whilst all the concentrations tested were cytotoxic, they did not all incur transformation. The LTC observed for B(a)P was 0.02 µg/ml, and therefore this concentration was selected for testing in the CTA for the Transformics Assay.

A concentration of 0.2µg/ml B(a)P was used in the subsequent transformics CTA, as a positive control.

Cells were treated 24 hrs after seeding. Following 72 hrs exposure, the medium was removed, and the cell cultures were incubated in a humidified incubator at 37°C with 5% CO₂. The time of exposure was chosen according to the EURL-ECVAM validated protocol ^{9–11}.

Total RNA extraction

Total RNA was isolated after 72 hrs of exposure with 0.02 µg/ml B(a)P, by using TRIzol Reagent (Invitrogen, San Diego, CA) followed by purification with Rneasy affinity column (Qiagen, Valencia, CA), according to the manufacturer's instructions. RNA quality was assessed by Agilent Bioanalyzer 2100 (RNA 6000 Nano LabChip, Agilent Technologies, Palo Alto, CA)⁵.

Four replicates were performed for each treatment (0.02 µg/ml B(a)P and 0.1% DMSO)⁵.

Total RNA labeling and hybridization

The cRNA was labeled, purified and hybridized on oligonucleotide slides (SurePrint G3 Mouse Gene Expression 8x60K Microarray Kit) using the Low Input Quick Amp Labeling Kit, versione 6.9.1, December

2015 (Agilent Technologies, Santa Clara, CA, USA) (www.genomics.agilent.com

["http://www.chem.agilent.com/".](http://www.chem.agilent.com/)

Statistical analysis of microarray data

Raw data were filtered for intensity and quality signal by using GeneSpring GX (Agilent Technologies).

A T-test unpaired $p \leq 0.05$, corrected by Benjamini–Hochberg, was used to select differentially expressed genes after B(a)P treatment in comparison with 0.1% DMSO.

Tools of biological interpretation

The list of differentially expressed genes was evaluated by using MetaCore, an integrated 'knowledge-based' platform with a manually annotated database of protein interactions verified by 'small experiment' data and gene–disease associations (<https://portal.genego.com/>). An enrichment analysis was performed to identify Pathways Maps and Process Networks altered after 72 hrs of 0.02 µg/ml B(a)P exposure.

Results

CTA

Results of the preliminary CTA and transformics CTA are shown in Figure's 2 and 3 respectively.

Statistically significant cytotoxic and transforming effects were observed at the selected B(a)P concentrations ($p \leq 0.01$), confirming the results from the preliminary tests (Fig. 3), and in accordance to the original requirements of the protocol ⁹.

Statistical analysis of microarray experiments:

The T-test unpaired ($p \leq 0.05$, Benjamini-Hochberg) 0.02 µg/ml vs 0.1% DMSO was performed using GeneSpring software, this indicated an extensive gene perturbation with B(a)P treatment on BALB/c 3T3 cells. Approximately 800 differently expressed genes were identified, amongst which 459 genes were over-expressed and 347 under-expressed.

Biological interpretation of microarray experiments

Application of MetaCore Pathway Maps, Process Networks and GO Processes resulted in a statistically significant false discovery rate ($FDR \leq 0.05$) (Table 1–2).

The statistically significant data were quantitatively dominated by the involvement of immune response and inflammatory processes. Additional key pathways and processes that were statistically significant were related to cell cycle regulation (proliferation / apoptosis), cytoskeletal adhesion and remodelling processes, cell differentiation and transformation (Figure S1).

Pathways and processes related to immune response

The immune response was characterized by the involvement of several cytokine-dependent pathways, interconnected by common modulated genes (Table 1–2).

Specific pathways of high relevance include: "Immune response_IFN-alpha / beta signaling via JAK / STAT", "Immune response_IFN-alpha / beta signaling via MAPKs", "Immune response_IFN-alpha / beta signaling via PI3K and NF-kB pathways". These pathways are activated as a consequence of alpha and beta interferons (IFNs) binding to the respective transmembrane receptors, which, through the activation of intermediate transcription factors such as STAT and IRF, determine inflammatory, immune and proliferative processes. To better address the characterization of single genes, the gene set "Process Network: Inflammation_Interferon signaling" was scanned (Table S1) and then two Networks were created using this dataset, in order to highlight relationships and interactions (Figure S2). By additionally using the building algorithms "Transcription regulation" and "Analyze network_Transcription factors" for Networks creation, and applying these to the "inflammation interferon signaling" Process Networks gene lists, we were able to highlight that STAT1 (Signal transducer and activator of transcription 1), was a pivotal player in the key transcription factors (Table S1; Figure S2). Whilst these algorithms were very useful for identifying principal genes of interest, not all genes of interest are included in this list, for example, genes related to antiviral and stress responses: *Cdkn1a* (also known as *p21*), *ifi15* and *Oas1*, are omitted.

In this inflammation-interferon molecular environment, IRF genes may function to propagate, amplify, and coordinate defence signals. This effect can be achieved both through the induction of IFN-alpha and IFN-beta (by binding with DNA IRF element in INFs promoters), and through binding with ISRE (IFN-stimulated response element) sequences that are found in most IFN-inducible gene (ISG) promoters.

The treatment with B(a)P appeared to induce the activation of the complement system, as highlighted by the significant modulation of the related Pathway Maps, in which a considerable over-expression of the *C3* gene is shown.

Furthermore, other immune response modulated pathways included interleukin-dependent pathways, associated with pro-inflammatory functions. Of particular interest is the over-expression of the interleukins *Il-22* and *Il-18* (Table 1; Pathway n.15;47).

Up-regulation of some negative regulators of cytokine signaling and immune responses, which prevent genes from being expressed, were also detected. In particular the over-expression of *Nfkbia*, which encodes a member of the NF-kappa-B inhibitor family (I-kb), and *Socs3*, which encodes the suppressor of cytokine signalling member 3 were highlighted. Both these genes may act in a classic negative feedback loop to regulate cytokine and JAK-STAT signal transduction^{12,13}.

Process Networks and GO processes analysis also confirmed the importance of the cytokine-mediated inflammation signals (Table 2–3).

Among the different significantly modulated Process Networks (P values and FDR provided in Table 2), the "Inflammation inflammasome" was of high interest, with the conspicuous presence of INF-related genes in the differential expressed gene list. A network is proposed to highlight connections within this gene set.

Within this gene set, RELA (also known as P65, a subunit of the NF- κ B transcription factor complex) is noted to be the key transcription factor using the building algorithm “Transcription regulation” (Table S2 and Figure S3).

Most of these reported genes have also been detected when applying Pathway Maps analysis, so here we are able to reinforce and consolidate the weight of evidence with respect to the crucial role of crosstalk between specific signaling pathways, with a very complex transcription regulation of downstream genes.

It has been reported that IL-22 can induce MAP3K8 signaling pathway and promoting cancer-associated inflammation in the tumor microenvironment ^{14,15}. Therefore, we manually added the “molecular node” *Map3k8* in the Networks creation dataset for the “inflammation_inflammasome” Process Network, in order to facilitate the identification of related interactions (Table S2 and Figure S3).

It is also worth noting the *Chil1* (Chitinase-like 1, also known as *Chi3l1*) differential gene expression (FC: +20.65; Table 3: GO processes i.e. n.1-3-5). This gene modulation has been already proposed in other studies as a potential link between the molecular microenvironment related to inflammation and cells transformation ^{16,17}. Moreover this gene has been shown to be secreted by Cancer Associated Fibroblasts (CAFs) in breast carcinomas during both early and advanced stages, and to play a central role in tumor promotion, producing a pro-inflammatory and immunosuppressed microenvironment ^{16,17}.

Consequently, the analysis presented here therefore further supports the utility of the BALB/c 3T3 cellular model to study molecular mechanisms of cancer, and the crucial role of fibroblasts in the transformation process.

Pathways and processes related to EMC modification and EMT/MET

Several statistically significant pathways and processes were linked to the “Epithelial to Mesenchymal Transition” (EMT), to processes of differentiation and modification of extracellular matrix (ECM) and cells adhesion (Table 1-2-3).

