Developmental Toxicity Testing from Animal towards Embryonic Stem Cells

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Summary
Developmental toxicology is the study of undesirable effects on the development of the organism, which may result from exposure before conception, from the period of prenatal development, or postnatally during the time of sexual maturation. The principal manifestations of developmental toxicity include: embryolethality, malformations, growth retardation, and functional impairment.
In 2001, the European Commission published the future chemicals policy entitled "White Paper: Strategy for a Future Chemicals Policy". The new regulation requires a toxicological evaluation with strong emphasis on reproductive toxicity, by using in vitro methods, especially for those chemicals marketed at more than 1 ton per year. For this reason, the establishment of in vitro models capable of detecting major undesirable manifestations in the fetus, are urgently required. The aim of the present review is to explore the capacity of existing in vitro systems, based on embryonic stem (ES) cells, to identify embryotoxicity with a focus on specific effects such as teratogenicity and growth retardation. In addition, we discuss the possibility to adapt the mouse ES cells based tests to human ES cells, avoiding inter-species variations in developmental toxicity studies and address related ethical issues. Considering the different manifestations of developmental toxicity, only a battery of in vitro tests will provide the necessary information for regulatory developmental toxicity assessment.

Zusammenfassung: Entwicklungstoxizitätstestung: Vom Tiermodell zu embryonalen Stammzellen
In der Entwicklungstoxikologie werden nachteilige Effekte auf die Entwicklung eines Organismus studiert, die sich vor der Befruchtung, während der pränatalen Entwicklung oder postnatal in der Zeit bis zur Geschlechtsreife auswirken können. Die wichtigsten durch Entwicklungstoxizität bedingten Symptome sind: Embryoletalität, Missbildungen, vermindertes Wachstum oder Funktionsstörungen.
Die Europäische Kommission veröffentlichte 2001 die zukünftige Chemikalienpolitik "White Paper: Strategy for a Future Chemicals Policy". Diese Regelungen fordern eine toxikologische Beurteilung mit Schwerpunkt auf Reproduktionstoxizität mittels in vitro Methoden, besonders für Chemikalien, deren jährliche Produktion ein Volumen von 1 Tonne überschreitet. Aus diesem Grund wird dringend die Entwicklung von in vitro Modellen benötigt, die die wichtigsten unerwünschten Symptome im Fötus abdecken. Die vorliegende Review-Arbeit untersucht die Fähigkeit von in vitro Systemen – basierend auf embryonalen Stammzellen –, Embryotoxizität nachzuweisen mit Fokus auf spezifische Effekte wie Teratogenität und Wachstumsstörungen. Zusätzlich werden aus ethischer Sicht die Möglichkeiten der Adaptierung von Mausstammzell-Tests auf humane Stammzellen diskutiert, um Interspezies-Variationen in Entwicklungstoxikologischen Studien zu vermeiden. In Anbetracht der unterschiedlichen Erscheinungsbilder einer Entwicklungstoxizität kann allerdings nur eine Batterie von in vitro Tests die nötigen Informationen zur regulatorischen Bewertung bezüglich Entwicklungstoxizität liefern.

Keywords: embryonic stem cells, teratogenicity, developmental toxicity, alternatives to animals experiments, malformations, growth retardation

1 Introduction

Developmental toxicology is an important area of toxicology. Rosano et al. (2000) showed that birth defects are the principle cause of infant mortality as assessed in 36 countries. Children born with major birth defects are normally affected by mental, physical and social disorders (Rosano et al., 2000; MacLeod, 1993; Yoon et al., 1997). To date, significant progress has been made in order to determine causes of birth defects. Birth defects is a term used to indicate those malformations observable at birth (congenital) or thereafter. Birth defect indicates structural or anatomical defects, but it can also include physiological or functional and behavioural defects. For the majority of those, the etiology is unknown, for some it is only poorly established. The causes of birth defects are various and can be summarised as genetic causes (autosomal genetic diseases, cytogenetic; 15-20%), environmental causes (maternal conditions and infections, mechanical problems, chemicals/drugs/radiation; 10%), preconception exposures (unknown percentage) and un-

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known causes (65%) (Brent and Beckman, 1990).

Wilson 1977 considered chemical agents responsible for 4-6% of the total birth defects. Nevertheless, in order to reduce risks of deviant development, it is a worthwhile goal to identify those chemical agents present in the environment and in consumer products to which the gravid mother and her conceptus are potentially exposed.

