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Original Article

Lithium influences differentiation and tissue-specific gene expression of mouse embryonic stem (ES) cells *in vitro*

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ABSTRACT The effects of lithium chloride (LiCl) on differentiation of mouse embryonic stem (ES) cells were investigated in order to evaluate the ES cell test (EST) used in a European Union validation study for screening of embryotoxic agents *in vitro*. We show that LiCl inhibited concentration-dependently the differentiation of ES cells into cardiac and myogenic cells. Whereas the inhibition of cardiac differentiation by high concentrations of LiCl was obvious at day 5 + 5, decreased skeletal muscle cell differentiation was observed only at day 5 + 8. Semi-quantitative RT-PCR analyses revealed significantly lower levels of mRNA encoding cardiac-specific α-*myosin heavy chain* and skeletal muscle-specific *myoD*. By morphological investigation, an influence of lithium on neuronal differentiation was not evident. However, mRNA levels of genes encoding synaptophysin and the 160 kDa neurofilament protein were increased by high LiCl concentrations, whereas mRNA levels of *mash-1* and *Engrailed-1* were decreased, suggesting a specific influence of lithium on neuronal differentiation. Furthermore, LiCl treatment resulted in a slight, but non-significant increase of β-catenin levels in ES cell-derived embryoid bodies. Our results demonstrate that the ES cell test, EST may be suitable to detect inhibitory effects of test compounds especially on cardiac differentiation, whereas effects on neuronal cells would not be detected. Therefore, we propose that morphological analyses of cardiac differentiation alone are insufficient to detect embryotoxic effects. The assay of other cell lineages at different developmental stages, and expression analyses of tissue-specific genes should also be employed.

KEY WORDS: *Lithium, embryonic stem cell differentiation, cardiac cells, neuronal cells, skeletal muscle cells.*

Introduction

Medical drugs and xenobiotics administered during pregnancy may interfere with embryonic development, and as a consequence induce embryolethal or teratogenic effects. Up to now, tests performed in laboratory animals are the only option currently available for assessing the possible effects of chemicals on reproduction. Routinely, test chemicals are analysed by segment studies, which cover preconceptional exposure and postnatal development including the lactation period (Spielmann, 1998). These *in vivo* tests are time consuming, expensive and have to be carried out on high numbers of laboratory animals. Therefore, there is a need for alternatives to living animals to test the potential reproductive toxicity of chemical substances by *in vitro* systems.

Over the past 20 years, different cellular systems have been proposed and were used as *in vitro* tests for developmental toxicity including established cell lines, such as 3T3 fibroblasts (Spielmann *et al.,* 1997), mouse ovarian tumour cells (Braun *et al.,* 1982), primary cultures of human embryonic palate mesenchymal cells (Pratt *et al.,* 1982), and limb bud cells in the 'micromass culture' test (Flint and Orton, 1984).

One of the recently developed *in vitro* approaches is based on blastocyst-derived pluripotent embryonic stem (ES) cells of the mouse, the so called ES cell test (EST; Spielmann *et al.,* 1997). ES cells have been found to differentiate *via* embryoid bodies (EBs) *in vitro* into differentiated cells of the cardiogenic, myogenic, neuronal, haematopoietic, adipogenic or chondrogenic lineage, as well as into endodermal, epithelial, endothelial or vascular smooth muscle cells (for reviews see Wobus and Guan, 1998; Wobus and Boheler, 1999).

The ES cell test is one of three *in vitro* embryotoxicity tests which are currently evaluated in an ECVAM (European Centre for the Validation of Alternative Methods) validation study of the

Abbreviations used in this paper: ES, embryonic stem; EST, embryonic stem cell test; MHC, myosin heavy chain.

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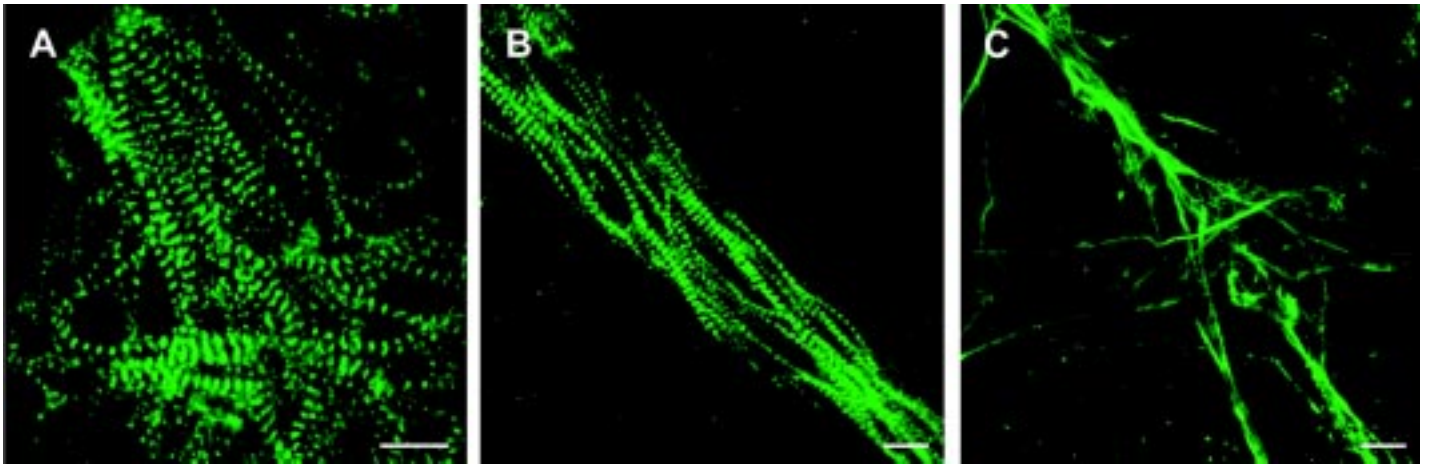

Fig. 1. Immunofluorescence analysis of cardiac, skeletal muscle and neuronal cells derived from embryonic stem cells. *Immunostaining with monoclonal antibodies labelling the Z-band specific epitope of the sarcomeric protein titin of* **(A)** *cardiac and* **(B)** *skeletal muscle cells, and* **(C)** *the 160 kDa neurofilament protein of neuronal cells in EB outgrowths at day 5 + 15. Scale bar, 10μm.*

European Union (Scholz *et al.,* 1999). In this test, ES cells are cultivated in 'hanging drops' as EBs for 3 days followed by suspension culture for 2 days, and plating of EBs at day 5. The influence of test chemicals on differentiating ES cells applied at days 0, 3 and 5 is analysed by the determination of beating cardiomyocytes in EB outgrowths in parallel to cytotoxic effects on ES cells and 3T3 fibroblasts (Spielmann *et al.,* 1997; Scholz *et al.,* 1999). The cytotoxicity data of ES and 3T3 cells on day 10 and the inhibition of cardiac differentiation of ES cells at day 5 after plating 5-day-old EBs (5 + 5 d) are the basis of a prediction model to classify test chemicals into three embryotoxicity classes, such as 'not embryotoxic', 'moderate embryotoxic' and 'strong embryotoxic' (Spielmann *et al.,* 1997; Scholz *et al.,* 1999).

