

MEETING REPORT

Cell transformation assays for prediction of carcinogenic potential: state of the science and future research needs

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Cell transformation assays (CTAs) have long been proposed as *in vitro* methods for the identification of potential chemical carcinogens. Despite showing good correlation with rodent bioassay data, concerns over the subjective nature of using morphological criteria for identifying transformed cells and a lack of understanding of the mechanistic basis of the assays has limited their acceptance for regulatory purposes. However, recent drivers to find alternative carcinogenicity assessment methodologies, such as the Seventh Amendment to the EU Cosmetics Directive, have fuelled renewed interest in CTAs. Research is currently ongoing to improve the objectivity of the assays, reveal the underlying molecular changes leading to transformation and explore the use of novel cell types. The UK NC3Rs held an international workshop in November 2010 to review the current state of the art in this field and provide directions for future research. This paper outlines the key points highlighted at this meeting.

Introduction

Assessment of the potential for a compound to induce carcinogenicity is a key consideration in the safety evaluation of chemicals, agrochemicals, consumer products and pharma-

ceuticals. The standard approach to carcinogenicity testing in some of these industries is to conduct 2-year bioassays in rats and mice. These assays use large numbers of animals and are time consuming and expensive: testing in both species can involve 600–800 animals per chemical, involves the histopathological examination of more than 40 tissues and costs ~€ 1 million (1). Cancer bioassays are therefore of limited practicality for use in large-scale chemical testing programmes such as the EU regulation REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) (2), while the Seventh Amendment to the EU cosmetics directive will ban the bioassay for cosmetic ingredients from 2013 (3).

For all these reasons, there is a need for alternative methods for carcinogenicity testing that are faster, more cost efficient and have reduced reliance on animals. *In vitro* assays for detecting potential genotoxicity and/or mutagenicity are available and accepted as part of regulatory test strategies, but they have a significant irrelevant positive rate (4,5) and follow-up animal testing is used in order to confirm whether such effects occur *in vivo*; this was banned in 2009 for EU cosmetic ingredients. Importantly, these assays do not detect non-genotoxic carcinogens. The animal testing ban under the revised cosmetics directive therefore presents a significant challenge to the cosmetics and consumer products industry; many valuable new ingredients could be excluded from development on the basis of a false positive *in vitro* genotoxicity result.

Several *in vitro* cell transformation assays (CTAs) have been developed as quicker and more cost effective alternative methods for detection of carcinogenic potential. These assays measure induction of phenotypic alterations characteristic of tumourigenic cells, and cells transformed *in vitro* have been shown to induce tumours when injected into immunosuppressed experimental animals (6,7). CTAs mimic some key stages of *in vivo* multistep carcinogenesis and have been shown to have a good concordance with rodent bioassay results, detecting both genotoxic and non-genotoxic carcinogens (8).

CTAs are currently used by the chemical, agrochemical, cosmetic and pharmaceutical industries and academia for screening purposes and to investigate basic mechanisms of carcinogenicity, but they are not widely accepted for regulatory purposes due to a number of reservations. Historically, three main concerns have been raised: reproducibility of results between laboratories, the subjective nature of using morphological characteristics for assessing transformation and a lack of understanding of the molecular mechanisms underlying transformation.

Interest in CTAs has fluctuated over the years but the recent drivers for developing faster non-animal methods for assessing carcinogenicity has led to a resurgence. The performance of the various methods has recently been reviewed (1,8), and several

lines of new research seeking to improve the objectivity of the assays, explore the use of novel cell types and reveal the underlying mechanistic changes are ongoing.

In view of these recent developments, the UK NC3Rs held an international workshop, sponsored by the UK Environmental Mutagen Society (UKEMS), to review the state of the science of CTAs and inform the direction of future research in this area. This paper sets out and expands upon the key themes that were discussed at the meeting.

Background: established CTAs

Malignant transformation of Syrian hamster embryo (SHE) cells by chemical carcinogens was first reported in the 1960s (6,7,9), and efforts have been ongoing since this time to develop *in vitro* assays for detection of carcinogenic potential and assess mechanistic events associated with neoplasia.

It has been reported that at least four stages seem to be involved in cell transformation (8,10). The stages are (i) a block in cellular differentiation (detected as morphological transformation in the SHE assay); (ii) acquisition of immortality expressed by unlimited lifespan and aneuploid karyotype and genetic instability; (iii) acquisition of tumourigenicity associated with foci formation and anchorage-independent growth obtained in the BALB/c 3T3, C3H10T1/2 and Bhas 42 assay systems and (iv) full malignancy when cells are injected in a suitable host animal. The Syrian hamster dermal (SHD) mass culture system was used to demonstrate that induction of cellular immortality is an early 'gatekeeper' event essential for transformation by powerful chemical carcinogens (11) and also by active oncogenes (12). Furthermore, using the cloned human oncogene *EJ-Ha-ras*, the phenomenon of oncogene-induced senescence, which must be bypassed for immortalisation to occur, was first described in the SHD assay (12).

