The efficiency of three shRNAs in silencing the galactose-1-phosphate uridyl transferase gene

BY

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BaccalareusTechnologiae: Biotechnology

Dissertation submitted in fulfillment of the requirements for the degree

MAGISTER TECHNOLOGIAE: BIOTECHNOLOGY

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2013

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Acknowledgements

I wish to express my gratitude to the following individuals who enabled this document to be successfully and timeously completed:

My supervisor: Professor M.Pillay thank you for all the time you dedicated to this work to help me finish.
Pillar of strength: Dr Phiyane Justice Lebea without you this journey was going to be endless. Thanks for all the sacrifices you made just to see me finish this study.
Financial Contributors: Research Department at Vaal University of Technology

Faculty : Faculty of Applied and Computer Sciences

Department : Biosciences

Institution : Vaal University of Technology and CSIR

Family: Thanks to Thulo Azael Nokoane, the late Mamotshabi Maria Nokoane, Mookho Sehume, Sammy Nokoane and Mokhele Ishmael Nokoane. I do not have words to express myself for your amazing support, love, and care during my studies. Thank you for babysitting Mokhele for me when I had to work on this Masters Degree and without your assistance I was not going to make it. God bless you.

Rand Water: Thanks to Professor Hein du Preez for believing in me, for motivating me and allowing me to use Rand Water resources.

ABSTRACT

Classic galactosemia occurs when an enzyme called galactose-1-phosphate uridyltransferase (GALT) is missing or not functional and causes serious health problems resulting in death.The long term prognosis of classical galactosemia, which results from inactivity of the protein product of galactose-1-phosphate uridyltransferase (GALT) has been poor even in patients that were treated early on in life. The reasons for this poor prognosis are ill-understood.

This study seeks to design and test specific short hairpin RNA (pshRNA) for their efficiency in knocking down the GALT gene RNA products thereby limiting the resultant enzyme activity. The following objectives were followed in designing the current study:

1. Designing a shorthairpin RNA (pshRNA) to target different regions of the coding sequence of the target GALT gene.
2. Propagating the pshRNAs in *Escherichia coli (E.coli)* and subsequently isolation of the respective plasmids for transfection.
3. Transfection of HeLa cells to test the efficiency of relevant pshRNAs in knocking down the GALT gene expression.
4. Transfection was followed by extraction of total mRNA, purification and quantification of total mRNA.
5. The GALT gene expression was qualitatively quantified against a house-keeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH) to evaluate efficiency of knockdown using real time PCR.

The three newly designed pshRNA (pshRNA2, pshRNA3 and pshRNA4) targeting the GALT gene expression showed a knockdown efficiency of 171 %, 48 % and 200 %, respectively.

The results of this study will be useful for future evaluation of the possible long term glycosylation patterns under proper UDP glucose/UDP galactose levels compared with variable defective GALT gene levels.

ABBREVIATIONS

Ago2	Argonaute
ATP	Adenosine triphosphate
cDNA	Complementary deoxyribonucleic acid
E.coli	*Escherichia coli*
Gal-1-P	Galactose-1-phosphate
GALE	UDP galactose 4-epimerase
GALK	Galactokinase
GALT	Galactose-1-phosphate uridylyltransferase
GAPDH	Glyceraldehyde phosphate dehydrogenase
GLUT	Glucose family of transporters
hUGP2	Human UDP-glucose pyrophosphorylase
mRNA	Messenger ribonucleic acid
NAD	Nicotineamide-adenine-dinucleotide
PCR	Polymerase chain reaction
pshRNAs	Plasmid shorthairpin ribonucleic acid
RBC	Red blood cell
RISC	RNA-induced Silencing complex

RNA	Ribonucleic acid
RNAi	RNA interference
UDP Gal	Uridine Diphosphate galactose
UDP-galactose	Galactose-1-phosphate uridylyltransferase
UDPGlc	Uridine diphosphate glucose
UDP-glucose	Glucose-1-phosphate uridylyltransferase
UMP	Uridylyl monophosphate
UTP	Uridine-5'-triphosphate

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GLOSSARY

Classic Galactosemia:	Classic galactosemia occurs when an enzyme called galactose-1-phosphate uridyltransferase (GALT) is missing or not functional and causes serious health problems resulting in death
Galactosemia:	Galactosemia is an inherited disease in which the transformation of galactose to glucose is blocked, allowing galactose to increase to toxic levels in the body and it is asymptomatic.

