Session 7.1
Stem cell technology in toxicity testing

A Perspective on Stem Cells as a Tool for In Vitro Testing

Aernout Luttun* and Catherine M. Verfaillie
Department of Medicine, Stem Cell Institute, University of Minnesota, Minneapolis, USA

Summary
Stem cells are on their way to becoming a central research tool in this and the next decades. Their differentiation potential promises replacement of lost tissue or provision of functional cells in degenerative diseases. Many of these treatment paradigms have been tested in animals. While stem cells have quickly moved to the frontline of research because they could revolutionise medical practice, they also could be very useful for purposes that do not require animal testing. Here we give an overview on how stem cells can be exploited as in vitro alternative to animals in addressing diverse biological questions.

Keywords: stem cells, differentiation, in vitro testing

Introduction
Like recombinant DNA in the 1980’s and transgenic animals in the 1990’s, stem cell technology is well on its way to becoming a central research tool in this and the next decades. Stem cells are generally derived from two main sources, embryos or adults, and can be further subcategorised according to their differentiation potential. The latter potential offers great promise for in vivo replacement of otherwise irreversible tissue loss seen in many degenerative diseases like myocardial infarction and stroke, or for provision of functional cells in many other disorders where a certain cell type has lost its function, like diabetes and Parkinson’s disease. Many of these treatment paradigms have been successfully tested in animal disease models. While stem cells have quickly moved to the frontline of research, mainly because they could revolutionise medical practice, they also could be very useful for other purposes that do not require the use of animals. Here we give an overview on how stem cells in general, and multipotent adult progenitor cells (MAPCs) in particular, can be exploited as in vitro alternative to animals in addressing diverse biological questions.

What are stem cells and why could they be a good alternative to animals?
A stem cell is defined by three main criteria: (i) self-renewal, (ii) the ability to differentiate into multiple cells in vitro and (iii) the ability to reconstitute a given tissue in vivo. Their self-renewal capacity and therefore unlimited availability without the need to sacrifice additional animals for derivation, makes stem cells a particularly useful alternative research tool. The second criterion can be used to divide stem cells into different categories depending on the extent of their differentiation repertoire. On top of the hierarchy is the “totipotent” fertilised egg that not only gives rise to all tissues from the three germ layers within the embryo (ectoderm, mesoderm and endoderm), but also to the supporting trophoblast required for the survival of the developing embryo. Embryonic stem (ES) cells and embryonic germ (EG) cells, isolated from the inner cell mass of the blastocyst or from primordial germ cells of an early embryo, give rise to mesoderm, endoderm, ectoderm and germ cells but not to extra-embryonic tissues, and are therefore termed “pluripotent”. Stem cells, isolated from various adult organs, that self-renew and dif-

* Aernout Luttun is a post-doctoral fellow sponsored by the American Heart Association
differentiate into multiple organ-specific cell types are termed “multipotent stem cells”. Committed cells that have limited or no self-renewal ability and differentiate into only one defined cell type are termed “progenitor cells” or “precursor cells”. The broader the differentiation potential, the more applications can be considered for a certain type of stem cell.

**Pluripotent embryonic or multipotent adult stem cells?**

While ES cells are considered pluripotent and therefore offer many potential applications, the differentiation repertoire of adult stem cells is limited and was long believed to be restricted to cell types of the tissue of origin. On the other hand, unlike stem cells from adult sources, the use of ES cells of human origin has met with significant ethical concerns. Over the last 3 years, however, several research groups have challenged the concept of restricted differentiation potential of adult stem cells and have independently identified adult stem cell populations able to differentiate into cells of all three germ layers, including osteoblasts, chondrocytes, adipocytes, neurons, and pancreatic islet-like cells (D’Ippolito et al., 2004). Kogler et al. also found a multipotent stem cell population in human cord blood, called “unrestricted somatic stem cell” or USSC, contributing to cell types from the three germ layers in vitro and in vivo.

<table>
<thead>
<tr>
<th>Name</th>
<th>origin</th>
<th>year</th>
<th>OCT4 expression</th>
<th>FACS phenotype</th>
<th>Differentiation</th>
<th>mesoderm</th>
<th>ectoderm</th>
<th>endoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKP</td>
<td>rodent skin, human scalp</td>
<td>2002</td>
<td>yes</td>
<td>Mouse: CD13^SSEA1^Fk1^low^CD90^low^Sca1^low^CD19^CD3^CD34^CD45^CD44^CD117^Gr1^-Mac1^-MHCclass I^-MHCclass II^-</td>
<td>yes</td>
<td>yes</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2002</td>
<td>yes</td>
<td>Mouse: CD13^SSEA1^Fk1^low^CD90^low^Sca1^low^CD19^CD3^CD34^CD45^CD44^CD117^Gr1^-Mac1^-MHCclass I^-MHCclass II^-</td>
<td>yes</td>
<td>yes</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MIAMI</td>
<td>human BM</td>
<td>2004</td>
<td>yes</td>
<td>CD29^CD49^-CD63^CD81^-CD122^+CD164^-CD36^-CD54^-CD56^-CD117^-CD45^-CD34^-HLA-DR^-</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>BMSC</td>
<td>human BM</td>
<td>2005</td>
<td>no</td>
<td>KDR^-CD90^-CD105^-CD117^-CD29^-CD44^-CD73^-CD45^-CD49^CD50^-CD34^-CD133^-HLA-ABC^-HLA-DR^-</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td></td>
</tr>
</tbody>
</table>

SKP: skin-derived precursors; MAPC: multipotent adult progenitor cells; MIAMI: marrow-isolated adult multilineage inducible; USSC: unrestricted somatic stem cells; BMSC: BM-derived multipotent stem cells; BM: bone marrow; CK: cytokeratin; glyA: glycoporphin A; ND: not documented.
vivo (Kogler et al., 2004). Finally, last year, Losordo’s group in Boston isolated multipotent cells from human bone marrow with a differentiation potential similar to MAPCs, but with different surface expression of several markers and lacking expression of the pluripotency marker Oct4 (Yoon et al., 2005). The broad differentiation potential of all these adult stem cell types along with their availability without ethical restrictions offers numerous possible applications in vitro and in vivo. Unlike ES or EG cells, none of these cell types have so far been documented to contribute to the germline.