Two predominant forms of CTA have emerged over the years: assays using primary normal diploid cells of which the SHE assay is the most established example and assays employing immortalised aneuploid mouse cell lines, in particular the BALB/c 3T3, C3H10T1/2 and Bhas 42 systems.

SHE cells have some metabolic competence and have a low rate of spontaneous transformation. Exposure to carcinogenic substances results in an increase in the number of morphologically transformed (MT) colonies, characterised by disorganised growth patterns, compared with controls. Over the years, a number of modifications to the SHE assay protocol have been made to optimise performance and improve transferability, including the use of culture medium with a reduced pH (6.7 versus 7) which enhances the sensitivity of the assay (13–16).

The endpoint assessed in the mouse cell line-based assays is the progression from immortality to tumourigenicity, evidenced by formation of foci of multilayered, disorganised anchorage-independent cells that grow over the surrounding contact-inhibited monolayer. The BALB/c 3T3 assay has been more extensively assessed than the C3H10T1/2 assay, and as with the SHE assay, a number of adjustments have been made to the protocol to improve its performance. This includes the option to use exogenous S9 metabolic activation (17), an amplification ('level II') procedure (18) and use of a two-stage assay to detect tumour-promoting chemicals or enhance sensitivity to weak initiators (19).

More recently, a further CTA has been developed using Bhas 42 cells, which were established by transfection of BALB/c 3T3 cells with v-Ha-ras. The Bhas 42 assay can detect tumour initiators or promoters, depending on the protocol used (20).

Recent validation work

Although CTAs have been in use for several decades, like many toxicology tests with a long history of use, they have never been formally validated. Over the last 10 years, several initiatives have reviewed the performance characteristics of CTAs with a view to considering their suitability for development into formal test guidelines that could be used for regulatory purposes.

The Organisation for Economic Co-operation and Development (OECD) coordinated the preparation of a detailed review paper (DRP) to retrospectively assess the performance of the SHE (pH 6.7 and pH \geq 7.0), BALB/c 3T3 and C3H10T1/2 assays using data from previously conducted studies (8). The aim of this project was to determine whether any of the assays were ready for development into OECD Test Guidelines.

Performance characteristics of the various assays are summarised in Table I. The overall performances of the two SHE assay protocols were considered to be equivalent. Although the sensitivity and specificity for the BALB/c 3T3 assay were lower than those for the SHE assay, it was suggested that this was due in part to the use of different protocols in the various studies. A limited assessment of reproducibility was also conducted, which indicated that consistency between laboratories was 87.7% for the SHE assay, 68.4% for the BALB/c 3T3 assay and 54.3% for the C3H10T1/2 assay. In terms of prediction of human carcinogenicity, the assays detected 90% of International Agency for Research on Cancer (IARC) group 1 (known human carcinogen) compounds and 95% of those in groups 2A and 2B (probable human carcinogen and possible human carcinogen, respectively). The ability to correctly identify human non-carcinogens is not discussed in the OECD DRP, but a previous evaluation of SHE assay performance reported a high rate of false positives (21).

The data presented in the DRP were considered at an OECD Expert Consultation Meeting in 2006. Overall, it was concluded that the SHE and BALB/c 3T3 assays had a strong ability to detect rodent carcinogens, with a good positive and negative predictive capacity. The performances of these two

Table I. Performance characteristics of the SHE, BALB/c 3T3 and C3H10T1/2 assays calculated for OECD DRP (8)

	SHE pH 6.7	SHE pH 7.0	BALB/c 3T3	C3H10T1/2
Concordance ^a (%)	74	85	68	84
Sensitivity (%)	66	92	75	72
Specificity (%)	85	66	53	80
Positive predictivity	88	88	77	95
Negative predictivity	62	75	50	34
False-positive rate	15	34	47	20
False-negative rate	33	08	25	28
Number of chemicals	88	204	149	96
Number of carcinogens (%)	54 (61)	142 (74)	100 (68)	81 (84)

^aConcordance = agreement between rodent carcinogenicity and CTA results.

assays were considered adequate for recommending they could be developed into official OECD Test Guidelines, but the data were considered too limited to draw conclusions for the C3H/10T1/2 assay.