The sciences of birth defects "teratology", or the process of induction of malformation "teratogenesis", from the Greek word root "teras" meaning malformation or monstrosity. Three fundamental principles of teratogenesis govern the chemical potential or the capability to induce congenital malformations in human or in other animal species. Paraphrasing Karnofsky (1965), these principles can be illustrated by the axiom: "A teratogenic response depends upon the administration of a specific treatment of a particular dose to a genetically susceptible species when the embryos are in a susceptible stage of development."

As assumed by Wilson 1973, the teratogenesis depends on the genotype of the conceptus, the way in which this interacts with environmental factors, the developmental stage and the mechanism (pathogenesis) as well. In addition, manifestation of adverse development increase in incidence and degree as dosage increases from no-effect to totally lethal level.

The four main manifestations of developmental toxicity are malformations, growth retardation, embryolethality and functional impairment (Wilson, 1973) (see Fig.1).

Malformations

Most types of malformations are observable in the majority of all species of animals, but specific malformations or their incidence are species-dependent. For example, eye defects, exencephaly, polydactyly, and cleft palate are most frequent in mice (Flynn, 1968; Kalter, 1968). In rabbits it is easy to observe limb defects, umbilical hernians, and craniofacial defects more frequently than other types of malformations (Chai and Degenhardt, 1962; Staples and Holtkamp, 1963). Nonetheless, some chemicals, capable of inducing malformations, may also produce other classes of developmental toxicity effects, consequently an agent that causes developmental toxic effects may not necessary be considered as teratogenic.

Growth retardation

Another aspect to be considered in developmental toxicity testing is intrauterine growth retardation (IUGR). A generally accepted definition for IUGR is: "Birth weight less than the tenth percentile for gestational age" (Lubchenco et al., 1963). An alternative definition is: "Birth weight more than two standard deviations below the mean for gestational age, corresponding to approximately the third percentile of the intrauterine growth curves" (Gruenwald, 1966). Numerous agents are known to cause IUGR. In a large human study, the incidence of IUGR was 5.3% and perinatal mortality among these infants occurred three times more often than normal (Low and Galbraith, 1974). In Callan and Witter (1990) 86% of perinatal deaths were found in the IUGR group. In addition, up to 20% of spontaneous abortions exhibit severe embryonic growth retardation. IUGR is common in infants that exhibit severe and multiple malformations (Warkany, 1971). In addition, congenital infections, neonatal deaths, and long term neurological and intellectual deficits have been noted more often in infants affected by IUGR (Miller, 1981).

Embryolethality

Death of the offspring is another aspect of developmental toxicity. Embryonic and early fetal death occurs in one out of every two pregnancies in humans (Shepard and Fantel, 1979). Fetal death is often associated with congenital malformations. The causes of mortality may be due to direct effects to the conceptus (alcohol, anticancer agents, lithium, methyl mercury and retinoids analogues) or indirect (maternally mediated) (Kalter, 1980). Maternally mediated effects are generally associated with: maternal homeostatic changes due to diabetes and phenylketonuria; and maternal toxicity resulting from alcohol abuse, use of aminopterin and trimethadione (Khera, 1985, 1987). Some teratogens cause primarily morphological modifications and in some situations this may lead to embryonic death. In humans, correlation between malformations and death has been demonstrated (Haas and Schottenfeld, 1979).

Functional impairment

Recently, parameters such as motor ability, sociability, emotional, and learning capacity are considered as effects in the developmental toxicity field. Indeed, some evidence indicates that specific types of behavioral alterations may result after exposure to certain agents, during critical periods in fetogenesis. These abnormalities are the result of modification occurring during development of specific neurotransmitter systems, after chemicals insult (Leonard, 1981). Many

