Embryonic Stem Cells *in vitro* - Prospects for Cell and Developmental Biology, Embryotoxicology and Cell Therapy

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Summary
Embryonic stem (ES) cells are able to differentiate in vitro via embryo-like aggregates, so-called „embryoid bodies", into derivatives of the endodermal, ectodermal and mesodermal lineage. We established standardised protocols for cardiogenesis, myogenesis, neurogenesis and vascular smooth muscle cell differentiation in vitro. The developmentally controlled expression of tissue-specific genes, proteins, ion channels and receptors during ES cell differentiation is the basis of several in vitro approaches: (1) „Loss of function" assays with ES cells containing homozygous mutations of specific genes, (2) „Gain of function" assays with ES cells overexpressing exogenous genes, (3) Developmental analysis of teratogenic/embryotoxic compounds in vitro, (4) Pharmacological assays and the establishment of model systems for pathological cell functions, and (5) The application of differentiation and growth factors for induction of selectively differentiated cells which, in the future, may be used as a source for tissue grafts. We propose the ES cell technology as valuable in vitro system to substitute and reduce the use of animals in basic and applied research.

Zusammenfassung: Embryonale Stammzellen in vitro - künftige Bedeutung für die Zell- und Entwicklungsbiologie, die Embryotoxikologie und die Zelltherapie
Embryonale Stamm (ES)-Zellen können in vitro in embryoähnlichen Aggregaten, sogenannten „embryoid bodies", in Derivate der endodermalen, ektodermalen und mesodermalen Linie differenzieren. Wir etablierten standardisierte Protokolle für die kardiogene, myogene und neuronale Differenzierung sowie die Entwicklung glatter Gefäßmuskelzellen in vitro. Die entwicklungskontrollierte Expression von gewebespezifischen Genen, Proteinen, Ionenkanälen und Rezeptoren während der ES-Zelldifferenzierung war die Grundlage für die Entwicklung verschiedener in vitro Modelle: (1) „Loss of function" Assays mit ES-Zellen, die homozygote Mutationen spezifischer Gene enthalten, (2) „Gain of function" Assays mit ES-Zellen, in denen definierte Gene überexprimiert werden, (3) die Analyse der Wirkung von teratogenen/embryotoxischen Verbindungen auf die ES-Zelldifferenzierung in vitro, (4) pharmakologische Assays mit Herzzellen und die Entwicklung von Modellsystemen für pathologische Zellfunktionen, und (5) die Differenzierungsinduktion mit Differenzierungs- und Wachstumsfaktoren zur selektiven Entwicklung von spezifischen somatischen Zellen, die in der Zukunft als Gewebeersatz bei Transplantationen eingesetzt werden könnten.
Die ES-Zell-Technologie wird dazu beitragen, den Einsatz von Tierversuchen in Grundlagen- und angewandter Forschung zu reduzieren.

Keywords: 3R, replace, reduce, pluripotent embryonic stem cells, in vitro differentiation, pharmacotoxicology, cardiogenesis, neurogenesis.

1 Embryonic stem (ES) cell technology

Embryonic stem (ES) cells, derived from the inner cell mass (ICM) of mouse blastocysts, are pluripotent cells that have the capacity to differentiate into cellular derivatives of endodermal, ectodermal and mesodermal lineages *in vitro* (Fig. 1; Martin, 1981; Evans and Kaufman, 1981; Wobus et al., 1984, 1991; Doetschman et al., 1985). After retransfer of ES cells into the mouse blastocyst, ES cells *in vivo* are able to generate somatic as well as cells of the germ line (Bradley et al., 1984). Therefore, ES cells provided the cellular system for the gene targeting technology to generate genetically deficient mice (Thomas and Capecchi, 1987). *In vitro*, undifferentiated ES cells, when cultivated as embryo-like aggregates, so called „embryoid bodies" (EBs), by using the „hanging drop" (Wobus et al., 1991; Rudnicki and McBurney, 1987), „mass culture" (Doetschman et al., 1985) or „methylcellulose" (Wiles and Keller, 1991; Hole and Smith, 1994) methods, are able to differentiate into cardiogenic (Wobus et al., 1991, 1997a, b; Maltsev et al., 1993, 1994; Hescheler et al., 1997; Miller-Hance et al., 1993; Wobus and Guan, 1998), myogenic (Miller-Hance et al., 1993; Rohwedel et al., 1994, 1995, 1998a; Rose et al., 1994), neurogenic (Strübing et al., 1995; Bain et al., 1995; Fraichard et al., 1995; Okabe et al., 1996), hematopoietic (Schmitt et al.,