Before OECD Test Guidelines could be developed for the SHE and BALB/c 3T3 assays, it was recognised that there is a need for further data on the assays' reproducibility and for the development of standardised test protocols. The European Centre for the Validation of Alternative Methods (ECVAM) therefore initiated a formal pre-validation study, addressing intra-laboratory reproducibility, transferability of the methods, inter-laboratory reproducibility and development of a standardised protocol.

This study has recently been completed and the findings are currently being evaluated by the ECVAM Scientific Advisory Committee (ESAC). The conclusions of the Validation Management Team are that the SHE pH 6.7 and pH 7.0 assays are transferable, reproducible within and between laboratories and standardised protocols have been developed that should be the basis for future use of CTAs (1). For the BALB/c 3T3 assay, an improved protocol which provides reproducible results has been developed, although further assessment of this method is recommended. The full results of this work are due to be published in a special edition of *Mutation Research* and are expected to support the development of OECD test guidelines for the SHE assays.

Validation studies have also recently been conducted for the Bhas 42 assay using both the initiation and the promotion protocols; three inter-laboratory studies (one Japanese and two international studies) coordinated by the Japanese New Energy and Industrial Technology Development Organisation (NEDO) and the Japanese Centre for Validation of Alternative Methods (JacVAM) and a single laboratory study of the assay characteristics and performance using 98 chemicals. The results of the inter-laboratory studies have recently been published, and the data demonstrate that the Bhas 42 assay is reproducible between laboratories (22).

In the single laboratory study with the Bhas 42 assay, results with 52 rodent carcinogens and 37 non-carcinogens were used to calculate performance characteristics (23). Concordance was 78%, sensitivity 73% and specificity 84%, while the false-positive and false-negative rates were 16 and 27%, respectively. Of the 52 carcinogens, 29 were negative or discordant in Ames tests; 18 of these 29 gave positive results in the Bhas 42 CTA, mainly using the promotion protocol. The authors of this study therefore concluded that the number of carcinogens escaping detection by *in vitro* screening would be reduced if the Bhas 42 CTA were included in the battery of *in vitro* assays used to predict carcinogenic potential.

New research to improve the objectivity and mechanistic understanding of CTAs

As noted in the Introduction, a number of reservations have limited the widespread acceptability and use of CTAs: questions over reproducibility, the subjective nature of scoring transformed cells and the absence of mechanistic understanding. Further understanding of these issues can be found in articles published in response to previous evaluations of the performance of the SHE assay (14,21).

The UK's Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) reviewed a 2001 evaluation of the SHE assay conducted as

part of an ILSI-HESI Collaborative Evaluation Program on Alternative Models for Carcinogenicity Assessment (21,24) and concluded that the assay should not be used for regulatory screening of chemicals for potential carcinogenicity (25). The critical problem identified was over the validity of using morphological assessment alone to identify transformed foci: it was suggested that this could limit the ability to obtain reproducible results [although the recent OECD review (2007) established the reproducibility of the assay], and the need for an objective molecular marker was highlighted.

An additional concern raised by the COM and others (26) is that while the assays can give a good indication of rodent bioassay results, they are not able to discriminate between human carcinogens and human non-carcinogens.

Perhaps, the greatest concern, however, relates to the lack of knowledge on the mechanisms by which CTAs are operating (25,27). CTAs have not been developed based on a theoretical correlation between carcinogenesis and a particular mode of action such as mutagenicity. Questions have been raised as to how the CTAs are able to detect chemical carcinogens that operate by a wide variety of mechanisms of action, both genotoxic and non-genotoxic (e.g. immune suppression, hormonal disequilibrium) and that can demonstrate species, gender and/or tissue specificity (25,27). Previously, at least within the pharmaceutical industry, these concerns have resulted in the general consensus that CTAs are rarely useful in predicting the potential carcinogenicity of drug candidates (28). The concerns were emphasized in a small study with five compounds that demonstrated an absence of any correlation between *in vitro* genotoxicity, cell transformation and rodent carcinogenicity (29).

Several suggestions have been made as to how CTAs may detect chemical carcinogens that operate by a range of mechanisms of action. It has been proposed that the assays may be detecting a basic carcinogenic change common to different modes of carcinogenesis, while an alternative proposal is that the success of the SHE assay may be due to the use of primary cells containing a wide variety of cell types susceptible to a range of different transformation pathways (21).

Concerns over reproducibility of the assays have been dealt with by the ECVAM-coordinated work to develop and validate standardised protocols for the SHE and BALB/c 3T3 assays discussed above. Research to improve objectivity and mechanistic understanding is underway, and this was the major focus of the NC3Rs workshop.

