

## Proceedings of German Society for Stem Cell Research (PGSSCR)

### **Reproducible and complete early separation of stem and feeder cells does not attenuate the differentiation potential of feeder-freed stem cells**

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Published on 16 May 2007

#### **Introduction:**

Mouse or human embryonic stem (mES/hES) cell lines are usually derived and propagated on inactivated feeder cell layers. Human feeder cell layers such as foetal human fibroblasts or post-natal human skin fibroblasts have lately been used in order to eliminate the risk of foreign protein contamination during hES propagation. However, it is impossible to conclusively evaluate the beneficial or detrimental effects of feeder cell layers on propagation and subsequent differentiation of stem cells without the possibility to dissociate feeder and stem cells. Furthermore, feeder cells themselves might have a detrimental effect in clinical application, since feeder cells might be carried along during *in vitro* differentiation of stem cells. We have therefore developed a methodology that allows for the mechanical separation of stem cell from colonies grown on feeder cell layers and the subsequent differentiation of feeder-freed colonies and cells.

#### **Materials and Methods:**

Stem cells (D3) were grown on neomycin resistant (*nr*) feeder cell layers derived from mouse embryos for 5 to 8 days. Stem cells from single colonies were harvested by using an automated cell selection system. Stem cells were aspirated and immediately transferred to a non-adhesive 96 well RT-PCR plates. Aspiration of stem cells but not feeder cells was achieved by carefully pre-defining aspiration pressure. Feeder cell contamination was detected by nested RT-PCR of the *nr* gene expressed in feeder cells only. Differentiation of feeder-freed stem cells was analysed by marker expression analysis (RT-PCR)

#### **Results:**

The sensitivity of the nested RT-PCR approach was demonstrated by amplifying *nr* mRNA from single feeder cells. Feeder cell contamination in aspirated stem cells was not observed, even when stem cells were successively harvested from the same colony. Propagation and differentiation of stem cells under standard conditions resulted in feeder cell contaminations that were still detectable

up to 20 days following initiation of differentiation, indicating the possibility of feeder cell contamination in subsequent stem cell based cell-replacement therapies. In a second step the differentiation potential of colony derived feeder-freed stem cells was examined. Embroid body formation of aspirated cells single colonies was allowed for 4-5 days in non-adhesive 96 well RT-PCR plates. >95% of cells or single colonies aspirated developed into single embroid bodies. Embroid body morphology closely resembled the spherical morphology of embroid bodies developed under standard conditions. Subsequently feeder-freed embroid bodies derived from defined single colonies were differentiated into cardiomyocytes or neuronal cells by standard protocols. Early differentiation events were detected by expression analysis of *gata6* (endoderm), *fgf5* (primitive ectoderm), *brachyury* (mesoderm). Differentiation into cardiomyocytes was evaluated through analysis of GATA-4, cardiac Troponin C, alpha-MHC expression. Neuronal differentiation was verified by RT-PCR analysis of *nestin*, *TH*, *GFAP*, *NSE* and neurofilament. The time dependent pattern of expression of stem cell differentiation in neuronal cells or cardiomyocytes was comparable and reproducible between stem cells differentiated under standard conditions and feeder-freed stem cells differentiated from single embroid bodies in PCR tubes.

#### **Discussion and Conclusion:**

We have therefore developed a method that for the first time allows the complete and reproducible separation from stem and feeder cells at an early time point and the analysis of distinct stem cell colonies. Complete separation from stem and feeder cells is essential in regenerative cell replacement therapies. Separation of stem and feeder cells is also an important prerequisite in the unambiguous analysis of differentiation mechanism.