CHAPTER 1
Introduction

Lactose is the predominant carbohydrate in mammalian milk, and its major role is that of osmole which assures the production of a fluid milk (Leslie 2003). Lactose is hydrolyzed into glucose and galactose by the reaction catalyzed by lactase. The lactase enzyme is located in the brush border (microvilli) of the small intestine enterocyte. The enzyme activity and the transit time of lactose through the jejunum mucosa are important for proper absorption. If lactase is absent unabsorbed sugars osmotically attract fluid into the bowel lumen. The fluid influx into the bowel is approximately triple the predicted amount based on the osmolality of the sugar content alone, because the intestine cannot maintain a high electrochemical gradient between contents and blood (Swagerty *et al.* 2002). Galactose is a six carbon simple sugar, an isomer of glucose and is also an aldohexose. Galactose is present in some glycoproteins and glycolipids. Glycolipids and glycoproteins are involved in the cell-cell recognition, regulation of the cell cycle, receptor function and other biological functions.

In most organisms, the conversion of D-galactose to the more metabolically useful glucose 1-phosphate is accomplished by the action of four enzymes that compose the Leloir pathway (Fig. 1). In the first step of this pathway, β-D-galactose is epimerized to α-D-galactose by galactose mutarotase. The next step involves the ATP-dependent phosphorylation of α-D-galactose by galactokinase to yield galactose 1-phosphate. Galactose-1-phosphate uridylyltransferase (GALT) catalyzes the transfer of a UMP group from UDP-glucose to galactose-1-phosphate, thereby generating glucose-1-phosphate and UDP-galactose. UDP-galactose is converted to UDP-glucose by UDP-galactose-4-epimerase. In humans, defects in the genes encoding for galactokinase, uridylyltransferase, or epimerase can give rise to the diseased known as galactosemia. The decreased GALT enzyme levels and elevated galactose-1-phosphate cause the disease galactosemia. Galactosemia is a disease associated with the inability to convert galactose to glucose. This disease may result in an increased concentration of the galactose in the blood.

Classic galactosemia it is often confused with milk allergy or intolerance, whereas it is a life time inherited genetic disease which affects many infants. Galactosemia is autosomal recessive in origin, that is, each parent carries a variant gene for the disorder. Although these individuals have less than normal enzyme activity, they appear clinically healthy with little or no symptoms. Since each parent contributes equally to the fetus gene pool, the possibilities that a child will be born with the disease are enhanced. Combination of two variant genes will result in a 25 % chance of producing an offspring with the true form of the disorder that is classic galactosemia. Males and females have an equal chance of having the condition (Chung 1997). Galactosemia was discovered in the year 1935 in different countries and in different populations.

To date there are different hypotheses for the actual biochemical consequence of long-term prognosis of galactosemia. There are suggestions that high levels of galactose-1-phosphate are responsible for the unsatisfactory outcome while some studies emphasize the role of UDP-galactose and glucose as playing a part in poor long-term prognosis. When GALT enzyme activity is deficient, galactose-1-phosphate, galactose, and galactitol tend to accumulate. Galactose-1-phosphate competes with the UTP-dependent glucose-1-phosphate pyrophosphorylase to reduce UDP-glucose production, thus both UDP-glucose and UDP-galactose are reduced, resulting in abnormally glycosylated proteins (Elsas 2000).

For the purpose of this study, short hairpin ribonucleic acid (pshRNAs) that can sufficiently knock down the transcription of GALT were investigated using RNAi. The aim of the study was to use pshRNAs with sufficient variable knockdown to ultimately be used to study the hypothesis that long-term factors that are responsible for poor long-term prognosis of classic galactosemia are related to the improper UDP-glucose/UDP-galactose. The following methodology was followed in designing the current study:

1. Designing a shorthairpin RNA (pshRNA) to target different regions of the coding sequence of the target GALT gene.
2. Propagating the pshRNAs in *Escherichia coli (E.coli)* and subsequently isolation of the respective plasmids for transfection.

3. Transfection of HeLa cells to test the efficiency of relevant pshRNAs in knocking down the GALT gene expression.
4. Transfection was followed by extraction of total mRNA, purification and quantification of total mRNA.
5. The GALT gene expression was qualitatively quantified against a house-keeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH) to evaluate efficiency of knockdown using real time PCR.

In summary, the three newly designed pshRNA used in this study was for targeting the GALT gene expression knockdown, the results obtained indicate the efficiency of knockdown was 171 %, 48 % and 200 % for pshRNA2, pshRNA3 and pshRNA4, respectively. This translates to an addition of one effective pshRNA that can be used in conjunction with those already described by Lebea (2006) and used in this study as pshRNA1 with 22 % knockdown efficiency. This will help with future evaluation of the possible long term glycosylation patterns under proper UDP glucose/UDP galactose levels comparing with variable defective GALT gene levels. A spectrum of complication associated with classic galactosemia still persists. The complications include ataxia, learning disabilities, and verbal dyspraxia in >30 % of patients and primary ovarian failure in >80 % of females (Waggoner *et al*. 1990). The proposed future work includes evaluating the possible effect of glycosylation of proteins and/or lipids in relation to these symptoms.