Improving performance, objectivity and mechanistic understanding in the SHE assay

Recent collaborative research aiming to simplify the SHE assay protocol and improve objectivity of scoring transformed colonies has focused on performing the assay without a layer of feeder cells and the use of infrared spectroscopy.

Conditioned media protocol. The SHE assay has traditionally been conducted using a feeder layer of X-ray-irradiated cells to support growth of the target cells, which are seeded in low numbers. The presence of a feeder layer has an impact on the identification of MT colonies: scoring requires determination of whether cells are stacked on top of each other or just on the feeder layer and whether there are patterns of criss-crossing growth. The background of feeder cells also limits the ease of distinguishing normal colonies from transformed.

A revised method has been developed, where target cells are seeded without a feeder layer using conditioned media prepared from the medium used to grow the cells in stock culture. Studies using this method demonstrate that plating efficiencies of vehicle controls are within the historical range for the standard SHE assay and test results are comparable to those with the standard protocol (30,31).

In the experience of the researchers, elimination of the feeder layer makes scoring of transformed and non-transformed colonies easier. It also has the advantage of removing the need for an X-ray machine and associated costs, making the assay more readily available to all testing laboratories.

Infrared spectroscopy to derive a 'biochemical-cell fingerprint'. While the conditioned media method makes scoring of transformed colonies more straightforward, it does not eliminate the potential for subjectivity. Research is currently ongoing to explore whether infrared (IR) spectroscopy can be used to provide a more objective determination of transformed colonies—and also provide mechanistic information (32).

Biomolecules absorb light in the mid-IR region and consequently derived vibrational spectra provide information on the structure, content and functionality of cellular components including protein conformation, DNA, RNA, lipids and glycogen content. Characteristics of or alterations in these constituents are often indicative of cell type and/or pathology (33). Attenuated total reflection Fourier-transform IR

(ATR-FTIR) spectroscopy is one such approach that is technically robust and readily applicable to support biochemical characterisation and classification. Other technologies include FTIR microspectroscopy and Raman spectroscopy; the major consideration towards future development of this approach will be the generation of novel computational algorithms to handle large datasets (34).

In a recent study, SHE assays were conducted whereby cells were grown on IR-reflective glass slides and exposed to a particular test compound (seven in total were examined; Figure 1). Resultant colonies were subsequently analysed using both ATR-FTIR spectroscopy (conducted in a blinded fashion) and conventional visual scoring (35). The sensitivity of this approach (i.e. true positives rate) allowed a correct predictive classification of $\geq 88\%$ of test agent treatment and $\geq 74\%$ of transformed colonies; in comparison, the specificity (i.e. true negatives rate) correctly classified vehicle control in $\geq 85\%$ and non-transformed colonies in $\geq 75\%$ of cases. The study authors suggested that it was not surprising that there was a lower rate of classification of transformed and non-transformed colonies in this study as progression from non-transformed to transformed would be expected to be a gradual process, and it is possible that partially transformed colonies may be detected using ATR-FTIR spectroscopy but may not necessarily be visually identified.

Interrogation of the biochemical profiles showed that each chemical treatment appeared to be associated with a different