How can (stem) cells be used as alternatives to animals?

Many in vitro techniques have been developed using different resources, including microorganisms, plants, invertebrates (i.e. Hydra attenuata), and embryos, tissues or cells from vertebrate animals and humans (Pearson, 1986). The best-known application of cells as an alternative to animal usage is the toxicity testing of drugs and chemicals. While numerous somatic cell lines (i.e. human keratinocytes for phototoxicity, lung cells for inhalation toxicity, etc.) have proven useful to test the toxicity or sensitivity to certain compounds, ES cells from animals are of particular interest in developmental toxicity testing. The embryonic stem cell test (EST) takes advantage of the potential of murine embryonic stem (ES) cells to differentiate in culture to test embryo toxicity in vitro and to screen for teratogenicity and growth retardation. The EST represents an in vitro system for the classification of compounds according to their teratogenic potential based on the morphological analysis of beating cardiomyocytes in embryoid body (EB) outgrowths compared to cytotoxic effects on undifferentiated murine ES cells and differentiated 3T3 fibroblasts. The test has recently been refined by defining more objective endpoints of differentiation other than the microscopic evaluation of “beating areas”, such as assessment by fluorescence activated cell sorting (FACS) and quantitative reverse transcription polymerase chain reaction (qRT-PCR), which evaluate expression of genes at the translational and transcriptional level, respectively (Seiler et al., 2004).

In addition, in order to address the potential concern of interspecies variations in developmental toxicity, some considerations have been made to adapt the mouse ES cell based tests to human ES cells (Pellizzer et al., 2005).

While embryonic stem cells have been very useful for in vitro developmental toxicity testing, in vitro adult stem cell research in general and testing with haematopoietic stem cells in particular, has been of tremendous importance for the unravelling of proliferation and differentiation mechanisms and for the discovery of several haematopoietic cytokines, which are now used worldwide for the treatment of (cancer) patients undergoing chemotherapy and radiation therapy. Two examples are the recombinant proteins erythropoietin and filgrastim (Neupogen).

Another major domain of cell-based in vitro testing is the biotransformation of drug compounds. Primary hepatocyte cell lines have frequently been used for that purpose. Gastric epithelial cells (i.e. Caco-2 cells) and co-cultures of brain microvessel endothelial cells with glial cells have been used for bioavailability and drug transport studies. Neurons derived from the embryonic rat spinal cord and dorsal root ganglia have been used as a supplement to in vivo testing of analgesic drugs. While the creation of knock-out animals has advanced the search for disease mechanisms tremendously, in vitro alternatives have now been developed, such as antisense nucleic acids or oligonucleotides and RNA interference to knock-down gene expression in specific cell targets in vitro. For a more extensive database of possible applications of cells as an alternative, the reader is referred to the following website: http://gripsdb.dimdi.de.

One major disadvantage of working with primary cell cultures is their limited proliferation ability, which necessitates repeated isolation and sacrifice of additional animals for their derivation. One possible solution to this problem is to immortalise the cells, which may however change their characteristics and function significantly. As mentioned above, stem cells are able to self-renew indefinitely, and therefore can be expanded without limitations. For many of the cell types mentioned above, adequate differentiation protocols have been established starting from stem cells, both embryonic and adult stem cells. Thus, stem cells could serve as a continuous source of such specialised cell types. When establishing a differentiation protocol, it is very important to document functionality of the resulting cell type. Functional cell differentiation has been elaborately documented for MAPC-derived cells such as endothelial cells (Reyes et al., 2002), hepatocytes (Schwartz et al., 2002), neurons (Jiang et al., 2003) and smooth muscle cells (unpublished results). Although in vivo transplantation into animal models is the most robust method to ultimately prove functionality, there are adequate in vitro tests available to document function. Endothelial function, for instance, was shown by uptake of acetylated LDL, by release of von Willebrand factor (vWF) upon histamine stimulation and by the formation of vascular tubes in a two-dimensional matrix (matrigel) system. Liver cell function was demonstrated by production of albumin and urea, by cytochrome P450 induction, glycogen storage and uptake of LDL. Neurons differentiated from MAPCs were shown to be functional by their electrophysiological properties similar to midbrain-derived neurons. Finally, (vascular) smooth muscle cell function is tested by responsiveness to smooth muscle cell agonists and by production of extracellular matrix components (fibrillar collagen and elastin). For other cell types, such as cardiomyocytes, differentiation has been suggested by the presence of MAPC-derived cells in the heart following blastocyst injection, but the conditions for in vitro (functional) differentiation require further optimisation.

Functional cardiomyocyte differentiation has been documented in ES cells (human and rodent), where function is assessed by electrophysiology (Boheler et al., 2004; Kogler et al., 2004; Lakshmipathy et al., 2004). A limitation to differentiation from ES cells is that in most cases differentiation requires the formation of three-dimensional structures (embryoid bodies or EBs), which often produce a mix of differentiated cell types instead of a pure culture of one differentiated cell type. Genetic manipulation protocols, using cell-specific promoters that drive a fluorescent colour, have been developed to generate purified cell populations by FACS sorting.
Examples of in vitro applications using MAPCs

In the following paragraph, we demonstrate how MAPCs or cells derived from MAPCs can be used for several in vitro applications. We also show how two different disciplines of science, stem cell biology and bioengineering, can join forces to find answers to biological questions.

