Chapter 3: HT1 hepatic cell model

Historically different models, i.e. fungal, nematode, and mouse models, that exhibit HT1 characteristics were used to investigate the pathophysiology of HT1. Although these models are useful, they, as all animal models, have limitations such as interspecies extrapolations (Mitchell *et al.,* 2001:1777). Despite experimental limitations of cellular models, the development of a cell culture model would be advantageous in that it is human genome based, the investigation of pathophysiological features are less expensive and less labour and time intensive (Schule *et al.,* 2009:1043), and the influence of external factors is minimised as growing conditions can be tightly regulated. This chapter describes the procedures followed and the results obtained during the development of a HT1 hepatic cell model.

3.1 Development of a HT1 hepatic cell model

To study the effects on DNA repair capacity and genome stability in HT1, a cell culture model (simulating the HT1 phenotype) was attempted by using RNA *interference* (RNAi) technology. The longevity of gene silencing by short hairpin RNAs (shRNAs) over small interfering RNAs (siRNAs) directed the choice of the targeting molecule (Sandy *et al.,* 2005:215). For this purpose the Knockout Inducible RNAi system (Clontech Laboratories, Inc.) which allows tight regulation of expression of shRNAs, and thereby the shRNA target gene, was used.

The Knockout Inducible RNAi system is a tetracycline-controlled expression system. A tetracycline-controlled transcriptional suppressor (tTS) regulates the expression of shRNAs from a tetracycline dependant mini-U6 promoter (Gossen and Bujard, 1992:5547). When doxycycline (dox, a tetracycline derivative) is added to the culture medium, the tTS protein is released from the U6-promotor allowing the expression of the shRNA thereby knocking down the expression of the target gene. By altering the concentration of dox, the expression of the shRNA can be tightly regulated, which is necessary for studies where complete loss of the target gene can be detrimental to the cell. To establish a cell culture model that simulates the HT1 phenotype, such tight control is essential. Complete knock-out of *fah* would be unfavourable, as is seen in the neonatal lethality of *fah*$^{-/-}$ mice (Grompe *et al.,* 1995:453).

3.1.1 shRNA oligonucleotide design, acquisition and annealing

Although several different companies (i.e. Sigma, Invitrogen, Promega, Dharmacon) had shRNAs available for knock-down of *fah* at the time when this study started, none of these were validated. The first validated shRNA for *fah* knock-down only became available late in 2009. With the above mentioned in mind and the fact that not all interfering RNAs possess the same potential for gene silencing (Boese *et al.,* 2005:71), four different shRNAs were designed. The guidelines for the successful design of shRNAs as given in the "Knockout RNAi Inducible Systems" user manual as well as guidelines that were given by Sandy *et al.,* McIntyre and Fanning, and Boese *et al.,* were followed (Boese *et al.,* 2005:71, McIntyre and Fanning, 2006, Sandy *et al.,* 2005:215). The target sense sequences were siRNA sequences designed and bioinformatically processed by the Broad Institute. For each target site, two complementary oligonucleotides (a top and bottom strand) were synthesized and PAGE purified by Sylvean Biotech. All the forward sequences or top strands had the same basic design i.e. (starting at the 5' end) a BamH I restriction site followed by the target sense sequence (21 nucleotides), a hairpin loop with a Xho I restriction site, the target antisense sequence, and a terminator sequence of 5 thymines. The reverse or bottom strand's basic sequence were, from the 5' end, an EcoR I restriction site, followed by 5 adenines, the target antisense sequence (21 nucleotides), the hairpin loop, the target site sense sequence and the sequences were terminated with a guanine. The sequences of the four shRNAs are given in table 3-1.

Table 3-1. Sequence information of the four shRNAs designed for *fah* knock-down.