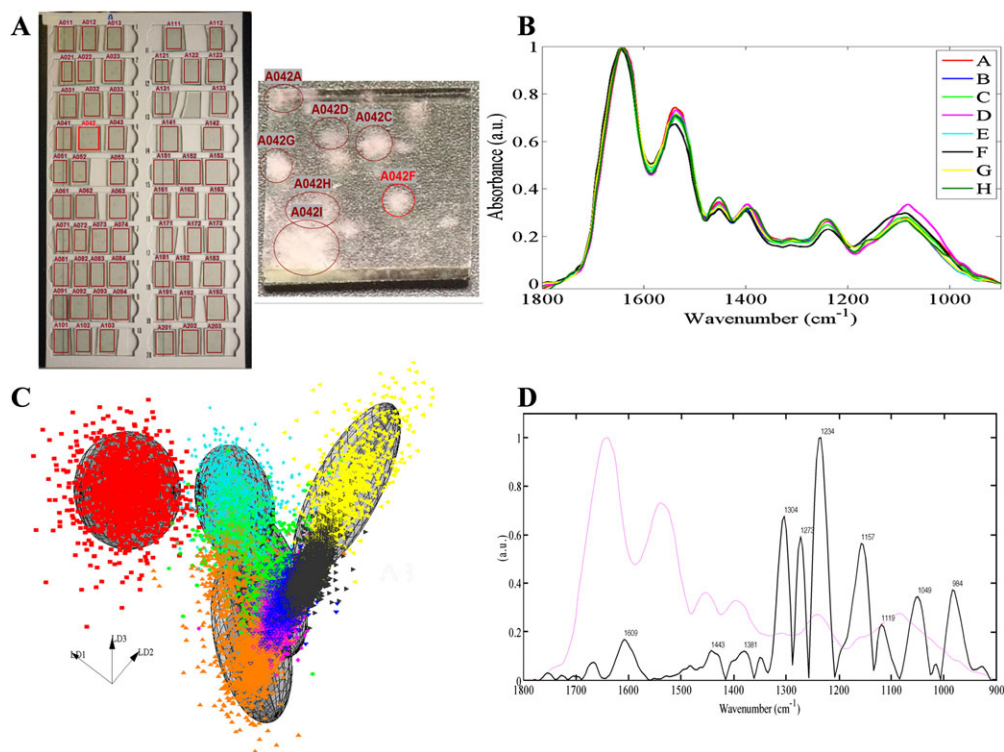


Fig. 1. Application of IR spectroscopy to objectively segregate SHE cells on the basis of test-compound treatment and mechanism. (A) SHE cells are grown so that they attach in culture to IR-reflective glass slides (1×1 inch) (34). Per test-compound treatment, a tray of such slides, is prepared to account for colony numbers and transformation rate associated with the assay. Post-treatment colonies are fixed on the slides and coded. (B) Average IR spectra (a profile of wavenumber-absorbance intensities giving rise to a 'biochemical-cell fingerprint') associated with eight particular treatments (A–H; one being a vehicle control and the seven others being test compounds). (C) Multiple IR spectra are derived from identified unstained SHE colonies; this gives rise to a complex dataset. To discriminate between different treatment groups, computational algorithms that allow for data reduction are required. In this case, linear discriminant analysis facilitates the reduction of each derived IR spectrum into a single point in a scores plot; points close to each other are similar, while increasing distance from each other implies dissimilarity. Each symbol colour represents a particular treatment category. (D) To identify a mechanistic basis for category segregation, a cluster vector identifies the wavenumbers responsible for segregating one particular category (i.e. test compound treatment group) from the vehicle control; importance of discriminating wavenumbers is proportional to the intensity of their individual weighting in the cluster vector plot (superimposed on an IR spectrum in this example).

spectral signature. For example, DNA/RNA alterations were predominant following benzo[*a*]pyrene (B[*a*]P) treatment while *N*-Nitroso-*N*-methylnitroguanidine (MNNG) was associated mostly with protein changes. Overall, transformed colonies were most clearly distinguished from non-transformed colonies by a high proportion of protein alterations, which the authors suggested could be linked to an increase in protein molecules signalling cell division in the transformed cells.

Gaining information on the biomolecular alterations associated with chemical treatment or morphological transformation of SHE colonies would be very valuable. Identifying biochemical effects indicative of treatment with specific chemicals or chemical classes could provide an indication of underlying mechanisms, while a more general identification of biomolecular signatures characteristic of genotoxic or non-genotoxic carcinogens would substantially enhance the utility of the SHE assay (35,36). These promising findings are being followed up with further larger scale research seeking to optimise classification of SHE cell colonies.

Improving objectivity in the Bhas 42 assay

Biomarkers associated with human cancer have been studied as a means of obtaining a more objective measure of cell transformation in the Bhas 42 assay. Three enzymes were selected for investigation: acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and alkaline phosphatase.

Several lines of evidence suggest that cholinesterases may be involved in tumour development: AChE and BChE are believed to play a role in cell proliferation and differentiation (37), cholinesterase genes have been found to be structurally altered or their products aberrantly expressed in tumours from a variety of tissues (38–40) and AChE has been shown to support anchorage-independent growth of tumour cells *in vitro* (41). Ectopic alkaline phosphatase expression has been found in tumours of the lung, testis, fallopian tube and gastrointestinal tract (42).

Further work is needed on a broader set of test compounds, but promising results have been obtained to date. Non-transformed Bhas 42 cells growing as a monolayer showed little or no expression or activity of the cholinesterases or alkaline phosphate, whereas expression was seen in transformed foci, with higher activity and intensity found in Type III foci (transformed foci characterised as dense multilayered and basophilic with random orientation at the focus edge and invasion into surrounding monolayer) compared with Type II (transformed foci with a more ordered and defined edge than Type III foci) (43). These biochemical markers could potentially also be applied to the BALB/c 3T3 CTA but are not suitable for the SHE assay as most tumour markers are also active in embryonic cells.

