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The cell transformation assay to assess potential carcinogenic properties of nanoparticles.

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Abstract

Nanoparticles (NPs) are present in many daily life products with particular physical-chemical properties (size, density, porosity, geometry ...) giving very interesting technological properties. Their use is continuously growing and NPs represent a new challenge in terms of risk assessment, consumers being multi-exposed. Toxic effects have already been identified such as oxidative stress, genotoxicity, inflammatory effects, and immune reactions, some of which are leading to carcinogenesis. Cancer is a complex phenomenon implying multiple modes of action and key events, and prevention strategies in cancer include a proper assessment of the properties of NPs. Therefore, introduction of new agents like NPs into the market creates fresh regulatory challenges for an adequate safety evaluation and requires new tools. The Cell Transformation Assay (CTA) is an *in vitro* test able of highlighting key events of characteristic phases in the cancer process, initiation and promotion. This review presents the development of this test and its use with NPs. The article underlines also the critical issues to address for assessing NPs carcinogenic properties and approaches for improving its relevance.

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I. Introduction

In Europe, **cancer** is the most frequently occurring form of non-communicable disease and the second most common cause of death, after cardiovascular diseases. Estimates for 2018 indicate 4.2 million new cancer cases and 1.94 million cancer deaths, as reported by the European Cancer Information System, in spite of major improvements in diagnosis and therapy and a slight decrease in deaths linked to certain cancers (Global Cancer Observatory, 2020). In 2017, the 70th World Health Assembly of the World Health Organization (WHO) adopted a resolution on cancer prevention and control, with a broad consensus that “Cancer is a growing public health concern which requires increased attention, prioritization and funding” (WHO Assembly Resolution, 2017).

Nanomaterials (NMs) are any type of material of nanosized thickness, where at least one dimension is less than approximately 100 nanometers (PAS71, 2005). They are widely used in different technologies and across almost all industrial sectors and product categories, whether cosmetics, industrial chemicals, food contact materials, or medicines because at this scale, unique optical, magnetic, electrical, and other properties emerge.

The term **nanoparticles** (NPs) is used for constituent particles of NMs or for nanoscale particles generated by different sources (natural or engineered, e.g. biotechnology, in healthcare technology, medicinal imaging applications, in food, gas sensing, electronic computer, energy harvesting (Kahn et al., 2019)).

Due to the advances in nanoscience and the NPs widespread use increasing in the last two decades, consumers, workers, and the environment are exposed to them in many different ways.

This exposure makes it urgent to study more deeply the hazardous effects of NPs to assess the risk for human and animal health, and the environment.

Nanotoxicology represents a complex and growing research area in toxicology. Inherent properties of NPs as well as environmental factors collectively influence NPs behavior, fate and transport, available fraction for uptake into the cells (bioavailability) and ultimately toxicity. Then, nanotoxicology needs multidisciplinary knowledge such as biology, toxicology, chemistry and physics to be able to get a proper characterization of NPs (Donaldson *et al.*, 2004, Oberdorster *et al.*, 2005, Donaldson and Poland, 2013, Caballero-Díaz and Valcárcel Cases, 2016).

Introduction of NPs into the market creates fresh regulatory challenges for an adequate, low cost and rapid safety evaluation using alternative methods such as *in vitro* testing strategies, which requires new

tools to better understand the NP mode of actions. Moreover, the 3R rule (Replace, Reduce and Refine) preconized by the European commission to avoid animal testing involves alternative strategies such as *in vitro* testing usually used as first intention tier to study NPs, with mechanistic information to support a weight of evidence for further use and application of NMs (Savage *et al.*, 2019).

Alternative methods have been tentatively used to predict the toxicity of a new NP. Among them, QSAR techniques are extensively applied in the area of toxicology and drug design to predict physicochemical properties of chemicals and their effects on the human organism and in the environment. But the development of QSAR models dedicated to NMs and NPs requires both the development of nano-specific descriptors and curated experimental datasets (Burello, 2017). Read-across could also be used to assess NP toxicity as a technique for predicting endpoint information for one or more substances (target substance(s)) by using data from the same endpoint from (an) other substance(s) provided that these substances are similar, i.e. have similar physicochemical, toxicological and ecotoxicological properties, or follow a regular pattern as a result of structural similarity that allows them to be considered as a group (REACH Annex XI, Stone *et al.*, 2020). However, the identification of structurally similar substances is more challenging for NMs and/or NPs than regular chemicals because NMs do not have a uniquely defined structure (Lamon *et al.*, 2018). The European Chemicals Agency (ECHA) has recently released guidance on how to justify grouping for read-across between nanoforms of the same substance (Echa, 2017) as differences in the toxicological behavior of NPs at different sizes have been reported (Li *et al.*, 2011, Sahu *et al.*, 2016). However, to date, the read-across approach has only been used with a few NPs, among them the nanoTiO₂ NPs (Lamon *et al.*, 2018, EFSA, 2021).

I.1. Nanoparticles and toxicological endpoints

NPs can have toxicological impacts in many ways. Toxicity of NPs can be tested using, *in vivo* and/or *in vitro* studies with the particularity that it will be a case-by-case approach. In contact with organisms, they can trigger inflammatory process, immune reactions, oxidative stress, DNA damage, cell death... (for general review, Ajdary *et al.*, 2018).

NMs are used in every field of manufacturing with different regulations, and requests for carcinogenicity assessment could vary from one to another. For the food and feed area, EFSA has published in 2018 a guidance on nanotechnologies and has proposed a stepwise framework for nano-related hazard identification and characterization. In this decision tree, carcinogenicity studies are asked only if there are indications for slow elimination or accumulation and distribution to specific tissues (EFSA, 2018). In this case, targeted in-depth investigations are required e.g. additional toxicokinetic study (optionally in human studies), reproductive and developmental toxicity, immunotoxicity, neurotoxicity, carcinogenicity/mutagenicity, endocrine properties, and effects on gut microbiome.

In the cosmetic field, a guidance on the Safety Assessment of Nanomaterials in Cosmetics has been published in 2019 (SCCS/1611/19). A carcinogenicity study is in general only submitted when already available; e.g., when carried out before the animal testing ban or when generated for compliance under a different (non-cosmetic) legislative framework. The decision on the carcinogenic potential of mutagenic or genotoxic substances may thus be made on the outcome of *in vitro* mutagenicity tests. A positive *in vitro* result in mutagenicity tests is then seen as indicative of the carcinogenic potential of the substance (SCCS/1602/18).

In regulatory toxicology, *in vitro* evaluation methods need to be faster cost-effective alternative methods, robust, reliable, and standardized (Dusinska *et al.*, 2017). In addition, in nanotoxicology, compared to standard chemicals, a proper characterization of NPs is essential, as it will determine the testing strategy due to potential interferences between NPs and the selected assay (Ahmed *et al.*, 2017). Furthermore, NMs are engineered to have various unique material properties. These properties will also affect their possible direct or indirect interaction with the DNA. Depending on the structure, size: surface ($\mu\text{g}/\text{cm}^2$) and volume ($\mu\text{m}^3/\text{cell}$), hydroscopic properties of the NP may have an impact on the genotoxicity test results. Catalytic activity and inflammation potential (including but not limited to ROS generation) may have an impact on general toxicity (Landsiedel *et al.*, 2009).

Moreover, it is also recommended for testing NPs *in vitro*, to perform a battery of tests on cells of human origin, either cell lines or primary cells, in conditions as close as possible to an *in vivo* situation (Dusinska *et al.*, 2017).

Damage to DNA leading to gene mutations, larger-scale chromosomal aberration, and aneuploidy (numerical chromosome changes) are generally considered as the first step in the multi-stage process of malignancy (Erickson, 2010). NP genotoxicity can be a consequence of three mechanisms: a direct interaction of NPs with genetic materials, an indirect action *via* ROS damage, due to toxic lesions (e.g. transition of metal's ions, *via* Fenton type reaction, due to binding with DNA bases, released from soluble NP) or a result of oxidative DNA damage by ROS during NP elicited inflammation (for review, see Magdolenova *et al.*, 2013). Indeed, the induction of **oxidative stress** induced by NPs due to an overproduction of reactive oxygen species (ROS) is also often searched as it has been shown to be an underlying mechanism of possible toxicity of NPs leading to immunotoxicity, genotoxicity (Dusinska *et al.*, 2017) and carcinogenicity (Guyton *et al.*, 2018).

However, **carcinogenesis process** involves sequential genetic alterations in a single target cell corresponding to several steps known as initiation, promotion, and progression events that are not necessarily dependent on mutations in the genomic material. Smith *et al.* (2016) identified 10 key events for pertinent mechanistic studies characteristics for human carcinogenesis. For example, events can trigger carcinogenesis, such as involvement of growth factors-like mechanisms in this process leading to an uncontrollable proliferation of cells corresponding to a promotion process. Those alterations cause a growth disturbance leading the cells to form malignant tumors (Jacobs *et al.*, 2016).

Moreover, epigenetic phenomena, including changes to the DNA methylome and chromatin compaction states, along with histone modification can also impact the carcinogenic process by affecting gene expression and DNA repair dynamics (Herceg *et al.*, 2013), epigenetic process being relevant for both genotoxic and non-genotoxic agent (You and Jones, 2012).

I.2. Genotoxic and non-genotoxic carcinogens

Carcinogenic agents (natural, chemicals, drugs, contaminants) are considered to fall into two broad categories, depending on their mode of actions.

