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Food for Thought ... on Cell Culture

Cell culture technologies form the basis of most alternative methods. They have matured over the last decades, but severe limitations remain, which – to say it positively – leave room for improvement. This second article of the Food for Thought series wants to raise awareness of current limitations that impact on the predictive value of test systems based on cell cultures.

The critical question is, how well the cell in culture reflects the cell in the organism, especially with regard to its state of differentiation and its response patterns in isolation in an artificial environment. Differentiation can be seen as the cellular equivalent of the phenotype at the level of an organism: Which genes are expressed at a given time point and which functionality do they allow? This critical appraisal of the problems of current cell culture techniques does not mean that *in vitro* systems are not useful, but stresses the need to critically evaluate the predictive value of these models in each and every case. This emphasises the need for validation, either in a formal sense for standardised tests or as a quality assurance process for the *in vitro* models in research and cell-based screening approaches.

Problem 1: From agony to atrophy – our cells are bored to death

It might well be that we make life too easy for our cells in culture. An organism works highly efficiently. Cell mass that is not required is removed. Everybody who has lost muscle mass after ceasing sport has experienced this. Atrophy is the reduction of mass when no continuous request for function is sensed. There is some evidence in many toxicity tests that something similar occurs in cell culture: A common observation is, for example, that cell vitality increases at the begin-

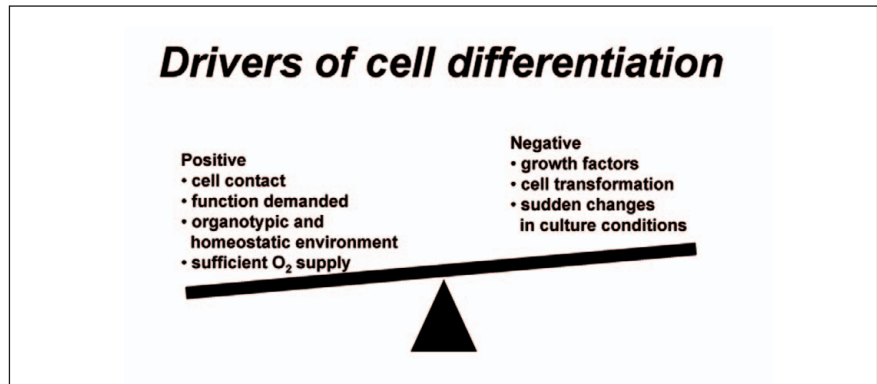


Fig. 2: Drivers of cell differentiation

ning of a concentration/response curve, i.e. a little bit of toxin keeps cell numbers higher than in untreated controls. Usually, this is perceived merely as a problem for curve fitting, e.g. to calculate IC₅₀ values. It might, however, in fact reflect the challenge to the system to either metabolise or defend to stop the agony, giving the cellular “life a sense”. What is true for cell viability might well translate to specific cell functions. Why should a cell maintain functions that are not requested? This might be much more a driving force for dedifferentiation than commonly acknowledged. We want our cells to perform optimally in the experiment, but we do not give them training before: why should they, for example, maintain metabolism of xenobiotics if they are ex-

posed to none? Figure 1 aims to illustrate that a common regulation of capabilities by demand of function might occur at the three levels of integration, strongly affecting cell culture both at the level of viability and functionality.

Problem 2: The cellular baby-boom – driving cells into growth and division results in less competent cells

Shorter doubling times mean less time and work before the next experiment. By adding growth factors or, even easier, the cell culture wonder cocktail foetal calf serum, we force cells into proliferation (Fig. 2). Human endothelial cells are supposed to have a half-life of 20 years *in vivo* – in culture it’s one day! How can we expect the cell to take the time and energy to express its specific functions when signals drive it toward multiplication not toward function? By this treatment we keep them in a baby state – able to grow, demanding in feeding but not really fully functional. In fact, not only do these pampered cells lack adult functions, they also often express genes typical for embryogenesis.