SHD cells: insights into mechanisms of transformation

Researchers at Brunel University have been using primary SHD cells as an alternative to SHE cells in studies investigating the mechanistic steps involved in cell transformation. SHD cells offer some advantages over SHE cells for such studies as they have a less mixed cell population than SHE cultures, it is possible to obtain large numbers of cells and they have a shorter lifespan in culture, reducing the time needed to perform transformation studies.

Malignant transformation of SHE and SHD cells occurs in a stepwise fashion. Immortalisation is an early and essential event in this process and involves bypass of oncogene/stress-

induced senescence (OSIS). OSIS refers to senescence induced by oncogenes or other cellular stressors such as DNA damage or culture conditions such as high oxygen or high concentrations of growth factors and is regarded as a primary tumour suppression barrier in rodent and human cells. Two critical tumour suppressor pathways are involved in establishing and maintaining OSIS in mammalian cells: the p16/pRb and ARF/p53 pathways (44,45).

The group at Brunel has been investigating the molecular changes underlying OSIS bypass in primary SHD cells following exposure to a range of genotoxic and non-genotoxic chemical carcinogens. This work has generated a near-complete molecular description of the mutational and epigenetic alterations leading to carcinogen-induced bypass of OSIS and immortalisation. These data indicate that different carcinogens show a distinct preference for inactivating different elements of the ARF/p53 and p16/pRb senescence effector pathways by mutational and/or epigenetic means, in some cases leaving a clear molecular fingerprint characteristic of the mechanism of action of the particular class of carcinogen.

Work is ongoing to further characterise the molecular events leading to OSIS bypass in SHD cells and to apply the analysis to transformed SHE cell colonies. The aim is to provide a mechanism-based validation of the SHE assay, so that OSIS bypass could be used as a mechanistically relevant endpoint for carcinogen screening.

Human CTAs

In addition to rodent cell transformation models, human CTAs may serve as an ideal system for identifying carcinogens that promote transformation in a more physiological context. However, the number of genetic events required to transform human cells is greater than in rodents. For example, the introduction of oncogene pairs, such as SV40 large T antigen (LT) and an oncogenic allele of *H-RAS*, is sufficient to render rodent cells transformed but has consistently failed to induce tumorigenic transformation in human cells. This discrepancy perhaps explains why a CTA based on human cells has not yet been established for routine use.

In addition to stress-induced senescence, human and rodent cells undergo replicative senescence, a restricted proliferative capacity in cells that results from shortening of telomere length (44,46). Telomeres are DNA–protein complexes that cap the ends of chromosomes, protecting them from degradation. Telomeres progressively shorten in length with each round of cell division due to incomplete replication of the chromosome ends and insufficient activity of the telomere-producing enzyme telomerase. This attrition leads to telomere dysfunction and genomic instability, which triggers a DNA damage response that promotes cell cycle arrest and replicative senescence (44,47,48). Rare cells may escape replicative senescence by gaining telomerase expression, which enables them to maintain telomere length and become immortalised, with further clonal evolution leading to the acquisition of genetic changes that result in malignancy (48).

Although human and rodent cells undergo replicative senescence, telomere length in cells from inbred rodent strains is significantly greater than that observed in human cells (44,46). In addition, telomerase expression is more robust in rodent cells (49). Many reports suggest that the differences in telomere biology between humans and rodents contribute to the threshold variation for rodent and human cell transformation (50,51) (Figure 2).