The first one is called **initiators**, they are DNA reactive carcinogens being able to initiate cells to carcinogenesis through direct interaction with DNA. These interactions result in DNA damage and/or structural/numerical chromosomal aberrations, which can be detected by genotoxicity tests. However, not all genotoxic agents are mutagenic; apoptosis and DNA repair can occur. To date, the standard approach to identify this class of carcinogens is an evaluation of mutagenic potential focused on the assessment of gene mutations and structural/numerical chromosomal aberrations and is generally carried out using a battery of *in vitro* bioassays with high specificity and sensitivity according to OECD guidelines. Recommended test battery strategy for genotoxicity evaluation has been issued by regulatory agencies. The bacterial reverse mutation assay (Ames test, OECD 471), which is the keystone test of the evaluation of chemicals, is in contrast not considered suitable for NPs owing to the inability of bacterial cells (unlike mammalian cells) to internalize particles. The use of mammalian cell models, especially human with a functional p53 is then recommended as a suitable battery of tests dedicated to NP assessment proposed by EFSA (2021) to address the critical genotoxicity endpoints. Both the *in vitro* mammalian cell gene mutation tests using the HPRT and XPRT genes (OECD TG 476) and the thymidine kinase gene (OECD TG 490) are appropriately completed with the use of a test for structural and numerical chromosome damage, i.e. the *in vitro* mammalian cell micronucleus test (OECD TG 487). To note, regarding to NPs, the most performed *in vitro* genotoxicity assays are micronucleus test and comet assay (Magdalena *et al.*, 2013).

A second category of carcinogens is non-DNA reactive agents called **promoters**. It has been estimated that 10-20% of recognized human cancer carcinogens are classified as class I by the IARC act by non-genotoxic mechanisms (Hernandez *et al.*, 2009). These carcinogenic agents are, at least initially, devoid of direct interaction with DNA. Promotion, the second step of carcinogenesis process is reversible, and induced by different events. Indeed, the indirect modifications of DNA structures or function may induce altered gene expression and/or signal transduction. Then, non-genotoxic carcinogens refer to carcinogens negative in genotoxicity assays.

To date, there are no specific requests (and no OECD *in vitro* guidelines) to identify non-genotoxic mechanisms of carcinogenicity. In several chemical regulatory frameworks, then, for now, the risk posed by non-genotoxic carcinogenic agents for the consumer cannot be managed.

The only way to detect compounds with non-genotoxic action is to perform long-term *in vivo* study but animal testing can be waived when results from genotoxicity are clearly negative and when no structural alerts are identified for most of regulations. Limited assessment of carcinogenicity increases the

probability of consumers to be exposed to agents able to promote tumors and alter hormone responsiveness; this is also true for NPs as demonstrated by Fontana *et al.* (2017) with silica NPs.

I.3. Carcinogenicity studies and their drawbacks

In vivo studies are essential for a complete hazard evaluation of carcinogens; they are used in the risk assessment process, with internationally agreed testing methods, which rely on rodents assays. The standard approach is to conduct a 2-year study on two different rodent species according to the OECD guidelines (OECD TG 451 or OECD TG 453). But, these assays have some limitations; using a large number of animals (600-800 per chemical), they have a prolonged duration (2 years), are very expensive (at least one million euros), raised ethical concerns, and gave scarce mechanistic information making difficult to completely understand the human relevance (Mascolo *et al.*, 2018, Madia *et al.*, 2019).

Lifetime rodent cancer bioassays have to be performed if any *in vivo* genotoxicity tests are positive but under most chemical regulations except pharmaceutical, plant protection, and biocide safety this is rare (Jacobs *et al.*, 2020). Moreover, retrospective analysis of carcinogenicity data revealed that mouse studies have not a substantial benefit in regulatory decision-making (Annys *et al.*, 2014).

In accordance with the 3R rules which support efforts to find alternative methods for testing on animals, the development of an *in vitro* strategy based on mode of action to evaluate carcinogen potential is becoming increasingly an appreciated aspect of hazard assessment urgently required, especially for the promotion step, as the best strategy to drive new developments (Corvi *et al.*, 2017).

To identify promotion property, cell transformation assays, as an *in vitro* alternative approach, were developed, using Balb/c 3T3 derived cells or SHE cells.

Sasaki *et al.* (1988) developed a cell transformation assay (CTA), based on the ability of normal cells to transform into malignant cells detecting both genotoxic and non-genotoxic carcinogens, mimicking, by the way, some key stages of the *in vivo* multistep carcinogenesis.

Although being validated by the European Center for Validation of Alternatives Methods (ECVAM), the CTA is not currently used for regulatory purposes. CTA is often performed to clarify *in vitro* genotoxicity results, evaluate classes of chemicals that have a low predictive capacity in traditional *in vitro* genotoxicity assays (e.g. aromatic amines), and investigate tumor initiation/promotion activity (mechanisms of action of specific carcinogens) (Creton *et al.*, 2012). It is used by academia, and by chemical, agro-chemical, cosmetic, tobacco and pharmaceutical industries and as part of weight of

evidence assessment (EFSA, 2011). Furthermore, CTA is recognized as a useful assay for substances with structural alerts for carcinogenicity to demonstrate similarities (or not) across a chemical category (Creton *et al.*, 2012). The scientific committee on consumer safety (2018) mentioned the use of CTA in its guidance for testing cosmetic ingredients, as a possible alternative to animal models to detect genotoxic and non-genotoxic carcinogens. A tiered approach based on Ames test and the SHE assay demonstrated detection of up to 90% of carcinogens (Benigni et Bossa, 2011). EFSA (2011) in its scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment, recommended also CTA as a promising new test method.

However, CTA by measuring induction of phenotypic alterations does not provide any information on molecular key events, nor mechanism of action making this bioassay not fully contemplated in the regulatory context. Three main concerns were raised for final OECD acceptance: the reproducibility of the data between laboratories, the subjective nature for assessing transformation (morphological characteristics), and the lack of knowledge on the molecular mechanisms involved in the transformation.

This article reviews the use of CTA with NPs and highlights important key points to perform and improve this bioassay for a relevant assessment of the NP carcinogenic potential. To our knowledge, no study using SHE was performed with NPs, then this review will focus only on the Balb/c 3T3 cells-based assays.

2. Cell transformation assay methods

CTA measures the change in phenotype between normal and malignant cells in mammalian cells after exposure to genotoxic or non-genotoxic carcinogens (Sakai *et al.*, 2002). The cell transformation assay was demonstrated to involve a multistage process that closely models key stages of *in vivo* carcinogenesis (LeBoeuf *et al.*, 1999). Formation of transformed foci is the consequence of the complex process of transforming cells into a malignant state. Transformed cells can lead to tumor formation *in vivo* when injected into a suitable host, underlining their biological relevance (Mascolo *et al.*, 2018). This assay was developed on different cell lines (Creton *et al.*, 2012).

2.1. Cell transformation assay on immortalized Balb/c 3T3 cells

The history of the CTA development is presented in Figure 1 and the development on Balb/c 3T3 cells has been reviewed by Schechtman (2012).

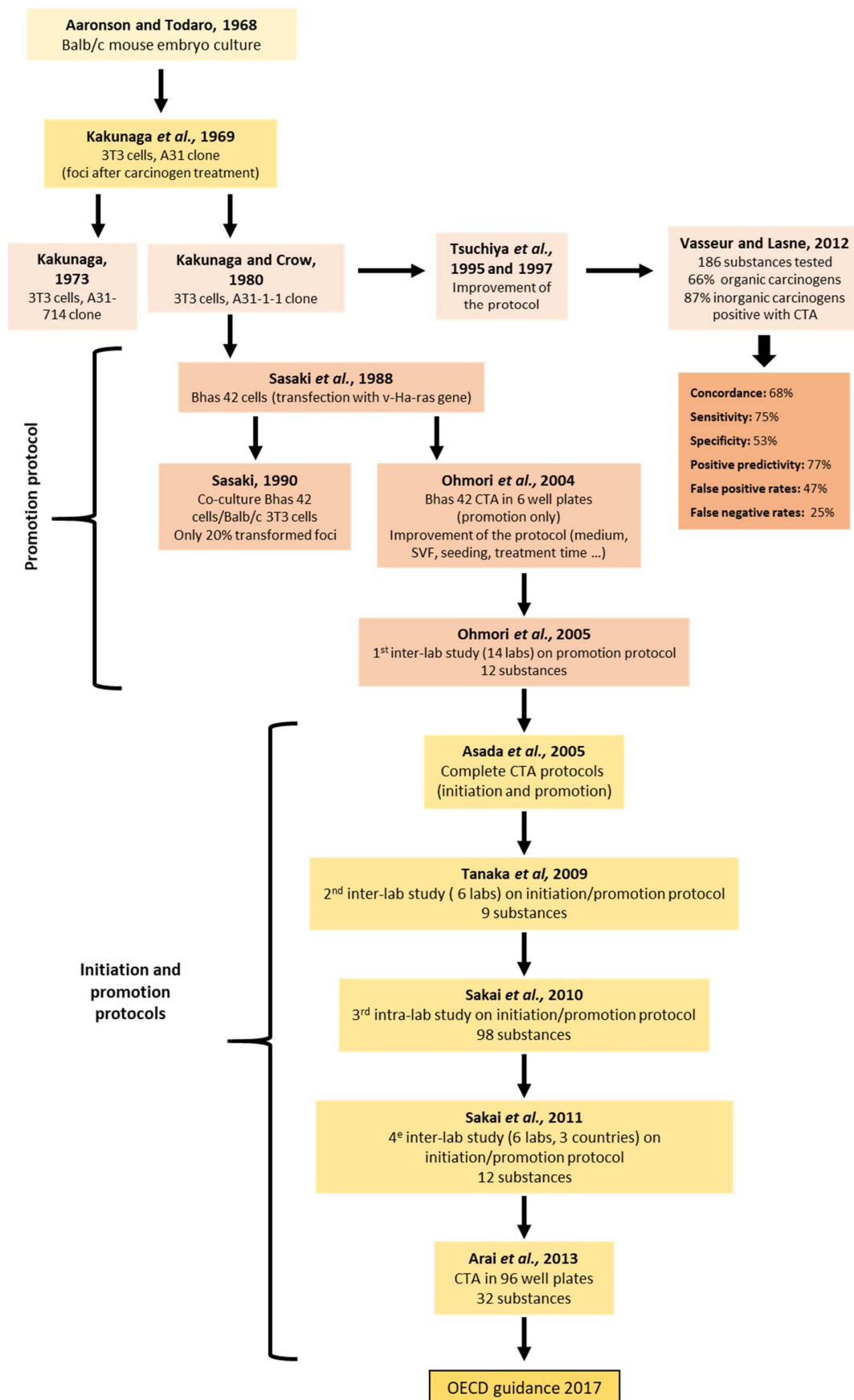


Figure 1: Overview of the development of the cell transformation assay from derived Balb/c 3T3 cells.