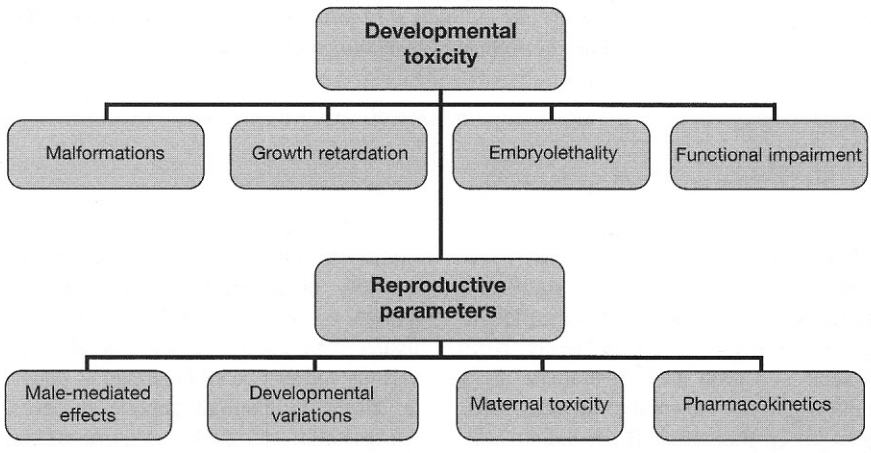

Fig. 1: Principal manifestations in developmental toxicity.
Embryolethality, malformations, growth retardation, and functional impairment, are the most important manifestations in developmental toxicity to be considered in an *in vitro* test strategy. Some other reproductive parameters should be taken into account.

compounds are well-known causes of functional impairment, for example aspirin (Okamoto et al., 1986), ethoxyethanol (Nelson et al., 1981) and hydroxyurea (Vorhees et al., 1979). In recent years, additional behavioural tests have become constituents of developmental toxicity assessment, especially in the field of pharmacology.

In addition to the classes described above, there are some other parameters to consider in developmental toxicity studies (see Fig. 1). These are reproductive parameters that may have relevance to humans. Male-mediated effects are one of those parameters, e.g. when defects to offspring result from sperm cells. For example, several studies in the progenies that came from male rabbits treated with colchicin or thalidomide, showed severe malformations (Chang, 1944; Lutwak-Mann, 1964). According to a review proposed by Davis et al. (1992) some chemical agents were classified according to their capacity to cause male-mediated adverse reproductive outcomes.

Another parameter is the developmental variation. Minor aberrations in structure and variations in ossification are related to this factor. These effects take place more often than malformations, and the common consequences to the fetus are delay in growth, minor changes in structure and alteration in the normal differentiation.

The influence of maternal toxicity is another factor to be taken into account in teratology assessment. It becomes obvious that toxicity to the embryo may be modified or influenced by toxicity to the mother. Nevertheless, it is poorly or not yet understood why, and how toxicity to the mother has direct effects on fetus development.

Some pharmacokinetic considerations are essential in teratology assessment. Pregnancy itself alters pharmocokinetics through physiological changes. For this reason, the study of pharmocokinetic properties of chemicals or drugs becomes essential, in order to quantify the real amount of exposure to the fetus and the resulting toxicological effect.

The aim of the present review is to give an overview regarding the use of *in vitro* systems, based on mouse embryonic stem cells, in developmental toxicity testing. The application of these methods to identify phenomena such as teratogenicity and growth retardation is discussed. In order to avoid inter-species variations, the possible use of human embryonic stem cells in developmental toxicity studies is explored and related ethical issues are taken into account.

2 Animal models used in developmental toxicity studies

Mouse, rat and rabbit are the generally accepted species of laboratory animals. However in recent years, other species has been proposed, such as hamster, guinea pig, dog, swine, ferret, and various species of primates. Nevertheless, the results obtained from testing these species in developmental toxicity studies, demonstrate that few proved human teratogens. Following the advice made by Wilson (1978) a critical point to be considered for safety evaluation, is the presence, in the model used, of the maternal-placenta-embryo relation. In addition Wilson (1975) advised the use of animal models able to metabolise, distribute and transfer the compound across the placenta in ways similar to humans.

In one large comparative study (Schardein, 1983), the rabbit and the primate were considered the most suitable species for general screening. In Schardein and Keller (1989) some other animal model were evaluated and rat, mouse, hamster, several species of primate and rabbit showed quite reliable ability to identify human developmental toxicants, in descending order. To date, no single species has been identified as most promising animal model for developmental toxicity testing.

The embryotoxic and in particular the teratogenic potential of a substance is tested using the Prenatal Developmental Toxicity Study (OECD, Organisation for Economic Co-operation and Development, Test Guidelines 414, Anon, 2001b). Other OECD guidelines are used, in order to evaluate effects on pre-/postnatal development and on fertility (see Tab. 1). Such studies, especially the two- or multigeneration study, are cost- and time-consuming. They consume up to 3,200 animals per substance.