Oligonucleotide name	Sequence
FAH_sh1_FRW	5'-**GATCC**[1]GGCTACCATATGCAAGTCCAA***TCTCGAGA***[2]TTGGACTT GCATATCCTAGC**TTTTT**[3]G -3'
FAH_sh1_REV	5'-**AATTC**[4]AAAAAGCTACCATATGCAAGTCCAA***TCTCGAGA***[2]TTGGAC TTGCATATGGTAGCCG -3'
FAH_sh2_FRW	5'-**GATCC**[1]GGAGAGTGTTCTTGCAGAACT***TCTCGAGA***[2]AGTTCTGC AAGAACACTCTC**TTTTT**[3]G -3'
FAH_sh2_REV	5'-**AATTC**[4]AAAAAGAGAGTGTTCTTGCAGCT***TCTCGAGA***[2]AGTTCTGC AAGAACACTCTCCG -3'
FAH_sh3_FRW	5'-**GATCC**[1]GCAGCATCATCAAGCACCTCT***TCTCGAGA***[2]AGAGGTGC TTGATGATGCTG**TTTTT**[3]G -3'
FAH_sh3_REV	5'-**AATTC**[4]AAAAACAGCATCATCAAGCACCTCT***TCTCGAGA***[2]AGAGG TGCTTGATGATGCTGCG -3'
FAH_sh4_FRW	5'-**GATCC**[1]GTGAAGTCATCATAACAGGGTA***CTCGAG***[2]TACCCTG TTATGATGACTTCA**TTTTT**[3]G -3'
FAH_sh4_REV	5'-**AATTC**[4]AAAAATGAAGTCATCATAACAGGGTA***CTCGAG***[2]TACCCTG TTATGATGACTTCACG -3'

[1]BamH I restriction site; [2]Hairpin loop; [3]Terminator thymines; [4]EcoR 1 restriction site

The lyophilized oligonucleotides obtained from Sylvean Biotech were resuspended in PCR grade water to a final concentration 100 µM each.

The forward and reverse complementary oligonucleotides (top and bottom strands) were mixed in a 1:1 ratio, which yielded 50 µM double stranded oligonucleotide (assuming 100% annealing). The mixtures were placed in a Eppendorf® thermalcycler and the following program run, 95℃ for 30 seconds, 72℃ for 2 min, 37℃ for 2 min, 25℃ for 2 min. The annealed oligonucleotides were stored at -20℃ until use.

3.1.2 Cloning shRNAs into pSIREN-RetroQ-Tet vector

The annealed oligonucleotides were thawed and diluted with PCR grade water to a concentration of 0.5 µM. For each ligation reaction, 1 µl RNAi-Ready pSIREN-RetroQ-Tet vector (50 ng/µl) (see Appendix A for vector map), 1 µl diluted, annealed oligonucleotide (0.5 µM), 1.5 µl 10X T4 DNA ligase buffer (Fermentas), 0.5 µl BSA (10 mg/ml; New England Biolabs), 10.5 µl nuclease-free H_2O and 0.5 µl T4 DNA ligase (400 U/µl; Fermentas) were combined in a microcentrifuge tube. The mixtures were incubated at room temperature for 3 h.

JM109 cell aliquots were then separately transformed with the different ligation mixtures. Briefly, competent JM109 bacterial cells (in house stock) were thawed on ice for 10 min. After gentle resuspension of the cells, 2 µl of a ligation mixture was added to 50 µl of the bacterial cell suspension. The transformation mixtures were kept on ice for 30 min, followed by heating in a water bath at precisely 42℃ for exactly 90 sec. The mixtures were immediately placed on ice for 2 min. Subsequently, 950 µl of SOC broth (Appendix D) were added to each transformation mixture and the tubes incubated at 37℃ for 60 min while shaking at 250 rpm. Of each transformation mixture, 50 µl and 100 µl were separately plated on LB agar plates containing 100 µg/ml ampicillin. All plates were incubated overnight at 37℃. For each recombinant plasmid, 8 small scale liquid cultures were inoculated with a single, well-isolated colony. After overnight incubation, plasmid DNA was isolated using the PureYield™ plasmid miniprep system from Promega. The correct plasmids were identified by restriction fraction length polymorphism (RFLP) analysis. To verify the sequences of the chosen plasmids, the plasmids were sent for sequencing. Dideoxy sequencing was performed by Onderstepoort Veterinary Institute. The raw sequencing results were received from Onderstepoort Veterinary Institute and analysed with DNAMAN software version 4.1.2.1.