Example 1: Creating artificial vascular grafts for bypass surgery
Myocardial infarction is one of the leading causes of morbidity and mortality worldwide. Its underlying cause is atherosclerotic degeneration of arteries that normally supply the heart muscle with oxygenated blood. As a result of inadequate arterial blood supply, part of the heart muscle dies and is replaced by dysfunctional scar tissue. The area around the dead tissue, however, can be potentially prevented from dying by restoring blood supply to the heart. Stem cells have now been tested for their ability to induce the formation of new blood vessels (arteries) and to replace the dead cardiac muscle by new cardiomyocytes, both in animal models and in patients. Although the initial results show promise, it is unlikely that stem cells alone will be sufficient to treat every single patient. Rather, stem cell transplantation could be a useful addition to and be combined with already existing approaches such as bypass surgery. The latter technique restores blood flow to the oxygen-deprived heart muscle by replacing one or more segments of the atherosclerotic arteries by vascular grafts, usually taken from the veins of the patient. Stem cells could be useful to create artificial vessel grafts. The latter would require differentiation of the stem cells into endothelial cells and vascular smooth muscle cells and their correct positioning following differentiation (i.e., endothelial cells lining the inside of the graft and smooth muscle cells surrounding the endothelial layer). Importantly, since these grafts need to be implanted into arteries, it would be appropriate to generate endothelial cells that have characteristics and functions of arterial endothelial cells. Indeed, it has recently been documented that endothelial cells from arteries and those from veins express different sets of genes, some of which may translate into different functions (Chi et al., 2003; Torres-Vazquez et al., 2003). Therefore, one of the current challenges in vascular repair and regeneration is to develop protocols that generate arterial endothelial cells. We have recently shown the ability of human MAPCs to be induced towards an arterial endothelial phenotype (unpublished results in collaboration with F. Prosper, University of Navarra, Spain). We also successfully generated artificial grafts in which human MAPC derived ECs lined the inside of a vascular graft made up of rat aortic smooth muscle cells (unpublished results in collaboration with B. Tranquillo, University of Minnesota, Minneapolis). Finally, appropriate communication between smooth muscle cells and endothelial cells may be required to generate vascular grafts as they may co-influence their proper differentiation. Indeed, it has been shown that co-transplantation of endothelial and smooth muscle cells results in the formation of much more stable, functional and durable vessels in vivo than transplantation of endothelial cells alone (Koike et al., 2004). We recently developed a three-dimensional matrix system in which both cell types can be co-cultured to study their interaction (unpublished results).

Example 2: Three-dimensional tissue engineering
For a system to be as adequate as animals to study certain biological phenomena, it should mimic the in vivo microenvironment as closely as possible. One of the goals of bioengineering is to reconstruct tissue according to its native architecture. All tissues in a living higher organism are dependent on blood flow for their proper oxygenation and nutrition. Moreover, the presence of blood vessels and vascular cell-derived molecules has been shown to be essential for tissue development and differentiation, as shown for the pancreas and the liver (Cleaver and Melton, 2003; LeCouter et al., 2003). Creating three-dimensional artificial liver constructs in vitro could be a valuable alternative to animals for drug toxicity testing and for biotransformation studies. One of the structural elements of the liver is the sinusoid, composed of a fenestrated vascular structure decorated with hepatocytes. In the adult liver, sinusoids are aligned radially, running from the lobule periphery to the central vein. This sinusoidal organisation is important for the function of the liver. Recently, the group of D. Odde (University of Minnesota, Minneapolis) developed a technique called “laser-guided direct writing” (LGDW). Using this method, they were able to pattern multiple cell types with micrometer precision onto biological matrices (Nahmias et al., 2004; Odde and Renn, 2000). In a first approach, they could lay down MAPCs into a matrix in a three-dimensional pattern without significant negative effect on cell viability (Nahmias et al., 2004). Subsequently, in an effort to recreate the liver tissue architecture in vitro, they patterned human umbilical cord endothelial cells (HUVECs) in several linear formations. Like for MAPCs, this did not compromise the viability of the endothelial cells but instead stimulated tube formation. Co-culture of these vascular tubes with hepatocytes resulted in an aggregated tubular structure, similar in organisation to a hepatic sinusoid (Nahmias et al., 2005). As the development of liver sinusoids takes several weeks in vivo, these endothelial-hepatic aggregates formed after a few days in culture are likely not fully developed sinusoids. Nevertheless, these results suggest that LGDW can be used to create a vascular backbone for ex vivo liver morphogenesis. Alternatively, LGDW could be used to lay down beads coated with different growth factors onto stem cells in order to induce their differentiation in a specific spatial pattern.

Conclusion
Many academic institutions have become aware of the excessive use of animals in their research programmes and have therefore started to implement policies to reduce the number of animals, to refine methodologies to diminish pain and suffering of animals and to encourage the development of alternative research tools that can answer biological questions equally well. Stem cell technology is one of these alternatives. The unlimited availability of stem cells from different species and our increasing knowledge on how to generate functional cell types from them
opens many possibilities for the design of stem cell-based *in vitro* test protocols, some of which are documented in this short overview.

**References**


**Correspondence to**

Catherine M. Verfaillie

Department of Medicine, Stem Cell Institute

University of Minnesota

2001 SE 6th Street

MN55455 Minneapolis, USA

e-mail: aernout_luttun@yahoo.com
Current Status of the Embryonic Stem Cell Test:
The Use of Recent Advances in the Field of Stem Cell
Technology and Gene Expression Analysis

Andrea Seiler, Roland Buesen, Katrin Hayess, Katharina Schlechter, Anke Visan, Elke Genschow, Birgitta Slawik and Horst Spielmann

Federal Institute for Risk Assessment (BfR), National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

Summary
All guidelines that are currently used for regulatory developmental toxicity testing of chemicals and drugs are based on animal experiments. The most promising alternative is based on embryonic stem cells of the mouse (mESC). Their ability to differentiate into numerous cell types have made ES cells a popular system to study gene function and developmental processes during differentiation in vitro. The embryonic stem cell test (EST) makes use of this capacity to detect developmental toxicants during differentiation of stem cells into cardiomyocytes.

In the present study our investigations were aimed at the further development of the validated EST protocol. We present improvements that focus primarily on (i) the quantitative assessment of drug effects at the cellular level, using a novel approach in which the expression of tissue-specific marker proteins under the influence of the test chemical is quantified by intracellular flow cytometry in ES cells, (ii) the development of protocols for ES cell differentiation into various cell types other than cardiomyocytes, e.g. neural cells, and (iii) the standardisation and optimisation of ES cell culture and differentiation conditions in chemically defined serum-free medium. An important strength of the molecular approach in combination with serum-free culture conditions is that in this way the ability of the test to monitor the cellular response to toxins could be expanded to proteins of many signal transduction pathways in a highly standardised form. Furthermore, these improvements now allow testing of substances known to interact with serum proteins.