The chemical transformation of 3T3 cells resulting in the induction of morphologically aberrant foci was reported by Kakunaga and Kamahora (1969) from immortalized cells of the BALB/c 3T3 A31 subclone issued from whole embryo cultures of BALB/c mice, established by Aaronson and Todaro (1968). These cells grow as a monolayer, they stop dividing when the culture surface is covered. However, upon treatment with carcinogens at the beginning of growth, some cells do not stop proliferation and grow as morphologically aberrant foci over the monolayer of normal cells. They grow as multilayers, are spindle-shaped, dense with basophilic staining, and are able to induce tumor if injected in nude mice (Dipaolo *et al.*, 1972).

Several sub-clones of the original clone A31 were generated and some were analyzed for their suitability for cell transformation. The sub-clone, A31-714 isolated by Aaronson and Todaro (1968) got also good properties such as contact-inhibition after reaching confluence, strong cell-to-substrate adhesion, high cloning efficiency, cell doubling time < 20h, high sensitivity to transformation induced by chemical, and low background of spontaneous transformation (Kakunaga, 1973).

Later, Kakunaga and Crow (1980) established an A31-1-1 clone, stable for transformation after chemical treatment and UV sensitive, and they recommended its use for the cell transformation assay. This clone has erroneously been considered to be derived from Balb/c 3T3 A31. A subsequent characterization performed by Uchio-Yamada *et al.* (2016) confirmed that this clone is from Swiss mouse.

Among all the clones studied, those exhibiting low frequencies of cell transformation appeared to have a reduced sensitivity to certain chemical carcinogens. They differ also substantially both in their capacities to endogenously metabolize test compounds, and in their spontaneous transformation frequencies (Schechtman, 1985a)

Indeed, *in vitro*, different parameters could influence the cell proliferation and the transformation frequencies such as cell culture method (pH, medium, serum, seeding density), cell passage number (Landolph, 1985, Schechtman, 1985b), treatment period and time, cell division time required for fixation and expression of the foci.

Some authors demonstrated that treatment with well-known tumor agents after exposure to chemicals enhanced the transformation potential of cells (two-step method) (Mondal *et al.*, 1976). Cell trypsinization at confluence increased the number of transformed foci (Schechtman, 1985b) and increasing the seeding rate and processing the cells one day after seeding gave better cell transformation (Matthews *et al.*, 1993). Lastly, decreasing the FBS content in the culture medium improved the sensitivity of the test (Hayashi *et al.*, 2008).

A new protocol was developed to address some of these items, especially to shorten the duration of the assay (Tsuchiya *et al.*, 1995). This protocol involves clone A31-1-1 cells, recommended by Kakunaga and Crow (1980) and a modified medium, in conjunction with a new treatment schedule. This resulted in a substantial improvement in the transformation frequency, the formation of clearly transformed foci at an earlier stage of incubation, an enhanced responsiveness of the assay to several carcinogens, and a new protocol suitable for investigating promoting activity (Tsuchiya *et al.*, 1997).

In parallel to the development of the protocol on the clone, a set of 186 chemicals tested in the Balb/c 3T3 CTA were reviewed by Vasseur and Lasne (2012). It includes 165 organic chemicals (112 classified as rodent carcinogens, 53 as rodent non-carcinogens) and 21 inorganic chemicals (15 rodent carcinogens, 6 rodent non-carcinogens). Sixty-six percent of the rodent organic carcinogens and 87% of the inorganic carcinogens were positive in the assay giving a concordance of 68%, a sensitivity, specificity and positive predictivity of 75%, 53% and 77%, respectively. False-positive and negative rates were 47% and 25% respectively.

However, there are still some drawbacks with this assay, notably the time (4-6 weeks of cell culture) required for the development of unequivocally transformed foci; the relatively low frequency of transformation making this assay quite labor-intensive and relatively time-consuming.

2.2. Cell transformation assay on Bhas 42 cells

2.2.1. Origins and characteristics of Bhas 42 cells

From the subclone A31-1-1 of Balb/c 3T3 cells, Sasaki *et al.* (1988) established a new cell line by stable transfection using v-Ha-*ras* gene, with a few copies of v-Ha-*ras* genes integrated into the chromosomal DNA (chromosomes 17 and 19), (Sasaki, 2015).

Bhas 42 (clone n°42 of v-Ha-*ras* transfected Balb/c 3T3 cells) are morphologically non-transformed cells sensitive to contact inhibition (Sasaki *et al.*, 1988). They do not form foci but they can be converted to transformed foci after treatment with a well-known promoter, e. g. 2-*O*-tetradecanoylphorbol-13-acetate (TPA). To check the tumor-inducing capacity of transformed cells, they were inoculated subcutaneously into nude mice and form tumors (latent period: 20-60 days) in contrast to non-transformed cells by 90 days. Thus, Bhas 42 cells are regarded as being initiated cells (Sasaki *et al.*, 1990).

This assay is a surrogate system to observe the transition of cells from premalignant to malignant stages. The new method on Bhas 42, markedly scaled down from the Balb/c 3T3 one, has many advantages, e.g. a high sensitivity, a shorter experimental period, and the use of smaller amounts of materials with a simplified procedure and a more efficient cost (Asada *et al.*, 2005; Sakai, 2007).

2.2.2. History of the test improvement

The first study of transiently transfected Bhas 42 cells showed their ability to transform after 2 weeks of treatment with TPA, followed by 3 weeks of growth to allow the developing of foci, instead of 4-6 weeks for the Balb/c 3T3 CTA. This transformation is characterized by significant morphological changes not so easy to quantify by the scorer under microscope after Giemsa staining (spindle shape, loss of contact inhibition, criss-cross of cells and appearance of multilayers) (Sasaki *et al.*, 1988).

The second experiment used the previously stably transfected cells established by Sasaki *et al.* (1988) in co-culture with Balb/c 3T3 cells. Inoculated at 100 cells/dish for 10⁴ Balb/c 3T3 cells, they were treated for 2 weeks with TPA and foci were counted 6 weeks after seeding (Sasaki, 1990). However, only 20% of the Bhas 42 cells formed foci suggesting that normal cells prevent division of the transformed cells.

Thus, Bhas 42 cells have been used alone to develop a specific CTA (Ohmori *et al.*, 2004), with only a protocol for the detection of chemical promoting activity. Many parameters were studied to determine the conditions allowing the maximum number of well-identifiable foci and a cell growth assay was developed in parallel to choose the concentration range to test in the CTA. For this, media with different percentages of fetal calf serum, cell seeding rates, promoter treatment and post-treatment culture times were compared. Finally, the percentages in the culture medium of solvents (DMSO, ethanol, acetone) used to dissolve compounds were also studied on the foci appearance. The main improvements were: omission of Bhas42 cell treatment with an initiating agent and with a subsequent cultivation for an expression period, a shortened period to 3 weeks, with a half of the time needed for the Balb/c 3T3 assay. The assay was also miniaturized: the size of the plates and the volume of medium used were then decreased to 6-well dishes and 2 mL, respectively. Only 3-6 wells were mandatory to test one dose instead of 10-20 dishes and the percentage of fetal bovine serum in the medium decreased from 10 % to 5 %.

Ohmori *et al.* (2005) published the first inter-laboratory study with this optimized protocol. Fourteen laboratories tested 12 compounds in the project. The first step was to implant the cell growth test and the Bhas 42 promotion assay in the different laboratories using two tumor promoters, TPA and

lithocholic acid (LCA) treatment. The latter was to test positive controls for the ability of the laboratories to perform the test. Finally, 12 compounds were tested and analyzed by 4 laboratories. Same results were obtained by the 4 labs with eight substances (4 α -phorbol, 17 β -estradiol, mezerein, phorbol 12,13-didecanoate, progesterone, diethylstilbestrol, insulin, sodium saccharin) giving a quite high consistency rate. For two substances (okadaic acid, catechol), only one of the four labs presented discordant results. Inconsistent results for two chemicals (dexamethasone, anthralin) required further improvements and technical refinement of the protocol.

In 2005, Asada *et al.* published the first study using a complete CTA test (detecting both the initiating and promoting activities of chemicals), using two protocols performed in parallel and based on a difference in the cell seeding number and in the chemical treatment time. In the promotion assay, Bhas 42 cells were exposed to chemicals when cells were near confluence. Since initiators need several cell divisions for gene mutation fixation, a new treatment protocol was designed: cells were seeded at a lower rate to divide several times in culture dishes after chemical treatment. After checking the feasibility of this new protocol using positive controls (MMNG, MCA for initiation and TPA, LCA, and okadaic acid for promotion), they tested 16 PAHs. Then, PAHs were divided into 4 groups according to the results. Benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene induced only foci formation in the initiation assay (group 1) in contrast to benzo[e]pyrene, benzo[gly]perylene, 1-nitropyrene and pyrene inducing foci only in the promotion assay (group 2). Positive answers were observed with benz[a]anthracene, benz[b]anthracene, chrysene and perylene in both initiation and promotion (group 3). For acenaphthylene, anthracene, coronene, 9,10-diphenylanthracene, naphthalene and phenanthrene, results were negative or equivocal (group 4). These findings suggested different modes of action of PAHs and an increased risk when people are exposed to PAH mixtures.