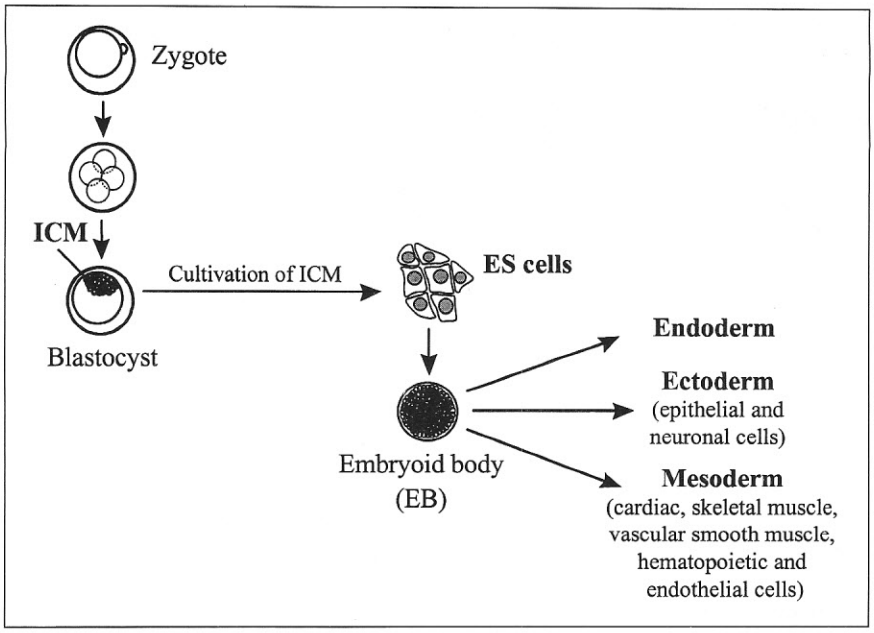

Figure 1: ES cell technology *in vitro*.
Permanent ES cell lines were cultivated from the inner cell mass (ICM) of mouse blastocysts. These pluripotent ES cells, when cultivated as embryoid bodies (EBs), are able to differentiate into cells of the endodermal, ectodermal and mesodermal lineage.

1991; Keller, 1995; Wiles and Keller, 1991; Hole and Smith, 1994) or adipogenic (Dani et al., 1997) lineage. ES cells were found to differentiate also into endodermal (Sauer, 1998), epithelial (Bagutti et al., 1996), endothelial (Risau et al., 1988) and vascular smooth muscle cells (Risau et al., 1988; Weitzer et al., 1995; Drab et al., 1997). During *in vitro* development of EBs, the expression of cardiac-, skeletal muscle-, neuron-, epithelial and vascular smooth muscle-specific genes was analysed by RT-PCR (Wobus et al., 1997). The occurrrence of action potentials and ion channels was studied by patch clamp analysis (Maltsev et al., 1993, 1994). The formation of proteins in differentiated cells of EB outgrowths was investigated by immunofluorescence with conventional fluorescence or confocal laser scanning microscopy (Rohwedel et al., 1998a, b). We found that during ES cell differentiation *in vitro*, tissue-specific genes, proteins, receptors and ion channels were expressed in a pattern closely resembling the pattern observed during mouse embryogenesis *in vivo* (reviewed by Guan et al., 1999). This controlled *in vitro* developmental pattern was the basis for the establishment of several approaches using the ES cell *in vitro* differentiation model: (1) „loss of function" assays (Fässler et al., 1996; Rohwedel et al., 1998b), (2) „gain of function" assays (Rohwedel et al., 1995), (3) developmental analysis of teratogenic/ embryotoxic compounds *in vitro* (Wobus et al., 1994a; Rohwedel et al., in press, Schmidt, in press), (4) pharmacological assays using cardiac cells (Wobus et al., 1991; Wobus et al., 1994b, Pich et al., 1997) and the establishment of model systems for pathological cell functions, and (5) the specific application of differentiation and growth factors for selective cell differentiation (Wobus et al., 1997b, unpublished data).