In the present study, lithium was chosen as test compound, because teratogenic effects of lithium have been demonstrated in mice (Szabo, 1970; Smithberg and Dixit, 1982), rats (Marathe and Thomas, 1986; Hoberman *et al.,* 1990) and human after lithium exposure during the first trimester of pregnancy (Jacobson *et al.,* 1992). Lithium is therapeutically used in the treatment of manic depressive psychosis and psychiatric disorders and is able to cross the placenta (Schou and Amdisen, 1975).

Aim of our study was to analyse the effects of lithium on ES cell differentiation and expression of tissue-specific genes to evaluate the ES cell test according to Scholz *et al.* (1999). We show that lithium, a substance known to induce dorso-anterior patterning in *Xenopus* embryos (Kao *et al.,* 1986) also affected mouse ES cell differentiation *in vitro* in a tissue-specific manner.

Results

Influence of lithium on cardiogenic, myogenic and neuronal differentiation of ES cells

The differentiation of mouse ES cells into cardiac, skeletal muscle and neuronal cells was shown by immunofluorescence analysis using antibodies against the sarcomeric protein titin (Fürst *et al.,* 1988) for cardiomyocytes (Fig. 1A) and skeletal muscle cells (Fig. 1B) and the 160 kDa neurofilament protein (Strübing *et al.,* 1995) for neuronal cells (Fig. 1C).

By morphological analysis, cardiac clusters were identified by their spontaneous contractions (Fig. 2A). Differentiation of ES cells into skeletal muscle cells was revealed by the formation of myoblasts which fuse into multinucleated myotubes during terminal differentiation (Fig. 2B). Neuronal cells forming a network of neuronal extensions were identified using phase contrast microscopy (Fig. 2C).

To analyse the influence of lithium on differentiation of ES cells, EBs were cultivated in the presence of LiCl at concentrations of 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M and 10^{-4} M for 15 days (0 - 5 + 10 d). We found that LiCl inhibited cardiogenic and myogenic differentiation in a concentration-dependent manner (Fig. 2 D,E): Whereas cardiac and skeletal muscle cell differentiation was significantly inhibited by 5×10^{-3} M and 10^{-3} M LiCl at developmental stages from 5 + 2 to 5 + 10 days, no obvious differences were found after treatment with 5×10^{-4} M and 10^{-4} M LiCl, respectively, in relation to the control (Fig. 2 D,E).

Beating clusters in EBs of the control variant already developed at day 5 + 2, whereas first cardiac clusters in EBs treated with 5×10^{-3} M LiCl were found only six days later (5 + 8 d). The number of EBs containing spontaneously beating cardiomyocytes after 5×10^{-3} M LiCl treatment was significantly lower (20% and 35%) compared to the control (97% and 97%) at days 5 + 8 and 5 + 10, respectively. In addition, the number of EBs containing cardiac cells after 5×10^{-3} M LiCl treatment was significantly lower compared to 10^{-3} M LiCl treatment at days 5 + 5 to 5 + 10 (Fig. 2D).

The first myoblasts in EBs of the control variant were observed at day 5 + 5, however, myoblasts in EBs treated with 5×10^{-3} M LiCl appeared only at day 5 + 8. The number of EBs containing differentiated skeletal muscle cells decreased from 38% and 67% (control) to 1 % and 4 % (5×10^{-3} M LiCl), respectively, at days 5 + 8 and 5 + 20. 10^{-3} M LiCl reduced the number of EBs containing skeletal muscle cells to 17% (5 + 8 d) and 58% (5 + 20 d; Fig. 2E). By morphological analysis of neuronal differentiation, we found less than 10% of EBs containing neuronal cells either in the experimental or in the control group, and no differences in the background levels between LiCl-treated EBs and control variants were observed (Fig. 2F).

In summary, morphological analysis revealed a lithium-induced concentration-dependent inhibition of cardiac and myo-

genic differentiation, whereas no influence on neuronal differentiation was evident.

Influence of lithium on the expression of tissue-specific genes

To test whether lithium treatment of ES cell-derived EBs influenced the expression of tissue-specific genes, mRNA levels of the following genes were analysed by semi-quantitative RT-PCR: The cardiac-specific *α-myosin heavy chain (α-MHC*), the skeletal muscle-specific *myoD*, and neuron-specific genes encoding the synaptic vesicle protein synaptophysin, the 160 kDa neurofilament protein (NFM), transcription factors Engrailed-1 (En-1) and mash-1. Treatment with 5 x 10^{-3} M and 10^{-3} M LiCl resulted in a significant downregulation of *α-MHC* mRNA levels at developmental stages of 5 + 5 and 5 + 10 d (Fig. 3A), whereas lower concentrations of LiCl did not affect mRNA levels compared to control variants. Similarly, skeletal muscle-specific *myoD* mRNA levels were significantly inhibited by 5 x 10^{-3} M LiCl applied during EB differentiation (Fig. 3B). Lower concentrations (10^{-3} M, 5 x 10^{-4} M and 10^{-4} M) of LiCl did obviously not affect *myoD* mRNA levels compared to the control variant (Fig. 3B).