From these Pathway Maps analyses, the disruption of cell junctions (i.e. up-regulation of *Lef-1*, downstream effector of the WNT signaling), the cytoskeletal remodelling (i.e. down-regulation of *Actb*, actin beta) and the modification of cell-adhesion and extracellular matrix composition (i.e. up-regulation of *Mmp-2* and *Mmp-13*, *Lama 4*, *Col16a1*, *Col5a1*, *Emlin2*, *Vcam1*, down-regulation of metalelastase *Mmp-12*, *Adamts1* and *Adamts5* also known as *Aggrecanase-2*) emerged as the main mechanistic outcomes.

The significance (FDR = 5.307e-5) of the Process Network “Signal transduction_WNT signaling” (Table 2; Process Network n. 2; Table S3) and the up-regulation of *Wntb5* (FC: +2.037) and *Lef-1* (FC: +1.45) together with the down regulation of *Fzd2* (FC: -1.8), support the WNT pathway deregulation.

In addition, the modulation of “Signal Transduction_TGF-beta, GDF and Activin signaling” and “Signal transduction_NOTCH signaling” are presented in the Process Networks list (Table 2; Process Network n 15;18).

The down-regulation of *Jag1* (*Jagged 1*, NOTCH ligand), *Smad2* and *Nanog*, crucial transcriptional factors in the TGF- β signaling, were detected, in addition to *Edn1* (*Endothelin-1*), gene located downstream in this pathway. The up-regulation of the *Tgfb β 3* gene (*Tgf- β type 3 receptor*, FC: +1.49) (Process Network n. 15) and *Tgf- β i* (*Tgf- β -induced protein*, also known as *Beta-ig-h3*) (Table 2 Process Network n.3; Table S4) confirmed the TGF- β signaling deregulation (Bissey et al., 2018). The TGF β 3 can interact with all three members of the TGF- β family (I II and III), and consequently interfere with the activation of the associated pathways.

Furthermore the up-regulation of *Dcn* (*Decorin*) (Table 2; Table S4.) is proposed to inhibit the TGF- β .

The downregulation of *Wisp1* and *Jag1* could be also evidence of NOTCH1 signaling pathway depression, an event which may occur in sustaining fibroblast proliferation^{18,19}.

Moreover, the up-regulation of *Lif* (*leukemia inhibitory factor*, FC + 2,55) (Table 1; pathway n.40) is noted to be an important regulatory factor^{20–22}.

Genes involved in the metabolism of Benzo (a) pyrene

Extensive literature evidence defines B(a)P (also 3-MCA and 2,3,7,8-tetrachlorodibenzo-p-dioxin) as a bifunctional AhR inducer^{23 24}, which, upon receptor ligand binding and heterodimerization with the heterodimeric transporter protein ARNT, is able to induce both Phase 1 oxidative enzymes (such as the cytochrome P450 enzymes CYP1A1 and CYP1B1) and Phase 2 conjugating enzymes (such as glutathione transferase and UDP-glucuronosyltransferase)^{25 24,26}.

In this study, a remarkable up-regulation of the *Cyp1a1* (FC = + 8.17) and *Cyp1b1* (FC = + 2.76) genes were observed. Positive regulation of the *Cyp1a1* and *Cyp1b1* genes was also identified in the significantly modulated Process Network "Signal transduction ESR1 nuclear pathway" (Table 2; Process Network n. 25), as was *Ugt1a6b*, over-expression, this gene encodes for UDP-glucuronosyl-transferase (UGT).

Phase II enzymes, such as UGTs, are necessary for the transformation of non-polar, exogenous and endogenous compounds into their hydrophilic derivatives. With respect to B(a)P, the activity of UGTs is fundamental for preventing the formation of epoxide BPDE (B(a)P-7, 8-dihydrodiol-9, 10-oxide), a potent mutagenic metabolite Fang^{27 28}. Indeed our group have previously noted that the loss in expression of UGTs associated with cell transformation is a crucial event to discriminate the adaptive from the adverse response that leads to transformation⁵.

Unlike UGT1A7, UGT1A9 and UGT1A10, UGT1a6 is unable to detoxify the intermediate metabolite BPD (B(a)P-trans-7,8-dihydrodiol), and thus is unable to prevent its oxidation and consequent epoxide formation^{27,28}. Moreover, down-regulation of some UGTs (but not *UGT1A6*) have been observed in human hepatocarcinogenesis, and the up-regulation of *Ugt1a6* has been observed in the rat hepatocarcinogenesis process²⁸.

With regard to inducible enzymes, the up-regulation of *Ncf4* (*Neutrophil cytosolic factor 4*, also known as *P40phox*, FC: +5.070; Table 2; Process Network n. 16) is notable. As a member of the NADPH oxidase subunits, this enzyme plays an important role in NADPH enzyme regulation of oxidase activity and the subsequent production of reactive oxygen species (ROS). A direct transcription regulation of *P40phox* by AhR has been proposed ²⁹.

Table 1

First 50 statistically significant Pathway Maps of the Enrichment Analysis; Upregulated and downregulated objects (individual and family of genes) are identified.

Enrichment for Pathway Maps						
	Pathway Maps	pValue	FDR	Gene ratio	Up regulated network Objects	Down regulated network Objects
1	Immune response_IFN-alpha/beta signaling via JAK/STAT	1,419E-11	9,964E-09	15/64	IRF1, Mx1, SOCS3, USP18, IRF7, p21, IRF9, IP10, GBP1, OAS1, ISG15, GBP4, RIG-G, RSAD2	HIF1A
2	Immune response_IFN-alpha/beta signaling via MAPKs	2,414E-08	8,472E-06	13/77	Irgm2, IRF7, Oasl2, p21, IRF9, IP10, Oas1g, ISG15, PL scramblase 1, Ku80, RIG-G, RSAD2	JNK(MAPK8-10)
3	Transcription_HIF-1 targets	1,353E-05	3,019E-03	11/95	WT1, Angiopoietin 2, MMP-2, p21	HIF1A, Endothelin-1, PGK1, NIP3, GLUT1, Nucleophosmin, NANOG
4	Development_Regulation of epithelial-to-mesenchymal transition (EMT)	1,720E-05	3,019E-03	9/64	Lef-1, PDGF-R-alpha, TCF8, MMP-2, WNT	Jagged1, Endothelin-1, Frizzled, SMAD2
5	Cell adhesion_ECM remodeling	3,966E-05	5,305E-03	8/55	Versican, MMP-2, LAMA4, MMP-13,	VIL2 (ezrin), Actin cytoskeletal, MMP-12, IGF-1
6	Development_YAP/TAZ-mediated co-regulation of transcription	4,534E-05	5,305E-03	8/56	Lef-1, Bcl-XL, ID3,	HIF1A, Endothelin-1, NANOG, p73, SMAD2
7	Glomerular injury in Lupus Nephritis	5,815E-05	5,831E-03	10/92	IRF1, Decorin, CX3CL1, RIG-I, IFI56, C3a, IP10	HIF1A, VCAM1, JNK(MAPK8-10)
8	Immune response_IFN-alpha/beta signaling via PI3K and NF-kB pathways	6,997E-05	6,140E-03	10/94	IFIT1, Mx1, IRF7, p21, GBP1, ISG15, I-kB, RSAD2	PI3K cat class IA, IRS-1
9	Development_TGF-beta-dependent induction of EMT via SMADs	1,474E-04	1,150E-02	6/35	Lef-1, TCF8, MMP-2	Jagged1, Endothelin-1, SMAD2