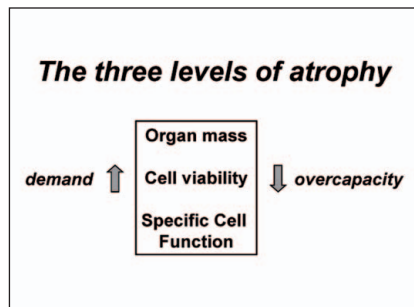


Fig. 1: The three levels of atrophy



**Problem 3:
A shock a day keeps
differentiation away – the
non-homeostatic
culture conditions**

The organism maintains a tightly controlled, stable environment for most cells: The composition of blood and extracellular fluids is intensively regulated. In contrast, cell cultures experience dramatic changes: At the time of culture medium change, nutrients are exhausted and waste has accumulated. From one moment to another, they are covered by fresh medium and often associated temperature and pH changes occur. The phenol red, a common additive to media, nicely shows the pH change: Old, yellow medium is replaced by fresh, red medium, demonstrating that the buffer capacity of the medium had long been exhausted. In human blood, pH varies only between 7.35 and 7.45 – more change leads to serious, even life-threatening conditions. Unavoidably, this shocking treatment favours cells that are able to adapt, not specialised cells maintaining sophisticated functions (Fig. 2).

**Problem 4:
Breathless cells – the lack of
oxygen**

Standard test systems supply only oxygen dissolved in the culture medium. At typical cell densities this is exhausted after a few hours. Further supply by equilibration with air in the incubator is low due to the limited surface area, the high water column of culture medium, which limits diffusion, and the lack of air stream. The result is anaerobic metabolism with accumulation of lactate – the main reason for acidification of the medium. Suffocating cells cannot be expected to show normal physiological reactions. Very few cell types normally burn glucose anaerobically, which means that this represents a completely abnormal condition. The limited oxygen supply is also a reason for the disproportion of cells *versus* culture media. Cells in culture achieve maximally 1% of the cell density in organs, because enough dissolved oxygen and nutrients need to be added. The need for nutrients is

also increased dramatically by their inefficient, anaerobic use – roughly 20 times less ATP is gained from glucose when it is broken down anaerobically compared to normal aerobic metabolism. Therefore, owing to the dilution in medium, cell contacts are limited and secreted substances, like signal molecules, are strongly diluted. There is another problem linked to this, which we often overlook: Not all effects of compounds depend on their concentration only, some also depend on absolute amounts relative to the cell number (especially when cells accumulate the substance). Thus, large quantities of substance added with the large amount of medium necessary, will overload the cells and mislead the interpretation of concentration/response curves.

**Problem 5:
The cancer origin of cell lines –
working on zombies**

The origin of cell lines is normally either a cancer tissue or primary cells that have undergone a similar form of transformation in culture (Fig. 3). Tumour cells have been shown to have undergone ten thousands of mutations compared to their progenitors (for review see Ponten; 2001, Frank and Nowak, 2004). They have drastically reduced the expression of organotypic function and favoured cell growth and division over other cell functions. They often show chromosomal aberrations and losses. Karyotyping, i.e.

the control of the chromosomal set-up of cell lines, regularly leads to big surprises. Few laboratories carry this out regularly and over many passages of cells. How can we expect the cells to resemble normal tissue when their origin is already far away from normal or when severe transformation and selection occurred? The idea that a tumour could be transplanted as a substitute for an organ is absurd. However, we let tumour cells substitute for primary cells in our experiments, often without questioning this approach.

**Problem 6:
Cell authenticity and cross-
contamination – cells in disguise**

This problem has been addressed earlier by us in the context of developing a Good Cell Culture Practice (Hartung et al., 2002; Coecke et al., 2005; Stacey and Hartung, 2007). However, it remains a grave concern and, following Aldous Huxley in his famous book, *Brave New World*, “Sixty-two thousand four hundred repetitions make one truth”. Thus, I will continue repeating the issue, which has been put forward by many others as well (more recently see Markovic and Markovic, 1998; MacLeod et al., 1999; Buehring et al., 2004; Nardone, 2007). There is compelling evidence that 15 to 20% of the cells used in experiments are not what they are supposed to be. A main reason for this is the HeLa miracle: In 1952, this cell line was created from a tu-