3 Future European chemicals policy

Recently the European Commission reported to the Council and the European Parliament the statistics on the number of animals used for experimental and other scientific purposes (Anon, 2005). In 2002, it has been estimated, that in the European Members States, 10.7 million animals have been sacrificed. In toxicological and other safety evaluation, the number of animals used represents 9.93% of the total and about 12.1% of these animals are used for reproductive and developmental toxicity tests.

In 2001, the European Commission published a policy statement on future chemicals regulation and risk reduction entitled "White Paper: Strategy for a Future Chemicals Policy" (Anon, 2001a). The new chemicals policy aims to finalise the risk assessments within a 12–15 year period for all chemicals marketed at more than 1 ton per year. For this reason, it becomes urgent to identify data on the hazardous properties of these chemicals. In addition, for those chemicals not tested in the past it will be necessary to re-evaluate their toxicological potential.

Tab. 1. OECD guidelines used to evaluate pre-/postnatal and fertility effects.

OECD Guideline	Description	References
OECD TG415:	One-Generation Reproduction Toxicity Study	Anon, 1983
OECD TG416:	Two-Generation Reproduction Toxicity Study	Anon, 2001c
OECD TG421:	Reproduction/ Developmental Toxicity Screening Study	Anon, 2001d
OECD TG422:	Combined Repeated Dose Toxicity Study with the Reproduction/ Developmental Toxicity Screening Study	Anon, 1996

Therefore, the European Union is in the process of introducing a new system for the Registration, Evaluation and Authorisation of new and existing Chemicals (REACH) in order to harmonise the testing requirements for new and existing chemicals. The White Paper and the draft legislation suggest that in the first phase testing should be restricted to *in vitro* tests.

4 *In vitro* model for developmental toxicity assessment

In vitro systems for developmental toxicology testing can be subdivided into three different classes: cell cultures (e.g. Embryonic Stem Cells Test (EST)), organ cultures (e.g. Micromass assay), and embryo cultures (e.g. Whole embryo culture). Brown (1987) and Brown et al. (1995) presented an extensive overview of the existing *in vitro* systems.

Ease of performance and reduced or no animal use are the most important advantages of cell cultures and for this reason, these models not requiring the use of experimental animals are favoured. Systems like embryo cultures are complex systems which provide a greater understanding of the developmental mechanisms involved. However, these models are more laborious and animal consuming.

Other alternatives to animal models are the organ culture systems. They need primary animal material, and they require specific technical infrastructures, which make the standardisation of the model more difficult. Limb buds, digits, palatal shelves, lung, intestine, and sex organs are examples of such organ culture systems (Faustman, 1988).

It was estimated that several millions of animals would have to be sacrificed according to the needs of the White Paper, developmental toxicology consuming up to two thirds of these (van der Jagt et al., 2004). It becomes obvious that new predictive screening methods for developmental toxicity testing are urgently required, especially since this field will most probably consume the highest numbers of animals.

Numerous workshops have been held in order to evaluate the most promising alternative tests. An ECVAM (European Centre for the Validation of Alternative Methods) workshop was held in 1994 (Brown et al., 1995) and the participants suggested the use of Xenopus embryos, mammalian embryo as well as micromass cultures and murine embryonic stem (ES) cells. However, in May 2000 the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) after evaluating the Frog Embryo Teratogenesis Assay (Xenopus (FETAX)), which is considered a teratogenesis assay, reported that FETAX is not sufficiently validated or optimised for regulatory applications (Anon, 2000).

In addition, the participants of a workshop held in 1981 on *in vitro* teratogenesis, agreed that only a battery of test will provide relevant information in teratogenic assessment (Kimmel et al., 1982). Indeed, taking into account the complexity of embryogenesis, it is clear that a single *in vitro* model is not able to substitute *in vivo* testing. Therefore, only a strategy of *in vitro* tests will be able to cover the complete range of mechanisms of development and their interaction (Bremer and Hartung, 2004).

5 Development of *in vitro* systems by using embryonic stem cells for developmental toxicity testing

In order to detect chemical effects during the process of differentiation, several approaches based on embryonic stem cells have been used. The EST (Spielmann et al., 1997) is one of the *in vitro* validated systems and it is based on ES cells, which permit the classification of chemicals in strong, weak, and non-embryotoxic. Nevertheless, for regulatory acceptance, EST needs to be improved. During an ECVAM workshop held in January 2003, a group of experts have reviewed the EST and they advised ECVAM to include supplementary endpoints able to identify effects on nervous system (Strubing et al., 1995), skeletal system (zur Nieden et al., 2003) and cardio-vascular system (Boheler et al., 2002). The ability of the ES cells to differentiate into a wide range of tissues, which might be the target of a teratogen, provides the unique opportunity to use them in teratogenesis studies. Furthermore, the experts recommended to add metabolic competences to the EST as well as the other v alidated embryotoxicity tests.