CHAPTER 2

Literature review

2.1. Chronological review of galactosemia

Galactosemia is a disorder of galactose metabolism that can result in life threatening complications in infants. The first report of galactosemia was by von Reuss in 1908. It concerned an infant fed on breast milk with poor growth. In 1917, Goppert reported an infant with poor growth, lactose exposure, and hypergalactosuria. The first comprehensive description of the variant form of hereditary galactosemia in 1935 was described by Mason and Turner based on an African-American infant. It was also the first report of a patient with any form of galactosemia due to GALT deficiency in the American literature. This patient had not been placed on a lactose-restricted diet until 10 months of age. The diet treatment quickly reversed the complications of poor growth, developmental delay, liver disease, and anemia. The presence of a defective human transferase gene results in low enzyme activity that causes galactosemia, and this slows down the transformation of galactose into glucose-1-phosphate. Galactose increases and produces galactose-1-phosphate which is toxic. Toxic galactose-1-phosphate is associated with the following symptoms (Frey 1996), including enlargement of the liver and the spleen, proteinuria, aminoaciduria, the disorders of the central nervous system and cataracts.

The first clinically recognized form of galactosemia was reflective transferase deficiency known as classic galactosemia (Gitzelmann 1967). The second form of galactosemia to be recognized clinically was Galactokinase (GALK) deficiency and was first reported by Gitzelmann (1967). He reported that patients with GALK deficiency demonstrate none of the potentially fatal developmental, cognitive, or ovarian complications seen in classic galactosemia but do present with neonatal cataracts, which generally self-resolve upon dietary restriction of galactose. On the basis of biochemical ascertainment of carriers in a select white population, GALK deficiency was estimated to affect 1/40,000 to 1/50,000 new-born infants (Mayes & Guthrie 1968). One large population study, however, indicated that the frequency of detectable GALK deficiency at birth is significantly less than1/100,000 (Levy 1980).

The studies done years back indicate that this disease could not be cured or treated and that is why as years progressed further investigations were done on classic galactosemia. In the year 2000 Holton *et al*. stated that classic galactosemia occurred with a frequency of 1/30,000 to 1/60,000 live births in the United States and in other multi-ethnic populations, and it is pan-ethnic. It is therefore plausible that the frequency of classic galactosemia in the USA population is on average 1/45 000. The literature on the actual frequencies in South Africa is inconclusive since the studies performed usually with very small numbers compared to the total national population size. One study done on the black population in Cape Town by Henderson *et al*. (2002) showed that approximately 1/14 400 of newborn blacks in the Cape Metropole had classic galactosemia and this is much higher than the current detection rate. It is thus likely that many patients go undetected. They concluded that the true incidence of galactosemia in the South African population is unknown, as newborn screening programmes have not been introduced.

Another South African study was done by Lebea and Pretorius (2008), using hormone screening on newborn babies. The study found that in Mpumalanga an incidence of 0.1 % for congenital hypothyroidism occurred and no patients with classic galactosemia were detected. It is not surprising that more infants with classic galactosemia were not described by 1935 because it was thought that most untreated babies die of *E. coli* sepsis in the new-born period (Berry *et al*. 2000).

Untreated classic galactosemia can lead to rapid neonatal death. Its known symptoms are vomiting, diarrhoea, cataracts, failure to thrive, hepatomegaly, liver dysfunction with bleeding diathesis, *E. coli* sepsis, and ending in neonatal death. Classic galactosemia is often detected pre- symptomatically in most industrialized nations by new-born screening and is treated by lifelong dietary restriction of galactose; although removing galactose from the diet resolves the severe and possibly fatal symptoms of classic galactosemia it is not always an effective tool towards long term clinical effects of the disorder. The third and most poorly understood form of galactosemia is UDP-galactose-4-epimerase (GALE) deficiency. The great majority of patients with GALE deficiency are asymptomatic. These individuals have shown normal growth and development and

have not developed any other serious sicknesses compared to the patient with GALT and GALK deficiency enzyme *(*Holton *et al.* 2000*).*