Keywords: in vitro, embryotoxicity, embryonic stem cell test, differentiation, cytotoxicity, molecular endpoints, intracellular flow cytometry, developmental neurotoxicity testing, serum replacement

Introduction
Assessing the toxicity of chemicals for development and the reproductive cycle according to standardised OECD Test Guidelines requires extensive screening and multi-generation studies (OECD, 1983, 1996, 2001a, 2001b, 2001c). For chemicals used as drugs, “segment studies” covering three important phases of pre- and postnatal development including fertility have to be conducted (ICH, 1993). These in vivo test methods are time-consuming, expensive and have to be carried out on large numbers of laboratory animals. For example, a developmental toxicity study (OECD TG 414) requires 150 animals and a two-generation study (OECD TG 416) more than 3,000 animals, including pups.

According to the currently ongoing implementation of the new chemicals policy of the European Union (REACH, Registration, Evaluation, Authorisation of Chemicals) the toxicity of approximately 30,000 existing chemicals produced or marketed at more than one tonne per year has to be evaluated in the European Union within a period of 12-15 years. The procedures for assessing reproductive toxicity will have the strongest impact on the total number of animals used for testing under REACH (Höfer et al., 2004; see also Anon., 2005). Furthermore it has been estimated that the testing costs for the developmental toxicity study and for the two generation study will represent up to 57% of the total testing costs of REACH (van der Jagt et al., 2004; see also Eskes and Zuang, 2005). In addition, the 7th Amendment of the Cosmetic Directive demands fixed deadlines for phasing out animal experiments for safety toxicity testing including reproductive toxicity (Anon., 2003). Thus, predictive screens for the evaluation of reproductive toxicity need to be made available with the ultimate goal of reducing animal use and testing more chemicals than can be accommodated by conventional whole animal testing.

To obtain information on the toxic effects of chemicals and drugs on specific elements of the reproductive cycle, reproductive toxicity testing is either performed in vivo using pregnant animals or in vitro on cultured embryos or embryonic cells and tissues from pregnant animals. Both for in vivo and for in vitro testing pregnant animals have to be sacrificed to obtain embryonic cells, tissues or organs. Taking advantage of the potential of ESC to differentiate in culture into a variety of cell types (reviewed in Kirschstein and Skirboll, 2001; Smith, 2001) an in vitro developmental toxicity test with two permanent cell lines from the mouse has been proposed, i.e. the Embryonic Stem Cell Test (EST) (Spielmann et al., 1997). Using the ability of mESC
to differentiate spontaneously into contracting cardiomyocytes, a reliable assay system has been designed for the assessment of embryotoxic/teratogenic properties of chemicals and drugs in vitro.

The validated EST

Murine ESC of the permanent cell line D3 (Doetschmann et al., 1985) are maintained in an undifferentiated state in culture in the presence of the leukemia inhibitory factor (mLIF; Williams et al., 1988). Differentiation of mESC is induced by the withdrawal of mLIF. Using the "hanging drop" culture technique described by Rudnicki and McBurney (1987) mESC form multicellular aggregates called embryoid bodies (EBs). Within these aggregates, complex interactions between heterologous cell types result in the induction of differentiation of stem cells to derivatives of all three embryonic germ layers (Martin et al., 1977), including contracting myocardial cells (Doetschmann et al., 1985; Rudnicky and McBurney, 1987; Maltsev et al., 1994; Hescheler et al., 1997; Guan et al., 1999; Boheler et al., 2002; Sachinidis et al., 2003).

The EST benefits from the fact that differentiation into contracting cardiomyocytes can be detected easily by microscopic inspection of EB outgrowths at day 10 of differentiation. In addition to the differentiation analysis, cytotoxic effects of the test substance on mESC and 3T3 fibroblasts are assessed (fig. 1). By using mESC and differentiated (adult) fibroblasts, the assay takes embryonic as well as maternal toxicity into account. To assess the concentration of a substance that inhibits differentiation (ID50) and proliferation (IC50D3 and IC503T3) by 50% compared to the untreated control, concentration-response profiles are generated. To predict the toxic potential of a test compound in vivo from in vitro data, a biostatistical prediction model (PM) was developed to assign the test compounds to three classes of embryotoxicity: non-embryotoxic, weakly embryotoxic and strongly embryotoxic (Genschow et al., 2002 and 2004).

In an international ECVM validation study on three in vitro embryotoxicity tests, the EST was scientifically validated using a set of 20 reference compounds characterised by high-quality in vivo embryotoxicity data assessed in laboratory animals and humans. The EST predicted the embryotoxic potential of the 20 reference compounds with an accuracy of 78%. Remarkably, a predictivity of 100% was obtained for strongly embryotoxic chemicals (Genschow et al., 2002 and 2004). According to the ECVM Scientific Advisory Committee (ESAC), the three in vitro methods for embryotoxicity testing (EST, micromass test; and postimplantation rat whole-embryo culture test) are scientifically validated and ready for consideration for regulatory acceptance and application (Balls and Hellsten, 2002).

Current improvements of the EST

Since the EST has been demonstrated to be a reliable alternative method for embryotoxicity testing, several attempts have been made by our group and others to improve the EST protocol. Our recent improvements were predominantly focused on the following objectives: (i) the quantitative assessment of drug effects at the cellular level to allow a more objective measurement, (ii) the establishment of additional tissue-specific endpoints to increase the precision of the assay and (iii) the replacement of foetal calf serum (FCS) in the EST to further standardise the test system.

The successful establishment of new molecular endpoints in the EST, including the expansion to further cell-type specific endpoints like neural cells and the successful implementation of serum-free culture conditions in the EST were presented in an oral presentation at the 5th World Congress on Alternatives & Animal Use in the Life Sciences in Berlin, Germany (September 21st-25st, 2005).
Methods

Murine ESC were differentiated into beating cardiomyocytes using the hanging drop method (Rudnicki and McBurney, 1987). Test substances were applied throughout differentiation. Differentiation was determined by microscopic analysis of beating cardiomyocytes at day 10 of development (validated endpoint) and by quantitative gene expression analysis of selected tissue-specific marker genes using flow cytometry (day 7 of differentiation, new molecular endpoint). Concentration-response curves were generated to determine the concentration of a substance which inhibits the development of contracting cardiac muscle cells by 50% (ID$_{50}$). Efficient differentiation into contracting cardiomyocytes from mESC (cell line D3), chemical treatment and the quantitative intracellular flow cytometry approach were performed as described previously (Seiler et al., 2004, Seiler et al., 2006, in press).