In the last few years, teratogenic effects of chemicals occurring during ES cells differentiation have been analysed by using molecular biological and immunological endpoints. Cultivation of transgenic ES cells with green fluorescence protein (GFP) driven by a specific tissue gene promoter is another elegant method used to detect modification in the normal ES differentiation (Metzger et al.; 1996, Kolossov et al.; 1998, Bremer et al., 2001; Paparella et al., 2002). In Wobus et al. (1997), Bigot et al. (1999), zur Nieden et al. (2001), Bremer et al. (2001), Schmidt et al. (2001) and Seiler et al. (2002) a molecular approach was used in order to monitor changes in the expression of specific tissue genes, during cardiac ES cells differentiation after chemicals exposure. In these studies only tissue specific markers were analysed. In Seiler et al. (2002, 2004) an immunological approach was proposed based on intracellular staining and flow cytometry to detect changes in sarcomeric myosin heavy chain (MHC) and alpha-actinin, during ES cells cardiac differentiation. In order to obtain more detailed information at which developmental stage the chemical is active, a different approach was proposed recently (Pellizzer et al., 2004a, 2004b) (see Fig. 2). The described method aims to analyse the expression of genes involved in early and later stages of cardiac and skeletal ES cells differentiation, in order to detect multiple of specific malformations after chemicals exposure. The *in vivo* data of the chemicals tested were correctly confirmed by the *in vitro* tests using the mentioned approach, demonstrating the usefulness of the ES system in teratogenicity studies. A suitable list of genes which may be useful in the identification of teratogenicity effects in the principal target tissues are summarised in Table 2.

Considering the molecular biological approach, array technologies now offer the opportunity to detect simultaneous changes in thousands of genes (Lockhart and Winzeler, 2000) or protein expression (Pandey and Mann, 2000) after chemicals exposure. This technology might be helpful in order to identify teratogenic effects and also in the description of toxicological mechanisms of developmental toxic agents. Nevertheless, much effort must be spent in order to validate these methodologies for regulatory acceptance.

These considerations lead to the conclusion, that the ES cell is a promising model suitable for the identification teratogenic effects in the major target tissues. As before, much effort must be spent in order to harmonise and standardise the methodologies to consider them for regulatory acceptance.

As described above, detection of malformations is only one of the aspects that have to be analysed in developmental toxicity studies. IUGR is considered one of the principal developmental toxicity manifestations. Several agents are known to cause IUGR (anti-convulsant drugs, alcohol, anticancer agents, smoking and illicit drugs) and often a potent teratogen produces IUGR effects. Indeed, IUGR is common in infants with severe and multiple malformations (Warkany, 1971).

Nonetheless, no *in vitro* systems capable of identifing IUGR effects are available to date. Only animal models (rat, mouse and pig) are used to clarify pathogenetic and pathophysiologic aspects of IUGR manifestations.

Recently, the capacity of mouse ES cells to generate embryoid bodies (EBs) in culture was used to develop a methodology for IUGR detection *in vitro*. After chemical exposure, the EBs growth was monitored by using image analyses to identify possible deviants to the normal size of EBs. In order to control the reliability of the test, the effects of 5 different compounds were investigated (Boric Acid, 5-Fluorouracil, 6-Amminonicotinamide, Methotrexate and Saccharin). The results obtained demonstrated that the EBs monitoring growth is a potential endpoint for the IUGR evaluation (manuscript in preparation). However, to verify the reliability of the test more compounds have to be evaluated. The monitoring of the EBs growth could be used as a toxicological endpoint in developmental toxicity testing. In order to exclude specific effects to the embryo, the test could be performed for those substances with unclear embryo-toxicity data as proposed (Bremer and Hartung, 2004).