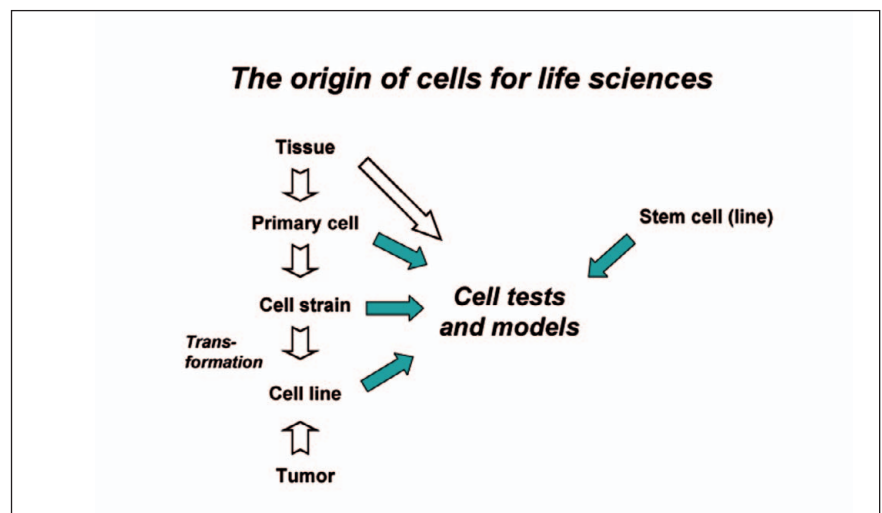


Fig. 4: The test-across concept



mour and has given rise to literally tons of cells since. I termed it a miracle, because still today the creation of human tumour cell lines is difficult and rare, and the growth rate of HeLa has not been met. Thus, cross-contaminating any cell line with a single HeLa cell will result after a while in overgrowth by HeLa, which has taken place unnoticed in many cases. Buehring et al. (2004) identified 220 scientific papers, where cell line results were reported with cells known today to be nothing but HeLa cells. In a survey involving more than 400 researchers, 9% were unknowingly using HeLa contaminants. MacLeod et al. (1999) analysed cells submitted to cell culture banks – a situation where researchers will be most certain about the authenticity of their cells – and found 18% of these cross-contaminated, often even with cells from other species. These cell lines had already been used for hundreds of papers and most probably continue to be used. Action is required as called for again recently by Nardone (2007).

Problem 7: DMSO & Co – what additives really add

Imagine a doctor telling you: “Take this pill in half a glass of dimethyl sulphoxide (DMSO) or a beer glass full of whisky.” (I prefer the latter.) That's how we offer most agents to our cells: 0.1% DMSO or ethanol. The problem is clear: We often deal with substances that are not soluble in water, and we cannot mimic the uptake and transport of the substance in the organism. The amount of solvent we have to use is often borderline toxic itself, with uncontrollable cocktail effects with the compound under study.

Another concern with regard to additives is the prophylactic use of antibiotics in cell culture and their effects on cells. The influence of antibiotics on cell morphology, cellular degeneration, cell death and cellular function was summarised by Kuhlmann (1993 and 1995). We often forget that these compounds, added at high concentrations (up to 100 µg/ml) and interfering, e.g. with protein biosynthesis also of mammalian cells, affect our test systems.

Last, but not least, the addition of serum: typically 10% and from a different species: a hardly standardisable, complex biological product and not even at relevant concentrations. To every pharmacologist, the binding of compounds to serum proteins and the fact that only the free portion in this equilibrium is available to act on its cellular target, is common knowledge. However, how do we interpret than 10% binding capacity? Astonishingly, higher serum conditions are not very well tolerated by most cells and especially human serum, even after complement inactivation, appears to be toxic to human cells. I am not aware of a sufficient explanation of this phenomenon.

Problem 8: Lack of activating metabolism – we miss the toxicants that need to be activated

Yes, we know it. We have heard this a thousand times, questioning the use of *in vitro* systems to substitute for animal tests. A few thoughts on it:

- Technical solutions to increase metabolic competence (viral transfection, stably transfected cell lines, maintenance of metabolic capacity of primary cells, cells from stem cells, etc.) are (slowly) increasing.
- The contribution of metabolism to the toxicity of general chemicals is not really known; it is likely that it is overestimated because of the predominance of drugs in the literature and a publication bias towards toxic compounds and complex mechanisms.
- Often the biological effect of the metabolite is stronger than that of the parent molecule, but does not represent a completely new quality; thus the hazard can be picked up also from the parent compound, though at misleadingly high concentrations.
- The main activating mechanism is the P450 system, i.e. oxidation; I am not aware of studies addressing the spontaneous oxidation taking place in our cell culture chambers, which might also give rise to some of the metabolites.
- The species differences in metabolism are well known; in most cases metabolism is protective (that's why it

represented a competitive evolutionary advantage). It is thus quite likely that we miss hazards by using animals, which do not show the activating metabolism of humans.

