



Funded by the Seventh Framework
Programme of the European Union



Project full title:

Hepatic and Cardiac Toxicity Systems modelling

Project acronym:

HeCaTos

Collaborative project

HEALTH.2013.1.3.-1:

Modelling toxic response in case studies for predictive human safety assessment

FP7-HEALTH-2013-INNOVATION-1-602156-HeCaTos

Deliverable Report D7.10:

Report on RNA interference analysis in in vitro 3D models

Edited by

Twan van den Beucken - Partner UM

Ralph Schlappbach - Partner ETH Zurich

Work package 7

Due date of deliverable: M48

Actual submission date: October 2017

Start date of project: October, 2013

Duration: 60 months

Maastricht University (UM)

Project co-funded by the European Commission within the 7th Framework Programme (2013-2018)		
Dissemination Level		
PU	Public	X
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

Contributions to deliverable - Internal review procedure

Deliverable produced by:	Date:
Twan van den Beucken - Partner UM	October 2017
Ralph Schlapbach - Partner ETH Zurich	October 2017
Deliverable internally reviewed by:	Date:
Jos Kleinjans - Partner UM	October 2017

Contents

Publishable Summary.....	3
Objective	3
Introduction	3
Results.....	4
Lentiviral transduction of primary human hepatocytes	4
Lentiviral transduction of a 3D cell model	4
Proof of concept study.....	5
Difficulties	5
References	5

PUBLISHABLE SUMMARY

We have developed a strategy to perform RNA interference (RNAi) analysis on in vitro 3D cell models. This strategy is based on short hairpin RNA (shRNA) that is introduced into the desired cells using lentiviral particles. Several optimization steps were carried out to ensure successful implementation of the selected RNAi technology:

- First, we were able to show that non-dividing primary human hepatocytes (PHH) could be transduced using green fluorescent protein (GFP) expressing lentiviral particles at a high efficiency;
- Second, we developed a strategy to efficiently produce 3D transduced spheroid cell models;
- Third, we provided proof of concept by using RNAi to knocked-down the expression of 30 transcription factors (TFs) in liver hepatocellular carcinoma cell line HepG2, cultured in 3D configuration, and link this to a functional phenotypic endpoint. All together RNAi technology is now set in place to perform functional studies on candidate genes identified in liver and heart microtissues.

OBJECTIVE

The objective of Deliverable 7.10 is to develop a strategy to perform RNAi analysis on in vitro 3D cell models.

INTRODUCTION

RNA interference is a very useful technology to study the functions of specific genes and pathways¹. RNAi aims to reduce the expression of selected genes to study their effect on a genome-wide scale or on phenotypic endpoints of interest. Although different strategies exist to perform RNAi studies, they all rely on small RNA molecules (~21 nucleotides) that are homologous to the mRNA intended to deplete (target mRNA). Binding of the RNAi molecules to the target mRNA causes the degradation of the mRNA target molecule (also referred to as knock-down)

The available RNAi strategies vary in the way these small RNAi molecules are introduced into the cell model. Two important aspects need to be considered when choosing the right RNAi strategy:

- (i) The duration of the experiment, and
- (ii) The efficiency at which the RNAi molecules can be introduced into the cell model of interest.

Within the HeCaToS project cell models are repeatedly exposed to compounds over a 14 day period. This requires an RNAi strategy that ensures knock-down of the desired genes over a prolonged period of time. Therefore a vector-based RNAi strategy is needed that allows stable expression of RNAi molecules. This is achieved using short hairpin RNAs (shRNAs) that are naturally converted into RNAi molecules within cells. Since the genetic information for the RNAi molecules is present within the cell, the RNAi can be expressed for an infinite time. In addition, the information to generate the RNAi molecule (shRNA) needs to be efficiently introduced into the desired cell model. For that we choose a lentiviral system that can achieve 100% efficiency in introducing the shRNA construct into each cell. Moreover, the lentiviral system allows transduction of non-dividing cells, which is particularly important as the 3D model systems used under HeCaToS are based on non-dividing cells.

RESULTS

Lentiviral transduction of primary human hepatocytes

First, we wanted to demonstrate that we can successfully introduce lentiviral constructs into non-dividing PHHs. For that we generated virus particles from a lentiviral construct that allows the expression of GFP from a CMV promoter. Using these lentiviral particles PHHs grown between collagen layers were transduced overnight. As control a non-GFP expressing construct was used. The next day GFP-positive cells were detected using fluorescent microscopy (Figure 1). On the left GFP expression was assessed in PHHs transduced with lentiviral constructs containing no GFP (pLKO.1-No GFP). On the right PHHs are shown that were transduced with the GFP expressing construct (pLKO.1-GFP). It is clear that PHHs were successfully transduced. This illustrates that non-dividing PHHs can indeed be transduced by means of lentiviral constructs at high efficiency.

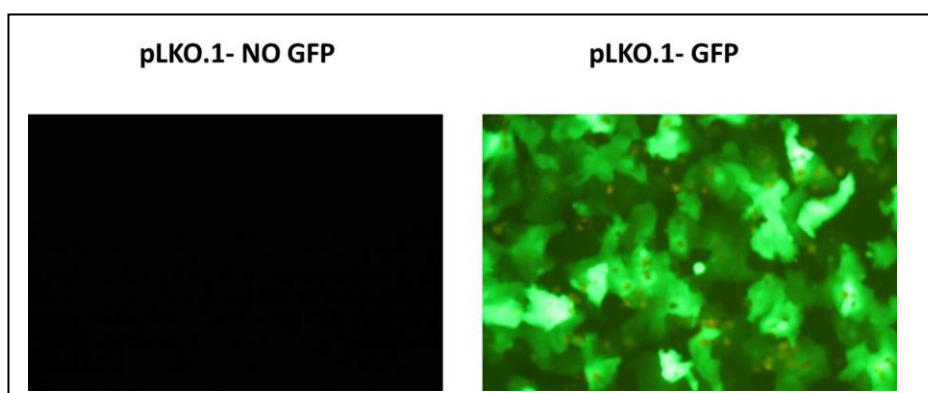


Figure 1: Successful transduction of PHHs with lentiviral particles.