2 „Loss of function" and „gain of function" assays

We successfully applied the *in vitro* ES cell technology to the analysis of loss of β_1 integrin function on differentiation. *In vivo*, the lack of β_1 integrin function resulted in embryonic death shortly after implantation (Fässler and Meyer, 1995). By using the ES cell differentiation approach *in vitro*, we have found that $\beta_1^{-/-}$ ES cells differentiated *in vitro* into cardiomyocytes, skeletal muscle and neuronal cells, but the normal differentiation process was severely impaired (Fässler et al., 1996; Rohwedel, et al., 1998b). Our data showed that loss of β_1 integrin function resulted in a delayed cardiogenic and myogenic differentiation and a delayed expression of cardiac and skeletal muscle-specific genes. The specification of cardiac precursor cells into pacemaker-, atrium- and ventricle-like cells was impaired and the sarcomeric architecture was disarranged in $\beta_1^{-/-}$ cells (Fässler et al., 1996, Guan et al., submitted). Myotube formation was reduced and the assembly of sarcomeric structures was retarded. In contrast, neurogenic differentiation of $\beta_1^{-/-}$ ES cells showed an accelerated expression of neuron-specific genes and an increased number of neuronal cells (Rohwedel et al., 1998b). The lineage-specific effects of loss of β_1 integrin function were reflected by the differential expression of genes encoding lineage-specific transcription factors (Brachyury, Pax-6, Mash-1) and signaling molecules (BMP-4 and Wnt-1). Because of the reduced and delayed expression of the BMP-4 encoding gene in $\beta_1^{-/-}$ cells, the regulatory role of exogenously added BMP-4 was analyzed during differentiation of wildtype and $\beta_1^{-/-}$ cells. An accelerated/ upregulated expression of Brachyury and a shift of Wnt-1 expression to terminal stages was found in both wildtype and $\beta_1^{-/-}$ cells. The data suggested that the reduced expression of BMP-4 may account for the accelerated neuronal differentiation in $\beta_1^{-/-}$ ES cells (Rohwedel et al., 1998b).

In conclusion, *in vitro* differentiation of mutant ES cells served as an excellent alternative strategy in all those cases where the gene defect resulted in early embryonic lethality to unreveal the function of those genes which are indispensable *in vivo*.

The *in vitro* differentiation of ES cells is also used in the „gain of function" strategy. Constitutive expression of exogenous genes in ES cells was found to influence the *in vitro* differentiation pattern (Rohwedel et al., 1995; Helgason et al., 1996; Dinsmore et al., 1996). For example, overexpression in ES cells of the gene encoding the myogenic transcription factor M-twist, a negative regulator of myogenic differentiation, resulted in a delayed and reduced myogenic differentiation and skeletal muscle-specific gene expression depending on the level of exogenous M-twist expression (Rohwedel et al., 1995). Thus, *in vitro* „gain of function" studies using ES cells may provide an alternative to transgenic animals.