2.2. Galactose metabolism and different types of galactosemia

Galactose metabolism from bacteria to humans is carried out in three enzymatic steps that are catalyzed sequentially by galactokinase (GALK), galactose-1-phosphate uridyl transferase (GALT), and UDP-galactose-4-epimerase (GALE) as shown in Fig.2.1 (Petry & Reichardt 1998).The metabolism process takes place through the initial phosphorylation of galactose to galactose-1-phosphate by the action of the galactokinase. Galactose-1-phosphate is transformed into glucose-1-phosphate and UDP-glucose by the action of the galactose-1-phosphate-uridylyl transferase. One molecule of ATP is required to convert galactose to glucose-1-phosphate. Glucose-1-phosphate is transformed into glucose-6-phosphate by the action of the enzyme phosphoglucomutase. The metabolism of glucose through glycolysis requires one molecule of ATP to phosphorylate it to glucose-6-phosphate (Frey 1996). Galactose can be converted into energy by entering the glycolytic pathway because glucose-1-phosphate is one of the GALT reaction products (Petry & Reichardt.1998).

The function of the GALT is to incorporate the uridine nucleotide moiety into the substrate by producing UDP-galactose and glucose-1-phosphate from UDP-glucose and galactose-1-phosphate which are needed for cell glycosylation. Glucose-1-phosphate enters glucose metabolism and UDP-galactose undergoes epimerization to UDP-glucose (Frey 1996). GALT is the second enzyme in the Leloir pathway, and is essential in human infants who consume lactose as their primary carbohydrate source. In infants with classical galactosemia, who have near or total absence of GALT activity, exposure to dietary galactose results in acute deterioration of multiple organ systems, including liver dysfunction, coagulopathy, poor feeding and weight loss, renal tubular dysfunction, cerebral edema, vitreous hemorrhage and *E.coli* sepsis (Leslie 2003).

When GALT is not functioning at all, there is another enzyme named pyrophosphorylase which does the same function as GALT but at a lower rate of 1 %.

Infants with total or absent GALT activity can still survive high levels of galactose-1-phosphate as it can still convert galactose to UDP-galactose and UDP-glucose through the GALE enzyme in Lelior pathway (Holton *et al*. 2000). The catalytic activities of UDP-galactose pyrophosphorylase and UDP-glucose pyrophosphorylase reside in the same protein (Leslie 2003).

GALE catalyzes the interconversion of UDP-glucose and UDP-galactose and is expressed in most tissues and cells, with greatest expression in human placenta and lungs as well as in several transformed cell lines. In humans with GALE deficiency, galactose is never the sole carbon source, the Leloir pathway intact through GALT, and UDP glucose can be synthesized normally through the pyrophosphorylase pathway. The action of the epimerase depends on the binding of substrate, reduction of carbon through a bound NAD cofactor, followed by rotation of substrate with the active site and positioning of a new hydroxyl group on the opposite site (Leslie 2003).

As mentioned above, galactose metabolism from bacteria to humans is carried out in three enzymatic steps and there is one other enzymes galactokinase (GALK) which is associated with galactosemia. However, this enzyme does not cause the severe clinical outcome as compared to GALT impairment. Galactokinase catalyzes a bidirectional reaction, but the equilibrium favors formation of galactose-1-phosphate. High levels of galactose-1-phosphate inhibit the forward reaction, and in the presence of the blocked Leloir pathway there is sufficient GALK inhibition to allow inhibition of free galactose (Leslie 2003).

Galactokinase deficiency galactosemia is a rare autosomal recessive inborn error of galactose metabolism. Cataract and sometimes pseudotumor cerebri appear to be the complication of galactokinase deficiency. The clinical outcome for patients with galactokinase deficiency is much better than for patients with classical galactosemia. Inhibition of galactokinase may be a promising approach for controlling damage in GALT deficiency patients. Cataracts caused by galacticol accumulation seem to be the only consistent abnormality in galactokinase deficiency and this can be prevented with a galactose restricted diet (Bosch *et al.* 2002). Galactose may be reduced to galacticol in a sorbitol pathway as shown in Fig.2.1 through the action of aldose reductase. Aldose

reductase is the first enzyme in the sorbitol pathway. It catalyzes the reduction of sugars to alcohol, particularly glucose to sorbitol and galactose to galacticol. Cataracts are induced by GALK deficiency, the phenotype is expressed only when aldose reductase is over expressed by a transgene (Leslie 2003).

Free galactose may be reduced to galacticol through the action of aldose reductase. Galacticol is poorly diffusible and may lead to significant tissue accumulation of galacticol in the cell (Leslie 2003). Free galactose is transported into cells by many transporters that are members of the glucose family of transporters (GLUT). Once inside the cell, galactose is trapped as galactose-1-phosphate in cells that express galactokinase.