In addition, the expression of four neuron-specific genes known to be expressed during ES cell differentiation *in vitro* (Rohwedel *et al.,* 1998) were analysed (Fig. 4). Treatment of ES cells with 5 x 10^{-3}, 10^{-3} and 5 x 10^{-4} M LiCl, respectively, significantly increased *synaptophysin* mRNA levels at stage 5 + 5 d, however, decreased mRNA levels were detected by LiCl-treatment (5 x 10^{-3} M) at 5 + 10 d (Fig. 4A). *NFM* mRNA levels were significantly enhanced by 5 x 10^{-3} M LiCl at days 5 + 5 and 5 + 10, and by 10^{-3} M LiCl at day 5 + 10 (Fig. 4B). In contrast, significantly decreased *mash-1* and *En-1* mRNA levels were observed by 5 x 10^{-3} M LiCl at days 5 + 5 and 5 + 10 (Fig. 4 C,D), and in the case of *mash-1*, by 10^{-3} M LiCl at day 5 + 10 (Fig. 4C).

These findings demonstrate that in accordance with the morphological differentiation analysis LiCl decreased cardiac and myogenic mRNA levels, whereas in contrast to the morphological analysis of neuronal differentiation, LiCl affected positively or negatively expression levels of neuron-specific genes.

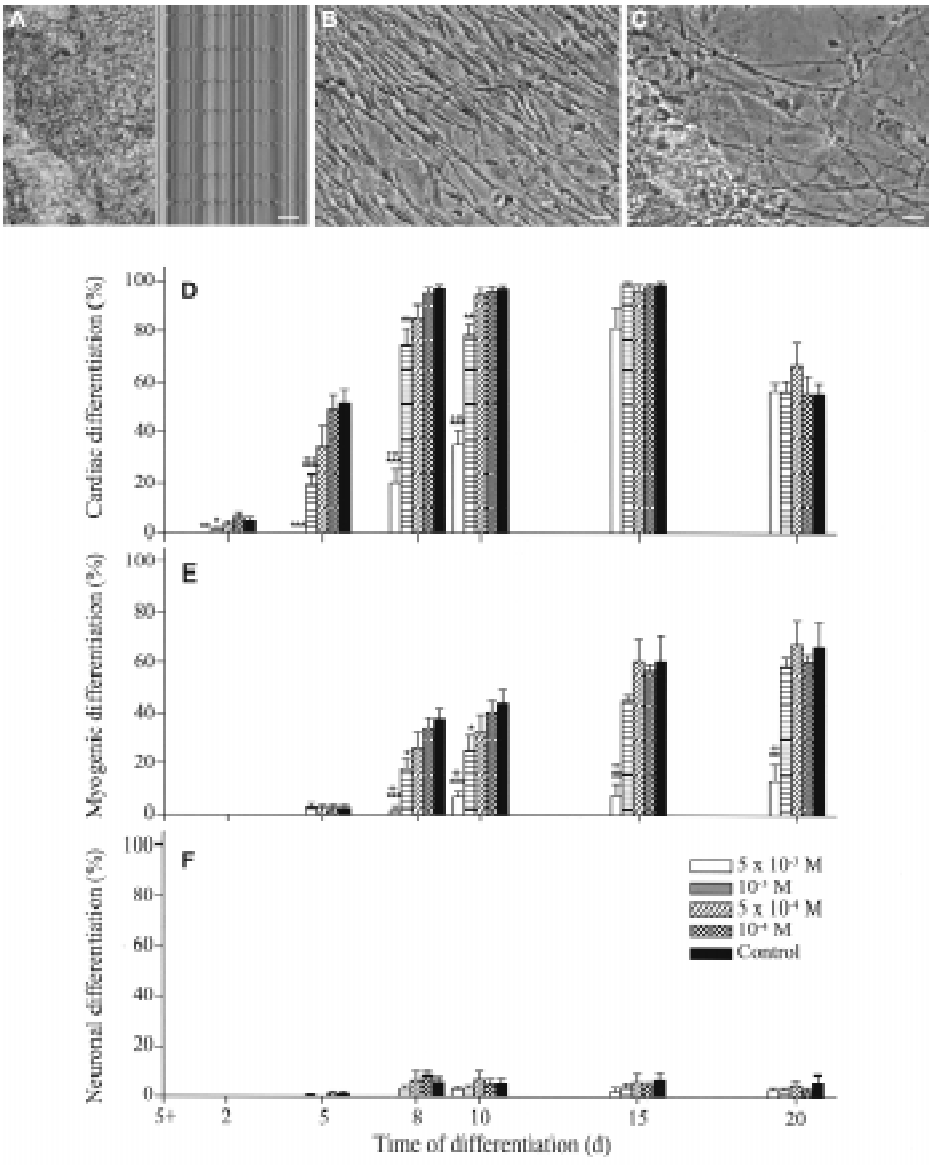

Fig. 2. Morphology and differentiation patterns of cardiac, skeletal muscle and neuronal cells derived from ES cells. *Morphology of* **(A)** *cardiac,* **(B)** *skeletal muscle and* **(C)** *neuronal cells differentiated from ES cells at day 5 + 15. Scale bar, 40μm. Differentiation patterns of ES cells into* **(D)** *cardiac,* **(E)** *skeletal muscle and* **(F)** *neuronal cells after cultivation of EBs in the presence of LiCl at concentrations of 5 x 10^{-3}, 10^{-3}, 5 x 10^{-4} and 10^{-4} M in comparison to the control. EBs were treated with LiCl between day 0 and 5 + 10 and differentiation was analysed from day 5 + 2 up to 5 + 20 in the EB outgrowths. Significance was tested by the Student t-test with significance levels: ***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05 for comparison between 5 x 10^{-3} or 10^{-3} M LiCl-treated and control variants, respectively and +++, p ≤ 0.001; ++, p ≤ 0.01; +, p ≤ 0.05 for comparison between 5 x 10^{-3} M and 10^{-3} M LiCl-treated variants.*

Influence of lithium on β-catenin levels in EBs and cellular distribution of β-catenin

Because lithium has been found to interfere with the Wnt-signaling pathway resulting in the intracellular stabilisation of β-catenin (Lucas and Salinas, 1997; Hall *et al.*, 2000), after LiCl treatment of EBs we analysed the levels and intracellular distribution of β-catenin by quantitative immunofluorescence, Western blot and confocal laser scanning microscopy (CLSM).