3 Analysis of embryotoxic/teratogenic factors

A new *in vitro* embryotoxicity test is the ES cell test (EST) established by Spielmann et al. (1997). In the EST, besides other parameters, the influence of test chemicals on the differentiation of ES cells into contracting cardiomyocytes at day 10 is used as indicator for the classification of the test chemicals into different embryotoxicity classes (Scholz et al., 1998; Spielmann et al., 1997).

We used mouse ES cells to investigate the teratogenic capacity of lithium. Lithium therapy is common in the treatment of manic depressive psychosis and other psychiatric disorders. However, there are several associated drawbacks. Lithium has a small therapeutic/ toxic ratio and must be taken over a long period of time. Teratogenic effects of lithium have been demonstrated in mice and rats (Marathe and Thomas, 1986; Hoberman et al., 1990).

In our study, we found that treatment of EBs with LiCl resulted in an inhibition of cardiac and myogenic differentiation, but not of neuronal cells (Fig. 2a and b; Schmidt, 1999). The expression of the cardiac-specific gene α-MHC was significantly inhibited under LiCl treatment (10^{-3} M) during ES cell differentiation *in vitro* (Fig. 2c). 10^{-3} M LiCl treatment also resulted in a slightly decreased expression of the skeletal muscle-specific gene myoD (Fig. 2d). However, the expression of the gene encoding the synaptic vesicle protein synaptophysin was accelerated/ increased by LiCl treatment during ES cell differentiation *in vitro* (Fig. 2e), whereas the expression of the Mash-1 gene encoding a transcription factor required for development of multiple neuronal lineages was remarkably decreased (Fig. 2f).

In conclusion, our results demonstrated that LiCl treatment influenced the differentiation of ES cells into cardiac and myogenic cells and the expression of cardiogenic, myogenic and neurogenic genes. Therefore, we propose to include into the EST not only a morphological differentiation marker (= cardiac differentiation), but also specific gene expression profiles after treatment with test substances (see Wobus et al., 1996).

In our former study, we found that retinoic acid (RA) influenced the *in vitro* differentiation of ES cells in a time- and concentration-dependent manner (Wobus et al., 1994a). Treatment with high concentrations of RA (10^{-7} and 10^{-8} M RA) during the first two days or between day 2 and 5 of EB differentiation increased neurogenesis (Strübing et al., 1995), but significantly inhibited cardiogenesis (Wobus et al., 1994a). In addition, incubation of EBs with RA (10^{-8} M) between days 2 and 5 of EB development resulted in an induced myogenic (Wobus et al., 1994a) and adipogenic differentiation (Dani et al., 1997). In contrast, treatment of EBs with 10^{-8} M RA after day 5 resulted in an induced cardiac (Wobus et al., 1994a and 1997b) and vascular smooth muscle cell differentiation (Drab et al., 1997), but an inhibited myogenic and adipogenic differentiation.