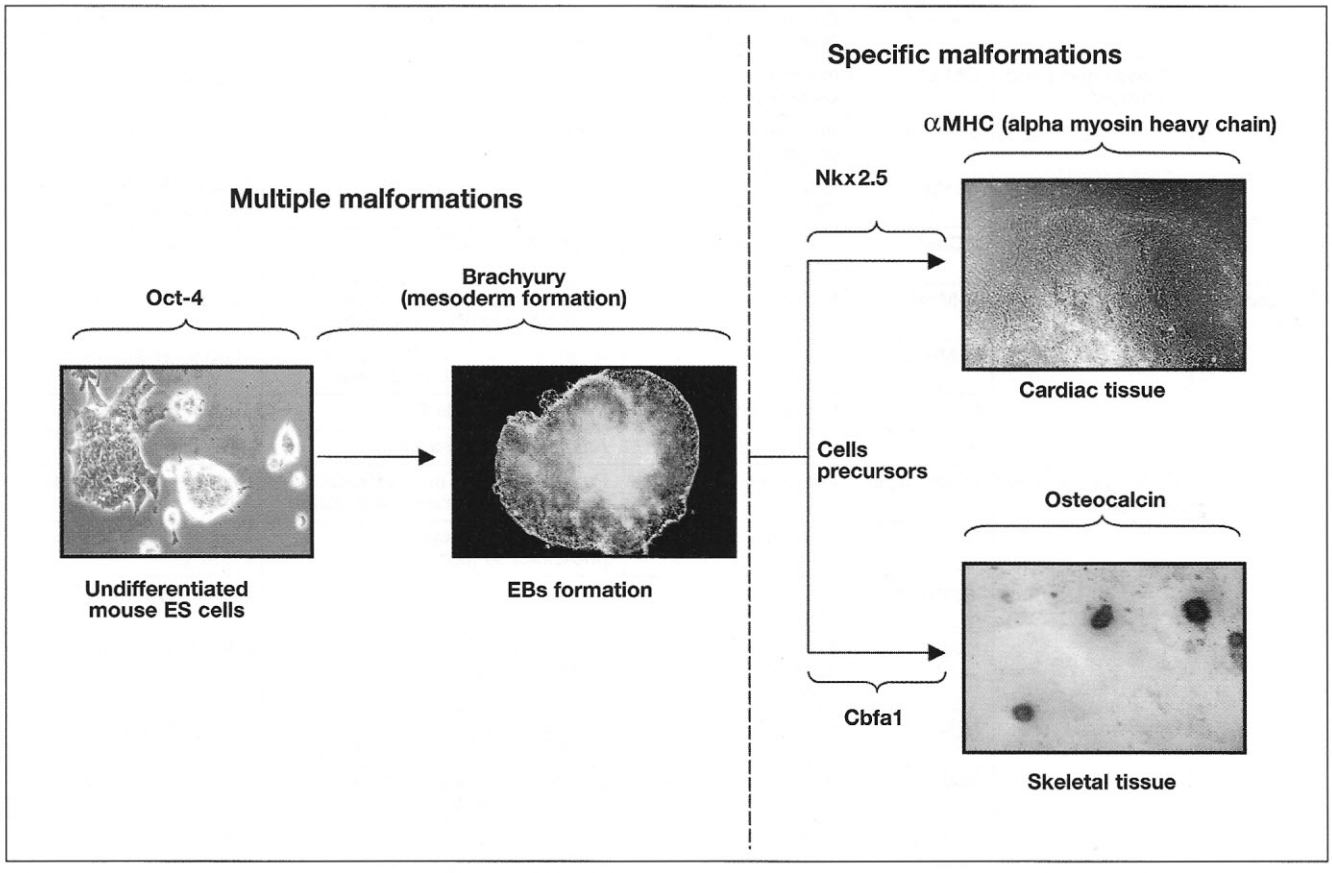

Fig. 2: Detection of multiple or specific malformations *in vitro* by using mouse ES cells.
In the scheme a gene expression analysis, performed during mouse ES cells differentiation into cardiac and skeletal tissue is proposed. If the chemical tested interferes with Brachyury and/or Oct-4 expression multiple malformations are expected. In contrary, if the chemical acts later, during cell precursors formation or even later during tissue formation, specific malformation occur.

Tab. 2: List of suitable genes helpful in *in vitro* teratogenicity studies.