For neural cell differentiation adherent monocultures in a defined medium according to Ying and Smith (2003), were generated with some modifications (K. Hayess, A. Visan and A. Seiler unpublished data).

To investigate cardiac ESC differentiation under serum-free conditions, mESC were adapted to serum-free medium in steps by raising the concentration of the serum replacement (Gibco, Karlsruhe, Germany) supplemented with specific factors in the medium (Advanced-DMEM; Gibco, Karlsruhe, Germany) while simultaneously reducing the foetal calf serum (FCS) concentration (Schlechter et al., manuscript in preparation). The adapted mESC were differentiated into beating cardiomyocytes according to the validated EST protocol (Spielmann et al., 1997; Seiler et al., 2006, in press).

Results

Improvements of the Embryonic Stem Cell Test (EST)

Implementing molecular endpoints of differentiation

In order to identify more objective endpoints other than the microscopic evaluation of “beating areas” and to follow cardiac differentiation at the cellular level, we improved and expanded the EST protocol by establishing molecular endpoints of differentiation. The quantitative expression of sarcomeric myosin heavy chain (MHC) and α-actinin genes under the influence of selected reference compounds was studied employing intracellular flow cytometry (Seiler et al., 2004). These results indicated that structural proteins of the sarcomere apparatus are promising candidates to predict developmental toxicity in vivo from in vitro data (Seiler et al., 2004).

To prove the general applicability of the new molecular endpoint in comparison to the validated microscopic evaluation, we are currently performing a prevalidation study using a set of ten chemicals with different embryotoxic potentials. For each chemical the new molecular endpoint and the validated endpoint – the microscopic analysis of beating cardiomyocytes – were assessed from concentration response curves. Results for two strongly embryotoxic, two weakly embryotoxic and one non-embryotoxic compound are presented in figure 2. Almost identical ID$_{50}$ values were obtained with both methods. These data indicate that the molecular approach is as predictive as the validated endpoint and that gene expression analysis using intracellular flow cytometry can serve as a new toxicological endpoint in the EST (Seiler et al., 2004; Buesen et. al., manuscript in preparation).

Differentiation of mESC into neural cells

The current experimental design of the EST involves differentiation of mESC into contracting cardiomyocytes. However, potentially embryotoxic drugs may target tissues other than the myocardium. This consideration prompted us to expand the EST to other major target tissues. To create an efficient in vitro approach for developmental neurotoxicity, we established a protocol for the differentiation of mESC into neural cells designed with special regard to the testing of chemicals and drugs.

In the past, for neural cell differentiation from pluripotent embryonic stem cells we followed the protocol published by Okabe et al. (1996) (lineage selection) with minor modifications or stimulated the induction of neural cells by retinoic acid treatment (Bain et al., 1996). Although the protocols were successfully established in our lab, they are not ideal for use in in vitro
The differentiation times are quite long and the growth factors are quite expensive. In the case of the second protocol mentioned above, the inducer substance, retinoic acid, is itself a strongly embryotoxic compound. To find an alternative, we studied the protocol published by the group of Austin Smith (Ying et al., 2003; Ying and Smith, 2003). The main advantage of this neural cell differentiation protocol is that differentiation can be easily achieved with adherent monolayer cultures in a defined medium.

Recently, our efforts were focused on the modification of this protocol resulting in a simple procedure for the efficient and reproducible development of neural cells in a comparatively short time. Flow cytometry analysis of a neuron-specific marker protein (microtubule-associated protein 2; MAP2) showed that at as early as day 16 of differentiation the major cell type was the MAP2-positive cell (34% of the total cell population, fig. 3). Studying the influence of selected reference compounds on neural development is currently in progress.

Fig. 3: Differentiation of neuronal cells from mESC using adherent monolayer cultures and quantification of neural marker protein expression by intracellular flow cytometry. Representative immunofluorescence staining of neural marker protein with anti-MAP-2, stained at day 16 of differentiation (A) with corresponding nuclear DAPI (4',6-diamino-2-phenylindole) staining. (B) Representative flow cytometry analysis of anti-MAP-2 stained cells at day 16 of differentiation (murine ES cell line D3). The x-axis corresponds to the fluorescence intensity and the y-axis to the number of cells per channel (events) (D). Control lacking primary antibody (C).

Establishment of serum-free culture medium in the EST
Serum represents an almost universal growth supplement that is effective in most cells. It contains most of the factors required for cell proliferation and maintenance. Using serum-supplemented media therefore reduces the need to spend time developing a specific, optimised medium formulation for every cell type under investigation.

In the EST, for an efficient differentiation of mESC into contracting cardiomyocytes as well as for routine maintenance of the cells, we usually use a cell culture medium supplemented with 15-20% foetal calf serum (FCS). Unfortunately, different serum batches vary considerably with respect to their chemical composition. Certain factors may be deficient in some batches while others may be present at excessive, inhibitory levels. For these reasons serum batches have to be pre-tested for how well they support differentiation and proliferation of mESC before chemical testing in the EST can be performed. In order to further standardise the EST protocol, our aim was to establish...
serum-free culture conditions. Chemically defined serum-free culture medium would provide several advantages: (i) improved protocol transfer to other laboratories (ii) improved reproducibility of the differentiation assay, (iii) no interference of undefined serum components with the test substance (reproducible bio-availability) and (iv) application of the EST in automated screening systems.

The effects on proliferation and differentiation of chemically defined serum-free media in combination with selected components have been investigated. Mouse ESC were adapted to various serum-free culture conditions and tested in the validated in vitro differentiation assay (Seiler et al., 2006, in press) in comparison to the control using the standard culture medium containing 15% FCS.

Using serum-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with specific factors known to promote cardiac development, we were able to exclude FCS completely from the culture medium (Schlechter et al., manuscript in preparation). As shown in figure 4, the differentiation of mESC into contracting cardiomyocytes was as efficient as in the presence of serum.

Conclusions

Reproductive toxicity is one of the most serious side effects a compound can have. Therefore, an important objective for toxicological safety assessment of chemicals and drugs is to evaluate adverse effects on reproduction and embryonic development. The complexity of the reproductive system and the multiple targets for exogenous induction of malformations during embryonic development are the rationale for highly standardised animal experiments, e.g. screening tests or multigeneration studies according to OECD test guidelines.

