IEF – Final Report

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BACKGROUND AND SUMMARY OF THE STUDY PLAN

Human embryonic stem cells (hESC) are pluripotent cells derived from preimplantation stage human embryos. Representing the earliest possible cell population in embryo proper, they have the capability to differentiate into any cell type found in adults and hence, they are anticipated to open important new avenues in biomedical sciences. Human embryonic stem cells are expected to become significant tools in discovering and testing new drugs as well as in studying the early embryonic development. Most importantly, their well-documented ability to differentiate into various cell types found in adults hold huge potential in cell replacement therapies in a number of severe degenerative human diseases such as age-related macular degeneration and type 1 diabetes.

Genetic manipulation of hESC will be a crucial issue in a variety of future studies. To elucidate the role of any given gene for example in directed differentiation, controlled loss- and gain-of gene function approaches are of great importance. A major problem in generating stable transgenic cell lines in any mammalian cells, including hESC, is the silencing of the transgene by the epigenetic modification of the genomic integration site. Understandably, variable or changing transgene expression levels may severely affect the results obtained in functional studies. The TransHesc project was designed to evaluate the possible advantageous role of the specific chicken β-globin insulator sequences in preventing transgene silencing in hESC. Recent findings in different mammalian cell lines (including human cell lines) have shown that two pairs of insulator sequences flanking the transgene can substantially prevent the lost of its activity. Whether the insulator sequences are functional also in hESC has not been elucidated so far.

SCIENTIFIC OUTCOME

Several test constructs were generated to demonstrate the role of insulator sequences in protecting transgenes in stably transfected hESC. As a positive control I first cloned the insulators in a well characterised pCAG-plasmid where GFP and puromycin resistance genes are expressed from the single CAG-promoter (Fig 1). Previous studies have shown that the CAG-promoter is strong and stable in hESC and the present results can confirm this. The insulator sequences did not change the stability of the expression as compared to non-insulated construct indicating that as such insulation does not prevent transgene transcription in hESC. However, the intensity of the GFP expression was clearly lower from insulated transgene which could indicate that the insulator sequences might negatively interfere with insulated promoter activity.

As a next step I generated test constructs with widely used CMV and ROSA26 promoters that are known to be silenced in hESC (Fig 2). The test constructs were composed of either insulated or non-insulated CMV/ROSA26 driven GFP expression unit and a separate SV40-promoter driven neomycin resistance unit for the initial selection of the transgenic cells. The hypothesis was that the insulated

units show stable expression while the non-insulated units will lose the expression during the culture. All the constructs were first tested in HeLa cells to confirm their proper function in a model cell line. As expected, in HeLa cells the activity of the CMV promoter was rapidly silenced if the cells were transfected with non-insulated construct. In contrast, the insulators flanking the transgene were able to prevent the loss of expression indicating that the insulator sequences do function in HeLa cells. The ROSA26 promoter showed strong expression without a significant lost of GFP positive cells during the culture independent of the insulation. However, the intensity of the GFP expression in cells transfected with insulated never reached the level seen in cells transfected with non-insulated constructs, again suggesting that insulators might negatively interfere with promoters inside "protected" area.

Next the CMV and ROSA26 test constructs were transfected in hESC (Fig 3 and 4). All the CMV-driven constructs showed in general very weak and rapidly down regulated expression and the insulator sequences did not restore the initial expression pattern. The ROSA26 promoter showed stronger expression than CMV but nevertheless the expression was silenced within a few passages after initial selection of the neomycin resistant clones. Similar to results with CMV promoter, insulator sequences did not protect the GFP expression from silencing. To further confirm the findings we sorted the ROSA26 transfected cells based on their GFP positivity and cultured the GFP-positive cells with or without selective antibiotic (Fig 5). Independent of the insulation of the test construct the constant antibiotic selection was able to restore the GFP expression while without selection the expression was lost. The results above indicate, that in contrast to original hypothesis, in randomly integrated transgenes the chicken β-globin insulator sequences are not able to prevent silencing in hESC

To evaluate whether the silencing of the test constructs was due to the certain types of DNA methylation or histone acetylation the cells were treated with specific DNA methylation and histone deacetylation inhibitors (Fig 6). Neither of the inhibitors (or their combination, not shown) could restore the silenced GFP expression from insulated or non-insulated transgenes.

CONCLUSION

Despite the chicken β-globin insulator sequences have been shown to protect transgenes from silencing in many cell types, based on this study they do not improve the generation of the stable transgenic in hESC. The reason for this is unknown, but it may be because of the specific targeting of the used promoter sequences to silencing. It is known that for example some viral promoters are recognized by host cell and silenced as unwanted "intruders". Also, the unique epigenetic status of hESC genome might explain the lack of insulation. The chicken β-globin insulators mostly protect silencing caused by heterochromatin spreading i.e. they define boundaries between open transcriptionally active chromatin (euchromatin) and closed transcriptionally depressed chromatin (heterochromatin). In general, this is highly important in terminally differentiated cells where the normal function of the cells necessitates the activity of only certain areas of the chromosomes while other areas must not be active. A number of studies have indicated that hESC chromatin is in relatively open conformation suggesting that the amount of classic heterochromatin would be low. However, several mechanisms for epigenetic modification of the chromatin exists in hESC and, indeed, it is likely that the chromatin structure in hESC is under the constant reorganisation not seen in differentiated cells. Hence, the failure of the chicken β-globin insulators to protect transgenes in hESC might be connected to atypical dynamics in the regulation of the hESC chromatin structure not "recognized" by the insulators. Further studies will be needed to confirm this.