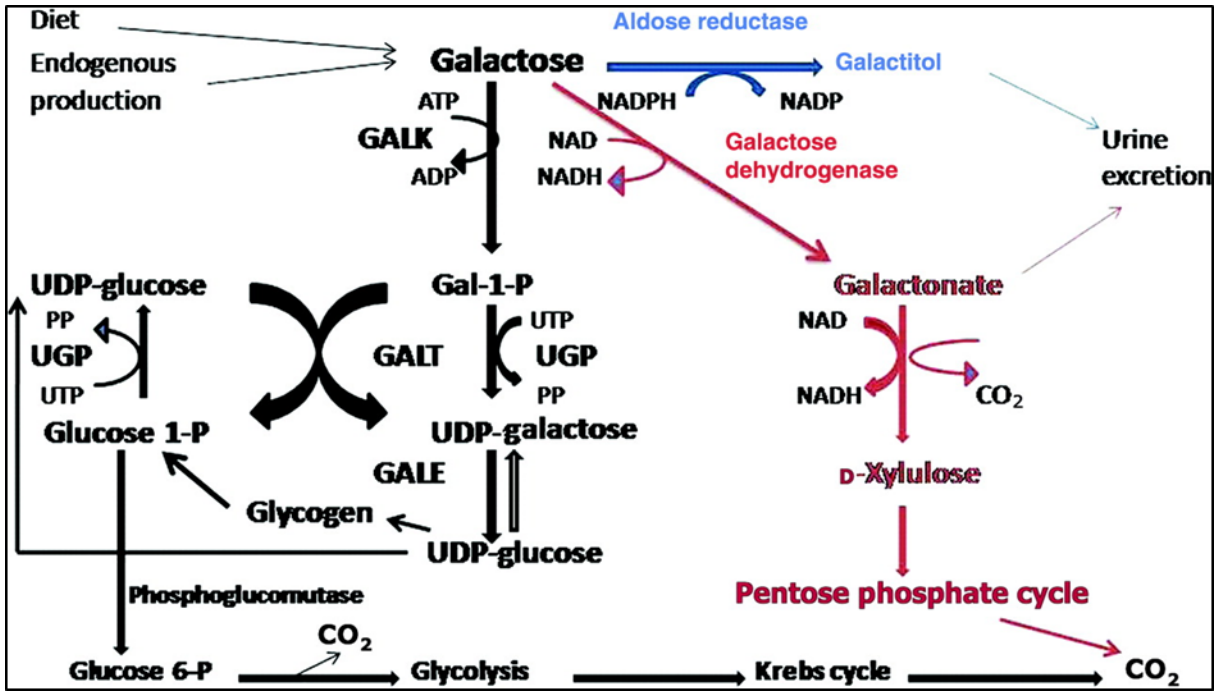

Figure 2.1: Pathway of galactose metabolism GALK, galactokinase; PP, pyrophosphate; UGP, UDP-glucose pyrophosphorylase; GALT, galactose-1-phosphate uridylyltransferase; GALE, UDP-galactose 4-epimerase. (Ficicioglu *et al.* 2010.)

2.3. Molecular tools used over time to study galactosemia

Various experiments have been conducted to investigate this disease. These include studies conducted to identify biomarkers of different types of galactosemia. The results of each study vary, depending on the mutations that have been identified as well as the general impairment of the resultant defective enzyme.

2.3.1. Reduced UDP galactose levels in classic galactosemia tissues.

Xu *et al.* (1989) assessed whether uridine diphosphate galactose (UDPGal) and uridine diphosphate glucose (UDPGlc) concentrations are altered in a galactosemic patient and if this may play a role in the pathogenesis of the disease. These nucleotide sugars were measured in various tissues including liver, erythrocytes and cultured skin fibroblasts from galactosemic patients and normal controls. Xu *et al.* (1989) found that in cells from normal individuals galactose-1-phosphate (Gal-1-P) was only detected in the presence of galactose in an incubated medium, while the levels of UDPGlc and UDPGal remained the same. When uridine was added to the incubation medium, the amount of both UDPGlc and UDPGal increased. In actual quantities, more UDPGlc was formed than UDPGal. When uridine and galactose were added at the same time, both UDPGlc and UDPGal increased, substantially more UDPGal was formed. It was also noted that more Gal-1-P was present in the presence of uridine but in contrast with intact cells from three galactosemic patients when galactose was added Gal-1-P increased 7-10 folds, while the levels of UDPGlc and UDPGal remained unchanged. In the presence of uridine alone, the Gal-1-P remained the same but UDPGlc and UDPGal increased to the normal extent as in the normal control cells. In the presence of both uridine and galactose, Gal-1-P rose markedly, while the change in UDPGlc was about the same as that seen when uridine alone was used. In these cases, there were no differences in Gal-1-P concentrations in the presence or absence of uridine (Xu *et al.* 1989). It was therefore concluded that there are several mechanisms which may contribute to the decrease in UDPGal in tissues of patients with classical galactosemia, which are lower UDPGal-4-epimerase and UDPGlc pyrophosphorylase activity and complete absence of transferase activity. It is also possible that the decreased level in UDPGal may be due to inhibiton of UDPGlc pyrophosphorylase by residual Gal-1-P. It is also possible that a