Sequencing results showed that *fah* knock-down shRNA constructs 1 and 3 each had a single basepair change from the designed shRNA constructs. Sequencing of *fah* knock-down shRNA construct 2 were unsuccessful, visual inspection of the electrophoretogram with Chromas showed that the sequencing reaction was performed with a mixed colony. *Fah* knock-down shRNA

construct 4 was identical to the designed shRNA construct (basepair changes can be seen in Appendix B.) Despite these small sequence changes, the *fah* knock-down shRNA constructs were used in subsequent experiments.

3.1.3 Pilot experiments

All cell culturing were done as described in section 4.1. Preceding the development of the single (ptTS expressing)- and double (ptTS and pSIREN-RetroQ-Tet-shRNA construct expressing)-stable cell lines, pilot experiments were performed to determine the optimal puromycin (Sigma-Aldrich) and G418 (Sigma-Aldrich) concentrations needed for selection and cell line maintenance, and to determine the optimal plating density for HepG2 cells.

To determine the optimal antibiotic concentration for selection, HepG2 cells were seeded at a fixed cell density of 2 x 10^5 cells per 25 cm^2 cell culture flask. In separate experiments, 10 ml selective medium containing varying concentrations of G418 (0; 800; 900; 1000; 1100; and 1200 µg/ml) and puromycin (0; 0.8; 1.0; and 1.2 µg/ml) were added to each flask. The cells were incubated for 14 days and the selective medium changed every two days. The lowest concentration of antibiotic that caused massive cell death within 5 days and complete cell death in 14 days were chosen for selection purposes. The respective selection concentrations that were chosen were 1200 µg/ml G418 and 1 µg/ml puromycin. Lower concentrations of 200 µg/ml G418 and 0.2 µg/ml puromycin were used for cell line maintenance.

After determining the optimal antibiotic concentrations, the optimal plating density of HepG2 cells were determined. Six different HepG2 cell densities (5 x 10^6; 1 x 10^6; 5 x 10^5; 2 x 10^5; 1 x 10^5; and 5 x 10^4) were seeded in six different wells of a 6-well cell culture flask. The cells were incubated in 2 ml of selective medium for 14 days. The selective medium was replaced every two days. The plating density of 2 x 10^5, which allowed the cells to reach 80% confluency before massive cell death at about day 5, was chosen.

3.1.4 Development of a single-stable ptTS expressing cell line

To develop a single-stable Tet HepG2 cell line that expresses the tTS regulator protein, HepG2 cells were transfected with the ptTS-Neo vector. Initially the CalPhos transfection reagent from Clontech Laboratories was used for transfections, but very low transfection efficiency was obtained. Also, the CalPhos transfection reagent caused a precipitate on the cells, which might have interfered with downstream applications. Washing of the cells after transfection with a 5 mM

EGTA solution, as suggested by Jordan and Wurm (Jordan and Wurm, 2004:136), only partially resolved this problem. Transfection reactions were then performed with FuGENE® (Roche Applied Science), as prescribed by the manufacturer. In short, 2 x 10^5 HepG2 cells were seeded in each of the six wells of a 6-well cell culture plate. One well was used as a negative control; another well as a transfection reagent control. A third well was transfected with a green fluorescent plasmid (eGFPN1; Clontech; see Appendix A for vector map) to determine transfection efficiency and the three remaining wells were transfected with the ptTS-Neo vector (Clontech; see Appendix A for vector map). The cells were incubated overnight in 2 ml complete growth medium. The complete growth medium was then replaced with 2 ml unsupplemented Dulbecco's modified eagles medium (DMEM) and the cells incubated for a further 3 h. In the meantime, for each transfection a transfection mixture was made up by adding 3 µl FuGENE® to 97 µl serum free medium (SFM) in a microcentrifuge tube, without touching the sides of the tube when adding the FuGENE®. The mixtures were quickly vortexed and incubated at room temperature for 5 min. Then 200 µg DNA (eGFPN1 or ptTS-Neo) were added to the appropriate microcentrifuge tubes containing the FuGENE® and SFM. The transfection mixtures were quickly vortexed and incubated for 15 min at room temperature. After incubation, the different transfection mixtures were slowly added to the assigned wells. The transfection reactions were incubated for 3 h at 37℃ and 5% CO_2, whereafter 10% fetal bovine serum (FBS) was added to each well. Selection for cells that stably express the ptTS protein was started after 48 h by changing the growth medium with G418 selection medium. Selection medium was changed every two days.