Further, 3 large-scale, intra- or inter-laboratory studies were undertaken to validate the complete test. Tanaka *et al.* (2009) tested 9 substances in 6 laboratories, and then Sakai *et al.* (2010) tested 98 compounds in his own lab. Performance indices were calculated from the results of 52 carcinogens and 37 non-carcinogens. The concordance was 78%, sensitivity 73%, specificity 84%, positive predictivity 86%, negative predictivity 69%, false-negative 27% and false-positive 16%. The concordance, specificity, negative and false-positive predictivity were superior while the other performance indices were equivalent to conventional genotoxicity test indices, identifying genotoxic carcinogens making the assay promising. In 2011, Sakai *et al.* published the results of an international validation study, carried out in 6 laboratories of 3 different countries (Japan, USA, Germany) with 12 compounds, positive or negative in the *in vivo* carcinogenesis assay. The Bhas 42 CTA correctly distinguished all six carcinogens and two

tumor promoters from four non-carcinogens, demonstrating the transferability, reproducibility between laboratories, and its applicability to predict a mode of action in the carcinogenicity.

Finally, in 2013, Arai *et al.* proposed a miniaturized version with the use of 96-well plates (instead of 6-well plates), for high-throughput automated applications. This protocol was validated using 32 compounds already tested in the 6-well method. The results were concordant between the two techniques, except for one compound, sodium arsenite.

Then, these data using 6-well and 96-well studies have led to an OECD guidance on the in vitro Bhas 42 cell transformation assay (OECD, 2017).

2.2.3 Protocols of Bhas 42 CTA according to the OECD guidance (2017)

In parallel to the CTA, a proliferation assay (Figure 2) is necessary to establish the concentrations affecting the cell growth. The OECD guidance recommends testing chemicals on a large concentration range: from weak or no toxicity to the highest acceptable level corresponding to less than 20% survival compared to the negative control. At least 5 concentrations should be tested: (a) one concentration below the non-toxic level (around 80-120% of cell growth), (b) two concentrations between the non-toxic level and the 50% inhibitory concentration (IC₅₀) and (c) two concentrations between the IC₅₀ and the IC₉₀ (OECD, 2017).

In the initiation assay, the cells are inoculated at a low density (4 000 cells/well in 6-well protocol) and exposed at the beginning of growth phase to a test chemical for 3 days, 24 h after seeding to detect initiating activity of the chemical.

For the promotion assay, the seeding is higher (14 000 cells/well in 6-well protocol) with cells being sub-confluent on day 4. Cells are treated continuously when they are sub-confluent for 10 days. This condition allows cell-to-cell communication and particularly favors the promoting activity of chemicals. The total culture duration is then, 21 days in each assay with a fixing and staining step at the end to observe the wells under light microscope. Representative protocols issued from the OECD guidance (2017) are shown in Figure 2.

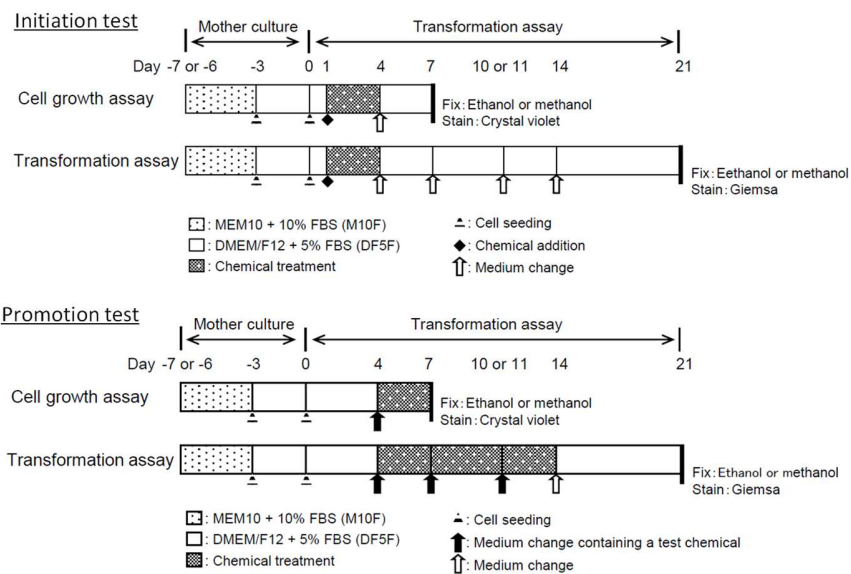


Figure 2: Timeline for the initiation and promotion test component of the transformation assay (6-well format) (OECD guidance, 2017)

Regards the application of this guidance for NPs testing, some adjustments are needed. It is discussed in the #4 “Keypoint to perform CTA for NPs testing”.

2.2.4. Controls (MCA/TPA)

Negative and positive controls must be included each time an experiment is performed. For negative controls, culture medium alone should not induce more than 10 foci/well (initiation test) and 12 foci/well (promotion test) in the 6-well protocol. Solvent used to dilute chemicals is always included for the positive controls (diluted in DMSO). The positive controls are depending on the protocol. The typical carcinogen 3-methylcholanthrene (MCA, 1µg/mL) is used for initiation assay. It induces many transformed foci (52) in the initiation assay, but none in the promotion assay (less than 10). For the promotion test, the well-known tumor promoter 2-O-tetradecanoylphorbol-13-acetate (TPA, 50 ng/mL) produces a great number of foci in the promotion assay (55), but none in the initiation assay (less than 5) (Sakai, 2007).

These positive controls were used in all the published studies but variabilities in the number of foci/well with the same chemical concentration were observed between laboratories (Ohmori *et al.*, 2005; Sakai *et al.*, 2010; Sakai *et al.*, 2011). This specific point is discussed later (see #4.3.).

3. Literature review on the use of CTA with NPs

Few articles were published regards to NP toxicity performing the CTA. NPs tested on Balb/c 3T3 and Bhas 42 CTAs are compiled thereafter (Table 1). In these articles, the NPs studied with CTA were all properly characterized before testing as *in vitro* assessment may vary according to physical-chemical parameters of NPs.

Table 1: Overview of the studies performing cell transformation assay for testing NPs.

Year	First author	Co-authors	Cell Model	Nanoparticles	Solvent	Concentrations	Treatment time	Results	Positive control
2009	Ponti	JRC	CTA Balb 3T3	cobalt NP cobalt ²⁺	Ultrapure sterile water	1, 7, 10, 30 μM 1, 7, 30, 70 μM	72h (initiation)	positive except 1 μM negative	$\text{Na}_2\text{CrO}_4 \cdot 4\text{H}_2\text{O}$ (50 μM)
2012	Uboldi	JRC	CTA Balb 3T3	amorphous SiO_2 NP	Ultrapure sterile water	100 $\mu\text{g}/\text{mL}$	72h (initiation)	negative	MCA (4 $\mu\text{g}/\text{mL}$)
2013	Ponti	JRC	CTA Balb 3T3	multiwall carbon nanotubes nude and functionalized	Dimethylsulfoxide	1, 10, 100 $\mu\text{g}/\text{mL}$	72h (initiation)	positive without dose effect at least at one concentration	MCA (4 $\mu\text{g}/\text{mL}$)
2014	Sighinolfi		CTA Balb 3T3	Fe_3O_4 , CeO_2 , Au NP	Phosphate Buffer Saline	10 μM	2 stage treatment (initiation: NP + TPA promotion: MCA + NP)	negative as initiators positive as promoter (Fe ₃ O ₄ and Au)	Co NP (10 mM)
2016	Stoccoro	JRC	CTA Balb 3T3	pristine, mediated citrate/silica ZrO_2 , TiO_2 NP	Ultrapure sterile water	10, 20, 40 $\mu\text{g}/\text{cm}^2$	72h (initiation)	positive for pristine, low and high citrate coated ZrO_2 NP positive for low and high citrate coated TiO_2 NP	MCA (4 $\mu\text{g}/\text{mL}$)
2016	Uboldi	JRC	CTA Balb 3T3	titanium dioxide nanoparticles	Deionized water	1, 5, 10 $\mu\text{g}/\text{mL}$	72h (initiation)	positive for nanosized and bulk rutile TiO_2 negative for nanosized and bulk anatase TiO_2	MCA (4 $\mu\text{g}/\text{mL}$)
2017	Choo		CTA Balb 3T3	AgNP	MEM medium	0.17, 0.66, 2.65, 5.30, 10.60 $\mu\text{g}/\text{mL}$	72h (initiation)	positive with dose effect	B[a]P 1 $\mu\text{g}/\text{mL}$
2011	Hasegawa		CTA Bhas 42	micro-sized In ₂ O ₃ , Dy ₂ O ₃ nano-sized In ₂ O ₃ , Dy ₂ O ₃ WO ₃ et Mo	Ultrapure sterile water	0.01 to 5 $\mu\text{g}/\text{mL}$	10 days (promotion)	positive for nano-sized Ln ₂ O ₃ and Dy ₂ O ₃ , negative for WO ₃ and Mo	not mentioned
2016	Gabelova		CTA Bhas 42	nanosilver (AgNIM300), AgNIM302 MNP differing in surface modifications	MilliQ water	0.1-10 $\mu\text{g}/\text{cm}^2$ for nanosilver 0.25-50 mM for MNPs	3 days (initiation)	negative for spherical AgNIM300, SO-MNP, SO-PEG-MNP positive for nanorods/wires Ag NIM302 and SO-PEG-PGLA-MNP	MCA (3 $\mu\text{g}/\text{mL}$)
2017	Fontana		CTA Bhas 42	SiO_2 (NIM 200, NIM 201, NIM 202, NIM 203), Min-U-Sil, diatomaceous earth	Sterile deionized water	2 to 80 $\mu\text{g}/\text{cm}^2$	3 days (initiation) 10 days (promotion)	negative as initiators positive as promoters	MCA (1 $\mu\text{g}/\text{mL}$) TPA (0.05 $\mu\text{g}/\text{mL}$)
2020	Jalili	Fontana	CTA Bhas 42	$\text{Al}^{\text{IV}}\text{Al}_2\text{O}_3$ and AlCl_3	Distilled water	0.3 to 28 $\mu\text{g}/\text{cm}^2$	3 days (initiation) 10 days (promotion)	negative as initiators and promoters	MCA (1 $\mu\text{g}/\text{mL}$) TPA (0.05 $\mu\text{g}/\text{mL}$)

Blue : positive in initiation protocol

Red : positive in promotion protocol

3.1. Studies with Balb/c 3T3 protocol

All the articles published with the Balb/c 3T3 protocol only focused on the initiation step, except one (Sighinolfi, 2014), where the two-steps were performed. The Joint Research Center of the European Commission published the 5 following articles using the Balb/c 3T3 assay to assess NPs to support the European strategy to develop *in vitro* systems and to understand the manufactured NP mechanisms of action:

Ponti *et al.* (2009) were the first authors to use the CTA for studying NPs. They assessed the morphological transforming activity of **cobalt NPs** (Co-nano) and **cobalt ions** (Co²⁺) in Balb/c 3T3 cells after 72h treatment (initiation assay) using sodium chromate (50 µM) as a positive control (Elias *et al.*, 1991). A total of 1.5x10³ cells/ml in 10 ml of complete culture medium were seeded in each 100 mm dish (10 replicates for each treatment). After 24 h, the medium was changed and replaced with one containing selected concentrations of Co-nano or Co²⁺. Co-nano was tested at 1, 7, 10, 30 µM and Co²⁺ at 1, 7, 30, 70 µM corresponding respectively to IC20, 30, 50, 80, determined by Colony-Forming Efficiency (CFE). After 72h treatment, cultures were maintained for 5 weeks, with the culture medium renewed twice a week. At the end of the experiment, the cells were fixed and stained. Co-nano were characterized in water and in complete culture medium for their morphology by Scanning Electron Microscope/energy dispersive using X-ray (SEM-EDX) technique and size distribution by NP tracking analysis.