For reproductive toxicity testing, the estimation of the number of laboratory animals needed under REACH for conventional implementation of the existing OECD guidelines is very high. Thus, testing of these chemicals can hardly be accommodated without the use of predictive cell-based screening systems.

In the field of developmental toxicity testing, the most promising cell-based assay to date is based on ESC of the mouse. As an in vitro system, which mirrors both differentiation and growth, the EST is well suited for the evaluation of the teratogenic potential of a compound. Recently, through a number of studies, we were able to improve and expand the validated EST considerably.

New predictive molecular endpoints of differentiation have been effectively implemented in the EST by studying the quantitative expression of marker genes under the influence of the test compound by employing intracellular flow cytometry.

A new differentiation protocol for use in in vitro developmental neurotoxicity assays has been developed. Additional major target tissues such as the nervous system may now be included in the test in order to get more precise information on the embryotoxicity of a compound.

Serum-free culture conditions for cell maintenance and differentiation of mESC have been successfully established. Chemical testing can now be performed in a more highly standardised form.

Despite the progress made, there are still some unresolved problems. For regulatory acceptance, the EST requires the enlargement of the database, since the formal validation study focused on pharmaceutical testing. Thus, more industrial chemicals exhibiting different patterns of embryotoxic potential and also a broader range of compounds representing different toxicological mechanisms have to be tested and the database of test chemicals needs to be expanded. Furthermore, in its present form the EST is only applicable for compounds that do not require metabolic activation. However, the metabolic (in-)activation of xenobiotics is an important prerequisite for the accurate determination of the toxic properties of a compound. The introduction of a metabolising system as an adjunct to the vali-

Fig. 4: ESC differentiation assay under serum-free and serum-containing conditions. On day 12 and 14 of the assay, differentiation into contracting myocardial cells was determined under the light microscope. Each well of the 24-well plate was inspected, and the number of wells containing spontaneously contracting cells was recorded. According to the validated EST protocol, the assay is acceptable if at least 21 of the 24 EBs have differentiated into spontaneously contracting myocardial cells. The results from a representative experiment are presented.
dated EST protocol is essential. Finally, to improve the precision of the EST and, in particular, to prevent false negative classifications more cell type-specific endpoints of differentiation (e.g., osteoblasts and chondrocytes) have to be added to the validated EST protocol.

In conclusion, the improved and expanded EST promises to be the first in vitro assay for developmental toxicity that may be accepted by regulatory authorities. Furthermore, implementation of the EST in regulatory test guidelines would demonstrate the importance of in vitro assays as valuable components of the risk/hazard assessment process.

**References**


van der Jagt, K., Munn, S., Torslov, J. and de Bruijin, J. (2004). Alternative approaches can reduce the use of test animals under REACH. http://ihcp.jrc.cec.eu.int/DOCUMENTA-


**Acknowledgements**

We gratefully acknowledge funding of the projects by the German Federal Ministry for Education and Research (BMBF, grant no. 0312312) as well as the support of the FP6 Project ReProTect (LSHB-CT-2004-503257 ReProTect).

**Correspondence to**

Dr. Andrea Seiler
ZEBET at the BfR
Diedersdorfer Weg 1
12277 Berlin
Germany

e-mail: seiler.zebet@bfr.bund.de
In Vitro Multipotency of Human Bone Marrow (Mesenchymal) Stem Cells

Sarah Snykers¹, Tamara Vanhaecke¹, Peggy Papeleu¹, Tom Henkens¹, Mathieu Vinken¹, Greetje Elaut¹, Ivan Van Riet² and Vera Rogiers¹


Summary
The capability of mesenchymal stem cells (MSC) to differentiate in vitro into hepatocytes by exposure to liver-specific cytokines was investigated. Simultaneous exposure of MSC to a mixture of hepatogenic cytokines only stimulated neuroectodermal and mesodermal differentiation. However, sequential exposure, resembling the order of secretion during liver embryogenesis, induced both glycogen storage and expression of cytokeratin 18. In order to trigger further endodermal differentiation, cells were exposed to trichostatin A (TSA). The latter up-regulated albumin secretion, a typical functional property of primary hepatocytes. In conclusion, MSC acquire trilineage potential under “hepatocyte-specific conditions”. TSA improves differentiation of MSC towards hepatocyte-like cells.

Keywords: bone marrow stem cells, hepatocytes, embryonic development, histone-deacetylase inhibitor, in vitro

Introduction
The liver is the principal organ for xenobiotic biotransformation and thus a key target for drug-induced toxicity (Gibson and Skett, 1994). Isolated primary hepatocytes and their cultures are therefore widely used in preclinical pharmaco-toxicological research and testing. A problem encountered, however, is the relative short lifespan and the rapid decline of liver-specific functions as a function of culture time (LeCluysse et al., 1995; Rogiers et al., 1995; Papeleu et al., 2002). Attempts have been made to cultivate functional hepatocytes for longer periods by using soluble medium components and by affecting cell-cell and cell-matrix interactions (Guillouzo, 1998; Rogiers et al., 1995). The ideal in vitro model, however, does not yet exist. Different alternatives have been explored to overcome these difficulties. In recent years, adult-derived stem cells became a hot topic in the field of molecular and cellular biology, for clinical application as well as for pharmaco-toxicological purposes. Indeed, stem cells are clonogenic and capable of both self-renewal and multilineage differentiation (Krause et al., 2001; Huttmann et al., 2003). In vivo as well as in vitro studies have provided evidence that stem cells can overcome germ lineage restrictions and express molecular characteristics of cells of different tissue origin (Krause et al., 2001; Huttmann et al., 2003).

The best-characterised stem cell compartment is the bone marrow, which consists of two stem cell populations, referred to as haematopoietic and mesenchymal stem cells (Huttmann et al., 2003). The latter were first described by Friedenstein et al. as a population of cells isolated from the bone marrow and capable of differentiation into bone, adipocytes, chondrocytes, osteoblasts, osteoprogenitors, skeletal myocytes, tendon and bone marrow stromal cells (Friedenstein, et al., 1976, 1987).