After four weeks, each of the selected colonies stably expressing ptTS was transferred to a well of a 24 well cell culture plate. Each colony was covered with 500 µl G418 selection medium (changed every two days) and propagated for a further 11 weeks. Afterwards, the cells from each colony were visually inspected for morphology changes. Cells from colonies that exhibited morphology changes were discarded. From the remaining colonies, those colonies that proliferated at the same rate as unaltered HepG2 cells were selected. These colonies were labelled as HepG2tTS10 and HepG2tTS12. These single stable cell lines were henceforth proliferated in G418 maintenance growth medium.

3.1.5 Developing a double-stable cell line

Before the development of a double-stable cell line, the functionality of each of the shRNA constructs was determined. To this end, HepG2 cells were transiently transfected with each of the shRNA constructs, since the single stable ptTS-Neo expressing HepG2 cells were still under construction. Initially, transient transfections were also performed with FuGENE®, but variable results were obtained, most probably due to inefficient/variable transfection (figure 3-1).

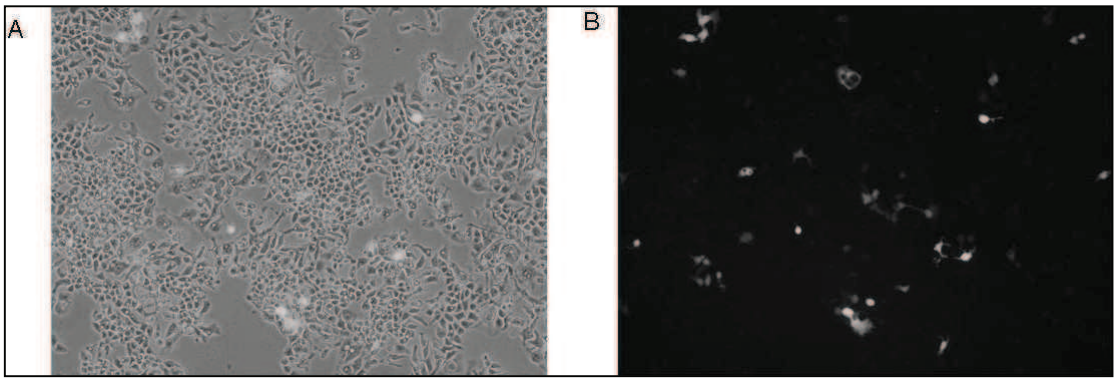

Figure 3-1. Efficiency of transfection with FuGENE®. A: HepG2 cells transfected with eGFPN1, 72 h after transfection. B: HepG2 cells transfected with eGFPN1, 72 h after transfection, UV light.

ExGen 500, a transfection reagent from Fermentas, yielded a much higher transfection efficiency, and was used from this stage onwards.

For the use of ExGen 500 the plating density of HepG2 cells were changed to 3 x 10^5 cells per well of a six well cell culture plate. The protocol as specified by the manufacturer was used for ExGen 500 transfections.