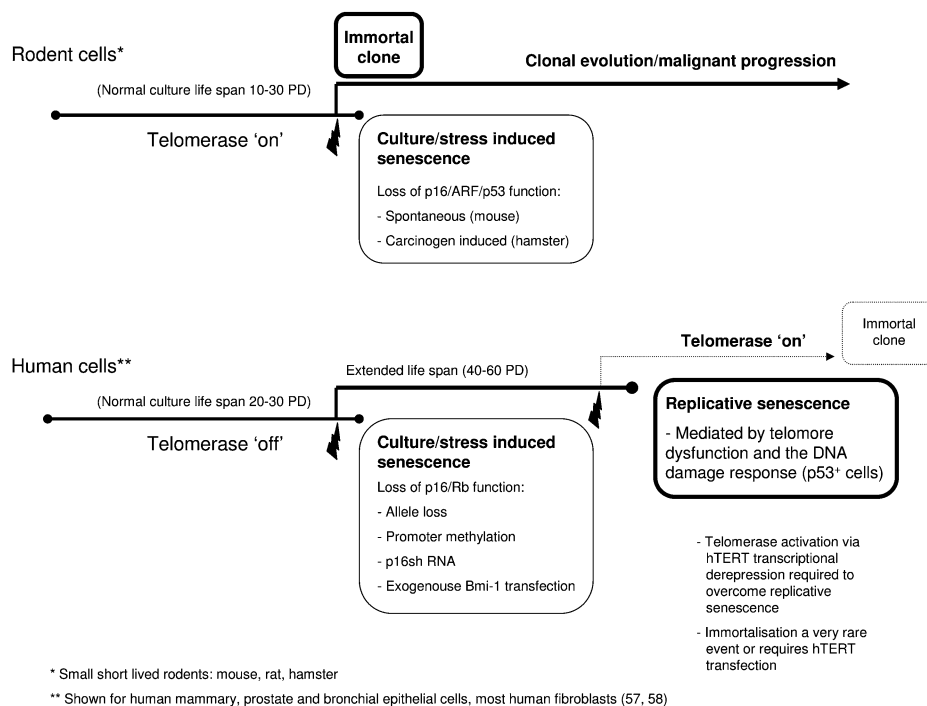


Fig. 2. Senescence barriers to immortalisation and malignant transformation in human and rodent cells. There are two major types of cellular senescence in mammalian cells: culture/stress-induced senescence (also known as OSIS) and replicative senescence. OSIS is induced by oncogenes or other cellular stressors, such as DNA damage or culture conditions, and is regarded as a primary tumour suppression barrier in rodent and human cells. Loss of p16/Rb function is required in order to overcome OSIS in human cells (52); loss of p16/Rb/ARF/p53 function overcomes OSIS in rodent cells (44,45). Replicative senescence results from shortening of telomere length, leading to telomere dysfunction and genomic instability and triggering a DNA damage response that promotes cell cycle arrest and replicative senescence. Telomere length is greater in rodent cells than human cells, and rodent cells also have more robust telomerase expression, whereas telomerase activation is required for immortalisation of human cells. It is suggested that these differences in telomerase biology contribute to the greater resistance to immortalisation and transformation in human cells compared with rodent cells: immortalisation of human cells requires bypass of two senescence barriers (53) whereas only one barrier must be overcome in rodent cells. PD, population doubling; shRNA, small hairpin RNA.

Human experimental models of transformation have been developed and used extensively to explore genetic and epigenetic events required for transformation and to screen for anti-cancer drug targets (47,54–56). These models are established in multiple tissue types, and the conversion of primary cells to tumour cells has been achieved in cells derived from the brain, breast, kidney, lung, ovary and prostate (51). Given the wide array of lineages used to model human cell transformation, these systems could potentially be employed in high-throughput screens as a means of identifying chemical carcinogens and their mechanisms in multiple tissue contexts.

A recent study examined the genetics underlying immortalisation and carcinogenesis to develop new human cell models for detection of genotoxic and non-genotoxic carcinogens (57). Human bronchial epithelial (HBE) cells were transduced with an oncogenic allele of H-Ras (HBER) or c-Myc (HBEM) to produce immortalised but untransformed cells (i.e. unable to induce anchorage-independent growth or form tumours in immunodeficient mice). Latency of cell transformation by benzo[*a*]pyrene diol epoxide (BPDE) and nickel sulphate (NiSO₄) was substantially reduced in the oncogene-transduced cells compared with carcinogen-treated control cells, with HBER cells having the shortest latency. Human embryonic kidney cells expressing SV40 LT and H-Ras (HEKR) also showed a similar or lower sensitivity to chemical carcinogens as HBER cells, depending on the carcinogen used.

A number of systems for metabolism of pro-carcinogens were also explored in this study, including transfection of cytochrome P450 enzymes, induction of P450s by pre-

treatment with the pro-carcinogen and addition of rat liver S9 fraction. In studies with aflatoxin B₁ and B[*a*]P, induction of P450 enzymes by prior chemical treatment had a similar effect on effectiveness of cell transformation as overexpression of specific P450 isoforms involved in bioactivation of these carcinogens. The finding that carcinogenic agents can facilitate tumorigenesis in these models suggests that human cell-based assays could be utilised more routinely in assessing the transformation potential of chemicals.

Current uses and future research needs

Despite their perceived shortcomings, CTAs are already being used by a range of chemical, agrochemical, consumer products and tobacco companies and contract research organisations. When employed in combination with other information such as genotoxicity data, structure–activity analysis and pharmacokinetic information, CTAs can facilitate a relatively comprehensive assessment of carcinogenic potential. Current uses of CTAs include:

- Clarifying *in vitro* genotoxicity results in a weight of evidence approach (58);
- Evaluating classes of chemicals that have a low predictive capacity in traditional *in vitro* genotoxicity assays (e.g. aromatic amines);
- Demonstrating differences or similarities across a chemical category;
- Screening for genotoxic and non-genotoxic carcinogens;

- Investigating tumour initiation/promotion activity;
- Studying mechanisms of action of specific carcinogens.