Still, metabolism is one of the key problems to work on for alternative methods (Coecke et al., 2006). However, let's do a small calculation of what its contribution might be based on prevalences (Hoffmann and Hartung, 2005): Most chemicals are not toxic. Percentages of toxic chemicals range between 2 (reproductive toxicity) and 20% (eye irritation). 10% prevalence of toxic effects should thus represent a fair estimate for a general calculation, i.e. 100 out of 1000 compounds need to be identified. Examples in which compounds become more toxic by metabolism are rare; examples of completely non-toxic compounds that have a new toxic property after metabolism are even rarer, together definitely less than 10% of the cases. Thus a test lacking metabolism would identify only 90% of the 10% toxic substances. In other words, a maximum of 10 out of 1,000 compounds are missed, i.e. <1%, and 99% are correctly classified. Whether this is an acceptable level and how this can be further reduced, e.g. by structure/function analysis, is beyond the scope of this article. Certainly, any toxic substance missed is a possible disaster, but whether any toxicological test scheme can produce certainties that are higher than 99% is worth discussion.

Problem 9: Lack of defence mechanisms – are our cells too sensitive?

Baby cells, heavily mutated by the loss of many genes and not trained to react to a toxic attack (see above) – this is a scenario of vulnerability. Indeed, we observe often that cell lines are much more sensitive than primary cells. We can now take either of two positions, i.e. praise this as precautionary or damn it as an overestimation of toxicity. I clearly favour the first view because of the most common purpose of such tests, i.e. hazard identification and classification and labelling. If the cell studied could protect itself against the toxicant successfully



within the organism but its descendant in culture cannot, this might indicate that the parent cell might be overwhelmed chronically or that there are other tissues and circumstances where the protective measures would fail. Better to know and flag an alert.

**Problem 10:
All cells are equal? – The
standardisation of culture
conditions**

Why do we culture murine cells at 37°C? Body temperatures of rodents are several degrees higher. Why do we force a pH of 7 while inflamed tissue can go down to a pH of 4? (The tight pH control mentioned above holds true for blood only.) Why do we use the osmotic strength of human blood in our cell culture media? Rodents have a higher salt concentration. It is all a matter of convenience and common practice. However, neither ensures the most physiological response or predictive test outcome.

**Problem 11:
Lack of quality assurance in cell
culture – “sorry, there was no
time to do a high-quality
experiment”?!**

We have elaborated on this before (Hartung et al., 2003; Coecke et al., 2005) in the context of developing the Good Cell Culture Practice guidance. To some extent all previous points discussed here give further reason for quality assurance. In systems that are highly artificial and reductionistic, not only the quality of procedures but also the predictive value of the results needs to be assured. At least in basic research this is not commonly done. When cell models “appear” to reflect our understanding, we are prepared to interpret new results. And if the results do not meet our expectations, we usually change the starting hypothesis and publish happily... This is definitely no solution for industrial R&D or regulatory testing, where economical and safety decisions need to be taken. Validation of test systems - to whatever extent - safeguards the quality of decisions. To some

extent basic research should also learn from this. It might be better to invest more upfront into the optimisation and evaluation of test systems than to progress towards generating results, which might represent artefacts.

**Problem 12:
The lack of standard protocols –
let many flowers bloom?**

We do not lack protocols – there are far too many – but they are far too brief and heterogenous. What we typically report in a scientific journal is not sufficient for another party to redo the cell culture experiment. The restriction in print space and writing effort is understandable from the side of the author and the editor, but their scientific customers need something different. Scientific publishing has not really fully embraced the opportunities of communication in the www-age. Internet depositories of detailed factual protocols are mushrooming, but links to them from scientific publications are rare. It would be desirable that reference was made to a publicly available standard protocol and only the deviations were detailed. For the restricted field of regulatory testing, ECVAM is aiming with its INVITTOX protocols at dbAlm (the ECVAM database for alternative methods, <http://ecvam-dbalm.jrc.cec.eu.int/>) to provide a repository of quality controlled protocols, but this concept deserves a broader application. It would be desirable to develop standardised formats and reference styles for the different providers in agreement with the journals in the life sciences.

Where is the upside?