An increase in morphological transformation was observed for all concentrations of Co-nano (except for Co-nano at 1 µM); while no statistically significant morphological transformation was observed for Co²⁺. These results agree with Lison *et al.* (2001), who demonstrated a genotoxic potential for cobalt *in vitro* without testing the morphological transforming potential. Concerning the morphological transformation by cobalt and its compounds, the IARC (IARC, 1991) concluded that they are possibly carcinogenic to humans (Group 2B) since there was sufficient evidence in experimental animal studies but inadequate evidence for carcinogenicity in humans (lung cancer) (Kuo, *et al.*, 2006).

In 2012, Uboldi *et al.* analyzed the initiation property of **amorphous silica NPs** (aSiO₂NPs) with the Balb/c 3T3 CTA. Compared to the previous article, the protocol was used with minor modifications such as the seeding number (3.3x10³ cells/mL instead of 1.5x10³) and the positive control (MCA at 4 µg/mL

instead of sodium chromate at 50 μM). NM-200 and NM-203 belong to the list of NMs of interest to the OECD and were delivered in powder form, while the synthesis of NRT-808, NRT-817, NRT-820 and NRT-944 was performed in the laboratory. Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM) were used to study the size distribution and morphology of the particles, respectively. NPs were not cytotoxic after 72h as determined by CFE. aSiO_2NPs (100 $\mu\text{g}/\text{mL}$) did not induce any morphological transformation in the Balb/3T3 cells despite the internalization (uptake and intracellular distribution) of the NPs confirmed by fluorescence microscopy following exposure to 85 nm fluorescently labeled aSiO_2NPs . They were located exclusively in the cytoplasmic region. Neither small (16–80 nm) water dispersed aSiO_2NPs , nor larger (NM-200; 200 nm size diameter) silica particles, nor pyrogenic aSiO_2NPs (NM-203; 300 nm size diameter) showed any carcinogenic potential in CTA, suggesting that size or synthesis method of aSiO_2NPs had no impact.

Few years later, **Ponti *et al.* (2013)** studied the morphological transformation of Balb/c 3T3 induced by **multiwall carbon nanotubes** (mw CNTs) mainly used for the production of smart textile. They investigated the toxicity of nude and chemically functionalized (-NH₂, -OH and -COOH groups) mw CNTs. Minor modifications of the protocol were applied compared to Uboldi *et al.* (2012): after 72 h of exposure, the suspensions were changed with fresh M10F instead of directly adding the DMEM/F12 medium and only from day 7, DMEM/F12 medium was used till day 24 and the medium was not changed during the last culture week.

The size distribution of the mwCNTs suspended in complete culture medium at the highest concentration tested (100 mg/mL) was measured by DLS. The sedimentation of different types of mw CNTs (nude, -OH, -COOH and -NH₂), dispersed as described above, was characterized by visible and near-infrared light absorption (VIS-NIR). Mw CNTs interaction with cells was investigated, using a Scanning Electron Microscopy (SEM) both in reflection and transmission configurations, and using a Transmission Electron Microscope (TEM). In this case, some mw CNTs were internalized by the cells, but they strongly interacted with cells and cell membrane with persistence.

The authors observed morphological transformation of mw CNTs in four independent experiments and the average data showed statistically a significant increase at least at one concentration for all the nude forms or functionalized mw CNTs but without any dose-response.

Three non-cytotoxic concentrations were tested (1, 10 and 100 $\mu\text{g}/\text{mL}$). Transformed foci increased at 1 mg/mL ($p < 0.001$) and 100 $\mu\text{g}/\text{mL}$ ($p < 0.01$) for -NH₂; at 1 $\mu\text{g}/\text{mL}$ ($p < 0.05$) for -COOH; at 10 $\mu\text{g}/\text{mL}$ ($p <$

0.01) for –OH and at 100 mg/mL ($p < 0.001$) for nude mw CNTs. However, as recommended by the OECD guidance, a second experiment is necessary to confirm the positivity.

Stoccoro et al. (2016) studied the morphological neoplastic transformation potential on Balb/c 3T3 cells of **titania** (TiO₂) and **zirconia** (ZrO₂) NPs, either in their **pristine** (uncoated) form or **modified with citrate and/or silica** on their surface. As benchmark material, commercial TiO₂ preparation (Aeroxide®P25) was used. The chemical-physical characterization of the pristine and modified ZrO₂ and TiO₂ samples was performed by TEM, DLS and Zeta-potential. No or low cytotoxicity (<30%) was noted by the authors, whatever the concentrations and treatment time tested.

CTA was carried out as reported by Ponti *et al.* (2013) on Balb/3T3 cells treated with ZrO₂ and TiO₂ NPs at concentrations of 10, 20 and 40 µg/cm², corresponding to 91.6, 183.3 and 366.6 µg/ml, respectively.

The pristine and low/high citrate coated **ZrO₂** NPs induced statistically significant morphological transformations with dose-effect suggesting initiation properties. In contrast, the silica-coated form did not induce any cell transformation suggesting the silica coating able to preserve cells from the foci formation and hence from morphological transformation.

For **TiO₂** NPs, only citrate-coated and P25 induced the formation of foci while pristine and silica-coated NPs did not induce any cell transformation. In 2010, TiO₂ was recognized as a possible carcinogen for humans (group 2B) (IARC, 2010). After France in 2019, since January 2022, TiO₂ is now forbidden in Europe as a food additive (Regulation 2022/63).

Uboldi et al. (2016) examined the effects of **nanosized and bulk titanium dioxide** (TiO₂) **in two different crystalline forms** (anatase and rutile) with CTA, performed according to Ponti *et al.* (2013). Inductively Coupled Plasma-Mass Spectrometry (ICPMS) was used to study the uptake of titania in Balb/3T3 cells as it allowed to relate the amount (expressed as mass and number of particles) of TiO₂ NPs internalized and their toxic potential. DLS and TEM were applied to characterize stock suspensions and Zeta potential was also measured at pH 7 for all NPs. Nanosized rutile TiO₂ (Ru10), bulk rutile (BRU), nanosized anatase (An-10) and bulk anatase (BAN) were tested at 1, 5, 10 µg/mL, as low cytotoxic concentrations (<30%).

Crystalline forms had no impact on the internalization; in contrast, nano-titania NPs were more internalized than their respective bulk materials suggesting that the uptake was size-dependent.

Ru-10 induced a statistically significant dose-dependent increase in cell transformation. Similarly, BRU resulted in transforming Balb/c 3T3 cells at all concentrations. In contrast, although An-10 and BAN slightly enhanced foci formation whatever the concentrations, their effect was not statistically

significant. In fact, while a significantly higher internalization of anatase TiO₂ NPs was measured, only the rutile crystalline form induced morphological transformation in Balb/c 3T3 fibroblasts. These results might be due to different reactivity of the two investigated crystalline forms. Actually, rutile TiO₂ NPs are more photocatalytic than anatase and consequently, are able to produce greater numbers of oxygenated free radicals on their surface (Fenoglio *et al.*, 2009; Lipovsky *et al.*, 2012) leading to indirect genotoxicity and then mutations. Recently, EFSA (2021) published a scientific opinion on the safety assessment of TiO₂ with TiO₂ particles having the potential to induce DNA strand breaks and chromosomal damages but not gene mutations and by the way, EFSA concluded that genotoxicity cannot be ruled out due to uncertainty concerning the mode of action involved (threshold or not).

Independently from the JRC, two teams published results on the Balb/c 3T3 CTA performed with NPs:

Choo *et al.* (2017) carried out the CTA to assess carcinogenic potential of **silver NPs (AgNPs)**, after a 72h treatment. The Ag nanopowder (<100 nm), was completely dispersed in an experimental medium by sonication and was characterized by energy dispersion X-ray spectrum (EDS) analysis, TEM, SEM, DLS, and Zeta potential. Protocol was the same as described previously with minor modifications compared to Uboldi *et al.* (2012). 2×10³ cells/mL were seeded in M10F complete medium, after 24 hours of incubation, medium was changed with media containing benzo[a]pyrene (B[a]P) as a positive control (instead of MCA in Uboldi *et al.*, 2012), anthracene as a negative control, or AgNPs at five concentrations (0.17, 0.66, 2.65, 5.30, and 10.60 µg/mL). Two cytotoxicity tests were performed, Cristal Violet and CFE with different determinations of t IC₅₀ (0.17 µg/mL and 5.91 µg/mL respectively). The chosen concentrations were as requested by the OECD guidance (from no observable adverse effective concentration to IC₉₀) corresponding to CV cytotoxic results.