Schwartz et al. described a population of cells in postnatal rat bone marrow named multipotent adult progenitor cells (MAPC) that were not only able to differentiate into most mesodermal cell types, but also into neuroectodermal and endodermal (hepatocytes) cell types (Schwartz et al., 2002).

In the present study, we investigated whether human MSC also have the potential to differentiate in vitro into endodermal cells such as hepatocytes. Experiments were undertaken in the presence of liver-specific cytokines, added either as a cocktail [fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF), insulin-transferrin-sodium-selenite (ITS) and dexamethasone] or sequentially, in a manner that closely reflects their temporal expression during in vivo hepatogenesis (FGF-4, followed by HGF, followed by a combination of HGF, ITS and dexamethasone).

Materials and methods
Isolation and expansion of MSC
Expanded MSC were obtained from the Academic Hospital, Vrije Universiteit Brussel. MSC were collected from 5-30 ml aspirates from the sternum of haematologically healthy donors and expanded in vitro at low density for 4 passages in medium containing foetal calf serum (Friedenstein, et al., 1976, 1987).

Multilineage differentiation of MSC
MSC (CD45-, Thy1) were cultivated at 21.5 x 10³ cells / cm² on 1 mg/ml collagen gel type I in the presence of liver-specific cytokines, added either as a cocktail [basal medium (Jiang et al., 2003) + 10 ng/ml FGF-4, 20 ng/ml HGF (both from R&D Systems), 1x ITS and 20 µg/l dexamethasone (both from
Sigma) or sequentially (days 0-3: basal medium + 10 ng/ml FGF-4; days 3-6: basal medium + 20 ng/ml HGF; from day 6 on: basal medium + 20 ng/ml HGF + 1x ITS and 20 µg/l dexamethasone). Differentiation media were changed every 3 days. 1 µM TSA (Sigma) was added from day 6 on.

**Cytological staining**

Cells were fixed with 10% formalin for 10 min at room temperature (neuroectodermal, adipogenic, hepatocyte differentiation) or with MeOH for 2 min at -20°C (osteogenic differentiation). After fixation, nerve vessels were identified by Bodian staining (Wüllimann et al., 1999). Adipocytes were identified by red lipid droplets after staining with Sudan III (Reyes et al., 2001). Mineralised nodules were stained black with the von Kossa technique, whereas unmineralised nodules were stained yellow (Reyes et al., 2001). Periodic-acid-Schiff (PAS) staining was used to determine glycogen storage, a functional parameter of endogenic differentiation (Schwartz et al., 2002). As control, PAS staining was performed in the presence of amyloglucosidase (Sigma).

**Immunofluorescence**

Immunostaining was performed as previously described (Schwartz et al., 2002).

**Albumin ELISA**

Albumin concentrations, secreted into the culture media, were quantified by ELISA (Koebe et al., 1994).

**Statistics**

Results are expressed as means ± SD of five independent experiments. Statistical analyses were performed using one-way ANOVA followed by Student’s t-test. The significance level was set at 0.05.

**Results**

**Trilineage differentiation of low-density MSC cultures**

To evaluate whether MSC had trilineage potential like MAPC, specific cytological staining was performed.

**Adipogenic, osteogenic and neurogenic differentiation**

Undifferentiated MSC did not stain positive for any of the cytological markers.

Simultaneous exposure of MSC to a mixture of hepatogenic cytokines resulted in a heterogeneous population of cells (fig. 1). More specifically, adipogenic differentiation was seen from day 5 on (fig. 1B). After 17 days of cultivation, more than 80% of the cells were loaded with lipid droplets and stained red with Sudan III (fig. 1D). In addition, the presence of calcium phosphate, characteristic for osteoblasts, could be shown from day 5 on by von Kossa staining (fig. 1E). Bodian staining clearly revealed the presence of nerve fibres (black staining) on day 8 (fig. 1G).

In order to obtain more homogeneous MSC-derived cell cultures, MSC were separately treated with each of the cytokines. It appeared that only adipocytes formed in the pres-

![Fig. 1: Adipogenic, osteogenic and neuroectodermal differentiation was demonstrated by means of Sudan III (A-D), Von Kossa (E,F) and Bodian (G) staining, respectively. MSC were simultaneously exposed to all liver-specific cytokines. 10 x 10 magnification.](image)

![Fig. 2: PAS-staining showed glycogen storage (A-C) from day 9 on. MSC were sequentially treated with liver-specific cytokines for 9, 17 and 22 days, respectively. 10 x 10 magnification.](image)
ence of ITS and dexamethasone, whereas FGF-4 induced the appearance of osteoblasts and neuroectodermal-like cells (data not shown).

**Endogenic differentiation**

Since no cells with typical morphological characteristics of hepatocytes were seen upon treatment with the cocktail of liver-specific cytokines, MSC were sequentially exposed to FGF-4, HGF, ITS and dexamethasone in a time-dependent order that closely resembles their secretion pattern during *in vivo* liver ontogeny. Under these conditions, glycogen uptake was seen from day 9 on (fig. 2A) and was clearly up-regulated throughout the culture time (fig. 2B-C). Moreover, cells stained positive for cytokeratin 18 (fig. 3A), a cytoskeletal filament present in hepatocytes, while control immunoglobulins did not (fig. 3B). Nevertheless, the differentiated cells did not resemble hepatocytes morphologically.

**TSA, a trigger for endodermal differentiation of hMSC**

In an attempt to enhance endogenic differentiation of MSC, TSA, a selective and reversible histone-deacetylase (HDAC) inhibitor (Xu, et al., 1997) was added to the culture medium.

**Hepatic functionality**

The secretion of albumin, a typical functional property of primary hepatocytes, was taken as a first criterion for hepatic functionality of differentiated MSC. MSC were sequentially exposed to liver-specific cytokines, as during *in vivo* hepatogenesis. 1 µM TSA was added from day 6 on, corresponding to promising culture conditions used for hepatic differentiation of MAPC (unpublished data). Interestingly, addition of 1 µM TSA to sequentially-treated MSC induced a significant upregulation of albumin secretion from day 15 on (p<0.01, one-way ANOVA and Student’s t-test) compared with control cultures (sequential) (fig. 4). At day 17, albumin secre-
tion even reached comparable levels with those observed in 2-day old monolayer cultures of primary rat hepatocytes (Vanhaecke et al., 2004). Up-regulation of albumin secretion was also seen upon exposure to a cytokine-cocktail supplemented with 1 µM TSA. However, this result was not statistically significant and was less distinctive than that observed after sequential treatment. MSC cultivated without TSA did not secrete albumin (fig. 4).