Lentiviral transduction of a 3D cell model

Next, we wanted to determine whether lentiviral particles could also successfully introduce shRNAs into cells when grown in a 3D configuration. Since we anticipated problems with the penetrance of the viral particles into a 3D spheroid, we decided to try two strategies for establishing lentiviral transduced 3D cell models (figure 2A). The first strategy is based on the transduction of pre-established 3D spheroids. For this we generated 3D spheroids from HepG2 cells, using the hanging droplet method. We used HepG2 as a cell model for optimization of the RNAi strategy to keep the costs low. The next day the formed spheroids were transferred into a 96-well plate and transduced by lentiviral particles carrying a pLKO.1 vector with a shRNA targeting Drosha mRNA (shDROSHA). In the second strategy we added the lentiviral particles for shDROSHA during the generation of the 3D spheroids. This should eliminate potential problems related to penetrance of the 3D spheroid. Twenty-four hours after the transduction of the 3D spheroids, RNA was isolated and expression of Drosha mRNA was determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis. Drosha mRNA levels were very similar in non-transduced 3D spheroids and spheroids transduced with particles carrying a non-targeting shRNA vector (Figure 2B). This was independent of the strategy used to form the 3D spheroids. Nevertheless, Drosha expression was only reduced when lentiviral particles were added during the formation of the 3D spheroids (Figure 2B). The efficacy of the shRNA against Drosha is similar to that observed in 2D cell cultures (data not shown) and thus not reflect any problems related to the use of 3D spheroids. RNAi strategies are never 100% efficient in reducing gene expression. The remaining

expression depends on the design of that particular shRNA design. Therefore more than 1 shRNA construct is used in parallel to target a gene of interest.

This work demonstrates that our lentiviral RNAi strategy can be successfully used in 3D cell models.

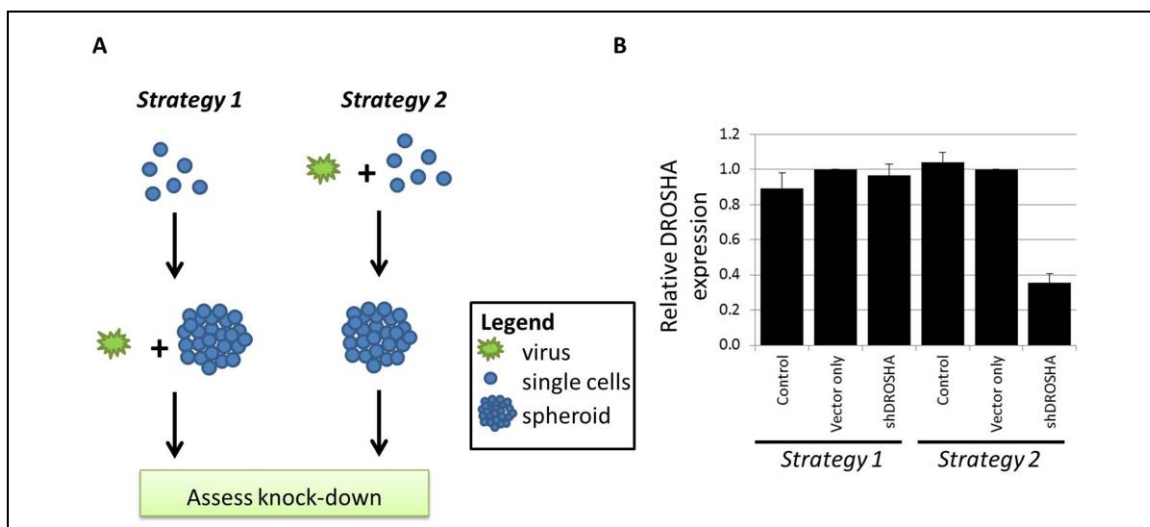


Figure 2: Lentiviral transduction of a 3D cell model. **A.** Two strategies to generate lentiviral transduced 3D spheroids. **B.** Knock-down efficiency of a shRNA targeting Drosha mRNA measured by qRT-PCR. Non-transduced 3D spheroids and spheroid transduced with lentiviral particles carrying vector control were used as controls. Drosha mRNA was normalized for housekeeping gene RPL13A.

Proof of concept study

Coordinating partner UM has been developing this lentiviral RNAi platform over the last 2 years as part of internal UM projects. This has resulted in a first publication on this technology from the department of Toxicogenomics (UM)². In this study we combined transcriptomics with RNAi and the assessment of phenotypic endpoints. Based on this experience we were able to adapt our protocols in order to apply them on 3D microtissues as required under HeCaToS.

DIFFICULTIES

A major hurdle in applying RNAi using 3D microtissues has been overcome. By using a lentiviral shRNA strategy, genes can be efficiently knocked-down in non-dividing target cells. Allowing 3D microtissue formation in the presence of the lentivirus enables high efficiency.

REFERENCES

1. Jason Moffat & David M. Sabatini. Building mammalian signalling pathways with RNAi screens. *Nature Reviews Molecular Cell Biology* 7, 177-187 (March 2006).
2. Smit E, Souza T, Jennen DGJ, Kleinjans JCS, van den Beucken T. Identification of essential transcription factors for adequate DNA damage response after benzo(a)pyrene and aflatoxin B1 exposure by combining transcriptomics with functional genomics. *Toxicology*. 2017 Sep 4;390:74-82.