The positive control (B[a]P) (1 µg/mL) showed a 21.33-fold increase of foci formation per plate in contrast to negative control (Anthracene, 20 µg/mL). Cells exposed to AgNPs for 72 hours showed significantly induced morphological transformation at all concentrations with a concentration dependency up to IC₉₀.

Sighinolfi *et al.* (2014) is the **only** study with the two-stage cell-transformation protocol using BALB/c 3T3 cells. They assessed the carcinogenic potential of **metal (Fe₃O₄, CeO₂, and Au) NPs**, used for medical applications as contrast agents in NMR, or as catalysts and UV adsorbent and tumor hyperthermia agents.

Treatments with a tumor initiator 3-methylcholanthrene (MCA, 0.5 µg/mL) at the beginning of cultivation and then with TPA (300 ng/mL), as tumor promoter to induce preneoplastic foci were used. This two-stage transformation assay was based on the improved method proposed by Kajiwara *et al.* (1997). In particular, it was used a special medium supplemented with a mixture of insulin, transferrin, ethanolamine, and sodium selenite (ITES) and a low concentration of fetal bovine serum (FBS). Then, it was demonstrated that this medium together with MCA+TPA treatment caused high-frequency transformation with foci appearing earlier (after 45 days instead of the 90 days). Cytotoxicity was studied on a range of concentrations from 1 to 100 µM with CFE and XTT assays, after 24h or 72h of treatment. Mild cytotoxic effect (<30%) was noted for all NPs at 50 and 100 µM after 24 or 72h of treatment, except for Co, which was concentration dependent cytotoxic, with high cytotoxic effect (IC₉₀) at the highest concentrations (50 and 100 µM).

As NPs were tested in various combinations as potential initiators, promoters or both, NPs were used at the concentration of 10 µM with/without MCA or TPA, following the two-stage protocol. Actively growing cells were seeded at a density of 5x10⁴ cells/dish in 5 mL of DMEM culture medium. Twenty-four hours after seeding, the cells were treated with/without the tumor initiator MCA at the concentration of 0.5 mg/mL with/without NPs. After three days, the medium was changed and ITES medium was added for another three days. Cells were then exposed (or not) to the tumor promoter TPA, at the concentration of 300 ng/ml with/without NPs. After two weeks, cells were normally seeded with ITES medium for another four weeks, fixed and stained.

Cobalt NPs at a 10 mM concentration was selected as reference carcinogenic compound to perform the two-stage transformational assay (Ponti *et al.*, 2009). The NP suspensions were characterized using the Zeta Potential (Z-Potential) and DLS to define the hydrodynamic diameter of the NP dispersion.

Data with NPs were depending on their respective chemical-physical properties. Indeed, while Fe₃O₄, Au, and CeO₂ NPs, when used as tumor initiators, did not give rise to neoplastic transformation. In contrast, Co NPs were able to induce foci formation, confirming the data of Ponti *et al.* (2009).

Fe₃O₄ and Au NPs, when tested as a promoter, showed a dramatic increase of neoplastic foci, as compared to MCA alone, reaching values similar to the well-known promoter TPA. The fact that Co NPs were not able to generate foci as a promoter could be attributable to its intrinsic cytotoxic activity.

Findings regarding the activity of CeO₂ NPs were quite interesting. In fact, CeO₂ NPs treatment after MCA treatment did not change the number of foci after treatment by MCA alone (17 and 19 respectively) but decreased significantly the foci formation when compared to the number of foci induced by treatments

with positive controls (MCA+TPA) (38), suggesting a potential antagonism in the promotion of cell transformation at non-cytotoxic concentrations.

3.2. Studies with the Bhas 42 protocol

To date, only four articles related to NPs and using the Bhas 42 protocol are available in the literature (Table 1).

Hasegawa *et al.* were the first to publish in 2012 a study on the tumor promotion activity of rare metals; **(molybdenum (Mo) and metal oxide (indium oxide (In_2O_3), dysprosium oxide (Dy_2O_3) and tungsten oxide (WO_3))** NPs according to the protocol of Ohmori *et al.* (2004).

Each metal was characterized. Purity, diameter, primary crystallite size, surface area and impurities were provided by the manufacturer, mean diameter in solution was measured by DLS and Zeta potential.

Growth inhibition of Bhas 42 cells was observed when more than 10 mg/ml of rare metals (micro-sized) were added in the medium, while a lower transformed colony number was observed in the group treated with the nano-sized In_2O_3 and Dy_2O_3 at the concentration of 5 mg/mL, which could be attributed to metal oxides cytotoxicity.

Both nano- and micro-sized In_2O_3 increased significantly transformed colonies at concentrations above 1 mg/ml and 2 mg/mL, respectively. For both **nano- and micro-sized Dy_2O_3** also the number of transformed colonies increased significantly at concentrations above 2 mg/mL. At the top concentration (5 mg/mL), the number of transformed colonies induced by the nano-sized In_2O_3 and Dy_2O_3 was lower than in the group treated with micro-sized NPs suggesting that size may play a role in the cytotoxicity. In contrast, **WO_3 or Mo** did not increase the number of transformed colonies in the concentration range of 0.01–5 mg/mL whatever the size (nano- or micro-particles). These results showed that particle sizes of these metals did not affect the promotion activity. However, in this study, six independent experiments were performed for each and in duplicate with a high standard deviation suggesting a poor reproducibility of the assay, perhaps due to the subjectivity of scoring and difficulties to detect unequivocally foci. Furthermore, no positive control for the CTA was used in this article.

Gabelova *et al.* (2016) investigated the capacity of two frequently used metallic engineered nanomaterials (ENMs), nanosilver and magnetite NPs (MNPs), to induce transformed foci in Bhas 42 cells using the tumor initiation protocol. Two types of nanosilver, **spherical NPs (AgNM300) and fibrous**

(AgNM302) nanorods/wires, and MNPs differing in surface modifications [MNPs coated with sodium oleate (SO-MNPs), MNPs coated with SO + polyethylene glycol (SO-PEG-MNPs) and MNPs coated with SO + PEG + poly(lactide-co-glycolic acid) SO-PEG-PLGA-MNPs] were included in this study. Particles were characterized in terms of particle size, magnetism and Zeta potential in stock and in culture medium. The silver NPs were tested at 0.1, 0.3, 1, 3 and 10 $\mu\text{g}/\text{cm}^2$ and the MNPs at 0.25, 0.5, 1, 2.5, 10, 25, 50 mM.

Two negative controls were used in these experiments: (i) Control 1, cells cultivated in medium supplemented with 5% serum during treatment with nanosilver and (ii) Control 2, cells cultivated in medium supplemented with only 2% serum during exposure to surface-modified MNPs. The average number of transformed foci per well determined in control 1 was 0.5 ± 0.5 , and in control 2 was 3.0 ± 2.1 . The average number of transformed foci per well determined in MCA-treated cells was 23.2 ± 2.4 .

Neither spherical AgNM300, nor SO-MNPs or SO-PEG-MNPs increased substantially the number of transformed foci. In contrast, a significant increase in the transformed foci number was observed when Bhas 42 cells were exposed to silver nanorods/wires (AgNM302) or SO-PEG-PLGA-MNPs, mainly at lower concentrations (1-10 mM) with the highest level of transformed foci with SO-PEG-PLGA-MNPs. These results showed that the carcinogenic potential of nanosilver is underlying by fibrous shape.

Fontana *et al.* (2017) analyzed the transformation initiating and promoting activities of four **synthetic amorphous silica (SAS)** NPs, two precipitated, NM-200 and NM-201, and two pyrogenic, NM-202 and NM-203. These NPs were characterized previously (Rasmussen *et al.*, 2013). The Bhas 42 CTA, with the initiation and the promotion assay, was performed in accordance with the OECD recommendations (OECD, 2017) and validated in the laboratory using known carcinogenic substances. The concentrations tested ranged from 2 $\mu\text{g}/\text{cm}^2$ to 80 $\mu\text{g}/\text{cm}^2$ for SAS. MCA (1 $\mu\text{g}/\text{mL}$) and TPA (0.05 $\mu\text{g}/\text{mL}$) were used as positive controls in the CTA performed with silica micro- and nano-particles. In this study, the number of transformed foci per well induced by MCA in initiation assays was 13.2 ± 3.1 versus 3.2 ± 1.6 transformed foci in the control (0.1% DMSO), and the number of transformed foci per well induced by TPA in promotion assays was 14.9 ± 3.0 versus 5.7 ± 2.0 transformed foci in the control (0.1% DMSO).

Transformed foci were observed with all SAS but only in the promotion assay, suggesting a tumor promoter mode of action of the silica NPs. NM-203 was active at lower concentrations compared to other SAS, due to high production of oxygen species in an aqueous solution (Rasmussen *et al.*, 2013).

Jalili et al. (2020) focused on initiation and promotion properties of aluminium NPs (Al^0 and Al_2O_3) by using CTA and Bhas 42 cells, as performed by Fontana *et al.* (2017). In this study, the supplier (IoLiTec (Heilbronn, Germany) provided the NP characteristics. The results for the positive controls were in agreement with the study of Fontana *et al.* (2017). The average number of transformed foci per well induced by MCA in initiation assays was 14.2 ± 2.9 versus 5.8 ± 1.9 transformed foci in the control (0.1% DMSO), and the average number of transformed foci per well induced by TPA in promotion assays was 20.3 ± 3.0 versus 8.8 ± 2.1 transformed foci in the control (0.1% DMSO).

Concerning cell proliferation, in the initiation assay, Al^0 induced a decrease on Day 7 at the two highest concentrations (1 (38%) and 3 $\mu\text{g}/\text{cm}^2$ (84%) for Al^0 NP). In contrast, Al_2O_3 in the initiation assay and the two forms of Al (Al^0 and Al_2O_3) in the promotion assay induced a moderate decrease in cell proliferation for all concentrations tested (<25% at the highest concentration, respectively, 3 $\mu\text{g}/\text{cm}^2$ for Al^0 and 9 $\mu\text{g}/\text{cm}^2$ for Al_2O_3), suggesting a weak cytotoxicity. No transforming activity was then observed with Al^0 and Al_2O_3 , in the initiation or promotion assays.