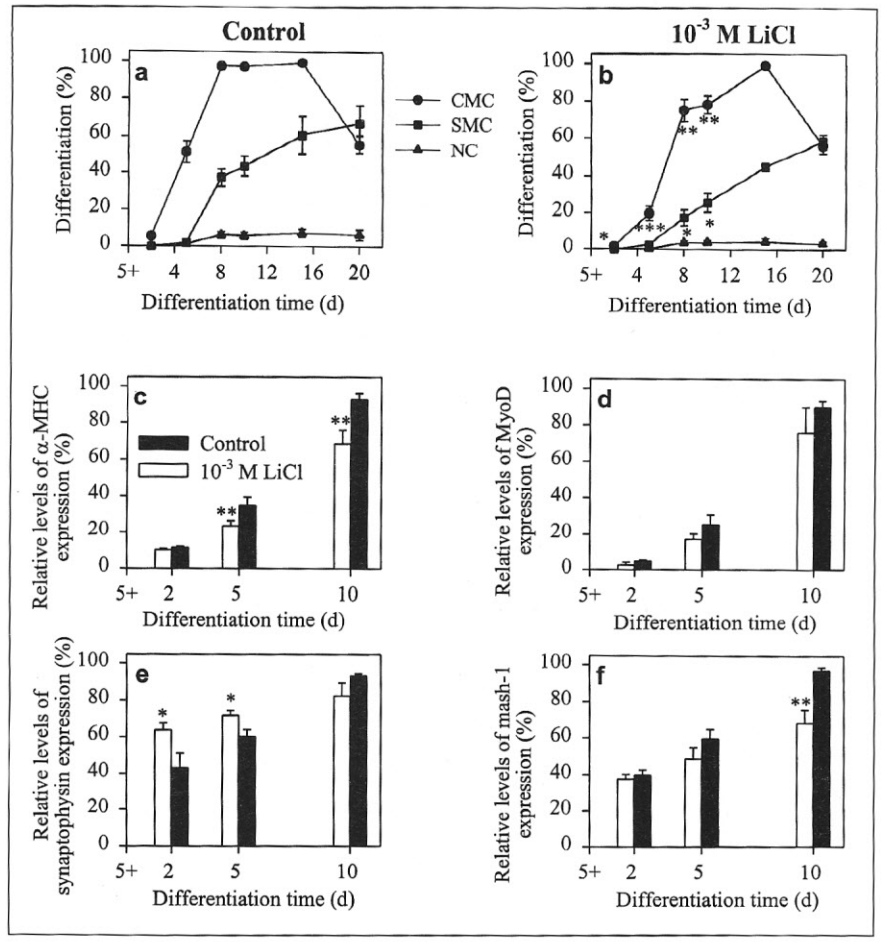

Figure 2: The influence of LiCl on cardiac, myogenic and neuronal differentiation of ES cells and the expression of tissue-specific genes.
(a, b): Percentage of EBs containing cardiac, skeletal muscle and neuronal cells during the differentiation of control (untreated, a) and LiCl-treated (b; 10^{-3} M, from day 0 to 5 + 10) EBs.
(c-f): Relative levels of the expression of cardiac-specific α-MHC (c), skeletal muscle-specific myoD (d) and neuron-specific synaptophysin (e) and Mash-1 (f) genes during the differentiation of both control and LiCl-treated EBs analysed by semi-quantitative RT-PCR (Methods see Wobus et al., 1997b). Significance was tested by the Student's t-test (*: $p \leq 0.001$; **: $p \leq 0.01$; *: $p \leq 0.05$).**

In summary, the concentration- and stage- specific effects of retinoic acid and lithium chloride on ES cell differentiation and gene expression demonstrated the suitability of the ES cell *in vitro* model for the analysis of teratogenic/ embryotoxic compounds during early development. The further improvement of the embryonic stem cell test (EST, Spielmann et al., 1997; Genschow et al., 1999) will be helpful to analyse, in the future embryotoxic/ teratogenic agents on a large scale *in vitro*.