Quantitative immunofluorescence analyses revealed increased β-catenin levels in EBs treated with 10^{-2} M, 5 x 10^{-3} M and 10^{-3} M LiCl in comparison to the control at days 5 and 10 (n = 60 EBs each; Fig. 5A), but differences in the relative β-catenin levels were relatively low. To ensure the quantitative immunofluorescence data, immunoblot analyses using antibodies against β-catenin and actin were performed in cell lysates of control and 5 x 10^{-3} M LiCl treated EBs (Fig. 5 B,C). Here, no significant increase of β-catenin levels in LiCl-treated variants in comparison to the control was found (n = 4 experiments; Fig. 5C).

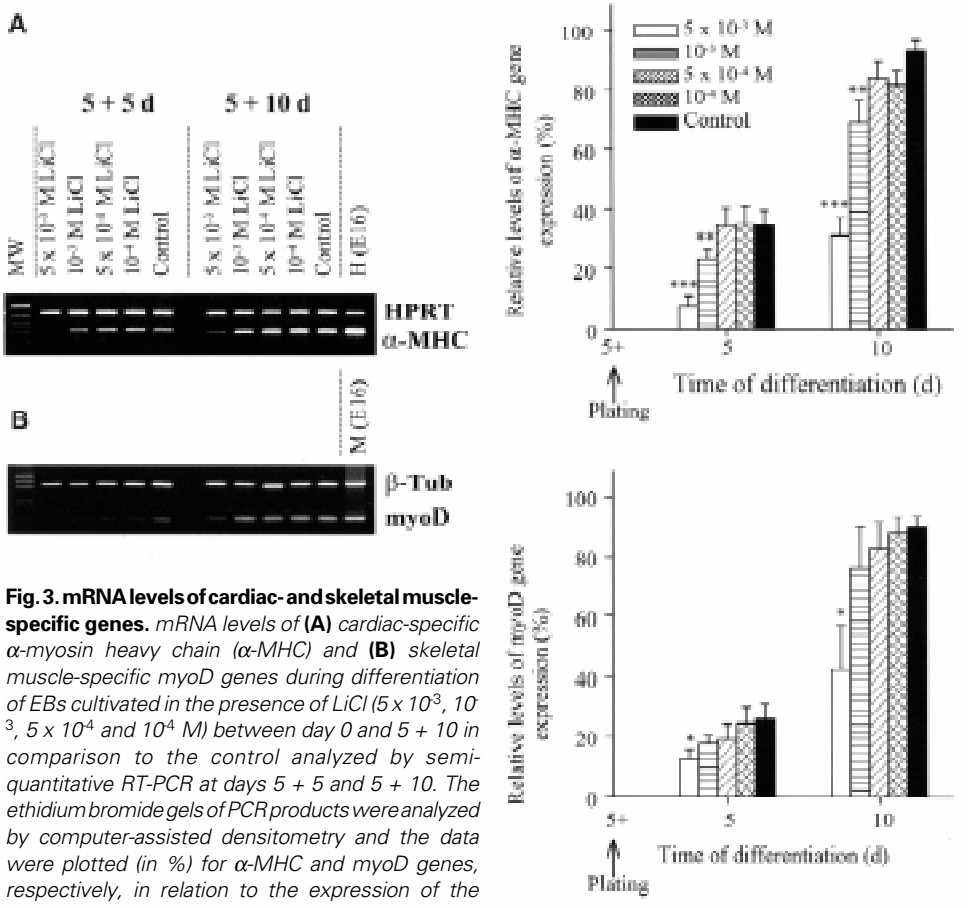

Fig. 3. mRNA levels of cardiac- and skeletal muscle-specific genes. *mRNA levels of* **(A)** *cardiac-specific α-myosin heavy chain (α-MHC) and* **(B)** *skeletal muscle-specific myoD genes during differentiation of EBs cultivated in the presence of LiCl (5 x 10^{-3}, 10^{-3}, 5 x 10^{-4} and 10^{-4} M) between day 0 and 5 + 10 in comparison to the control analyzed by semi-quantitative RT-PCR at days 5 + 5 and 5 + 10. The ethidium bromide gels of PCR products were analyzed by computer-assisted densitometry and the data were plotted (in %) for α-MHC and myoD genes, respectively, in relation to the expression of the housekeeping genes hypoxanthine-guanine phosphoribosyltransferase (HPRT) or β-tubulin (β-Tub) used as internal standards. Heart (H) and skeletal muscle (M) tissue of a 16 day old mouse embryo (E16) were used as positive controls. MW, molecular weight. Significance was tested by the Student t-test (***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05).*

Furthermore, by CLSM analysis of LiCl-treated and control EBs we found no differences in the distribution and intracellular localisation of β-catenin in control (Fig. 5D) and LiCl-treated cells (Fig. 5E). Similar results were found in 5 x 10^{-3} M and 10^{-3} M LiCl-treated EBs (not shown).

Although quantitative immunofluorescence analysis would suggest an influence of LiCl on intracellular β-catenin distribution in ES cell-derived EBs, Western blot and CLSM analyses did not reveal significant LiCl-induced effects with respect to the intracellular level and distribution of β-catenin.

Discussion

By using the ES cell differentiation model and in comparison to the embryonic stem cell test, EST (Spielmann *et al.*, 1997), cardiac, myogenic and neuronal differentiation and tissue-specific gene expression levels were analysed after LiCl treatment during ES cell differentiation. Lithium specifically affected ES cell differentiation and expression levels of tissue-specific genes at a concentration of 10^{-3} M, which is in the range of effective serum concentrations: The lowest therapeutically effective serum level of lithium used for the treatment of manic depressive psychosis is in the range of 0.6 x 10^{-3} M, which is below the toxic threshold value of 1.6 x 10^{-3} M (Schou, 1969). The Danish Register of 'Lithium Babies' (1968) reported data on lithium-exposed children: From a total of 225 cases analysed, 25 (11%) had major congenital malformations. From these patients, 18 (72%) developed cardiac anomalies, and one third of this group showed Ebstein's anomaly, a rare congenital heart defect with an incidence of 1: 20000 (Jacobson *et al.,* 1992). In two of the remaining malformed babies, the central nervous system was affected (Morton and Weinstein, 1976).