Transient transfections were performed as follows: a day prior to transfection 3 x 10^5 HepG2 cells were seeded and incubated with 2 ml complete growth medium. After 24 h, complete growth medium was replaced with 2 ml DMEM supplemented with only 10% FBS. The concentration of the different shRNA constructs, an untargeted control and the eGFPN1 plasmid was adjusted by diluting 3 µg of DNA to 200 µl with 150 mM NaCl. The dilutions were vortexed and centrifuged for a few seconds. To each of the DNA dilutions, 8.23 µl ExGen 500 was added and immediately vortexed for 10 sec. The reactions were incubated at room temperature for 10 min. After incubation, 200 µl of the transfection mixture was added to the assigned well. A negative control (no DNA or transfection mixture) and a transfection control (transfection mixture without DNA) were also included. After addition of the transfection mixtures, the six well cell culture plates were centrifuged for 5 min at 280*g* in a Heraeus Multifuge 1 L-R centrifuge. The transfections were incubated overnight at 37℃ and 5% CO_2. The following day the DMEM with 10% FBS was replaced with 2 ml complete growth medium and the cultures were incubated for 48 h. After incubation, RNA was isolated and *fah* expression was determined as described in sections 4.2 and 4.4.

The expression levels of *fah* by HepG2 cells after transient transfection with each of the shRNA constructs are given in figure 3-2.

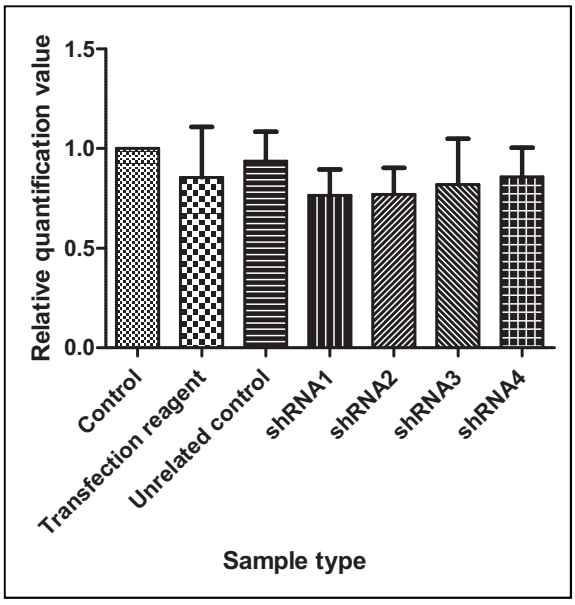

Figure 3-2. Expression of *fah* by HepG2 cells after transient transfection with each of the shRNA constructs. Expression values are the average for three separate transient transfection trials. Error bars indicate the standard deviation. Control: Untransfected HepG2 cells; Transfection reagent: HepG2 cells treated with transfection reagents but without DNA; Unrelated control: HepG2 cells transfected with an untargeted control shRNA construct; shRNA1: HepG2 cells transfected with *fah* knock-down shRNA construct 1; shRNA2: HepG2 cells transfected with *fah* knock-down shRNA construct 2; shRNA3: HepG2 cells transfected with *fah* knock-down shRNA construct 3; shRNA4: HepG2 cells transfected with *fah* knock-down shRNA construct 4.

The expression of *fah* is quantified relative to the expression of *18S* rRNA, by way of the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001:402). The *18S* rRNA gene was chosen as reference gene, since it has been reported that this gene has little variation in expression, and variations in mRNA levels are weak in comparison and will not greatly affect total RNA levels (Kamath-Loeb *et al.,* 2005:740, Thellin *et al.,* 1999:291, Vogel *et al.,* 2002:1505). Expression of *fah* in untransfected control HepG2 cells are normalised to one and the expression level of each of the other samples are expressed relative to this value. From the results in figure 3-2 it is apparent that, compared to untransfected control HepG2 cells, the expression level of *fah* is lower in all of the other samples. The lower expression levels of *fah* in cells transfected with an unrelated control shRNA construct as well as the cells subjected to transfection procedures, but without a shRNA construct, indicate that the process of transfection affects the expression of *fah* to some degree. Nonetheless, the expression of *fah* is lower in the HepG2 cells transiently transfected with the *fah* knock-down shRNA constructs, when compared to expression levels in untransfected control HepG2 cells and HepG2 cells transiently transfected with an untargeted control shRNA construct. The seemingly low level of knock-down (<25%) by the *fah* knock-down shRNA constructs is to be expected. With transient transfections, RNA is extracted from cells simply transfected with the shRNA constructs without selection for cells expressing the shRNA. RNA is therefore extracted from a mixture of cells that express and do not express the shRNA. Keeping in this in mind, it seems that transient transfection with the *fah* knock-down shRNA constructs resulted in no knock-down of *fah*.