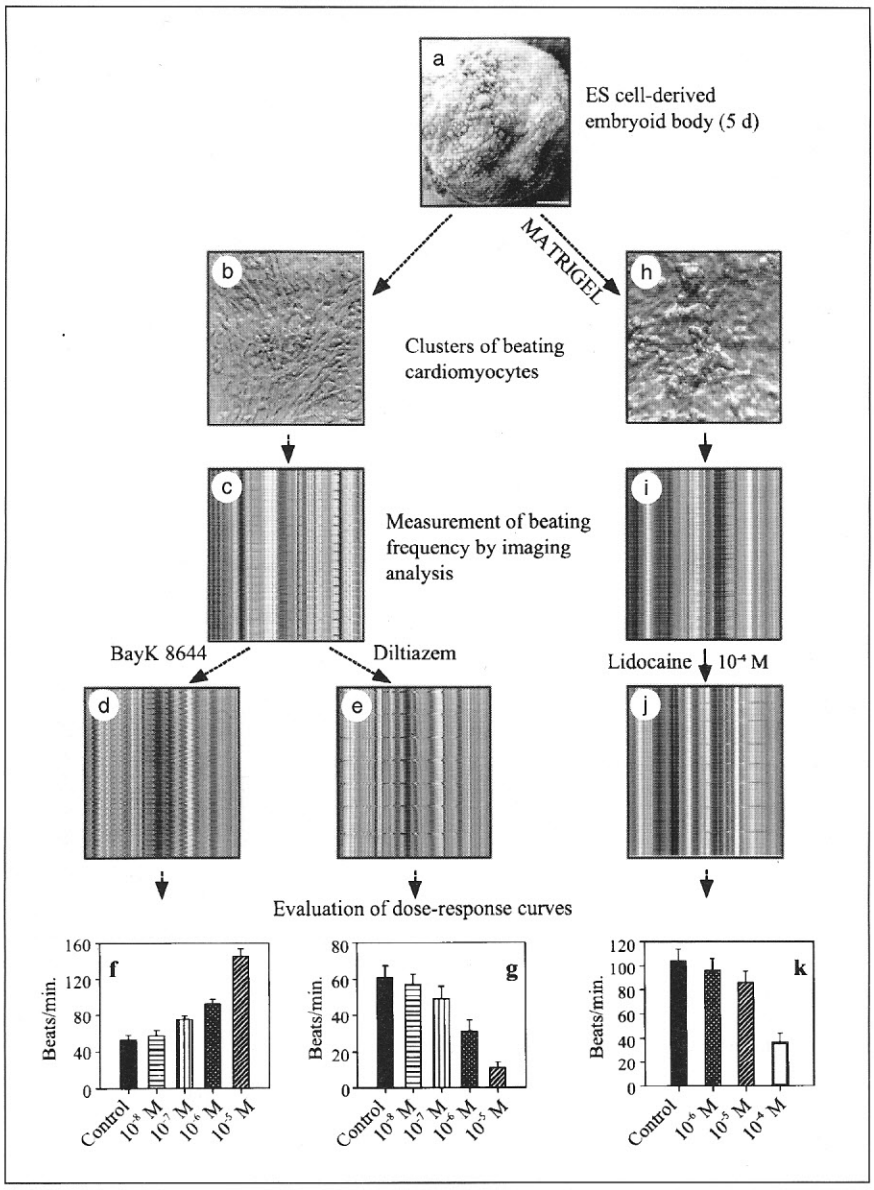

Figure 3: ES cell technology in vitro used for pharmacological investigations on cardiac cells.
ES cells were cultivated as embryoid bodies (EBs) in hanging drops for 2 days and in suspension for another 3 days. After plating of 5-day EBs (a; morphology is shown by SEM; bar = 50μm), spontaneously and rhythmically beating clusters of cardiomyocytes develop in the EB outgrowths (b). Beating clusters of cardiomyocytes were selected and the basal level of the frequency was measured and digitized by the LUCIA 'Heart' imaging system (c). Positively (BayK8644; d) or negatively (Diltiazem; e) acting chronotropic drugs were cumulatively added to the beating clusters and the resulting data used to establish dose-response curves (f, g).
Cardiac clusters differentiated in the presence of MATRIGEL (h) revealed higher beating frequency and arrhythmias (i) in comparison to control cells (c) at day 5 + 32. Lidocaine (10^{-4} M) significantly reduced the beating frequency and revealed an anti-arrhythmic effect on cardiomyocytes (j, k).

4 *In vitro* models for studying pharmacological responses and pathological cell functions

In our earlier studies (Wobus et al., 1991 and 1994b; Maltsev et al., 1993; 1994; Pich et al., 1997), we found that cardiomyocytes differentiated from ES or EC (embryonic carcinoma) cells displayed characteristic chronotropic responses to cardioactive drugs (Fig. 3b-g; Pich et al., 1997) comparable to heart cells that developed *in vivo*. The expression of pharmacological functions of *in vitro* differentiated cardiomyocytes in EB outgrowths enabled us to use a computer-assisted imaging device for the establishment of a semi-automatic screening method (Fig. 3) to study pharmacological effects of cardioactive drugs (Pich et al., 1997).