Our data show that by applying the EST *in vitro* screening system, a differentiation-inhibiting effect of LiCl would have been detected. High concentrations of LiCl inhibited cardiac and skeletal muscle differentiation and additionally, resulted in decreased mRNA levels of cardiac-specific *α-MHC* (see Wobus *et al.,* 1997) and skeletal muscle-specific *myoD* genes (see Rohwedel *et al.,* 1994). However, in comparison to cardiac differentiation, myogenic differentiation is accomplished at later developmental stages. An influence of LiCl on skeletal muscle cell differentiation would not have been detected by the EST, because it includes morphological analysis of cardiac cells only at day 5 + 5. It means that in addition to cardiac differentiation, other cell types and developmental stages have to be included into the EST.

Whereas morphological analysis revealed no effects of LiCl on neuronal cell differentiation (in fact, low spontaneous neuronal differentiation is found in most ES cell lines, see Strübing *et al.,* 1995), specific differences of mRNA levels of neuron-specific genes were evident. Our findings of increased *synaptophysin* and *NFM* mRNA, but decreased *mash-1* and *En-1* mRNA levels induced by high lithium concentrations may reflect different effects of lithium on neuronal patterning versus the induction of specific neuronal cell types and proteins.

Mash-1 is spatially and temporally expressed in distinct precursor cells of the neural crest-derived autonomic as well as central nervous system and is required for the development of multiple neuronal lineages during embryogenesis (Verma-Kurvari *et al.,* 1996). En-1 is required to maintain the ventral compartment and is necessary for the apical ectodermal ridge formation during embryogenesis (Logan *et al.,* 1997; Altabef *et al.,* 2000). Synaptophysin is an integral membrane protein of small synaptic vesicles and a marker for neuronal fiber outgrowth in addition to synapse formation (Bergmann *et al.,* 1991). *Synaptophysin* mRNA transcripts have been detected during early neurulation both in the CNS and PNS (Marazzi and Buckley, 1993). An inhibition of nervous system morphogenesis, but enhanced neuronal differentiation by lithium exposure was also found in *Xenopus* embryos (Kao *et al.*, 1986; Breckenridge *et al.,* 1987). Our results of enhanced *synaptophysin* mRNA levels after

lithium treatment are supported by data showing that 5 x 10^{-3} M LiCl induced neurite outgrowth and increased the number of synapsin I positive clusters in mouse cerebellar neurons (Lucas and Salinas, 1997; Hall *et al.,* 2000).

In addition to the analysis of different tissue-specific genes, various developmental stages and treatment schedules have to be taken into consideration in order to detect stage-specific effects of embryotoxic compounds. Whereas LiCl treatment between 0 to 5 + 2 d or 0 to 5 + 10 d, respectively, resulted in a concentration-dependent inhibition of cardiac differentiation, no significant effects on differentiation patterns were found after LiCl treatment from day 0 to 2 or day 2 to 5, respectively (data not shown). This is in contrast to stage-dependent developmental defects induced by lithium in *Xenopus* embryos (Breckenridge *et al.*, 1987) and to previous studies showing that retinoic acid concentration- and stage-dependently influenced ES cell differentiation patterns of cardiogenesis, myogenesis, adipogenesis and vascular smooth muscle differentiation (Wobus *et al.,* 1994, 1997; Strübing *et al.,* 1995; Dani *et al.,* 1997; Drab *et al.,* 1997; Rohwedel *et al.,* 1999). To detect embryotoxic and/or teratogenic effects of test substances, therefore, it may be necessary to evaluate additional application schedules.

The inhibitory effects of lithium on glycogen synthase kinase-3β (GSK-3β) in the Wnt-signaling pathway is resulting in an increased cytoplasmic pool of β-catenin, which after direct interaction with the transcription factor LEF-1/TCF may be translocated into the nucleus and activate gene expression in *Drosophila* (van Leeuwen *et al.,* 1994; Cavallo *et al.,* 1997; van de Wetering *et al.,* 1997), *Xenopus* (Behrens *et al.,* 1996; Yost *et al.,* 1996; Larabell *et al.,* 1997; Gradl *et al.,* 1999) and mouse cells (Danielian and McMahon, 1996). The data suggested that lithium, like Wnt-7a, could enhance the stabilisation of β-catenin and modulate synaptic functions in granule cells by inhibiting the activity of GSK-3β (Lucas and Salinas, 1997; Hall *et al.*, 2000). The slight increase of β-catenin levels in EBs after LiCl exposure observed in our immunofluorescence study could suggest an interference of lithium in the Wnt-signaling pathway. However, no increased β-catenin levels were found by immunoblotting and CLSM analysis. Therefore the slight increase of β-catenin levels seen by immunofluorescence analyses may reflect only a positive ('statistical') effect (i.e., 60 EBs by immunofluorescence instead of 4 experiments by Western blots were analysed).