complete block in the transferase reaction results in the decreased levels of UDPGal and that the reactions of UDPGlc pyrophosphorylase and UDPGal-4-epimerase are insufficient to generate enough UDPGal from glucose sources to maintain normal levels (Xu *et al.* 1989).

Charlwood *et al.* (1998) assessed the correlation between the red blood cell galactose-1-phosphate and the percentage of disialyted biantennary glycans present in serum transferrin from patients with galactosemia before and after treatment. They found that there was not a clear correlation between the level Gal-1-P and the length of treatment or state of glycosylation. They then concluded that the structures of the truncated glycans found on serum transferrin from untreated galactosemic patients, are consistent with a decreased capacity to galactosylate glycoproteins. This defect in galactosyltransferase activity could be due to the inhibition of the galactosyltransferase enzyme itself or decreased availability of the substrate, UDP-galactose. There was no clear correlation between the length of treatment and the fall in the level of Gal-1-P in the red blood cell and the normalization of glycosylation of serum transferrin. There are several possible explanations for this observation which are: there is a difference in response times for the drop in red blood cell Gal-1-P level and normalization of serum protein glycosylation. There is some evidence that galactose-1-phosphate inhibits galactosyltransferase when at high concentration in cells and it has been shown to inhibit milk UDP-galactosyltransferase activity. It is also a competitive inhibitor and substrate for UDP-glucose pyrophosphorylase, which catalyzes the formation of UDP-galactose from Gal-1-P and UTP. Galactose, Gal-1-P or other galactose derivatives may inhibit other enzymes involved in the synthesis of UDPgal, or its transport into the Golgi, thereby lowering the effective substrate concentration. Based on the two studies by Xu *et al.* (1989) and Charlwood *et al.* (1998) it seems that there is a correlation between defective galactosylation and glycosylation of proteins possibly due to decreased availability of UDP galactose in classic galactosemia tissues.

2.3.2. The need for a human galactosemia functional model.

Lai *et al*. (2003) developed a human cell model that was similar to the yeast system. They used SV40-transformed human fibroblasts that were derived from a patient with classic galactosemia. This cell line accumulated Gal-1-P and did not grow on galactose. However, when these GALT-deficient cells were transfected with human UDP-glucose pyrophosphorylase (hUGP2) or GALT they were rescued. They also tested the hypothesis that in classic galactosemia, Gal-1-P accumulated and reduced UDP-glucose synthesis by inhibiting hUGP. They quantified UDP-glucose and UDP-galactose and determined the kinetics of hUGP inhibition by Gal-1-P. They concluded that overexpression of hUGP2 in human GALT-deficient cells overcame inhibition of endogenous hUGP2 by Gal-1-P and restored normal UDP-hexose levels. Lai *et al*. (2003) found out that there are at least three possible mechanisms to explain why human GALT-deficient fibroblasts cannot grow in medium with galactose as the sole carbohydrate. They include lack of energy because galactose is not metabolized to form glucose-1-phosphate, toxicity resulting from accumulated toxins such as Gal-1-P in the blocked Leilor pathway and decreased production of essential uridylated hexoses such as UDP-galactose.

In a separate study, Leslie (2003) constructed a GALT double knockout transgenic mouse, but these mice were healthy and fertile despite being fed with high galactose diet. The absence of complications in the GALT-knockout mice suggested that human galactose metabolism is unique and emphasized the need to develop a human cell model system.