For the development of a double-stable knock-down cell line i.e. HT1 hepatic cell model, *fah* knock-down shRNA constructs 1 and 4 were chosen. Construct 1 was chosen since this construct gave the highest degree of knock-down in transient transfection trials, and construct 4 was chosen since dideoxy sequencing showed that this construct was the only construct with hundred percent sequence similarity to the intended target i.e. *fah*.

shRNA constructs 1 and 4 were separately transfected into HepG2tTS cell lines 10 and 12 by following the same transfection protocol that were used for transient transfections, with the difference that 24 h after transfection, the DMEM supplemented with 10% FBS was replaced by 2 ml of selection medium i.e. complete growth medium with 1 µg/ml puromycin and 200 µg/ml G418. Selection medium was then replaced every second day, until multicellular colonies was established for each selection clone. The surviving colonies were each transferred to a well of a 24 well cell culture plate and further propagated in maintenance growth medium. When cell quantities were sufficient, $2x10^5$ cells of each of the double-stable colonies were seeded in each of two wells of a six well cell culture plate. In one of the two wells, *fah* knock-down was induced by adding 1 µg/ml doxycycline to the growth medium. This 'induction' medium was changed every day. Seventy-two hours after the initiation of induction, RNA was isolated from the uninduced and induced *fah* knock-down cells, and the expression level of *fah* was determined as described in sections 4.2 and 4.4.

The expression level of *fah* in the transfected colonies is calculated against the level of *fah* expression in control HepG2tTS10 or HepG2tTS12 cells. The level of *fah* expression in the HepG2tTS10 and HepG2tTS12 cells are normalised to a hundred percent. The level of uninduced and induced *fah* knock-down in each of the double-stable colonies in the HepG2tTS10 and 12 cell lines are given in table 3-2.

The results show that in the HepG2tTS10 cell line a relatively high degree of knock-down of *fah* was achieved in all but one colony. The increase in *fah* expression in colony 10/4/5 after induction of *fah* knock-down with doxycycline is possibly due to an experimental error. The C_T value of *18S* is higher in this sample compared to all other *18S* C_T values. This lower amount of *18S* in the sample would lead to a higher relative expression value for *fah*. The small differences (<5%) between the knock-down values of the uninduced and induced colonies of the HepG2tTS10 cell line, indicate that induction of *fah* knock-down with doxycycline did not significantly effect the expression of *fah*.

Table 3-2. Percentage knock-down of *fah* in HepG2tTS cell lines 10 and 12.

Colony	Percentage knock-down		Colony	Percentage knock-down	
	Uninduced (-Dox)	Induced (+Dox)		Uninduced (-Dox)	Induced (+Dox)
10/1/1	67.2	70.4	12/1/1	17.5	22.9
10/1/3	74.4	74.0	12/1/6	21.0	25.7
10/1/24	77.8	81.2	12/1/8	49.3	1.2
10/1/25	72.3	70.0	12/1/9	53.4	38.4
10/4/2	N.D.	44.7	12/4/1	22.3	44.6
10/4/3	84.2	79.5	12/4/2	-7.9	13.5
10/4/4	N.D.	53.0	12/4/12	-99.7	-106.6
10/4/5	74.1	-302.4	12/4/13	-1.0	-0.3
10/4/6	76.2	78.4	12/4/15	56.4	-209.5
10/UTC/3	63.5	69.2	12/4/16	31.7	42.4
10/UTC/4	48.5	44.0	12/4/22	47.8	57.7
10/UTC/6	72.1	74.6	12/4/24	27.0	32.7
10/UTC/11	59.5	65.2	12/4/29	-5.08	-12.5
10/URC/1	N.D.	74.0	12/UTC/5	N.D.	-4.9
10/URC/3	N.D.	77.5	12/URC/3	24.9	47.2
10/URC/7	70.5	72.2	12/URC/7	N.D.	14.8
10/URC/10	N.D.	73.7			