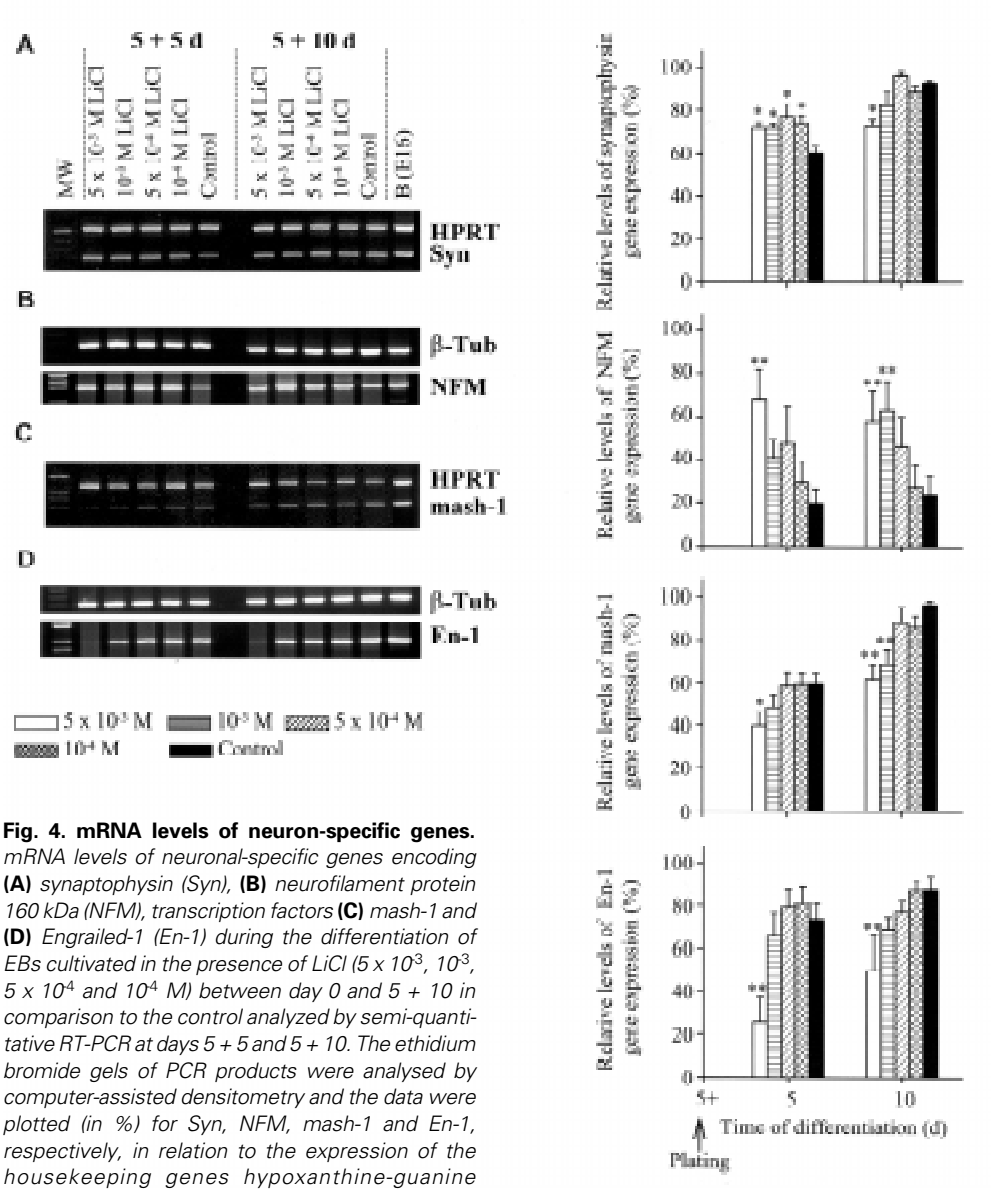

Fig. 4. mRNA levels of neuron-specific genes. *mRNA levels of neuronal-specific genes encoding* **(A)** *synaptophysin (Syn),* **(B)** *neurofilament protein 160 kDa (NFM), transcription factors* **(C)** *mash-1 and* **(D)** *Engrailed-1 (En-1) during the differentiation of EBs cultivated in the presence of LiCl (5 x 10^{-3}, 10^{-3}, 5 x 10^{-4} and 10^{-4} M) between day 0 and 5 + 10 in comparison to the control analyzed by semi-quantitative RT-PCR at days 5 + 5 and 5 + 10. The ethidium bromide gels of PCR products were analysed by computer-assisted densitometry and the data were plotted (in %) for Syn, NFM, mash-1 and En-1, respectively, in relation to the expression of the housekeeping genes hypoxanthine-guanine phosphoribosyltransferase (HPRT) or β-tubulin (β-Tub) used as internal standards. Brain tissue (B) of a 16 day old mouse embryo (E16) was used as positive control. MW, molecular weight. Significance was tested by the Student t-test (***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05).*

In addition, other explanations have to be discussed: (i) the concentration of lithium (5 x 10^{-3} M) used in our study in comparison to 2 x 10^{-2} M (Lucas and Salinas, 1997) is too low, (ii) the EB system is more complex than primary cultures (Lucas and Salinas, 1997; Ai *et al.*, 2000), and (iii) the effects of lithium on cells differentiating within the EB may be not mediated by the β-catenin/Lef-1 pathway. The latter explanation has been supported by recent studies showing that in the mouse LiCl-induced axial defects were not mediated by this pathway (Rogers and Varmuza, 2000) suggesting species-specific differences in the regulation of lithium via the Wnt-signaling cascade.

In conclusion, the concentration-dependent effects of lithium on ES cell differentiation and gene expression levels supported the suitability of the ES cell differentiation model for the analysis of teratogenic/embryotoxic compounds *in vitro*. By using the EST