Douglas & Hawthorne (1964) were the first to describe galactose sensitivity in the single celled eukaryote, *Saccharomyces cerevisiae*. They isolated mutants that were unable to grow in medium containing galactose (Gal+), as the sole carbon source. Douglas & Hawthorne (1964) also found that gal7-null and gal10- null cultures were unable to grow in medium containing different levels of galactose although another carbon source, ethanol, was present. But gal7-null yeast accumulated gal-1-phosphate (Gal-1-P) upon exposure to galactose. They also observed that spontaneous mutations in GAL1 restored growth of the gal10- null yeast on medium containing both galactose and

ethanol (Ross *et al*. 2004) and concluded that only the GALT-null appears to metabolize galactose, but at a slower rate. GALE-null yeasts arrest growth at galactose concentrations 10-fold higher than do their GALT-null counterparts. They concluded that although all galactose arrested cultures demonstrate abnormally high levels of Gal-1-P, there is no quantitative relationship between peak Gal-1-P attained and the degree of arrest observed in a given culture.

From these three studies, it is apparent that a human cell model for classic galactosemia would be desirable to further study the factors that contribute towards poor long term prognosis of classic galactosemia patients. This study will design and test more pshRNAs with sufficient variable knockdown to contribute to the available ones. In that manner a contribution will be made towards ultimately using these pshRNAs to knock down GALT mRNA sufficiently to study the hypothesis that long-term factors that are responsible for poor long-term prognosis of classic galactosemia are related to the improper UDP-glucose/UDP-galactose.

2.4. RNA Interference

RNA interference (RNAi) is the process that inhibits the flow of genetic information from messenger RNA (mRNA) to protein synthesis (Vilgelm *et al.* 2006).The process is also known as RNA silencing, co-suppression or post transcriptional gene silencing. The presence of small RNAs inside cells has been known for more than 20 years. Yet such RNAs did not attract much interest until recently, because they were considered as the degradation products of other RNAs. The role of eukaryotic small RNAs was fundamentally revised after the discovery of RNA interference. RNAi was first observed in transformation studies in the petunia flower when the introduction of a pigment producing gene (*CHS*) suppressed the expression of both the introduced and the homologous endogenous gene (Napoli *et al*. 1990). RNAi was first discovered in *Caenorhabditis elegans* animals by Fire *et al*. (1998).

When dsRNA was injected into *C. elegans* specific and effective gene silencing was observed. The double stranded RNA reduced the activity of the genes. Various hypotheses were advanced to explain the mechanism of RNA interference. It was found

that these small and, at first glance, insignificant molecules play a key role in eukaryotic development (Vilgelm *et al.* 2006).

2.4.1. Mechanism of RNA Interference

The basic mechanism of RNAi is a multistep process (Kabir *et al*. 2008). RNA interference in the cell is initiated when exogenous or endogenous (transcribed from cell genes) dsRNA is introduced into a cell. The efficiency of RNA interference directly depends on the dsRNA size.

The longer the dsRNA, the higher is the production of siRNA. When the dsRNA enters the cell, it is targeted by the enzyme Dicer. The Dicer cut the dsRNA into smaller segments of 21-25 nucleotides (Fig 2.2). The siRNA associate with RNA-induced Silencing complex (RISC) in the cell cytoplasm, interact with the catalytic RISC component which contains several proteins surrounding small interfering RNA (siRNA) (Caudy *et al*. 2003; Zeng *et al*. 2003). siRNA duplex then becomes single stranded and binds to the targeted mRNA, cleaving it in the region covered by siRNA (Parrish *et al*. 2000).

2.4.2. Dicer

Dicer is a ribonuclease in the type III RNase family. It is responsible for recognizing and cleaving dsRNA into short double stranded RNA fragments called small interfering RNA (siRNA) that are 20-25 bp in size. The size of these fragments is species specific. These siRNAs contain two protruding non-paired nucleotides with a hydroxyl group at the 3`end and a phosphate group at the 5` end. Such a structure is essential for further steps of the process leading to RNA silencing. Molecules with blunt ends or a modified 5`-terminal region lack the siRNA activity (Vilgelm *et al.* 2006).These siRNA are then incorporated into RNA-induced Silencing complex (RISC) which contains several proteins. RISC induces Argonaute (Ago2). RISC bound with double-stranded siRNA is inactive. For its activation it is necessary that the siRNA strands are separated by ATP-dependent helicase. When the RISC enzyme is activated it binds to the targeted mRNA using the siRNA as a guide to find the target sequence. The endoribonuclease enzyme

cleaves the mRNA which is then degraded by exoribonucleases resulting in a loss of expression of the gene (Rahman *et al*. 2008).