UTC: Untargeted control; URC: Unrelated control

The expression of *fah* in the HepG2tTS12 colonies are highly variable, as knock-down values differ from 106% higher to 57.7% lower than in uninduced control HepG2tTS12 cells. The same type of possible experimental error as in colony 12/4/15 +Dox is observed for colony 10/4/5 +Dox. As was observed for the HepG2tTS10 colonies, the addition of doxycycline to the HepG2tTS12 colonies did not significantly alter the level of *fah* expression in these cells. The percentage knock-down of *fah* in the HepG2tTS12 colonies was furthermore on average lower than in the HepG2tTS10 colonies.

User guidelines for the Knockout RNAi inducible system recommend the use of doxycycline (dox) instead off tetracycline (tet) as significantly lower concentrations of dox is required for complete activation and dox has a longer half-life than tet, but either can be used. Residual tetracycline in the FBS added to the growth medium may therefore cause a 'leakage' in the inducible system, resulting in the 'continuous' expression of the shRNA and consequently target knock-down. To assess whether any residual tet in the added FBS could be responsible for the same level knock-down seen in uninduced and induced cells, *fah* expression by HepG2tTS12 colonies was determined, expressing either an control untargeted shRNA or a FAH knock-down shRNA. To this end, from control HepG2tTS12, colony 12/4/22 and colony 12/UTC/5, 2 x 10^5 cells were seeded into a well of a six well cell culture plate. For each cell line four wells were seeded. Growth medium supplemented with regular FBS was added to two wells from each cell line. To

the other two wells, growth medium supplemented with special tet-system approved FBS was added. After 24 h, induction was initiated by adding doxycycline to the appropriate wells. Seventy two hours after the initiation of induction, RNA was isolated from all of the wells, and *fah* expression was determined as described in section 4.2 and 4.4. Results from this experiment are given in table 3-3.

Table 3-3. Percentage knock-down of *fah* by different colonies after incubation in different growth media.

Colony	**Percentage knock-down**	
	Regular FBS	**Tet-system approved FBS**
12/4/22 (-Dox)	55.2	71.6
12/4/22 (+Dox)	63.3	67.5
12/UTC/5 (-Dox)	63.7	48.7
12/UTC/5 (+Dox)	63.9	70.7

The results in table 3-3 indicate that the percentage of knock-down was slightly higher in the colonies grown in the tet-system approved FBS supplemented growth medium, than in the colonies grown in regular FBS supplemented growth medium. However, the percentage knock-down difference between induced and uninduced of each colony grown with tet-system approved FBS was comparable to the percentage knock-down difference of the colonies grown with regular FBS. The high level of knock-down in uninduced cells and the small difference in knock-down between induced and uninduced cells, was therefore not the result of residual tet in the regular FBS.

It is furthermore apparent from results in table 3-2 and 3-3 that in both HepG2tTS10 and HepG2tTS12 cell lines, the colonies obtained from transfection with an untargeted shRNA construct or unrelated control target shRNA construct, the expression of *fah* was at the same level as in the colonies transfected with *fah* knock-down shRNA constructs. In theory, these colonies should not exhibit the same or lower *fah* expression than colonies with *fah* knock-down shRNA constructs, as *fah* is not the intended knock-down target. The main question that arises from these results are therefore if only *fah* is affected or if gene expression in general is affected by the process followed for the establishment of the HT1 hepatic cell model. To elucidate this issue, further investigation is needed.