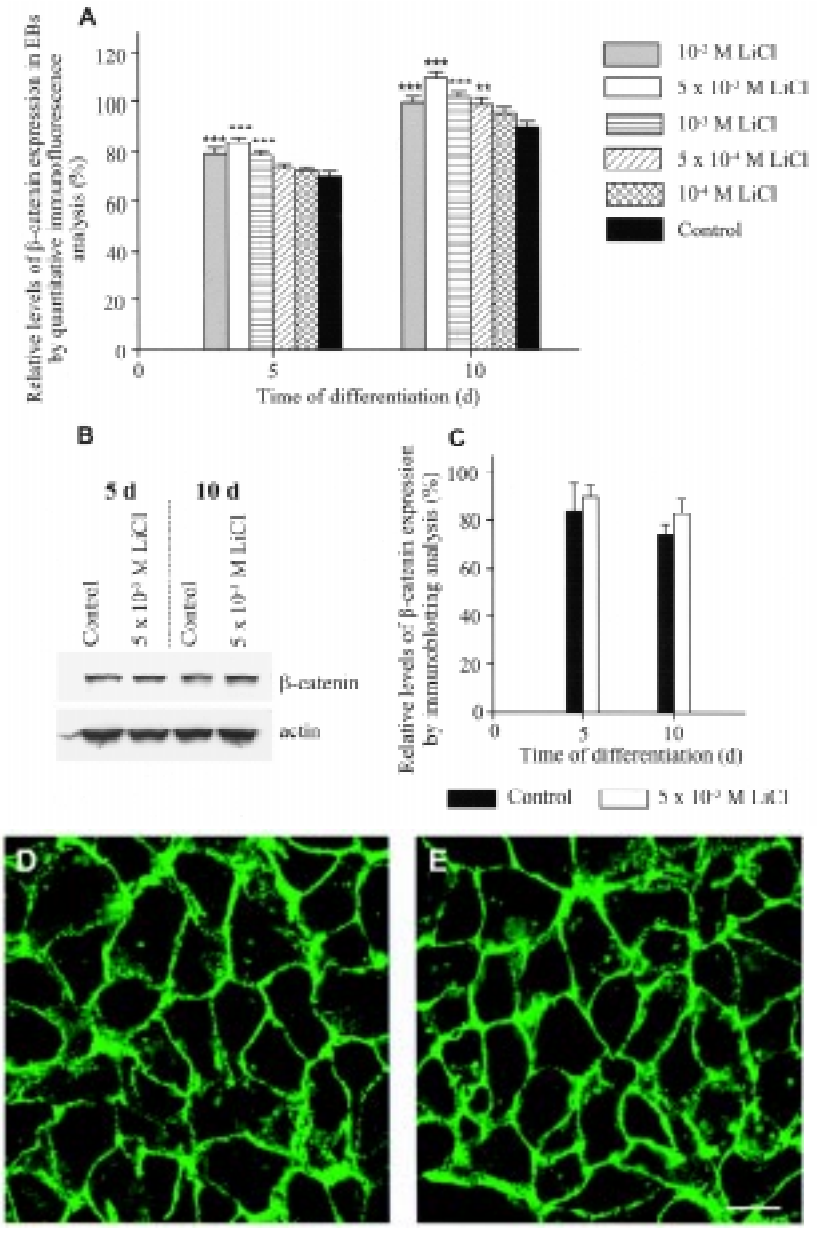

Fig. 5. Expression and localisation of β-catenin in EBs. (A) *Quantitative evaluation of relative β-catenin levels in EBs cultured in the presence of LiCl (10^{-2}, 5 x 10^{-3}, 10^{-3}, 5 x 10^{-4} and 10^{-4} M) between day 0 and 10 in comparison to the control by immunofluorescence analysis at days 5 and 10. Significance was tested by the Student t-test (***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$).* **(B,C)** *Immunoblotting of β-catenin and actin (for loading control) shown in whole-cell lysates from control or LiCl-treated EBs at days 5 and 10.* **(D,E)** *Confocal images of cellular localisation of β-catenin in* **(E)** *10^{-2} M LiCl-treated and* **(D)** *control EBs at day 10. No significant differences were found in the cellular distribution of β-catenin between control and LiCl-treated variants. Scale bar, 10 μm.*

and the 'Improved Prediction Model' (Scholz *et al.,* 1999), a high correlation has been found between *in vivo* embryotoxicity and *in vitro* EST data (Scholz *et al.,* 1999). Now, human pluripotent ES cell lines are being available (Shamblott *et al.,* 1998; Thomson *et al.,* 1998) which allow in the future, direct analyses of embryotoxic agents on human ES cells *in vitro*. On the basis of the present data, we propose to study embryotoxic substances on differentiation of ES cells *in vitro* by including analyses of various cell types including different developmental stages, and tissue-specific gene expression patterns.

Materials and Methods

ES cell culture and differentiation of embryoid bodies

ES cells of line D3 (Doetschman *et al.,* 1985) were cultivated on feeder layer of primary mouse embryonic fibroblasts (Wobus *et al.,* 1991) on gelatin (0.1%)-coated petri dishes (Falcon) in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Eggenstein, FRG) supplemented by 15% heat-inactivated fetal calf serum (FCS, selected batches, Gibco), L-glutamine (Gibco, 2 mM), β-mercaptoethanol (β-ME, Serva, Heidelberg, FRG, final concentration 50 mM) and nonessential amino acids (NEAA, Gibco, stock solution diluted 1:100) as described (Wobus *et al.,* 1991, 1997).

ES cells were differentiated as embryoid bodies (EBs) in hanging drops in DMEM supplemented with 20% FCS, L-glutamine, NEAA and β-ME. Differentiation medium (20 μl) containing 400 ES cells was placed on the lids of petri dishes filled with phosphate-buffered saline (PBS). After cultivation of EBs in hanging drops for 3 days and in suspension for 2 days, EBs were plated separately onto gelatin-coated 24-well microwell or 6 cm culture plates at day 5. Different concentrations of LiCl (10^{-2} M, 5 x 10^{-3} M, 10^{-3} M, 5 x 10^{-4} M and 10^{-4} M) were added to the cultures at days 0, 3, 5, 5 + 5 and 5 + 8 with fresh medium, and medium change without addition of LiCl was performed every second day after day 5 + 10.

For the analysis of cell differentiation, spontaneously beating cardiomyocytes, skeletal muscle and neuronal cells were morphologically investigated from the outgrowths of EBs cultured in two 24-microwell plates at various stages during differentiation. The percentage of EBs containing beating cardiomyocytes, skeletal muscle or neuronal cells was amounted as percent value of cardiogenic, myogenic or neuronal differentiation. At least three independent experiments were performed. Each data point represents the mean value ± standard error of the mean (SEM).

Morphology of beating cardiac clusters, skeletal muscle, and neuronal cells in EB outgrowths was analysed by a computer-assisted LUCIA imaging system (Nikon, Düsseldorf, Germany) including the 'HEART' application.

Immunofluorescence assay

For immunostaining of the sarcomeric protein titin (Z-disk) and neurofilament protein 160 kDa, EB outgrowths derived from D3 ES cells were analysed at day 5 + 15. For immunolabelling of β-catenin, EBs cultivated in hanging drops and in suspension in the presence of LiCl (10^{-2} M, 5 x 10^{-3} M, 10^{-3} M, 5 x 10^{-4} M and 10^{-4} M) were analysed at days 5 and 10. EBs and EB outgrowths were rinsed twice with PBS and fixed with methanol: acetone (7: 3) at -20°C for 10 minutes. After blocking with 10% goat serum in PBS for 1 hour, specimens were incubated at 37°C with mouse monoclonal antibodies anti-titin (Z-disk-specific epitope) clone T12 (Fürst *et al.,* 1988), anti-neurofilament protein 160 kDa (NFM) clone NN18 (Roche Diagnostics GmbH) and anti-β-catenin clone 14 (Transduction Laboratories, Dianova) for 1 hour. After rinsing in PBS three times, specimens were incubated for 1 hour at 37°C with the Fluorescein-conjugated goat anti-mouse IgG (Dianova). Specimens were rinsed with PBS (3x) and with distilled water (1x), embedded in Vectashield mounting medium (Vector

Laboratories Inc., USA) and analysed with the fluorescence microscope Optiphot-2 (Nikon) or the confocal laser scanning microscope (CLSM, Carl Zeiss).