Recently, we have analysed beating clusters in EB outgrowths treated by extracellular matrix proteins (MATRIGEL; Fig. 3h-k). Our studies showed that cultivation of EBs in the presence of MATRIGEL resulted in the development of cardiomyocytes characterized by high frequency and arrhythmias (Fig. 3i) compared to untreated cells (Fig. 3c). We found that lidocaine significantly reduced the beating frequency and revealed an anti-arrhythmic effect on cardiomyocytes which differentiated in the presence of MATRIGEL (Fig. 3j, k), while cardiomyocytes of the control were not significantly affected (data not shown).

In the future, the ES cell differentiation model may be used to study the regulation of development of pathological cell types. Both strategies, the pharmacological and the embryotoxicological screening systems may help to reduce the use of living animals in pharmacotoxicology.

5 Selective differentiated cells as cellular grafts

The ES cell technology has also been used to obtain cell populations of specific lineages *in vitro*. We used the ventricle-specific isoform of the regulatory myosin light chain (MLC-2v) promoter fused to the β-galactosidase gene (selectable marker) as an indicator for RA-induced ventricular differentiation (Wobus et al., 1997b). It was found that RA caused a significant acceleration of cardiac differentiation and an increase of β-galactosidase activity (Fig. 4a-c, see also Wobus et al., 1997b). These effects of RA were reflected by an enhanced expression of cardiac- and ventricle-specific genes and an increased number of ventricle-like cells (Wobus et al., 1997b).

For specific differentiation of neuronal cells, we applied growth and differentiation factors (Okabe et al., 1996) and cultivation conditions (unpublished data) during ES cell development *in vitro* to induce neuronal precursor cells and then spe-

cifically induce proliferation and differentiation of dopaminergic neurons (Fig. 5). Nestin-positve neuronal precursor cells were induced in more than 80% of the total cell population. These precursor cells are then differentiated into dopamine-2 receptor (D_2R)-positive neurons (Fig 5c). The maximal percentage of D_2R-positive cells (Fig. 5c) in neurofilament protein (160 kD)-labelled neuronal cells (Fig. 5b) could reach about 50 % (unpublished data).

6 Conclusions and future prospects

The establishment of the various ES cell differentiation models allows us to study cellular differentiation processes during early embryonic development *in vitro* and permit the analysis of differentiation of early embryonic cells via progenitor cells into highly differentiated and specialized cells of many lineages. In addition, the differentiation of genetically modified cells by "gain of function" (Rohwedel et al., 1995) and "loss of function" (Fässler et al., 1996; Rohwedel et al., 1998b) assays *in vitro* is an excellent substitute and alternative to the use of transgenic animals.

These techniques are especially relevant with respect to the use of human pluripotent embryonic stem cells which are now being available (Thomson et al., 1998). In addition, pluripotent primordial germ cell-derived, so-called embryonic germ (EG) cells have been isolated from human fetal tissue and cultivated as permanent undifferentiated cell lines (Shamblott et al., 1998). In the future, human ES/EG cells may be of special interest for studying early embryonal development by differentiation analysis *in vitro*. In the future, xenobiotics acting as embryotoxic or teratogenic factors may be analysed on human cells *in vitro* for their capacity to affect developmental processes. The ES cell differentiation system is also suitable for a routine screening of pharmacological functions on differentiated cardiomyocytes (Wobus et al., 1991; Maltsev et al., 1994; Pich et al., 1997). The embryotoxicological approaches together with pharmacological, electrophysiological and confocal imaging analyses of differentiated ES cell-derived phenotypes (Hescheler et al., 1997) might help to reduce the use of laboratory animals in pharmacotoxicology.