The successful outcome of the recent ECVAM pre-validation studies with the SHE, BALB/c 3T3 and Bhas 42 assays, and ongoing work to develop OECD Test Guidelines may be expected to promote further interest in the use of these assays, as will the regulatory and financial pressures to find alternative methods for carcinogenicity testing outlined in the introduction.

Clearly, the utility of CTAs will vary between sectors, and also on a case-by-case basis, but they can be considered as a useful tool to have in the carcinogenicity testing toolbox. At the present time, they may be of greater value in the evaluations where carcinogenicity testing is not practical (e.g. large-scale testing programmes such as REACH) or for compounds where carcinogenicity testing is typically not conducted or restricted (e.g. cosmetics). Areas where CTAs could be particularly valuable are in the identification of non-genotoxic carcinogens and the mechanisms by which they operate and providing mechanistic understanding to support the characterisation of genotoxic compounds as thresholded at low doses (59).

However, many scientists continue to have reservations about the use of CTAs and they are still not accepted under many regulatory testing frameworks. If widespread adoption and acceptance in international regulatory safety testing schemes is to be achieved, there remains a need for further research to increase the objectivity of scoring and mechanistic understanding. The recent progress presented at the workshop in developing objective measures of transformation in SHE cells and understanding of the molecular events involved in overcoming the OSIS barrier to transformation in hamster cells provides encouragement that CTAs can be enhanced to improve their predictive power. The work to identify biochemical markers of transformation in the BALB/c 3T3 and Bhas 42 assays is also encouraging and further research in this area would likewise be very valuable.

Research on the use of FTIR spectroscopy in the SHE assay and on mechanisms of OSIS bypass in SHE and SHD cells is ongoing. A number of additional areas for further work were suggested at the workshop, including evaluation of a wider set of chemicals with a range of known modes/mechanisms of action to better define which mechanisms the assays are sensitive to, and genomic analysis following treatment with known non-carcinogens and genotoxic and non-genotoxic carcinogens to gain an insight on mechanisms and markers of transformation. Two further key research needs were raised and are outlined below.

Characterising cells susceptible to morphological transformation

Recent work aimed at understanding CTAs has focused on the biochemical and genetic alterations associated with transformation. Another relatively underexplored area that would merit further investigation is characterisation of the cell population that undergoes morphological transformation in the SHE assay.

SHE cell isolates comprise a complex mixture of embryonic cells, and it has been shown that only a sub-population of cells is capable of undergoing morphological transformation (60). Studies in the 1990s demonstrated that this susceptible population consists of undifferentiated and committed progenitor stem cell-like cells of both mesenchymal and epithelial lineages and that transformation occurs via a block in the differentiation of these cells (10,60). Studies in animals and

humans into cells of origin in cancer (i.e. the normal cell that acquires the first cancer-promoting alteration) have suggested that the cell of origin may often correspond to the normal tissue stem cells or committed progenitor cells (61). Thus, it is intriguing to speculate that this may be further evidence of the *in vivo* relevance of the SHE assay. Research applying modern analytical techniques to better characterise the susceptible sub-population involved in SHE cell transformation and confirm the potential role of stem cells and progenitor cells was therefore advocated at the workshop.

Developing human cell-based carcinogenicity assays

While current CTAs show good concordance with rodent bioassay data, they have a low specificity for human carcinogens, resulting in an inability to distinguish between human carcinogens and human non-carcinogens. This limitation is also the case for rodent bioassays and should not preclude use of the CTAs, but the development of human-based assays would be desirable for improved prediction of human carcinogenic potential, in particular for the detection of human non-genotoxic carcinogens.

As discussed previously, replicative senescence is the primary barrier against immortalisation and malignant transformation in human cells, and the robustness of this barrier has prevented the development of human CTAs. Rodent CTAs provide a useful tool for exploring the events involved in bypass of OSIS that could contribute to malignant transformation, but they do not cover replicative senescence. Understanding of the genetic events required for overcoming replicative senescence is increasing, and this should support the development of human assays that can model telomerase activation and replicative senescence bypass. The recent work by Pang *et al.* (54) provides promising evidence that useful human-based cell transformation systems could be developed with sufficient research investment.

It is evident that a weight of evidence approach making use of all available data sources and taking into account modes of action of chemical carcinogenesis is important to support scientifically based decisions for assuring public safety. Development of a human mechanisms-based cell transformation system would be extremely valuable in contributing to such an approach and the NC3Rs plans to invest in further research in this area.

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