Stage of development		Gene name	Expression	Description	References
Undifferentiated state		Oct-4	In pluripotent embryonic stem cells	POU transcription factor, essential for the initial formation of pluripotent founder cell population in the mammalian embryo	Scholer, 1991
		Tert	In pluripotent embryonic stem cells	Telomerase reverse transcriptase	Meyerson et al., 1997
Germ layers formation	Mesoderm	Brachyury	During mesoderm formation	Transcription factor necessary for normal mesoderm formation	Cunliffe and Smith, 1992
		BMP-4	Induces bone formation in vertebrates	Bone morphogenetic protein 4	Rohwedel et al., 1998
	Ectoderm	ASH-1	Early neuronal crest development, autonomic neuronal progenitors	Achaete-scute homologue, transcription factors	Rohwedel et al., 1998
		Pax6	During ectoderm formation	Trascriptional factor (Paired box gene6)	Rohwedel et al., 1998
	Endoderm	HNF-4	During endoderm formation	Hepatocyte nuclear factor 4, transcription factor	Abe et al., 1996
		AFP	During endoderm formation	Alpha-fetoprotein, transcription factors (AT motif-binding factor)	Abe et al., 1996
Progenitors cells	Cardiac	Nkx2.5	During cardiac precursors formation	Transcription factor	Lints et al., 1993
		GATA-4	During cardiac precursors formation	GATA binding protein, transcriptional factor	Leahy et al., 1999
	Chondrogenic and Osteogenic	CbFa1	In early osteoblast progenitor cells	Transcription factor	Ducy et al., 1997
		Scleraxis	In mesenchymal progenitors	Transcription factor	Asou et al., 2002
	Neuronal	Nestin	In the neurons progenitors	Intermediate filament protein	Suarez-Rodriguez and Belkind-Gerson, 2004
		NCAM	In the neurons progenitors	Neural cell adhesion molecule	Bain et al., 1995
ES-derived cells	Cardiac	A-MHC	In the cardiac cells	A part of heavy chains myosin involved in kinetic processes	Maltsev et al., 1993
		B-MHC	In the cardiac cells	A part of heavy chains myosin involved in kinetic processes	Maltsev et al., 1993
		MCL-2V	In the cardiac cells	Myosin light chain 2	Fassler et al., 1996
		ANF	In the cardiac cells	Atrial natriuretic factor	Fassler et al., 1996
	Chondrogenic and osteogenic	Osteocalcin	In mature osteoblast	Protein secreted in to extracellular matrix during bone mineralization	Desbois and Karsenty, 1995
		BAP	In mature osteoblast	Bone specific alkaline phosphatase (enzyme)	Xiao et al., 2004
		CollagenIIB	In mature chondrocytes	Cartilage matrix protein	Kramer et al., 2000
		Sox-9	Expressed at high levels in chondrocytes	HMG-box containing transcription factor	Asou et al., 2002
	Neuronal	NFL	In mature neurons	Gene encoding structural neurofilament protein (68-kDa)	Bain et al., 1995
		MAP-2	In mature neurons	Microtubule-associated protein-2. Dendritic-specific MAP	Wiche et al., 1986
		Synapto-physin	In mature neurons	Gene enconding integral membrane protein of small synaptic vesicles	Singec et al., 2002
		Neuronal tubulin	In mature neurons	Important structural protein for differentiated neuron	Menezes and Luskin, 1994

6 Humanisation of mouse ES cell existing model and ethical aspects

An advantage of the use of the ES cells for developmental toxicity testing is the availability of human ES (hES) cells (Thomson et al., 1998). In fact, the existing test based on murine ES cells could be adapted to hES cells. Not all mammalian species are equally susceptible or sensitive to the toxic influence of a chemical and for this reason a humanised system should be more predictive. Indeed, a chemical can be teratogenic in one species but not in other species. Human models will avoid interspecies variations, which are a crucial factor in developmental toxicity testing, hence no extrapolations to human might be necessary any more.

Recent publications have demonstrated that hES cells are able to produce EBs, expressing genes involved in all three germ layers formation and in differentiation of several cell types (Schuldiner et al., 2000). In addition, the ability of hES cells to differentiate *in vitro* into neuronal, skin, adrenal and keratinocyte (Reubinoff et al., 2000; Zhang et al., 2001; Green et al., 2003), blood, endothelial, kidney, bone, muscle, heart (Kaufman et al., 2001; Kehat et al., 2002, 2003; Levenberg et al., 2002; Xu et al., 2002; He et al., 2003), pancreas and liver cells (Schuldiner et al., 2000, Assady et al., 2001; Eiges and Benvenisty, 2002) has been demonstrated. Moreover, several studies have shown the capacity of hES to generate cardiomyocytes (Kehat et al., 2002; Kehat et al., 2003; He et al., 2003; Xu et al., 2002) electrophysiologically similar to normal human cardiomyocytes, and foetal ventricular myocytes (Mummery et al., 2003). In the validated EST cardiomyocytes differentiation represents an endpoint and hES cells could substitute mouse cells in the test.