Enrichment for Pathway Maps						
10	Cell adhesion_Alpha-4 integrins in cell migration and adhesion	1,734E-04	1,187E-02	6/36	VCAM1, ITGB7	ERM proteins, F-Actin cytoskeleton, Actin cytoskeletal, PI3K cat class IA
11	Signal transduction_Additional pathways of NF-kB activation (in the cytoplasm)	2,006E-04	1,187E-02	7/52	PKC-zeta, Adenylate cyclase, TPL2(MAP3K8), alpha chain (CSNK2A1), I-kB, NFKBIA	PI3K cat class IA, Casein kinase II
12	Development_Growth factors in regulation of oligodendrocyte precursor cell survival	2,030E-04	1,187E-02	6/37	PDGF-R-alpha, Bcl-XL, NFKBIA	PI3K cat class IA, IRS-1, IGF-1
13	Immune response_Classical complement pathway	2,266E-04	1,223E-02	7/53	C3c, C3a, C3b, C3dg, C3, iC3b, C1s	
14	Apoptosis and survival_APRIL and BAFF signaling	2,739E-04	1,373E-02	6/39	NF-AT2(NFATC1), Bcl-XL, I-kB, RelB (NF-kB subunit)	JNK(MAPK8-10), TACI(TNFRSF13B)
15	Development_The role of GDNF ligand family/RET receptor in cell survival, growth and proliferation	3,062E-04	1,386E-02	9/92	GFRalpha2, Bcl-XL, NFKBIA, IL-22	HIF1A, F-Actin cytoskeleton, PI3K cat class IA, JNK2(MAPK9), IRS-1
16	Development_Role of Activin A in cell differentiation and proliferation	3,159E-04	1,386E-02	6/40	Adenylate cyclase, p21, MAD, LHX3	NANOG, SMAD2
17	Immune response_TNF-R2 signaling pathways	6,077E-04	2,396E-02	6/45	TRAF1, Bcl-XL, I-kB, BMF	PI3K cat class IA, JNK(MAPK8-10)
18	Signal transduction_Additional pathways of NF-kB activation (in the nucleus)	6,143E-04	2,396E-02	5/30	PKC-zeta, STO, Adenylate cyclase, I-kB, NFKBIA	
19	Immune response_IL-1 signaling pathway	6,724E-04	2,405E-02	8/82	IRF1, PKC-zeta, IP10, TPL2(MAP3K8), I-kB, MMP-13	PI3K cat class IA, JNK(MAPK8-10)
20	Transcription_Androgen Receptor nuclear signaling	6,853E-04	2,405E-02	6/46	MMP-2, p21, WNT,	VIL2 (ezrin), Frizzled, IGF-1

Enrichment for Pathway Maps						
21	Immune response_Histamine H1 receptor signaling in immune response	7,703E-04	2,458E-02	6/47	NF-AT2(NFATC1), VCAM1, I-kB, NFKBIA, MMP-13	JNK(MAPK8-10)
22	Development_PIP3 signaling in cardiac myocytes	7,703E-04	2,458E-02	6/47	PKC-zeta, Bcl-XL	G-protein alpha-12 family, PI3K cat class IA, IRS-1, IGF-1
23	Muscle contraction_Relaxin signaling pathway	8,632E-04	2,635E-02	6/48	PKC-zeta, MMP-2, I-kB, NFKBIA	PI3K cat class IA (p110-beta), Endothelin-1
24	Ligand-independent activation of Androgen receptor in Prostate Cancer	9,668E-04	2,795E-02	7/67	Tcf(Lef), Bcl-XL	PP2A regulatory, Frizzled, PI3K cat class IA, IRS-1, IGF-1
25	Immune response_Lectin induced complement pathway	1,075E-03	2,795E-02	6/50	C3c, C3a, C3b, C3dg, C3, iC3b	
26	G-protein signaling_RhoA regulation pathway	1,111E-03	2,795E-02	5/34	ARHGEF3	VIL2 (ezrin), PRK1, G-protein alpha-12 family, IGF-1
27	Androgen receptor activation and downstream signaling in Prostate cancer	1,135E-03	2,795E-02	9/110	ER81, Bcl-XL, p21, IL6RA	VIL2 (ezrin), Versican, PI3K cat class IA (p110-beta), IRS-1, IGF-1
28	Signal transduction_PKA signaling	1,195E-03	2,795E-02	6/51	LBC, Adenylate cyclase, NFKBIA	PP2A regulatory, AKAP12, G-protein alpha-12 family
29	Development_IGF-1 receptor signaling	1,195E-03	2,795E-02	6/51	PKC-zeta, Bcl-XL, I-kB,	PI3K cat class IA, IRS-1, IGF-1
30	Some pathways of EMT in cancer cells	1,195E-03	2,795E-02	6/51	Lef-1, PDGF receptor, PDGF-R-alpha, I-kB	PI3K cat class IA, Endothelin-1
31	Immune response_IL-9 signaling pathway	1,449E-03	3,115E-02	5/36	SOCS3, I-kB, IL-22	PI3K cat class IA, IRS-1
32	Apoptosis and survival_Role of PKR in stress-induced apoptosis	1,464E-03	3,115E-02	6/53	IRF1, p21, I-kB, NFKBIA	PACT, PP2A regulatory
33	Immune response_Alternative complement pathway	1,464E-03	3,115E-02	6/53	C3c, C3a, C3b, C3dg, C3, iC3b	

Enrichment for Pathway Maps						
34	Immune response_IL-3 signaling via JAK/STAT, p38, JNK and NF-kB	1,540E-03	3,179E-02	8/93	SOCS3, Spi2a, Bcl-XL, I-kB,	PI3K cat class IA, DHA2, MKP-1, TACI(TNFRSF13B)
35	Immune response_Platelet activating factor/ PTAFR pathway signaling	1,778E-03	3,374E-02	6/55	NF-AT, NF-AT2(NFATC1), Adenylate cyclase, NFKBIA	F-Actin cytoskeleton, PI3K cat class IA,
36	Immune response_CD28 signaling	1,778E-03	3,374E-02	6/55	NF-AT, NF-AT2(NFATC1), Bcl-XL, I-kB	PI3K cat class IA, JNK(MAPK8-10),
37	PGE2 pathways in cancer	1,778E-03	3,374E-02	6/55	Tcf(Lef), Lef-1Adenylate cyclase, COX-1 (PTGS1)	HIF1A, PI3K cat class IA (p110-beta)
38	G-protein signaling_G-Protein alpha-12 signaling pathway	1,856E-03	3,429E-02	5/38	LBC	G-protein alpha-12 family, PI3K cat class IA, JNK(MAPK8-10), M-Ras
39	Transcription_P53 signaling pathway	2,089E-03	3,578E-02	5/39	XPA, MMP-2, p21	JNK(MAPK8-10), MKP-1
40	Development_Cytokine-mediated regulation of megakaryopoiesis	2,141E-03	3,578E-02	6/57	IRF1, Bcl-XL, p21, LIF, sIL6-RA	PI3K cat class IA,
41	Immune response_Role of PKR in stress-induced antiviral cell response	2,141E-03	3,578E-02	6/57	IRF1, IRF7, I-kB, NFKBIA	PACT, JNK(MAPK8-10),
42	Immune response_TLR5, TLR7, TLR8 and TLR9 signaling pathways	2,141E-03	3,578E-02	6/57	IRF1, IRF7, TPL2(MAP3K8), I-kB	PI3K cat class IA, JNK(MAPK8-10)
43	Development_Prolactin receptor signaling	2,341E-03	3,737E-02	6/58	IRF1, SOCS3, Bcl-XL, OAS1	PI3K cat class IA, IRS-1
44	Apoptosis and survival_NGF activation of NF-kB	2,342E-03	3,737E-02	5/40	PKC-zeta, Bcl-XL, I-kB, NFKBIA	PI3K cat class IA
45	Main growth factor signaling cascades in multiple myeloma cells	2,617E-03	3,994E-02	5/41	I-kB	HIF1A, PI3K cat class IA, IRS-1, IGF-1
46	Development_Regulation of lung epithelial progenitor cell differentiation	2,617E-03	3,994E-02	5/41	Tcf(Lef), WNT	Jagged1, FZD2, Frizzled,
47	Immune response_IL-18 signaling	2,785E-03	4,074E-02	6/60	VCAM1, IL-18, Bcl-XS, I-kB	PI3K cat class IA, JNK(MAPK8-10),

Enrichment for Pathway Maps						
48	Immune response_LTBR1 signaling	2,785E-03	4,074E-02	6/60	PKC, Adenylate cyclase, I-kB	PI3K cat class IA, JNK(MAPK8-10), YES
49	Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	2,915E-03	4,176E-02	5/42	PKC-zeta, Bcl-XL, I-kB, RelB (NF-kB subunit),	TACI(TNFRSF13B)
50	Signal transduction_AKT signaling	3,236E-03	4,544E-02	5/43	Bcl-XL, p21, I-kB	PI3K cat class IA IRS-1

Table 2
Statistically significant Process Networks (FDR $\leq$ 0,05) of the Enrichment Analysis