Morphological features
This inductive effect of TSA on the differentiation process of MSC to hepatocyte-like cells was well supported by microscopic analysis of the cell morphology. Indeed, upon exposure to TSA, a complete transformation of the cell morphology could be observed, regardless of the experimental set-up (fig. 5). After 3-5 days TSA-treated MSC formed epithelioid cells with clear, round nuclei. Fibroblastic cells, however, persisted throughout the culture time, particularly upon exposure to the cytokine-cocktail (fig. 5A2).

Discussion and conclusions
Hepatocyte-based in vitro models are important tools for pharmacotoxicological research and regulatory testing of xenobiotics (Gibson et al., 1994). A serious drawback, however, is their limited lifespan and rapid loss of differentiated hepatic properties (LeCluysse et al., 1995; Rogiers et al., 1995; Papeleu et al., 2002). The use of stem cells could offer a solution. It is well recognised that in adult mammals, including humans, a number of tissues are continuously regenerated from immature cells (i.e. skin, intestinal epithelia, blood cells, the olfactory bulb in the brain) (Krause et al., 2001; Huttman et al., 2003; Friedenstein et al., 1976; Friedenstein et al., 1987; Schwartz et al., 2002; Jiang et al., 2003; Reyes et al., 2001). The interest in adult stem cells has in particular been triggered by the numerous ethical dilemmas surrounding the use of embryonic stem cells in preclinical and clinical research (Henningson et al., 2003). Therefore, the ability to isolate, cultivate and manipulate multipotent stem cells from non-embryonic origin would provide researchers with an unlimited source for cell and organ development studies, pharmacotoxicological research and regulatory testing.

In the present study, we investigated whether MSC, like MAPC, are able to differentiate in vitro into cell types of all three germ layers. In particular, the endodermal differentiation received most attention. MSC were hence exposed to well-defined hepatogenic cytokines, added either as a cocktail or sequentially, in a time dependent order as seen during liver embryogenesis.

Upon exposure to a cocktail of liver-specific cytokines, low-density MSC differentiated into a heterogeneous population of

![Fig. 5: Light-microscopic analysis of 17-day old differentiated MSC upon simultaneous (A) or sequential (B) exposure to liver-specific cytokines in the absence (1) and presence (2) of 1 µM TSA, respectively; 20 x 10 magnification, phase contrast.](image-url)
mesodermal (adipocytes and osteoblasts) and ectodermal (neuroectodermal-like cells) cells. Although lipid droplets (adipogenic differentiation) also appear in hepatic epithelium, the differentiated cells did not display an epithelial-like morphology. They neither expressed other hepatocyte-specific markers such as alphafetoprotein, albumin or cytokeratin 18, nor did they take up glycogen (data not shown).

Conversely, sequential exposure of MSC to hepaticogenic cytokines induced not only expression of the mid-late hepatic marker cytokeratin 18 from day 7 on, but also caused an upregulation of glycogen storage from days 9 to 22. However, cells did not morphologically resemble adult hepatocytes. As a trigger for further differentiation of MSC towards endodermal lineage, TSA, a drug candidate for hyperproliferative disorders, was introduced into the present culture media. TSA causes an hyper-acetylation of histone proteins, resulting in an increased accessibility of target DNA for transcription factors, and thus also liver-enriched transcription factors, thereby facilitating transcription of the target genes (Marks et al., 2001; Yoshida et al., 1990). As such, TSA and HDAC-inhibitors in general, have been shown to induce differentiation, apoptosis and cell cycle arrest in tumour cells (Yamashita et al., 2003). In primary cells, e.g. cultured hepatocytes, it was found that 1 µM TSA induced cell cycle arrest during G0/G1 and G1/S phase in EGF-stimulated cells but it did not induce apoptosis (Papeleu et al., 2003). These findings triggered us to add TSA to the culture media in order to positively affect the differentiation process towards the endodermal lineage. Indeed, upon sequential exposure to liver-specific cytokines, TSA caused a significant upregulation of albumin expression in cultured hepatocytes, it was found that 1 µM TSA induced cell cycle arrest during G0/G1 and G1/S phase in EGF-stimulated cells but it did not induce apoptosis (Papeleu et al., 2003). These findings triggered us to add TSA to the culture media in order to positively affect the differentiation process towards the endodermal lineage. Indeed, upon sequential exposure to liver-specific cytokines, TSA caused a significant upregulation of albumin secretion, a typical functional property of hepatocytes, to levels similar to those found in monolayer cultures of 2-day old primary rat hepatocytes (Vanhaecke et al., 2004). Furthermore, microscopic analysis supported these observations. TSA-treated MSC adopted an epithelial-like morphology, regardless of the culture conditions. However, a higher number of fibroblastic cells persisted throughout the culture time upon exposure to the cytokine cocktail.

Currently, the culture conditions for advanced differentiation of MSC into mature hepatocytes are being optimised.

References

**Acknowledgements**

Research was funded by a Ph.D. grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) and grants from the Research Council (OZR) of the Vrije Universiteit Brussel, Belgium. The authors thank Prof. Y. Vander Heyden and B. Dejaegher for their valuable statistical advice and G. De Pauw, E. Desmedt, B. Degreef, H. Mertens and S. Coppens for their dedicated technical assistance.

**Abbreviations:**

- fibroblast growth factor-4 (FGF-4)
- hepatocyte growth factor (HGF)
- histone-deacetylase (HDAC)
- insulin-transferrin-sodium-selenite (ITS)
- mesenchymal stem cells (MSC)
- multipotent adult progenitor cells (MAPC)
- Periodic-acid-Schiff (PAS) staining
- trichostatin A (TSA)

**Correspondence to**

Sarah Snykers  
Dept. Toxicology  
Vrije Universiteit Brussel  
Laarbeeklaan 103  
1090 Brussels  
Belgium  
e-mail: Sarah.Snykers@vub.ac.be.