Additionally, it was observed that cell proliferation for all of the double-stable colonies were exceptionally slow and after some time cell proliferation stopped. These double-stable colonies were, therefore not viable. It was previously reported that genes involved in signal transduction, protein turnover and cell growth and proliferation is affected in HT1 cells, and that treatment with

NTBC do not correct these changes (Luijerink *et al.,* 2003:901). Although a high level of knock-down of *fah* was achieved in the HepG2tTS10 cell line, the current version of the HT1 hepatic cell model is not yet ideal for the intended purpose, i.e. determining the mechanisms underlying the mosaicism seen in HT1.

3.2 Future considerations

Although an ideal HT1 hepatic cell model has not yet been achieved, the strive to create the ideal HT1 hepatic cell model should not be diminished. A different approach might result in creation of an ideal HT1 hepatic cell model. As mentioned earlier, tight control of *fah* knock-down is essential, necessitating the use of shRNA or miRNA and excluding the use of siRNA for target knock-down. Hence, things to consider in future:

1. Instead of the use of an inducible system to switch *fah* knock-down on and off, a different vector system that continuously express a *fah* knock-down shRNA could be used. As in the mouse model of Grompe *et al* (Grompe *et al.,* 1993:2298), NTBC can then be added to rescue the phenotype. Recently, some companies (Sigma-Aldrich, OriGene) produced such shRNA vector constructs, validated for *fah* knock-down. Currently there are no reports of studies where these specific products have been used.[1]

2. As in the mouse models by Endo *et al* (Endo *et al.,* 1997:24426) and Manning *et al* (Manning *et al.,* 1999:11928), the simultaneous knock-down of *fah* and *hpd* or *hgd* could be attempted.

3. Use of miRNA as an alternative to shRNA. Some reports suggest that miRNA is less toxic to cells than shRNAs and is therefore safer to use (Boudreau *et al.,* 2009:169, Mcbride *et al.,* 2008:5868).

Of these possibilities, the first is perhaps the most viable, since the corresponding mouse model closely resembles the disease progression in humans (Mitchell *et al.,* 2001:1777). In those mouse models that have two genes simultaneously knocked down, hepatocellular carcinoma did not develop (Nakamura *et al.,* 2007:1556S).

[1] Based on Google (http://www.google.com), ScienceDirect (http://www.sciencedirect.com) and Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) search with the following keywords: shRNA fumarylacetoacetate hydrolase; shRNA tyrosinemia; tyrosinemia RNA interference; fumarylacetoacetate hydrolase RNA interference; shRNA OriGene knock-down model tyrosinemia; shRNA Sigma-Aldrich knock-down model tyrosinemia (Date of access: April 2011).

A further consideration for the development of a HT1 hepatic cell model is the cell line to be used. HepG2 cells are commonly used for research purposes in HT1 (Jorquera and Tanguay, 1999:2284, van Dyk and Pretorius, 2005:815) and knock-down studies (Lin *et al.,* 2010:361, Liu *et al.,* 2008:7175). However, the cells are already carcinoma cells. If HepG2 cells are used for the development of a HT1 hepatic cell model, the already underlying cell changes due to the carcinoma could influence results obtained from research in these cells. Nonetheless, if appropriate controls are included in experiments, HepG2 cells may be an option for the development of a HT1 hepatic cell model. Another liver cell line that might be considered is the THLE2 cell line, which is SV40 transformed liver cells. The ATCC recommends use of these cells for pharmatoxicological studies as well as for the investigation of the aetiology and pathology of hepatocellular carcinoma. However, extensive literature research revealed no current use of THLE2 cells for knock-down studies. The use of a different cell type might also be considered. As FAH deficiency also affects the kidneys and peripheral nerves (Mitchell *et al.,* 2001:1777), kidney or nerve cell lines could be considered. Another possible approach for the development of a HT1 hepatic cell model, would be the use of disease-specific induced pluripotent stem cells (iPSCs) as recently suggested by Ghodsizadeh and colleagues (Ghodsizadeh *et al.,* 2010:622).

In conclusion, although the development of an ideal HT1 hepatic cell model for the intended use was not achieved in the current study, the development of such a model is still appealing. Apart from the major advantage of a HT1 hepatic cell model being human genome based, basic advantages of use of cell cultures, such as less ethical limitations, are also included.