Enrichment for Process Networks					
	Process Networks	p-value	FDR	Gene ratio	Network Objects from Active Data
1	Immune response_Phagocytosis	6,475E-07	5,307E-05	24/223	MANR, ERM proteins, VIL2 (ezrin), Actin cytoskeletal, Actin, PI3K cat class IA, PI3K cat class IA (p110-beta), JNK(MAPK8-10), JNK2(MAPK9), MRLC, C3b, C3dg, C3, iC3b, p40-phox, ENDO180, BLNK, HDL proteins, Casein kinase II, alpha chain (CSNK2A1), Casein kinase II, alpha chains, I-kB, NFKBIA, Casein kinase II, alpha' chain (CSNK2A2), C/EBP
2	Signal transduction_WNT signaling	6,760E-07	5,307E-05	21/177	PP2A regulatory, NF-AT, NF-AT2(NFATC1), Tcf(Lef), Lef-1, AKAP12, FZD2, Frizzled, JNK(MAPK8-10), JNK2(MAPK9), MMP-2, WISP1, Adenylate cyclase, NANOG, p73, Casein kinase II, alpha chain (CSNK2A1), Casein kinase II, alpha chains, Casein kinase II, WNT5B, WNT, Casein kinase II, alpha' chain (CSNK2A2)
3	Cell adhesion_Cell-matrix interactions	3,832E-05	2,006E-03	20/211	Perlecan, ADAM23, ECM1, Aggrecanase-2, Versican, Decorin, COL16A1, BETA-IG-H3, COL5A1, Collagen V, MMP-12, MMP-2, WISP1, Connexin 43, LAMA4, EMILIN-2, ADAM-TS1, ITGB7, TSG-6, MMP-13
4	Immune response_BCR pathway	7,205E-05	2,494E-03	15/137	NF-AT, NF-AT2(NFATC1), Actin cytoskeletal, Actin, PI3K cat class IA, PI3K cat class IA (p110-beta), JNK(MAPK8-10), JNK2(MAPK9), C3dg, SHIP, Bcl-XL, BLNK, Casein kinase II, alpha chain (CSNK2A1), I-kB, NFKBIA
5	Inflammation_IFN-gamma signaling	9,114E-05	2,494E-03	13/109	PACT, IRF1, IFI16, IL-18, PI3K cat class IA, PI3K cat class IA (p110-beta), Bcl-XL, p21, IRF9, IP10, EIF2S3, I-kB, NFKBIA

Enrichment for Process Networks					
6	Immune response_Phagosome in antigen presentation	9,531E-05	2,494E-03	21/243	ERM proteins, VIL2 (ezrin), Actin cytoskeletal, Actin, PI3K cat class IA, PI3K cat class IA (p110-beta), JNK(MAPK8-10), JNK2(MAPK9), PSMB9, C3dg, C3, iC3b, SEC15L, PSME3, ENDO180, BLNK, Casein kinase II, alpha chain (CSNK2A1), Casein kinase II, alpha chains, I-kB, NFKBIA, Casein kinase II, alpha' chain (CSNK2A2)
7	Inflammation_Interferon signaling	3,945E-04	8,848E-03	12/110	IRF1, SOCS3, IFI44, CCL8, IRF7, IFI56, MxA, Bcl-XL, IRF9, GBP1, GBP2, ISG15
8	Cell adhesion_Cadherins	5,422E-04	1,064E-02	16/182	Tcf(Lef), PKC, Actin cytoskeletal, Actin, Frizzled, G-protein alpha-12, PI3K cat class IA, WISP1, MAST205, Casein kinase II, alpha chain (CSNK2A1), Casein kinase II, alpha chains, Casein kinase II, ACP1, WNT, Casein kinase II, alpha' chain (CSNK2A2), YES
9	Development_Regulation of angiogenesis	6,553E-04	1,143E-02	18/222	HIF1A, PKC, PRK1, WT1, IL-18, TCF8, PI3K cat class IA, FXR, Endothelin-1, MMP-2, Cathepsin B, Connexin 43, Syndecan-3, Angiogenin, p21, SMAD2, I-kB, RelB (NF-kB subunit)
10	Inflammation_IL-6 signaling	8,080E-04	1,269E-02	12/119	SOCS3, PI3K cat class IA, PI3K cat class IA (p110-beta), SAA3, C3, p21, HDL proteins, I-kB, NFKBIA, C/EBP, IL-22, Alpha1-globin
11	Inflammation_MIF signaling	1,079E-03	1,500E-02	13/140	PLA2, PKC, VCAM1, JNK(MAPK8-10), JNK2(MAPK9), Adenylate cyclase type VII, Adenylate cyclase, Casein kinase II, alpha chain (CSNK2A1), Casein kinase II, alpha chains, Casein kinase II, I-kB, NFKBIA, Casein kinase II, alpha' chain (CSNK2A2)

Enrichment for Process Networks					
12	Cell cycle_G1-S Growth factor regulation	1,146E-03	1,500E-02	16/195	PP2A regulatory, IRF1, Tcf(Lef), PKC, PDGF-R-alpha, PI3K cat class IA, PI3K cat class IA (p110-beta), JNK(MAPK8-10), JNK2(MAPK9), p21, MAD, IRS-1, I-kB, NFKBIA, RelB (NF-kB subunit), IGF-1
13	Cell adhesion_Cell junctions	1,413E-03	1,707E-02	14/162	ATP1B1, Tcf(Lef), PKC-zeta, PKC, Actin cytoskeletal, Actin, PI3K cat class IA, Endothelin-1, Connexin 43, Casein kinase II, alpha chain (CSNK2A1), Casein kinase II, alpha chains, Casein kinase II, Casein kinase II, alpha' chain (CSNK2A2), YES
14	Inflammation_Amphoterin signaling	2,500E-03	2,678E-02	11/118	VCAM1, Actin cytoskeletal, Actin, PI3K cat class IA, PI3K cat class IA (p110-beta), JNK(MAPK8-10), JNK2(MAPK9), MRLC, I-kB, NFKBIA, MMP-13
15	Signal Transduction_TGF-beta, GDF and Activin signaling	2,559E-03	2,678E-02	13/154	PP2A regulatory, HIF1A, Lef-1, PKC, PI3K cat class IA, JNK(MAPK8-10), IRF7, UNRIP, p21, SMAD2, IRS-1, IGF-1, TGF-beta receptor type III (betaglycan)
16	Inflammation_Histamine signaling	2,852E-03	2,752E-02	16/213	PLA2, NF-AT2(NFATC1), VCAM1, Actin cytoskeletal, Actin, IL-18, JNK(MAPK8-10), JNK2(MAPK9), MDP1, p40-phox, Adenylate cyclase type VII, Adenylate cyclase, IP10, COX-1 (PTGS1), I-kB, NFKBIA
17	Inflammation_IL-10 anti-inflammatory response	3,016E-03	2,752E-02	9/87	SOCS3, Nucleolysin TIAR, PI3K cat class IA, PI3K cat class IA (p110-beta), MMP-2, Bcl-XL, I-kB, NFKBIA, MMP-13
18	Signal transduction_NOTCH signaling	3,155E-03	2,752E-02	17/235	Jagged1, HIF1A, PDGF receptor, PDGF-R-alpha, FZD2, Frizzled, PI3K cat class IA, JNK(MAPK8-10), JNK2(MAPK9), GLUT1, p21, p73, SMAD2, WNT5B, WNT, I-kB, NFKBIA
19	Inflammation_Innate inflammatory response	3,772E-03	3,117E-02	14/180	PLA2, PKC-zeta, IL-18, JNK(MAPK8-10), JNK2(MAPK9), IRF7, C3a, C3b, C3, PGRP-S, IP10, COX-1 (PTGS1), I-kB, NFKBIA