This capability of the hES cells to differentiate into all germ layer derivatives promises to provide a system able to identify possible effects on a wide range of target tissues. In addition, the systems based on hES will avoid interspecies differences.

Moreover, the use of hES in research will also offer the chance to study human development, providing more precise information concerning events that cannot be studied directly in the human fetus. Using this information, the pre-vention and the treatment of unusual human development might become possible. In addition, the reduction of risks related to drug exposure should be achievable.

The optimisation and the standardisation of hES cell *in vitro* models are the most important goals. However, at the moment hES cell research represents an ethical issue. In order to respect the ethical and moral value of the human embryo, many European countries have taken legislative actions (see Tab. 3). At the same time the European Community supports the expansion of funding for hES cell research. Indeed, in the contest of the 6th Research Framework Programme (FP6), adopted in 2002, the European Union (EU) provided financial support for hES cell research, especially to fight major human diseases.

In order to protect the human embryo, in 2003 guidelines on EU-funded hES cell research were adopted by the Commission (Anon, 2003a). The aim of the Commission proposal was not to create ethical principles that must be respected by the EU Member States, but to set up conditions for EU FP6 research, in which stem cells from human supernumerary embryos are involved.

The most important topics regulate by the Commission proposal are the following:

- "EU will not fund human embryonic stem cell research where it is forbidden by a Member State";
- "Human embryonic stem cells can only be derived from supernumerary embryos that are donated for research by parents and that were created before 27 June 2002, the date of the adoption of the Framework Programme";
- "Potential research project partners applying for EU funding must seek ethical advice at national or local level in Member States where the research will take place, even in countries where obtaining such ethical advice is not mandatory";
- "Research will be funded only when it is demonstrated that it meets particularly important research objectives";

Tab. 3: Regulation of the use of human embryonic stem cells in the European Member States (update September 2003).

Country	Policy adopted
Italy, Luxembourg, Portugal	No specific regulation concerning human ESC research
Austria, Spain, France, Ireland	Legal veto for the derivation of ES cells from human embryos
Austria, Denmark, Germany, Spain, Finland, France, Greece, Ireland, Netherlands, Portugal	Legal veto for the production of human embryos for research aims. Veto for the derivation of ES cells from human embryos, by law or by ratification of the Convention of the Council of Europe on Human Rights and Biomedicine (Oviedo on April 4, 1997)
Belgium, Denmark, Finland, Greece, Netherlands, Sweden, United Kingdom	Legal permission for the derivation of hES cells from supernumerary embryos
Germany	Legal veto for the derivation of ES cells from human embryos. Legal permission for the import of hES cell lines, which were created before January 2002
Belgium, United Kingdom	Legal permission for the production of human embryos for ES cells research

Compiled from Anon, 2001e and Anon, 2003b

- "Research will be funded only when there is no adequate alternative available. In particular, it must demonstrated that one cannot use existing embryonic or adult stem cell lines";
- "Traceability of stem cells will be required".

The employment of hES cells in the research field is encouraged by the EU. In fact, within FP6 the EU sponsors an Integrated Project titled "ReProTect", with a duration of 5 years and a budget of 9.1 millions of EURO. 35 different partners from academia, SMEs, governmental institutions and others are involved. The principal aim of ReProTect is to create new *in vitro* models and use existing *in vitro* models in order to set up a test strategy that will provide information on the chemical hazards to the mammalian reproductive cycle. In ReProTect the investigation of the potential of hES cells for embryotoxicity testing is adressed.

7 Conclusions

The main goal of the present review is to point out the potential of the ES cells in *in vitro* hazard assessment. Many studies have demonstrated the usefulness of ES cells in the identification of developmental toxicity effects of chemicals. Nevertheless, in order to replace the use of animals in laboratory studies, it is necessary to consider many aspects regarding the use of *in vitro* systems. Metabolism and biotransformation in general, are one of the most important topics. Due the complexity of the whole organism, not all aspects can be mimicked *in vitro*, but the creation of human metabolism based-models would be a potent tool that could support the ES cells and other *in vitro* systems. In addition, the understanding of the toxicological mechanisms of congenital defects and genetic and environmental factors in such mechanisms is a crucial topic in developmental studies. For this reason, it becomes obvious that both studies of biological mechanisms of developmental toxicants, their toxicological assessment and exposure to pregnant women will be essential in the future.

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