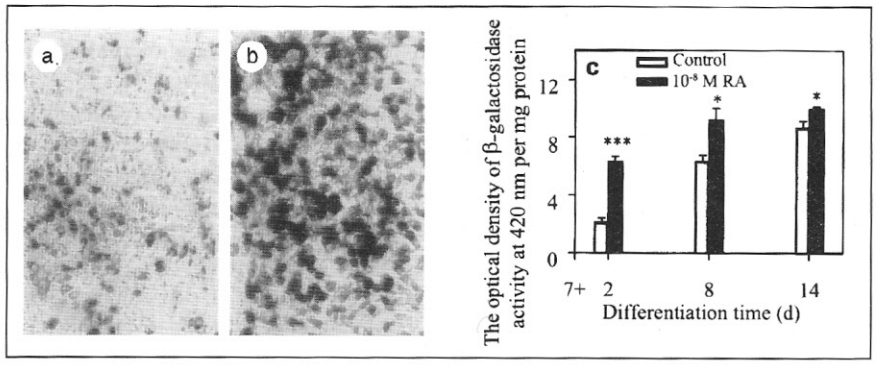

Figure 4: Increased number of b-galactosidase-positive cells in EB outgrowths derived from pGNA/MLC2.1-transfected ES cells after RA treatment (10^{-8} M; from day 5 to 15; b) compared to control (untreated; a) cells at day 7 + 8. The b-galactosidase activity in relation to cellular protein content (c) was evaluated in control and RA-treated variants. Each data point represents the mean value ± standard error of the mean. Statistical significance was tested by the Student's t-test, significance levels: *p ≤0.05; ***p ≤0.001. Bar = 40μm.

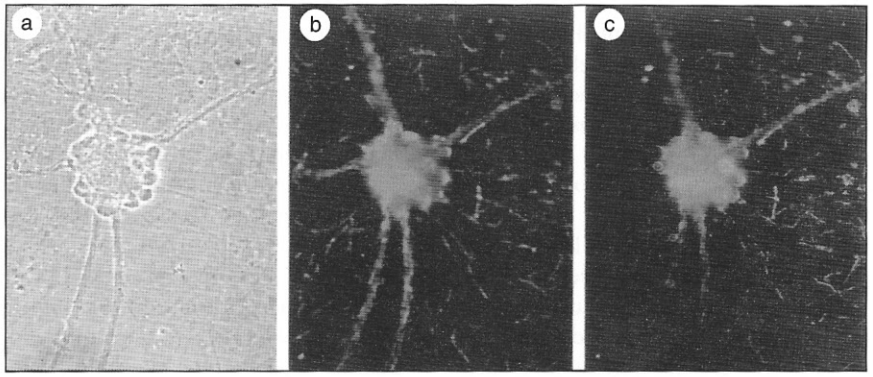

Figure 5: Double immunofluorescence analysis of D_2R-positive (c) and NFM (b) labelled neuronal cells at day 4 + 23 after selective differentiation of R1 ES cells into dopaminergic neurons. Phase contrast (a) of the same field shown in (b) and (c). Bar = 40 μm.

By using human stem cells, the ES cell *in vitro* technology may be of special interest to obtain homogeneous populations of differentiated cells to be used for cellular transplantations. For example, ES cells differentiated into GABAergic (Strübing et al., 1995; Dinsmore et al., 1996) or dopaminergic (Okabe et al., 1996; Dinsmore et al., 1998) neurons, cardiac ventricular (Wobus et al., 1997b) or haematopoietic cells (Hole and Smith, 1994; Potocnik et al. 1997) might be used as a novel source of cells for somatic cell therapy, to be used in degenerative disorders. Transgenic ES cell lines carrying tissue-specific promoters fused to selectable marker genes can be differentiated into the specific lineages *in vitro*, and after selection, the differentiated cell population might be used to reconstitute defective tissues (Klug et al., 1996; Dinsmore et al., 1996; Rust et al., 1997).

But, before future applications of the human ES cell technology, international rules should be worked out with respect to ethical implications and the commercial use of human ES cells.

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