6 Summary

Strategies to inhibit cell differentiation in long-term cultivation of embryonic stem cells D3 with regard to their use in the embryonic stem cell test.

The embryonic stem cell test (EST) is an *in vitro* embryotoxicity test that has been developed in order to classify test compounds for their teratogenic potential. The pluripotency of embryonic stem cells (ES cells) is a prerequisite for using these cells in the EST.
If their differentiation is inhibited, the self-renewal-capacity of the embryonic stem cells is almost unlimited. However, suboptimal culture conditions can cause differentiation of stem cells even under differentiation inhibiting strategies and may result in a change of the cell population.
This thesis is about the investigation of various methods of preventing ES cell differentiation. A method was looked upon positively if it allows to keep the undifferentiated status of the ES cells as well as their ability to differentiate into contracting cardiomyocytes.
The permanent embryonic stem cell line D3 was cultivated over a period of 50 passages under the influence of 1000 U/ml Leukemia Inhibitory Factor (LIF), 2000 U/ml LIF, 20 ng/ml human Oncostatin M or in co-culture with STO-fibroblast-feeder layer with and without supplementation of the culture medium with LIF.
Over the period of observation the cell populations were characterized for their differentiation status. In addition to the morphological investigation also the expression of the stem cell markers SSEA-1 (stage-specific embryonic antigen-1) and alkaline phosphatase activity (AP-activity) was investigated. These markers are typically expressed by undifferentiated stem cells and regulated down after the differentiation. Immunocytochemical and cytochemical techniques, FACScan-analyses and a photometrically determined enzyme-activity-test were performed. The influences of penicillin G and 5-fluorouracil on growth and differentiation of stem cells were investigated in proliferation and differentiation assays following the EST protocol.
On the one hand, the presented study aimed on comparing the various differentiation inhibiting strategies and on checking their suitability for the long term cultivation of ES cells, on the other hand on the evaluation of different methods of determining the SSEA-1-expression or the AP-activity and their correlation with the ability of the ES cells to be used in

the EST. The evaluation was based on the assumption that undifferentiated stem cells grow in typical colonies (when cultivated in culture flasks), that they express the surface antigen SSEA-1 and that they possess a high alkaline phosphatase activity. In the differentiation assay of the EST these cells are able to differentiate into contracting cardiomyocytes.

The study demonstrated that in long term cultivation of ES cells the sole supplementation of the culture medium by LIF is not sufficient to inhibit changes of the stem cell population manifesting in increasing numbers of differentiated cells. These cell populations displayed morphological changes and a reduced number of SSEA-1 positive cells, a reduced AP-activity and a restricted capacity to differentiate into cardiomyocytes.

In contrast the direct co-culture of ES cells with inactivated STO fibroblasts and the supplementation of the culture medium by Oncostatin M respectively, inhibited the differentiation for a long culture period. The stem cell markers SSEA-1 and AP-activity and the differentiation capacity of these cell populations remained on a high level.

By using feeder layer the present restriction of the stem cell population to 25 passages in the EST could be extended to 50 passages. This would allow the better exploitation of a suitable cell population.

A quantification of the SSEA-1 positive cells and the alkaline phosphatase activity respectively is required for the evaluation of the stem cell populations with regard to the performance of the EST. Of the various approaches the investigation of the colony morphology and immunocytochemical (SSEA-1 expression) or cytochemical analyses (AP-activity) proved to be no objective methods for evaluating the stem cell population according to their differentiation status. The suitability of the stem cell population for the EST is not predictable by the flowcytometric determination of AP-positive cells either. Whereas the flowcytometrically analyzed percentage of SSEA-1 positive cells as well as the photometrically determined AP-activity correlated with the differentiation capacity of the cell populations.

FACScan-analyses for SSEA-1 expression or the enzyme activity test for AP-activity present a simple and fast way to evaluate the stem cell population by quantifying these stem cell markers. The time consuming quality check of the ES cells as prescribed in the EST protocol could be replaced and the test for suitability could be carried out shortly before the EST.