Enrichment for Process Networks					
20	Reproduction_Male sex differentiation	4,441E-03	3,487E-02	17/243	PAFAH alpha (LIS1), PKC-zeta, PKC, PMEPA1, PDGF receptor, PDGF-R-alpha, WT1, BAPX1, AMH type II receptor, Adenylate cyclase type VII, p21, NANOG, STK39, Olfactory receptor, Casein kinase II, Casein kinase II, alpha' chain (CSNK2A2), IGF-1
21	Development_EMT_Regulation of epithelial-to-mesenchymal transition	4,682E-03	3,501E-02	16/224	Jagged1, HIF1A, NF-AT2(NFATC1), Lef-1, Actin, PDGF receptor, PDGF-R-alpha, Frizzled, TCF8, PI3K cat class IA, JNK(MAPK8-10), Endothelin-1, MMP-2, SMAD2, WNT, I-kB
22	Immune response_TCR signaling	7,223E-03	4,933E-02	13/174	NF-AT, NF-AT2(NFATC1), CD137(TNFRSF9), Actin cytoskeletal, Actin, PI3K cat class IA, JNK(MAPK8-10), JNK2(MAPK9), TRAF1, Bcl-XL, TPL2(MAP3K8), I-kB, NFKBIA
23	Inflammation_Inflammasome	7,597E-03	4,933E-02	10/118	PACT, RIG-I, IL-18, JNK(MAPK8-10), IRF7, TXNIP (VDUP1), EIF2S3, ISG15, I-kB, NFKBIA
24	Inflammation_IgE signaling	7,782E-03	4,933E-02	11/137	PLA2, NF-AT, NF-AT2(NFATC1), PI3K cat class IA, PI3K cat class IA (p110-beta), JNK(MAPK8-10), JNK2(MAPK9), MDP1, BLNK, I-kB, NFKBIA
25	Signal transduction_ESR1-nuclear pathway	7,855E-03	4,933E-02	15/216	SOCS3, WT1, PI3K cat class IA, MPG, SET, LBC, C3, Adenylate cyclase type VII, Adenylate cyclase, RAMP3, CYP1A1, p21, CYP1B1, IRS-1, IGF-1

Table 3
First 10 statistically significant GO Processes of the Enrichment Analysis.

Enrichment by GO Processes				
	Process	pValue	FDR	Gene ratio
1	Response to stress	3.713e-22	2.771e-18	233/4937
2	Cellular response to cytokine stimulus	3.510e-19	1.310e-15	98/1398
3	Response to cytokine	2.914e-18	7.248e-15	105/1611
4	Response to external stimulus	1.159e-17	2.163e-14	159/3089
5	Cellular response to organic substance	4.898e-17	6.115e-14	170/3457
6	Response to organic substance	4.917e-17	6.115e-14	207/4579
7	Cellular response to chemical stimulus	4.374e-16	4.663e-13	190/4141
8	Negative regulation of biological process	7.285e-15	6.795e-12	269/6845
9	Response to interferon-beta	5.701e-14	4.727e-11	17/57
10	Response to virus	1.456e-13	1.087e-10	41/403

Discussion

Here we have utilised the "Transformics Assay" to develop an evaluation and analysis of the effect of cell exposure to 0.02 µg/ml B(a)P, the lowest transforming concentration, using the BALB/c 3T3 A31-1-1 cell line. The study aimed to identify the molecular and cellular events necessary to sustain the transforming activity of B(a)P, at this threshold concentration.

The study results reported here consolidate and improve upon those previously reported in the BALB/c 3T3 model validation (ECVAM, 2010), where the validation report showed an increment in malignant foci formation at the concentration range 0.01-0.02 µg/ml.

The integration of the CTA with Transcriptomics (Transformics) allowed the identification of the correlation between the transformation outcome (focus) and the gene expression profile, following 72 hrs exposure.

A deep perturbation of a large set of immune and inflammatory pathways and processes was observed, showing cross-talk interactions and reciprocal control, essentially 'immune-editing'. Overall, the data obtained are consistent with principal key events related to the biological process "inflammation", regardless of the tissue type, specifically i) cell activation and phenotypic modification, that includes alterations in their secretory activity, activation of biosynthesis pathway, production of pro-inflammation

molecules, changes in metabolism and morphology; ii) increase of molecules with pro-inflammatory action; and iii) recruitment / activation of leukocytes³⁰. (See Tables and Supplementary Material for further information about some involved Differential Expressed Genes (DEGs)).

It is postulated that the first steps in the inflammation loop signaling could be: mitogen activated kinases (MAPKs) activity and pattern recognition receptors (PPRs) signaling activation³¹.

Several modulated pathways are sustained by mitogen-activated protein kinases (MAPKs) activity (Table 1: pathways 1-2-34).

The three families of MAPKs, extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal/stress-activated protein kinases (JNK/SAPK), and the p38s are fundamental intracellular signal transduction mediators. They control cytoplasmic signaling and gene expression through the phosphorylation of transcription factors and the modulation of their functions. Due to these mechanisms they also play important roles in the inflammatory response^{32 33 34}.

It has been proposed that the toxicity of B(a)P (and other similar AhR ligands), may be mediated by the crosstalk between the AhR and MAPKs activity.

Various MAPKs are involved in the ligand-AhR multicomplex formation in cytoplasm, in its nuclear translocation, as well as in AhR-DNA binding and consequent gene transactivation. Interestingly, the JNK and ERK activation by B(a)P treatment has been reported in both AhR-positive and AhR-negative mouse embryonic fibroblasts^{32,33}.

A deregulated MAPK activity could be an important non genotoxic Mode of Action (MoA) interplaying with the AhR signalling cascade, during early events leading to toxic degeneration and cell transformation. Many of the inflammation related pathways and processes observed are associated with a "cellular antiviral state", highlighting overlaps in some sterile and non-sterile stress responses.

Various innate immune receptors, also called Pattern Recognition Receptors (PPRs), have been reported to recognize both pathogens (PAMPs, Pathogen Associated Molecular Patterns) and endogenous ligands released by distressed or damaged cells, generally called DAMPs (Damage-Associated Molecular Patterns)^{35 36}.

In our study the modulation of the "[Immune response_TLR5, TLR7, TLR8 and TLR9 signaling pathways](#) (Toll-like receptors) (Pathway Map n. 42) and the up regulation of the *Rig-I* (*Retinoic acid-inducible gene 1*) together with the modulation of the Process Networks "inflammation inflammasome" and "inflammation interferon signaling" have been observed (Table 2-S1-S2 and Figures S2-S3).

The activation of such PPRs (RIG-I and TLRs), can initiate IRF- and NF- κ B- dependent signals, leading to the release of type 1 interferons and other cytokines and, consequently, to the JAK/STAT signaling cascade, leading to transactivation of ISG (interferons-stimulated genes) promoters, generating the INF response (Figure 4).

The concomitant up-regulation of *RIG-I* and some ISGs (such as *Oas1a*) are linked to a positive-feedback/feed-forward mechanisms and the formation of an auto-amplification loop RIG-I/IRF^{13,33}. Moreover, the overexpression of *RIG-I* has been connected to the relative inflammasome activation and mature pro-inflammatory interleukins release (such as IL-18)³⁷.

As the results show, several significantly modulated pathways are either directly or indirectly related to the cell transformation process, including: EMT related pathway modulation, ECM molecular signaling remodelling and TGF- β and WNT signaling modulations.

The EMT is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, gaining migratory and invasive properties to become mesenchymal stem cells. EMT is essential during embryo implantation and organ development, whilst in adults it mediates tissue regeneration during wound healing. EMT has also been shown to occur in organ fibrosis and cancer progression³⁸.

EMT is initiated by complex signaling networks, both dynamic stimuli triggered by stromal cells and ECM triggered by soluble factors (such as proinflammatory cytokines and growth factors).

It has been demonstrated that paracrine signaling from CAFs and myofibroblasts can regulate epithelial-mesenchymal plasticity, fibroblast activation, ECM modification and promote tumor progression and fibrosis^{16,20,22,39}.

Results from the Enrichment Analysis, as well as the directly observable morphological change in spindle-shaped cells, suggest that fibroblasts are undergoing several modifications, such that there is a “CAFs initiation phenotype”²¹. Taken in combination, these background signaling events can be considered a composite molecular signal of ECM remodeling, as indicated by the modulation of the relative Pathway maps (Table 1; pathways n 5;10), Process Networks (Table 2 and Table S4.) and fibrotic disease (fibrosis, data not shown; FDR 1,902E-17, Ratio 55/531).

Moreover, the scored *Lif* gene up-regulation has been noted to be associated with a constitutive activation of the JAK1/STAT3 signaling pathway, this sustains the contractile and pro-invasive abilities of fibroblasts^{20 21,22}.

Furthermore, these results suggest the inhibition of NOTCH 1 and TGF- β pathways and the deregulation of the WNT signaling pathway. The WNT and TGF- β pathways are both directly and indirectly involved in tissue homeostasis processes of self-renewal and proliferation, differentiation, cell movement and adhesion^{38 40 41 42}.

The product of *Wnt-5b*, which is up-regulated in our cell model, is a ligand regulating fibroblasts activation via noncanonical signalling, it occurs in inflammation responses mediating IL-6 and CXCL8 release and JNK, p38, and p65 NF- κ B activation⁴³.

