8 Summary

The Embryonic Stem Cell Test (EST) is an alternative method to animal experiments, which is intended for the prediction of an embryotoxic potential of substances (Spielmann et al., 1997). The EST is based on the differentiation of embryonic stem cells of the mouse (line D3). Their ability to form contracting cardiomyocytes within 10 days of development is used as a model of embryonic development. For this purpose, the cells are cultured in aggregates called *Embryoid Bodies* (EBs), which can be examined microscopically on day 10 for the presence of contracting areas. As a parameter for embryotoxic effects, the concentration of a test substance, that causes an inhibition of cardiomyocyte differentiation in half of the EBs is determined (ID_{50}-value). To cut off general toxic effects, the halfmaximal inhibiting concentration on cell growth is established in D3 cells and the fibroblastoid murine cell line 3T3 ($IC_{50\ D3}$, $IC_{50\ 3T3}$).

The aim of the presented thesis was to study gene expression during cardiomyocyte differentiation, to select appropriate candidate genes and to establish molecular markers for embryotoxic substance effects.

During the 10 day differentiation period the expression of 18 genes with a known *in vivo* function in early mesoderm or heart differentiation was studied by means of quantitative RT-PCR. This selection contained heart muscle specific genes of the contractile apparatus α-MHC, β-MHC, MLC1, MLC2v, α-Actin, α-Actinin and Troponin T, transcription factors of early mesodermal and heart development GATA-4, Tbx5, Hand1 and 2, MesP1 and 2, as well as myocardin, the cytokine cardiotrophin-1, the growth factor cripto-1 and, in addition, genes, which are not heart specific (Oct4 and NF-L). During the differentiation of D3 cells to cardiomyocytes, the majority of gene specific transcripts was found in patterns that were in accordance with their expression *in vivo*. The expression of important transcription factors like MesP1 and 2 as well as Hand1 and 2 was determined during cardiomyocyte differentiation in D3 cells for the first time.

In order to establish molecular markers for a prediction of embryotoxic effects on the level of gene expression, the genes MesP1 and MLC1 were chosen as test candidates, whose expression was determined after substance treatment at appropriate time points (MesP1 on day 5; MLC1 on day 7 and in addition on day 10).

Eleven substances with known embryotoxic potential *in vivo* were selected and their concentration-dependent effects on differentiation of contracting cardiomyocytes and on expression of the marker genes were determined and compared. In addition, two substances without comprehensive *in vivo* data were tested. Comparison of the four determined parameters, microscopic evaluation on day 10, expression of MesP1 on day 5, expression of MLC1 on day 7

and expression of MLC1 on day 10, showed that the sensitivity of the molecular markers was comparable with the conventional microscopic endpoint in the majority of cases.

An analysis of the results of the 11 known substances with all four endpoints using a mathematic model which was developed for the prediction of an embryotoxic potential (Genschow et al., 2002) lead in all but two single tests to the correct classification.

Thus, out of the molecular markers that were established successfully, two lead to a correct prediction of the embryotoxic potential of the substances even earlier than the conventional endpoint.

The results of the presented work contribute to understanding the succession of molecular events during *in vitro* differentiation of cardiomyocytes and, therefore, to improve the possibilities of the EST to be applied as a screening test for embryotoxic effects in early substance development. The results allow to shorten the test duration and with the use of molecular endpoints automation of parts of the test procedure becomes possible. Furthermore, the fact that an early mesodermal gene like MesP1 allowed a correct embryotoxic prediction shows that the presented results can contribute to the selection of genes, which can be used as molecular markers for a wider choice of differentiation processes.