Briefly, to date, very few studies on NPs and CTA are published, seven articles using the Balb/c 3T3 and four with the Bhas 42 cells. Five of these articles were performed by the JRC, using only the initiation protocol and only three were carried out with the two-stage protocols (initiation and promotion). In all studies, as recommended, characterization of tested NPs was always performed in parallel using different techniques such as TEM, SEM, DLS, Zeta potential for a proper interpretation of the results. Different NPs (cobalt, silice, carbon, silver, aluminium...) were tested giving various results (positive or negative with initiation and/or promotion protocol and even some antagonism activities). No rules about NP mode of action can be established regards to these studies. For example, for Balb/c 3T3 initiation, 57% of the NPs were negative (8/14) and 43% positive (6/14). Taking into account the promotion studies, 44% of the NPs were negative (4/9) and 56% positive (5/9) leading to new information on NPs mode of action, compared to the genotoxicity test battery recommended. Regards to Bhas 42 protocols, 40% positive effects (6/15) were found for promotion step.

4. Key points to perform CTA for NPs testing

Some particular points could be specified for a CTA OECD guidance dedicated to NPs assessment.

4.1. Preparation of NPs before testing

NPs are prone to change properties in different media and treatments and many characteristics have to be taken into consideration to assess toxicity.

For any *in vitro* assessment regards to NPs, **a prerequisite step is to characterize first the NP suspension in the culture medium** and not just as supplied in powder form or water suspension. Properties of NPs change depending on the surrounding environment; a lot of reactions can occur when NPs are into biological media. Proteins can be adsorbed to the surface of NPs and Zeta potential can be modified when NPs are at physiological pH. NPs can precipitate/agglomerate/aggregate, changing then their toxic potential. Therefore, it is necessary to perform an accurate characterization of NPs before, during and after testing (DeLoid *et al.* 2014). Particle size, state of agglomeration, surface properties and stability of the dispersion stock solution as well as how the NPs dispersed in the final treatment medium should be measured and monitored.

The size and the shape must be determined as two crucial parameters for the internalization mechanism. Studies have shown that there is a size- and shape-dependent toxicology associated with NPs. For the size, smaller is the NP, higher is the toxicological effect and for the shape, nanotubes have a greater ability to translocate inside cells than nanospheres or nanocubes (Yuan *et al.* 2010). The **surface properties** have to be assessed, as this is the most critical parameter regarding interactions with the biological medium. **Surface area** has an important impact; larger is the surface, higher is the probability for biomolecules present in the medium to interact with NPs. The **surface chemistry** (nanoparticle functionalization) impacts also the toxic effect of NPs. Lastly, the **surface charge** of the NPs in the medium is required as it determines the NP stability. Indeed, if they are all negatively or positively charged, they are stable in suspension thanks to electrostatic repulsion, but when NPs have both positives and negatives charges on their surface, it conducts to their agglomeration/aggregation. Agglomeration or aggregation increases the size and changes the shape of NPs, influences NP interactions in biological medium and most importantly, modify the stability and homogeneity. It is

crucial then, to determine surface charge of NPs at culture medium pH (7.4) before *in vitro* assessment (Caballero-Díaz *et al.* 2016). The presence of mutagens and transition metals affiliated with the particles must also be identified (Schins *et al.*, 2002, Stone *et al.*, 2010).

Homogeneity of NP suspension is required to correctly investigate toxicological effects, but many parameters can affect it. NPs are simply nano-objects in suspension, a sedimentation phenomenon can occur in addition to agglomeration and aggregation. To avoid this phenomenon, an ultrasound step allows to homogenate NP suspensions. Ultrasounds are currently used with a bath or a probe depending on NP properties. Ultrasound probes break stronger aggregates or agglomerates, but this method has the drawback to heat the sample. Ultrasound bath has a weaker impact avoiding the increase of NP suspension temperature (Hartmann *et al.* 2015). Another drawback of the ultrasound step is the foam formation due to the presence of proteins and salts in the culture medium. Then, it is recommended to prepare NP suspension into water or phosphate buffer saline (PBS) and to homogenate with ultrasound before incorporating NPs into the culture medium (Jiang *et al.* 2009).

Properties of NPs and their toxic effects can also be influenced by the different physical and chemical characteristics of **solvents** used for dispersing or dissolving them. Factors such as pH, salinity, water hardness, temperature and presence of dissolved or natural organic particles in the medium can influence the biological reactivity of NPs. Thus, they may behave differently in water, culture medium, PBS and other solvents with pronounced effects on their uptake, cellular localization and hence the observed toxic response (Handy *et al.*, 2012).

Furthermore, in the culture medium, a selective set of proteins with a high affinity for the NP surface will adhere and remain more or less bound, forming a **corona** (Gonzalez *et al.*, 2010, Elsaesser *et al.*, 2012 ; Sakulkhu *et al.*, 2015). Protein corona could be schematically represented as a double-layer coating wrapping the NPs, consisting of a first tightly bound monolayer, usually defined as “hard” corona, composed of proteins with high affinity for the NP surface (Rocker *et al.*, 2009), whereas the outer layer, termed “soft” corona, consists of a loose protein layer reflecting serum proteins abundance, predominantly comprising albumin and derivatives (Walczyk *et al.*, 2010; Casals *et al.*, 2010) easy to be eluted (Sakulkhu *et al.*, 2014b). This protein corona will greatly modify NP parameters, their hydrodynamic diameter, surface charge and especially their surface chemistry. In addition, the proteins making this corona differ according to the type of NPs and the medium composition. These are just as many parameters that affect the ability of NPs to penetrate inside cells and therefore their toxicity.

Two effects of the protein corona can be distinguished. On one hand, the surface of the NPs coated with proteins allows better **stability** by steric hindrance as well as by the surface charges modification. It also enhances biocompatibility of NP by ligand-receptor recognition (Kobos *et al.*, 2018). On the other hand, these bound proteins with many antibodies at their surface can activate the mechanisms of cellular internalization (Sakulkhu *et al.*, 2014a) and increase NP uptake. Proteins (FBS, BSA...) in NP dispersions and cell cultivation may vary from 0 to 10 % (Magdalena *et al.*, 2012a). Toyooka *et al.* (2012) demonstrated that the genotoxic effect was less extensive with BSA coated than with uncoated TiO₂NP. With regards to **internalization**, the most cells concerned are immune cells. Among the proteins identified of the protein corona, many are proteins of the complement system (Sakulkhu *et al.*, 2014b). These proteins are then recognized by the different cells of the immunity system, which internalize the NPs. As the immune cells can move into the body through the lymphatic system, they carry with them NPs that can be internalized, degraded, or excreted (Zhu *et al.*, 2009). Indeed, it has been demonstrated that a lot of NPs have immunotoxic effects.

As shape, size, surface charge and surface chemistry can affect cellular uptake of NPs and not all *in vitro* models possess all the endocytic mechanisms, it is important to check the ability of the tested NPs to be internalized in the chosen cell model. Each type of NPs is internalized by the cell *via* preferentially uptake pathway (Rennick *et al.*, 2021). *In vitro*, the principal route of entry of NPs into the cells is *via* endocytosis. This uptake could be investigated by diverse analytical techniques based on transmission electron microscopy (TEM) and scanning electron microscopy (SEM), induced coupled-plasma (ICP)-based techniques and fluorescent-based methods such as microplate reader, flow cytometry and confocal imaging (Shin *et al.*, 2020).

Then, to conclude and for all these reasons, to adapt the CTA OECD guidance to NPs, a paragraph should be added to specify, using recognized methods, the characterization of the tested NPs as a pre-requisite step.

4.2. Selection of NP concentration range using CTA

In the studies on NPs, dose metrics can be expressed in mass ($\mu\text{g}/\text{mL}$), surface area (m^2/mL) or particle number (n/mL) (Park *et al.*, 2009). In the majority of studies published with CTA, concentrations are expressed in mass units, i.e. as NP dispersion concentration ($\mu\text{g}/\text{mL}$) or μM , or as NP mass per surface of exposure cells ($\mu\text{g}/\text{cm}^2$). Generally, regards to *in vitro* toxicity, a correlation with surface area has been

proposed as a recommended dose expression (Oberdorster *et al.*, 2005; Braakhuis *et al.*, 2016) as it can be compared to *in vivo* exposure results (even if attention to differences in the exposure method should be considered) (Elliott *et al.*, 2017). The use of mass, number or concentration per cell appears to be particularly appropriate for NP testing; however, it is recommended to express concentrations in the three different dose metrics (mass, surface area and number), which is not mentioned in the published articles. This allows conversion from one dose to another when needed for determination of the best fitting dose metric for a toxic effect. If the particles are thoroughly characterized, this should be anyhow feasible (Drasler *et al.*, 2017).

In the CTA guidance for standard chemicals (2017), the highest concentration of the test chemical in the medium should be 2 mg/mL or 10 mM or 2 µL/mL if no precipitate or limiting cytotoxicity is observed. Regards to the highest concentrations (table 1), for most of the studies, NPs were tested up to 100 µg/mL or to 70 µM, which is 20 or 142 fold respectively below the limits recommended in the guidance, suggesting that for NP testing, these limits could not be attained.

The cell growth assay (as a marker of cytotoxicity) associated with CTA enables to fix the concentration tested in the initiation and promotion test. Unlike conventional genotoxicity assays where concentrations to be tested are up to IC₅₀, in the CTA, the range of concentrations can be wider (up to IC₉₀). Nevertheless, this top dose does not appear easily achievable as in the previous mentioned published articles, NPs showed weak cytotoxic effects with a decrease of viability, only up to 30%, except for Co (Sighinolfi *et al.*, 2014) and Ag (Choo *et al.*, 2017) which were demonstrated strongly cytotoxic, allowing a IC₉₀ determination. Regards to the guidance, if the IC₉₀ could not be reached, then, the highest concentration to be tested should be determined by agglomeration of NPs.