Whilst the putative downregulation of TGF- β signaling has been previously considered controversial, with respect to the classical “molecular phenotype” of myofibroblasts related to CAFs, more recently, Biffi et al.

have highlighted differences in CAFs subpopulation in pancreatic ductal adenocarcinoma, discerning between two subtypes characterized by either myofibroblastic or inflammatory phenotypes, with the latter expressing *Lif* and JAK/STAT activation²¹.

Whilst this is a very specific example that so far has not been reported for other cancer types, the fact that this has been observed contributes to an improved weight of evidence for the down-regulation of TGF- β .

Interestingly, the LIF/STAT3 and WNT/ β -catenin, as well as the inhibition of TGF- β pathways are also all involved in the maintenance of pluripotency⁴⁴. Considering the final end-point of oncotransformation and not forgetting that EMT can be triggered in a tissue-specific manner, we consider that these combined molecular data illustrate the overall molecular dynamics of the evolving tumor “micro-microenvironment”.

The WNT, LIF-JAK/STAT signaling perturbations represent critical events supporting the hypothesis that the BALB/c 3T3 cell model is both a “transformation cell system” and a “transforming microenvironment system”.

The TGF- β signaling plays a key role in the regulation of cellular functions and tissue homeostasis. The inefficient activation of the TGF- β signaling pathway has been associated with the imbalance of the immune response such that it is unable to counteract the ongoing inflammation⁴⁵.

The TGF- β role is tissue-specific and depends on the tumor stage^{41 42}. Indeed, the TGF- β -induced pathway may act as tumor suppressor during the premalignant phase of carcinogenesis, promoting cell cycle arrest and apoptosis. On the other hand, it sustains tumor growth and progression at later stages of carcinogenesis.

Our results suggest that the inhibition of TGF- β signaling, could be a transient response to sustain the inflammation process. It is also noteworthy that TGF- β interplay with AhR signaling has been reported⁴⁶⁻⁴⁸.

The AhR involvement in cell adhesion, spreading, migration and modulation of ECM composition, either in physiological cell conditions or following the exposure to xenobiotics, and in particular to B(a)P, has already been shown²⁵. Moreover, the AhR is involved in autophagy and EMT processes, these functions can be altered by AhR transmigration and depletion in cytoplasm⁴⁹⁻⁵¹.

Our study gives clear evidence that the AhR-mediated signaling pathway has a major role in triggering the molecular and cellular response to B(a)P in this cell model, this is supported by the noticeable overexpression of phase I *Cyp1a1* and *Cyp1b1* genes and the *p40phox* enzyme^{5,29,52}

On the basis of the results described in this paper and our previous reports³ we can reasonably speculate that the sustained inflammation signal, following the exposure to a critical concentration of a carcinogen, may influence the B(a)P metabolic fate supporting the activation of steps leading to genotoxic damage/injury. This hypothesis is supported by a number of reports showing that exposure to

inflammation fostering molecules can slow down the B(a)P metabolism and generate higher levels of B(a)P-DNA adducts ⁵³⁻⁵⁵.

Several studies have proposed that a dysregulation of inflammation processes and competition between excess initial inflammation and pro-fibrotic signaling can contribute to chronic inflammatory diseases and cancer ⁵⁶⁻⁵⁸

Our data show an over-stimulation of the inflammatory response, which, together with oxidative stress effects and inhibition of healing, can build a self-sustaining inflammation system and a transforming microenvironment. At the same time these immune responses may alter the phase I/phase II metabolism of the carcinogen. Metabolic derived debris (products of oxidative stress and/or unstable metabolites activity) may accumulate in cells and induce the innate immune response to DAMPs, forcing the response to become chronic and maladaptive (Figure 5).

At the tissue level, a further pro-inflammatory response magnification can be inferred, as a consequence of the recruitment and differentiation of many immune cells (macrophages, mast cells, and various T cell populations).

The experimental evidence from this study points towards non-genotoxic carcinogenic events being active protagonists of neoplasia induction, driving cells towards transformation.

Our experimental study provides evidence that AhR could be involved in the aberrant immune response ^{5,59,60} triggering a reactive metabolite cascade that leads to genotoxicity and also promotes fibroblast motility ²⁵. Taken together, these biological perturbations sustain the toxic disturbance, cause modifications in cells secretory activity and in extracellular matrix composition, creating a dysfunctional loop driving oncotransformation.

However, further studies are needed to clarify the complex role of AhR in such processes.

Furthermore, with the identification of a LTC of 0.02 µg/ml B(a)P we have a preliminary threshold for the CTA assay when identifying whether a result for an AhR bin of test chemicals is positive or negative, within an IATA.

Interestingly, in vivo, endpoints predictive of carcinogenicity induced by B(a)P are not the most sensitive toxicity outcomes, and the LTC reported here improves sensitivity for the prediction of oncotransformation, as compared to the RCB. For example, with B(a)P in vivo rodent studies, the critical point of departure of 0.025 mg B(a)P /kg-bw-day for neurotoxicity is used, not the lowest observed adverse level of 0.54 mg B(a)P /kg-bw-day for rodent forestomach carcinogenicity ⁶¹

To conclude, we have demonstrated that the Transformics Assay has the ability to highlight mechanisms leading to cell malignant transformation following exposure to carcinogens, and that it can support the use of the CTA to model the carcinogenesis process in vitro within the IATA for NGTxC being proposed at the intergovernmental level ².

Declarations

Author contributions statement

GP carried out the experimental work, drafted the manuscript, with inputs from AC, MGM, and MNJ, and designed the figures. GP and MGM processed the experimental data from the transcriptomics experiments, performed the statistical and biological analysis and drafted the results. MGM coordinated and supervised the transcriptomics experiments. MV coordinated and supervised the preliminary cytotoxicity and cell transformation tests, processed the experimental data and drafted the results, with the contribution of SS. CZ, FR, SS and FB carried out the experimental work and contributed to the data analysis. AC conceived the study and contributed to the design and implementation of the research with inputs from SG, who passed away during the preparation of the manuscript. MNJ substantively revised the manuscript

References

1. Adler, S. *et al.* Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. *Arch Toxicol* **85**, 367-485, doi:10.1007/s00204-011-0693-2 (2011).
2. Jacobs, M. N. *et al.* Chemical carcinogen safety testing: OECD expert group international consensus on the development of an integrated approach for the testing and assessment of chemical non-genotoxic carcinogens. *Archives of Toxicology* **94**, 2899-2923, doi:10.1007/s00204-020-02784-5 (2020).
3. Jacobs, M. N. *et al.* International regulatory needs for development of an IATA for non-genotoxic carcinogenic chemical substances. *ALTEX*, doi:10.14573/altex.1601201 (2016).
4. Corvi, R. *et al.* Moving forward in carcinogenicity assessment: Report of an EURL ECVAM/ESTIV workshop. *Toxicology in vitro : an international journal published in association with BIBRA* **45**, 278-286, doi:10.1016/j.tiv.2017.09.010 (2017).
5. Mascolo, M. G. *et al.* The transformics assay: first steps for the development of an integrated approach to investigate the malignant cell transformation in vitro. *Carcinogenesis* **39**, 955-967, doi:10.1093/carcin/bgy037 (2018).
6. Malik, D.-e.-s., David, R. M. & Gooderham, N. J. Mechanistic evidence that benzo[a]pyrene promotes an inflammatory microenvironment that drives the metastatic potential of human mammary cells. *Archives of Toxicology* **92**, 3223-3239, doi:10.1007/s00204-018-2291-z (2018).
7. Zhao, L. *et al.* Toxicological effects of benzo[a]pyrene on DNA methylation of whole genome in ICR mice. *Cell Mol Biol (Noisy-le-grand)* **61**, 115-119 (2015).
8. Murray, I. A., Patterson, A. D. & Perdew, G. H. Aryl hydrocarbon receptor ligands in cancer: friend and foe. *Nature reviews. Cancer* **14**, 801-814, doi:10.1038/nrc3846 (2014).
9. Corvi, R. *et al.* ECVAM prevalidation study on in vitro cell transformation assays: General outline and conclusions of the study. *Mutation Research* **744**, 12-19, doi:10.1016/j.mrgentox.2011.11.009 (2012).
10. Sasaki, K. *et al.* Recommended protocol for the BALB/c 3T3 Cell Transformation Assay. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis* **744**, 30-35, doi:10.1016/j.mrgentox.2011.12.014 (2012).