For quantitative immunofluorescence analysis of β-catenin, the Axioscope microscope (Carl Zeiss) coupled to a microscope photometer system MPM20D (Carl Zeiss) was used. Before measurement, one EB with higher fluorescence intensity was used to define the control value of fluorescence as 100%. The same control and background values for all stages were used. Relative fluorescence intensities of EBs measured at different stages in relation to the control were given by the photometer system as percentage values. The appropriate aperture (Ø = 2.5 mm) to measure the fluorescence of different regions (varies according to the size) of EBs was selected and the mean value of each EB was calculated (n = 20 EBs per sample of n = 3 independent experiments). The mean values of relative fluorescence (in %) in graphs were calculated and given in correlation to the differentiation time of EBs.

For confocal laser scanning microscopy (CLSM), the detection of Fluorescein-labelled cells was performed by the following system configuration: excitation wavelength 488 nm, splitting wavelength 510 nm and barrier filter wavelength 515 to 565 nm). Single sections were taken for both control and LiCl-treated cells.

Detection of tissue-specific genes by semi-quantitative RT-PCR analysis

The expression of tissue-specific genes was analysed by semi-quantitative RT-PCR analysis as described (Rohwedel *et al.,* 1998). EBs collected at 5 and 10 days after plating (5 + 5 and 5 + 10 d) were suspended in lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl, 0.1 M β-ME). Total RNA was isolated using a single step extraction method (Chomczynski and Sacchi, 1987). mRNA was reverse transcribed using Poly dT tail primer Oligo $d(T)_{16}$ (Perkin-Elmer, Überlingen, FRG) and cDNA was amplified using oligonucleotide primers complementary and identical to the cardiac-specific gene *α-MHC*, skeletal mucle-specific gene *myoD* and neuron-specific genes *synaptophysin*, *NFM*, *mash-1* (see Rohwedel *et al.,* 1998) and

En-1 primer pair (390 bp)
5'-TCT CGT CTT TGT CCT GAA CCG T-3',
5'-TGG TCA AGA CTG ACT CAC AGC A-3';
(Joyner *et al.,* 1987), and primers to the housekeeping genes

hypoxanthine-guanine phosphoribosyltransferase HPRT;
primer pair 1: (447 bp)
5'-CGC TCA TCT TAG GCT TTG TAT TTG GC-3'
5'-AGT TCT TTG CTG ACC TGC TGG ATT AC-3';

primer pair 2: (502 bp)
5'-GCCTGTATCCAACACTTCG-3'
5'-AGCGTCGTGATTAGCGATG-3';

(Konecki *et al.,* 1982) as well as *β-tubulin* (see Rohwedel *et al.,* 1998) were used as internal standards. Reverse transcription was performed with MuLV reverse transcriptase (Perkin-Elmer) for 1 hour at 42°C, followed by denaturation for 5 min at 99°C and cooling to 4°C following the protocol supplied by the manufacturer. For the semi-quantitative determination of mRNA levels of *α-MHC*, *myoD*, *synaptophysin* and *mash-1* genes, multiplex PCRs were carried out with Ampli Taq DNA polymerase (Perkin-Elmer) using the 'Primer-dropping' method according to Wong *et al.* (1994) as described (Wobus *et al.,* 1997). 34 (*HPRT1*) and 35 cycles (*α-MHC*), 44 (*β-tubulin*) and 45 cycles (*myoD*), 40 (*HPRT2*) and 45 cycles (*synaptophysin*), and 40 (*HPRT1*) and 45 cycles (*mash-1*) of amplification were used. For determination of relative mRNA levels of *NFM* and *En-1* genes, two separate PCR reactions, either using primers of the analyzed gene or primers specific for *β-tubulin* were performed with 3 µl from each RT reaction, and 40 (*NFM*), 35 (*En-1*) and 28 cycles (*β-tubulin*).

One third of each PCR reaction was electrophoretically separated on 2% agarose gels containing 0.35 µg/ml of ethidium bromide. Gels were illuminated with UV light and the ethidium bromide fluorescence signals of gels were stored by the E.A.S.Y system (Herolab GmbH, Wiesloch, FRG) and analysed by using TINA2.08e software (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, FRG). The intensity of the ethidium bromide fluorescence signals was determined from the area under the curve for each peak and the data of the target genes were plotted as percentage changes in relation to the expression of the housekeeping genes. Each RT-PCR was performed twice, and at least three experiments were done. Each data point represents the mean values ± standard error of the mean (SEM).

Immunoblotting

EBs (n = 30 for each variant) cultivated without (control) or in the presence of LiCl (5×10^{-3} M) were analysed at days 5 and 10 (n = 4 independent experiments). EBs were washed twice with ice-cold PBS and subsequently lysed in ice-cold lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.02% NaN_2, 1 mM phenylmethylsulfonyl fluoride (PMSF, added before use)]. Cellular lysates were prepared by homogenisation and centrifugation at 500 g at 4°C for 5 minutes. Protein concentrations of the crude total cellular lysates were determined by using the protein assay kit (Bio-Rad). 2x sample buffer (125 mM Tric-HCl pH 6.8, 4% (w/v), 10% (v/v) glycerol, 2% (v/v) 2-ME, and 0.006% Bromophenol Blue) was added to each sample. Equal amounts of protein were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham Pharmacia Biotech) in a semi-dry system (transfer buffer: 25 mM Tris, 190 mM glycine, 20% methanol). Membranes were then incubated in blocking buffer (5% non-fat dry milk in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.1% Tween 20) for 1 hour at room temperature. β-catenin was detected using the mouse monoclonal antibody anti-β-catenin clone 14 (dilution 1:6000) and membranes were incubated overnight at 4°C. At the same time, the mouse monoclonal antibody anti-actin clone C4 (dilution 1:6000; Chemicon) was used as internal standard. After being washed in rinsing buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.1% Tween 20) for 30 minutes (change every 5 minutes) at room temperature, membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (diluted 1:20,000; Amersham Pharmacia Biotech) for 1 hour at room temperature. After further rinsing, the membranes were incubated in ECL detection reagents (Amersham Pharmacia Biotech) for 1 minute and exposed to X-ray film (Hyperfilm^{TM}ECL, Amersham Pharmacia Biotech).

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