In the international validation study (Sakai *et al.*, 2011), five or more concentrations were selected based on cell growth assay results, respectively performed for the initiation or promotion protocol.

The chosen concentration range will not be the same depending on the protocol studied, initiation or promotion. Indeed, in the initiation protocol, the cells are treated for only 3 days with NP while in the promotion protocol, the cells are exposed for 7 days to NP, resulting in a greater cytotoxic effect.

4.3. CTA reproducibility between laboratories (negative and positive controls).

A trend is observed with experiments: when negative controls are weak, positive controls induce also a lower number of transformed foci and conversely.

In 2005, Ohmori *et al.* published the first inter-laboratory study on the Bhas 42 CTA for tumor promoters. They observed a dose-dependent increase of transformed foci in cells treated with the positive control TPA in the 12 laboratories, compared to the control cells. However, there were variations in the results among them; the response was highest in two laboratories with many foci also observed in negative controls. In contrast, in two other laboratories, few foci appeared in control cultures.

The first inter-laboratory study for the complete (initiation and promotion) assay in 2009 (Tanaka *et al.*, 2009) reveals again some variability in the inducibility of transformed foci either in negative controls or after treatment with the same concentrations of the positive controls (MCA and TPA). Incidentally, laboratories with no experience in CTA reading, give lower results with positive controls than laboratories performing CTA routinely, suggesting an impact of the reading skills and experience. In 2011, in another inter-laboratory study with 6 laboratories, this finding was again noticed. There were always significant differences between negative and positive controls in both the initiation and promotion assays, but the number of foci induced by each positive/negative controls varied between laboratories and even sometimes within laboratories (Sakai *et al.*, 2011).

To minimize variability between experiments, it is of first importance to control the cell maintenance conditions (Ohmori *et al.*, 2005), especially serum batch and cell culture period. For example, the use of the same cell passage is recommended to obtain reproducible results of CTAs (IARC/NCI/EPA Working Group, 1985, Heidelberger *et al.*, 1983).

In the OCED guidance dedicated to NP assessment, nanoform positive controls could also be used instead of standard chemicals (MCA and TPA). Considering the results published in the previous mentioned articles, TiO₂ and SiO₂ could be potential candidate as initiator and promoter positive controls, respectively.

4.4. Subjective nature of using morphological characteristics for assessing transformation

The transformed foci of Bhas 42 cells are judged based on their morphological characteristics. The foci must have more than 100 cells, with spindle-shaped cells different from the contact-inhibited monolayer cells, a deep basophilic staining, a random orientation of cells at the edge of foci, a dense multilayering of cells and an invasive growth into the monolayer of surrounding contact-inhibited cells. As all

transformed foci do not have all of these characteristics, a certain level of subjective judgments cannot be avoided (Sasaki, 2014).

Photo catalogue were published with different cell models (Sasaki *et al.*, 2012, Maire *et al.*, 2012, Bohnenberger *et al.*, 2012). It is a practical tool to help with the foci identification and cell colonies classification after the staining step. Several conditions are necessary for a reliable transposition of the test in a new laboratory: staff training, detailed protocol and use of the photo catalogue.

4.5. Metabolic status of cells used in CTA

The cell model used for the CTA possesses limited metabolic status, Cyp isoforms, (except Cyp1A1) are not expressed (Hayashi *et al.*, 2012) and various approaches have been employed to enhance their endogenous metabolic activity (Huberman *et al.*, 1966; DiPaolo *et al.*, 1971). Later, Schechtman and co-workers developed methods for supplementing endogenous metabolizing activity of Balb/c 3T3 A31 cells, with a rat liver S9 metabolizing enzymes (Schechtman, 1985a, Asada *et al.*, 2005).

Regards to NP assessment, most poorly soluble NMs are not metabolised and the metabolic activation system (S9) may interfere with the assay reducing the NM bioavailability. Organic NMs or some inorganic NMs coated with organic functional groups may however exert their genotoxic effects in the presence of the metabolic activation system (Sharifi *et al.*, 2012). To date, S9 has never been used with CTA, however, it could be evaluated case by case,

5. Future improvement of the CTA

5.1. Improvement of foci scoring

The major drawback of the CTA is the visual detection of tumor foci, which impact inter-reproducibility data as discussed previously. They are manually scored for morphological transformation under a stereomicroscope as described by the International Agency for Cancer Research (IARC) Working Group (IARC/NCI/EPA, 1985).

Alterations in adhesion protein profiles or enzyme activities modulating cellular adhesion and migration are involved in the process of focus formation. These may be promising candidates for the identification of molecular markers leading to a more objective scoring of foci and a greater acceptance of cell transformation tests in general. In 2007, Poth *et al.* proposed to measure the butyrylcholinesterase

(BChE) activity in the type III foci induced by chemical treatment, as a biomarker. Indeed, Bhas42 cells growing as a monolayer of non-transformed cells showed no increased activity of BChE. Morphologically transformed cells issued from positive foci gave a marked response in the activity of BChE.

Sasaki *et al.* (2013) found that transformed Bhas 42 cells but not the non-transformed parental cells can survive in the presence of H₂O₂. They determined that, after treatment with H₂O₂, transformed foci are selectively stained with dyes used for monitoring cell viability, such as WST-8 or resazurin. The stained foci could then be quantified by absorbance with a microplate reader.

Using this analytical technique, they demonstrated that sodium arsenite in the 96-well method could be judged positive as transformed foci on the sidewall are stained and became measurable. Wells with transformed foci showed high OD values, whereas OD values of wells without transformed foci showed a blank level. The total OD values and the number of wells containing transformed foci in each plate increased dose-dependently with MCA concentration. Then, the Bhas 42 transformation assay in 96-well plates and subsequent treatment with H₂O₂ followed by the referenced dye staining may be a novel approach to the scoring of transformed foci and, as such, may be readily applicable as a rapid and objective high throughput screening system for the detection of chemical carcinogens.

Since 2013, an Italian team (Urani *et al.*, 2013, Callegaro *et al.*, 2015, Callegaro *et al.*, 2017) works on another solution to improve the last step of the Balb/c 3T3 CTA, namely the visual scoring of foci. This reading is based on morphology of foci under a light microscope by trained experts, which implies a fair degree of subjectivity. They proposed to use digital image analysis to support the scoring of malignant foci and they develop statistical descriptors, by translation of morphological features (foci size, multilayering and invasive cell growth into the background monolayer, spindle-shape of transformed cells and texture heterogeneity of foci). The aim is to use these descriptors to mimic the classification performances of the visual scorer to discriminate between transformed and non-transformed foci.

5.2. Use of CTA in the battery for potential carcinogenic properties of NPs

CTA offers a good phenotypic anchoring, which links the cause (exposure) to the event (oncotransformation) (Corvi *et al.*, 2017; Mascolo *et al.*, 2018).

The toxicogenomic approach applied to this transformation assay is an interesting way, for the identification of the transcriptional pathways, significantly modulated as the consequence of the agent

exposure and which are strictly related to the formation of the transformed phenotype. Colacci (2016) developed an experimental protocol, which combined the Balb/c 3T3 CTA and a global gene expression analysis to highlight the cross-talk between genotoxic and non-genotoxic carcinogenic mechanisms in the pathway leading to malignant cell transformation. The toxicogenomics approach applied to the *in vitro* CTA allowed the identification of the transcriptionally activated pathways (Mascolo *et al.*, 2018). This integrated approach has the potential to be considered part of an Integrated Approach for Testing and Assessment for non-genotoxic carcinogenesis (Corvi *et al.*, 2017). Transcriptomics may highlight the key events along the pathway to the adverse outcome. CTA can be considered to provide endpoints related to morphological transformation, if NPs induce also oxidative stress we can wonder if it is linked to morphological transformation, if both the transformation and oxidative stress is observed, level of concentration may inform as a possible link or not (Jacobs *et al.*, 2016). Then, CTA used as part of a testing strategy and/or in a weight of evidence approach, could contribute to assessing carcinogenic potential not only of chemicals (Creton *et al.*, 2012) but also of NPs. CTA combined with toxicogenomic profiling will be a way to better highlight underlying mechanism with information on molecular mechanisms of NPs as already demonstrated for chemicals (Mascolo *et al.*, 2018). Indeed, some authors propose a path for implementation of innovative cancer risk assessment including genomic signatures for mechanistic relevance of carcinogenicity. Transcriptomic signatures have been demonstrated to be powerful prediction of toxicological changes that can be integrated in risk assessment (Yauk *et al.*, 2020). More recently it was reported in a first article on gene expression over time during the process of cell transformation with only a tumor-promoting compound. Pathways that were activated or inactivated during the process of cell transformation in the Bhas 42 cells treated with TPA were related not only directly to *RAS* but also to various pathways in the hallmarks of cancer (Ohmori *et al.*, 2022).

Conclusion

Nanotechnology is regarded as the technology of the future, but a better understanding of NP mode of actions for their safe use is needed. Alternative *in vitro* testing has to be considered with NPs, carcinogenesis being even more challenging for the risk assessment. In the respect of the 3R rules, cell transformation assay is recognized to be able to detect various types of carcinogens, including those that are thought to act *via* genotoxic and non-genotoxic mechanisms. To date, this bioassay has been performed with a dozen of NPs either with the protocol of initiation or promotion alone or both.

Concerning the promotion assay, SiO₂, LnO₂, Dy₂O₃, Fe₃O₄ and Au showed non-promotor/active properties

The accuracy of prediction of NP carcinogenicity would be improved by using the CTA in the battery of *in vitro* assays for a weight of evidence approach and in an Integrated Approach for Testing and Assessment, CTA could be one of the possible building blocks. . As discussed earlier a further guidance regards CTA would b need to be adapted for testing specifically NMs.

This assay with future developments especially coupled to genomic approaches, accompanied regarding to the specific NP properties a complete characterization, internalization and stability studies in the experimental conditions, is a promising tool, especially for NPs, which will probably never be tested in an *in vivo* carcinogenic study according to OECD guidelines.

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