11. Tanaka, N. *et al.* Prevalidation study of the BALB/c 3T3 Cell Transformation Assay for assessment of carcinogenic potential of chemicals. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, doi:10.1016/j.mrgentox.2011.12.008 (2012).
12. Ivashkiv, L. B. & Donlin, L. T. Regulation of type I interferon responses. *Nat Rev Immunol* **14**, 36-49, doi:10.1038/nri3581 (2014).
13. Czerkies, M. *et al.* Cell fate in antiviral response arises in the crosstalk of IRF, NF- κ B and JAK/STAT pathways. *Nature Communications* **9**, doi:10.1038/s41467-017-02640-8 (2018).
14. Fukui, H. *et al.* IL-22 produced by cancer-associated fibroblasts promotes gastric cancer cell invasion via STAT3 and ERK signaling. *Br J Cancer* **111**, 763-771, doi:10.1038/bjc.2014.336 (2014).
15. Kim, K. *et al.* Interleukin-22 promotes epithelial cell transformation and breast tumorigenesis via MAP3K8 activation. *Carcinogenesis* **35**, 1352-1361, doi:10.1093/carcin/bgu044 (2014).
16. Cohen, N. *et al.* Fibroblasts drive an immunosuppressive and growth-promoting microenvironment in breast cancer via secretion of Chitinase 3-like 1. *Oncogene* **36**, 4457-4468, doi:10.1038/onc.2017.65 (2017).
17. Salomon, J., Piotrowska, A., Matusiak, Ł., Dzięgiel, P. & Szepietowski, J. C. Chitinase-3-like Protein 1 (YKL-40) Expression in Squamous Cell Skin Cancer. *Anticancer Res* **38**, 4753-4758, doi:10.21873/anticancer.12783 (2018).
18. Liu, Z.-J. *et al.* Inhibition of fibroblast growth by Notch1 signaling is mediated by induction of Wnt11-dependent WISP-1. *PloS one* **7**, e38811-e38811, doi:10.1371/journal.pone.0038811 (2012).
19. Shao, H. *et al.* Notch1-WISP-1 axis determines the regulatory role of mesenchymal stem cell-derived stromal fibroblasts in melanoma metastasis. *Oncotarget* **7**, 79262-79273, doi:10.18632/oncotarget.13021 (2016).
20. Albregues, J. *et al.* Epigenetic switch drives the conversion of fibroblasts into proinvasive cancer-associated fibroblasts. *Nature Communications* **6**, 1-15, doi:10.1038/ncomms10204 (2015).
21. Biffi, G. *et al.* IL1-Induced JAK/STAT signaling is antagonized by TGF β to shape CAF heterogeneity in pancreatic ductal adenocarcinoma. *Cancer Discovery* **9**, 282-301, doi:10.1158/2159-8290.CD-18-0710 (2019).
22. Yoshida, G. J., Azuma, A., Miura, Y. & Orimo, A. Activated fibroblast program orchestrates tumor initiation and progression; molecular mechanisms and the associated therapeutic strategies. *International Journal of Molecular Sciences* **20**, 2256-2256, doi:10.3390/ijms20092256 (2019).
23. Prochaska, H. J., De Long, M. J. & Talalay, P. On the mechanisms of induction of cancer-protective enzymes: a unifying proposal. *Proc Natl Acad Sci U S A* **82**, 8232-8236, doi:10.1073/pnas.82.23.8232 (1985).
24. Moffat, I. *et al.* Comparison of toxicogenomics and traditional approaches to inform mode of action and points of departure in human health risk assessment of benzo[a]pyrene in drinking water. *Critical reviews in toxicology* **45**, 1-43, doi:10.3109/10408444.2014.973934 (2015).
25. Pohjanvirta, R. *The AH Receptor in Biology and Toxicology*. (John Wiley & Sons, 2011).

26. Jacobs, M. N. in *Nutrients and cell signaling* (eds J. Zemplini & K. Dakshinamurt) Ch. I. Nuclear receptors, transcription factors, and signaling cascades, 35-90 (CRC Taylor & Francis, 2005).
27. Fang, J.-L. *et al.* Characterization of benzo(a)pyrene-trans-7,8-dihydrodiol glucuronidation by human tissue microsomes and overexpressed UDP-glucuronosyltransferase enzymes. *Cancer Res.* **53**, 1529-1533 (2002).
28. Bock, K. W. & Köhle, C. in *Methods in enzymology* Vol. 400 57-75 (2005).
29. Wada, T., Sunaga, H., Ohkawara, R. & Shimba, S. Aryl hydrocarbon receptor modulates NADPH oxidase activity via direct transcriptional regulation of p40phox expressions. *Molecular Pharmacology* **83**, 1133-1140, doi:10.1124/mol.112.083303 (2013).
30. Villeneuve, D. L. *et al.* Representing the process of inflammation as Key Events in Adverse Outcome Pathways. *Toxicological Sciences* **163**, 1-7 (2018).
31. Mescoli, A. *et al.* The Secretive Liaison of Particulate Matter and SARS-CoV-2. A Hypothesis and Theory Investigation. *Front Genet* **11**, 579964, doi:10.3389/fgene.2020.579964 (2020).
32. Puga, A., Ma, C. & Marlowe, J. L. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. *Biochemical Pharmacology* **77**, 713-722, doi:10.1016/j.bcp.2008.08.031 (2009).
33. Tian, B. *et al.* BRD4 couples NF- κ B/RelA with airway inflammation and the IRF-RIG-I amplification loop in respiratory syncytial virus infection. *J Virol* **91**, doi:10.1128/jvi.00007-17 (2017).
34. Vázquez-Gómez, G., Rocha-Zavaleta, L., Rodríguez-Sosa, M., Petrosyan, P. & Rubio-Lightbourn, J. Benzo(a)pyrene activates an AhR/Src/ERK axis that contributes to CYP1A1 induction and stable DNA adducts formation in lung cells. *Toxicol Lett* **289**, 54-62, doi:10.1016/j.toxlet.2018.03.012 (2018).
35. Anders, H.-J. & Schaefer, L. Beyond tissue injury-damage-associated molecular patterns, toll-like receptors, and inflammasomes also drive regeneration and fibrosis. *Journal of the American Society of Nephrology* **25**, 1387-1400, doi:10.1681/ASN.2014010117 (2014).
36. Bernardizzi, C., Castro, F. & de Souza, H. S. in *Fibrostenotic inflammatory bowel disease* (ed Florian Rieder) Ch. 8, 111-126 (Springer, Cham, 2018).
37. Chen, I. Y. & Ichinohe, T. Response of host inflammasomes to viral infection. *Trends in Microbiology* **23**, 55-63, doi:10.1016/j.tim.2014.09.007 (2015).
38. Cai, Z., Cao, Y., Luo, Y., Hu, H. & Ling, H. Signalling mechanism(s) of epithelial–mesenchymal transition and cancer stem cells in tumour therapeutic resistance. *Clinica Chimica Acta journal* **483**, 156-163, doi:10.1016/j.cca.2018.04.033 (2018).
39. Bissey, P. A. *et al.* Dysregulation of the MiR-449b target TGFBI alters the TGF β pathway to induce cisplatin resistance in nasopharyngeal carcinoma. *Oncogenesis* **7**, 40-40, doi:10.1038/s41389-018-0050-x (2018).
40. Reya, T. & Clevers, H. Wnt signalling in stem cells and cancer. *Nature* **434**, 843-850, doi:10.1038/nature03319 (2005).
41. Neuzillet, C. *et al.* Targeting the TGF β pathway for cancer therapy. *Pharmacology & Therapeutics* **147**, 22-31, doi:10.1016/J.PHARMTHERA.2014.11.001 (2015).