In vitro research has become the dominant technique in the life sciences. Nobel prizes are given for *in vitro* research. Companies have changed their screening programmes for new agents strongly to *in vitro*, and increasingly also regulatory decisions are based on *in vitro* tests. However, the many advantages of the technology have a price. We must not neglect that these technologies have their shortcomings or we will be disappointed

later. The measures to be taken are the same as for any type of technology: (1) standardisation, (2) quality assurance and (3) validation. The reflections in this article have mainly addressed (1) and (2) by illustrating the principal shortcomings of *in vitro* approaches.

Among the proposals to be made are:

- We need technological developments to overcome the current limitations, e.g. to provide homeostatic culture conditions, oxygen supply and to control for differentiation.
- More attention should be given to species-specific differences, not only with regard to culture conditions: We substitute rodent cells for human ones too easily. The MEIC study (Ekwall, 1983) for acute toxicity has already hinted that human lethal blood values of toxicants are better predicted by human cell systems. The recent validation of a myelotoxicity assay (CFU-GM) demonstrated very clearly that *in vivo* toxicity in mouse and man of different chemotherapeutic agents is reflected by the sensitivity of cells *in vitro*: This was possible by using both human and murine cells to establish a relative sensitivity, which allowed converting mouse LD₅₀ values into human maximal tolerated doses in clinical trials (Pessina et al., 2003). Thus, species differences in acute *in vivo* toxicity were reflected *in vitro*.
- Stem cells need to be exploited to produce better (human) cells, not only for developmental toxicity (Bremer and Hartung, 2004).
- The fate of test substances in cell culture needs to be studied more intensively; we have coined the term “*in vitro* biokinetics” to describe that a chemical, similar to its distribution and metabolism *in vivo*, has a fate in the cell culture dish, which determines the active concentrations acting on a cell over time; I strongly believe that an increasing understanding of these phenomena will help to interpret the results obtained *in vitro* in a more predictive manner. A report on a recent ECVAM workshop on this topic is currently being prepared.
- A very interesting approach called “read-across” is taken in the regulatory field. Here, results of sufficiently simi-

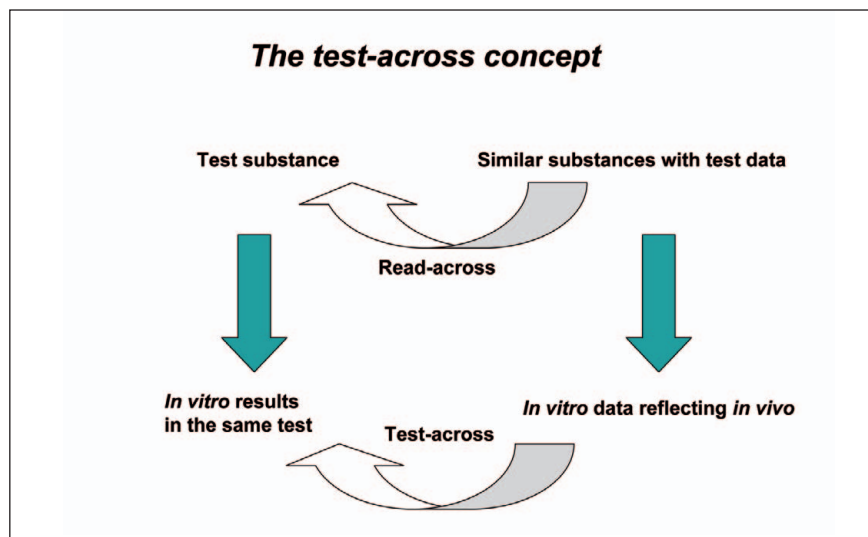


Fig. 3: The origin of cells for life sciences

lar chemicals, for which animal test data are available, are used to intrapolate for a non-tested substance. In a similar manner, it should be possible to minimize an *in vitro* test for a given substance, i.e. by showing that related compounds are judged correctly, the result for a substance where there are no *in vivo* data that can be relied on; I would suggest to call this “test-across” (Fig. 4); this clearly represents an advantage over mere structure/relationships, since in addition actual testing in a living system is carried out. This might at the same time represent a solution in cases where no formal validation for the respective part of the chemical universe has been done (applicability domain) or where a full validation is not (yet) possible.

In conclusion, these reflections encourage considering the shortcomings of cell culture systems: This should prompt more intense quality assurance, also in the context of an evidence-based toxicology (Hoffmann and Hartung 2006). We can learn from the role model of formal validation studies for regulatory testing. While these standards certainly exceed what can be typically done as quality control in other areas of the life sciences, they represent the gold standard that would be desirable.

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