42. Syed, V. TGF- β Signaling in Cancer. *Journal of Cellular Biochemistry* **117**, 1279-1287, doi:10.1002/jcb.25496 (2016).
43. van Dijk, E. M. *et al.* Noncanonical WNT-5B signaling induces inflammatory responses in human lung fibroblasts. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **310**, L1166-L1176, doi:10.1152/ajplung.00226.2015 (2016).
44. Tao, L., Huang, G., Haizhu, S., Chen, Y. & Chen, L. Cancer Associated Fibroblasts: An essential role in the tumor microenvironment. *Oncology letters* **14**, 2611-2620, doi:10.3892/ol.2017.6497 (2017).
45. Ihara, S., Hirata, Y. & Koike, K. TGF- β in inflammatory bowel disease: a key regulator of immune cells, epithelium, and the intestinal microbiota. *Journal of Gastroenterology* **52**, 777-787, doi:10.1007/s00535-017-1350-1 (2017).
46. Haarmann-Stemmann, T., Bothe, H. & Abel, J. Growth factors, cytokines and their receptors as downstream targets of arylhydrocarbon receptor (AhR) signaling pathways. *Biochem Pharmacol* **77**, 508-520, doi:10.1016/j.bcp.2008.09.013 (2009).
47. Gomez-Duran, A. *et al.* Fitting a xenobiotic receptor into cell homeostasis: How the dioxin receptor interacts with TGF β signaling. *Biochemical Pharmacology* **77**, 700-712, doi:10.1016/j.bcp.2008.08.032 (2009).
48. Nakano, K., Tsunoda, M. & Konno, N. Tributyltin (TBT) increases TNF α mRNA expression and induces apoptosis in the murine macrophage cell line in vitro. *Environmental health and preventive medicine* **9**, 266-271, doi:10.1007/bf02898141 (2004).
49. Giannone, J. V., Li, W., Probst, M. & Okey, A. B. Prolonged depletion of AH receptor without alteration of receptor mRNA levels after treatment of cells in culture with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biochemical Pharmacology* **55**, 489-497, doi:10.1016/S0006-2952(97)00493-0 (1998).
50. Tsai, C. H. *et al.* The inhibition of lung cancer cell migration by AhR-regulated autophagy. *Scientific Reports* **7**, 1-14, doi:10.1038/srep41927 (2017).
51. Raaschou-Nielsen, O. *et al.* Air pollution and lung cancer incidence in 17 European cohorts: prospective analyses from the European Study of Cohorts for Air Pollution Effects (ESCAPE). *The lancet oncology* **14**, 813-822, doi:10.1016/s1470-2045(13)70279-1 (2013).
52. Murray, I. A., Patterson, A. D. & Perdew, G. H. Aryl hydrocarbon receptor ligands in cancer: friend and foe. *Nature reviews. Cancer* **14**, 801-814, doi:10.1038/nrc3846 (2014).
53. Šmerdová, L. *et al.* Inflammatory mediators accelerate metabolism of benzo(a)pyrene in rat alveolar type II cells: The role of enhanced cytochrome P450 1B1 expression. *Toxicology* **314**, 30-38, doi:10.1016/j.tox.2013.09.001 (2013).
54. Shi, Q. *et al.* Altered gene expression profiles in the lungs of benzo(a)pyrene-exposed mice in the presence of lipopolysaccharide-induced pulmonary inflammation. *Toxicology and applied pharmacology* **336**, 8-19, doi:10.1016/j.taap.2017.09.023 (2017).
55. Shi, Q., Godschalk, R. W. L. & Van Schooten, F. J. Inflammation and the chemical carcinogen benzo(a)pyrene: Partners in crime. *Mutation Research* **774**, 12-24, doi:10.1016/j.mrrev.2017.08.003 (2017).

56. Colotta, F., Allavena, P., Sica, A., Garlanda, C. & Mantovani, A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* **30**, 1073-1081, doi:10.1093/carcin/bgp127 (2009).
57. Taniguchi, K. & Karin, M. NF- κ B, inflammation, immunity and cancer: coming of age. *Nature Reviews Immunology* **18**, 309–324, doi:10.1038/nri.2017.142 (2018).
58. Helm, J. S. & Rudel, R. A. Adverse outcome pathways for ionizing radiation and breast cancer involve direct and indirect DNA damage, oxidative stress, inflammation, genomic instability, and interaction with hormonal regulation of the breast. *Archives of Toxicology* **94**, 1511-1549, doi:10.1007/s00204-020-02752-z (2020).
59. Nguyen, N. T., Hanieh, H., Nakahama, T. & Kishimoto, T. The roles of aryl hydrocarbon receptor in immune responses. *International Immunology* **25**, 335-343, doi:10.1093/intimm/dxt011 (2013).
60. Goodson, W. H. *et al.* Assessing the carcinogenic potential of low-dose exposures to chemical mixtures in the environment: the challenge ahead. *Carcinogenesis* **36 Suppl 1**, S254-296, doi:10.1093/carcin/bgv039 (2015).
61. Chepelev, N. L., Moffat, I. D., Bowers, W. J. & Yauk, C. L. Neurotoxicity may be an overlooked consequence of benzo[a]pyrene exposure that is relevant to human health risk assessment. *Mutat Res Rev Mutat Res* **764**, 64-89, doi:10.1016/j.mrrev.2015.03.001 (2015).

Figures

Experimental Design

Figure 1

Transformics Assay experimental design Cell culture experiments were conducted in parallel. A set of plates for each treatment (NT, 0.1% DMSO, 0.02 µg/ml B(a)P, 0.2 µg/ml B(a)P, 4 µg/ml 3-MCA) were maintained in culture for the CTA (A-B). The foci formation served as the phenotypic anchoring for the microarray results. A) Experimental protocol of the Cytotoxicity assay to evaluate the cell survival after the chemical treatment. B) Experimental protocol of the Transformation Assay, with medium changes at specified time points C) Integrated experimental protocol: RNA was extracted from cells treated with the Lowest Transforming

Concentration (LTC) 0.02 µg/ml B(a)P and cells treated with 0.1% Dimethyl Sulfoxide (DMSO), as control. The microarray experiment was conducted with a glass slide containing 8x60K formatted arrays, four of which were hybridised with the treated cells lysate, and four with the control lysate. The GeneSpring GX software (Agilent Technologies) was employed for the statistical analysis of microarray experiments, whereas MetaCore (Thomson Reuters, <https://portal.genego.com/>) was used for the biological analysis. Figure created with BioRender.com

Figure 2

Preliminary CTA results. (A) Mean number of colonies/plate ± standard error (SE). ** p ≤ 0.01 vs vehicle control, T-test. (B) Relative Clonal Efficiency (RCE). ** p ≤ 0.01 vs vehicle control, χ² -test. (C) Transformation frequency, calculated on the basis of the foci number and the Absolute Clonal Efficiency (ACE) values. ** p≤0.01 vs vehicle control, Poisson rates comparison. (D) Number of foci ± standard error (SE). ** p ≤ 0.01 vs vehicle control, T-test.

Figure 3

CTA (Transformics Assay) Results (A) Mean number of colonies/plate $\pm$ standard error (SE). ** $p \leq 0.01$ vs vehicle control, T-test. (B) Relative Clonal Efficiency (RCE). ** $p \leq 0.01$ vs vehicle control, χ^2 -test. (C) Transformation frequency, calculated on the basis of the foci number and the Absolute Clonal Efficiency (ACE) values. ** $p \leq 0.01$ vs vehicle control, Poisson rates comparison. (D) Number of foci $\pm$ standard error (SE). ** $p \leq 0.01$ vs vehicle control, T-test.

Figure 4

Rig-I and TRLs crosstalk with INF signalling and autoamplification loop of JAK-STAT signaling: Rig-I and TRLs can induce interferons production (type I and probably type III) and subsequent INF signalling response with a positive loop regulation. This is achieved through STAT1 activation. STAT1 can induce the transcription regulation of several genes. IRF1, in turn can promote DNA binding of STAT1. STAT1 (STAT1-STAT2 Heterodimers) could also interact with IRF-9 and activate ISGs. Rig-I has also been described as an inflammasome activator, via caspase-1 activation and release of mature IL-1 β and IL-18. Created with BioRender.com

Figure 5

Summary MoA diagrams of B(a)P, from AhR binding and activation the Molecular Initiating Event leading to Genotoxic and Non Genotoxic MoAs. Several interactions between different events can be inferred, at cellular and molecular level, affecting the overall biological response. Abbreviations: Damage-Associated Molecular Patterns (DAMPs); Endoplasmic Reticulum (ER); Epithelial–Mesenchymal Transition (EMT); Extracellular Matrix (ECM); Mode of Action (MoA); Reactive oxygen species (ROS); Xenobiotic response element (XRE).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Pilloetal2021SupplementaryMaterialdocx.docx](#)