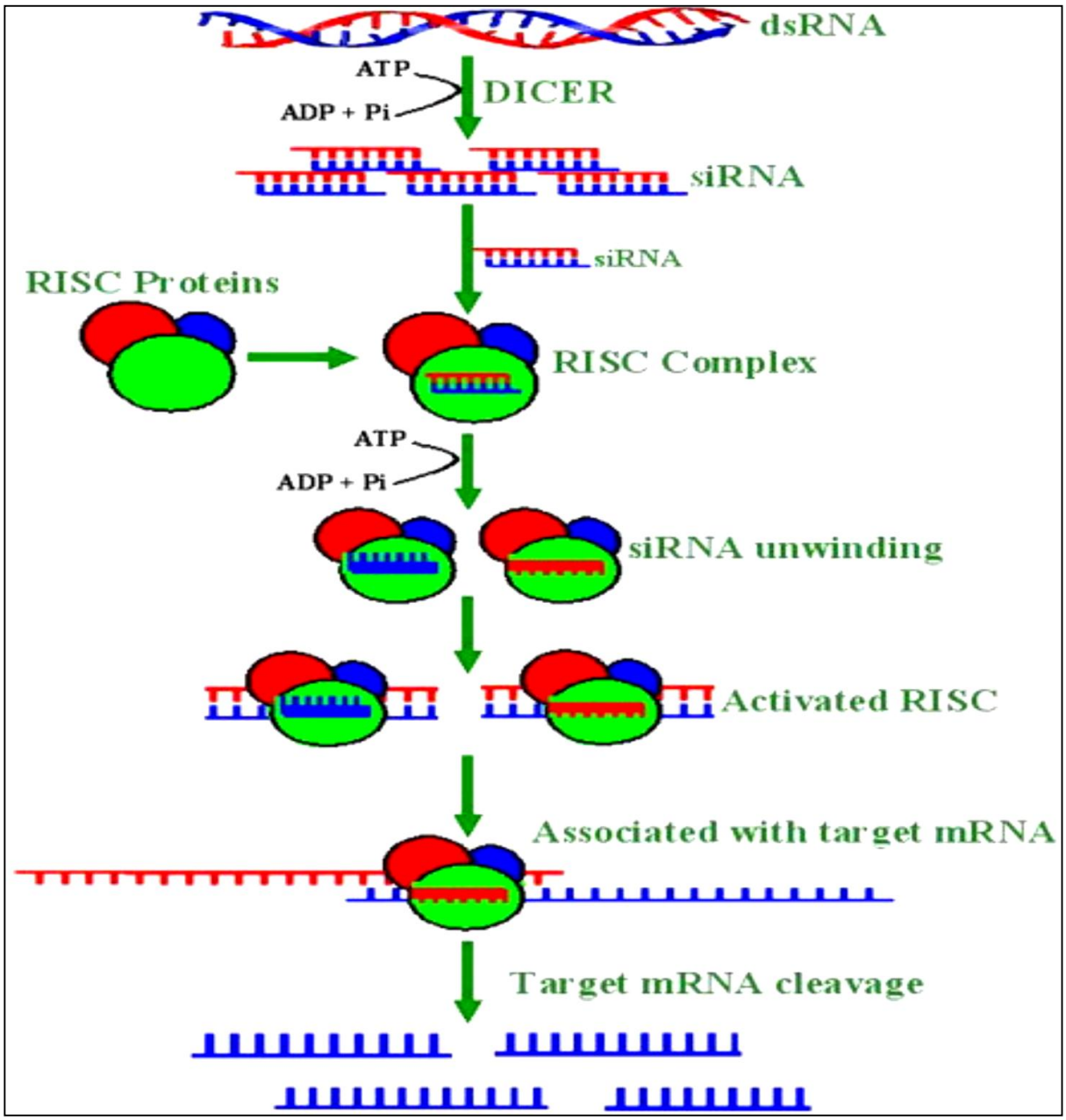

Figure 2.2: RNAi mechanism: silencing of viral genes through activation of RISC. siRNAs combined with RISC binds to mRNA causing cleavage of its sense strand (Rahman *et al*. 2008).

2.4.3. siRNA Design, optimization and delivery design of target sequences

Identifying an optimal target sequence is critical to the success of RNA interference experiments. Since it is not possible to predict the optimal siRNA sequence for a given target, multiple siRNAs are evaluated.

Recommendations for the design of siRNAs are constantly being improved upon as knowledge of the RNAi process continues to expand. The recommendations are as follows: siRNA target sequences should be specific to the gene of interest and have ~20–50 % GC content (Henshel *et al*. 2004). Ui-Tei *et al*. (2004) reported that siRNAs satisfying the following conditions are capable of effective gene silencing in mammalian cells: 1) G/C at the 5′ end of the sense strand; 2) A/U at the 5′ end of the antisense strand; 3) at least 5 A/U residues in the first 7 bases of the 5′ terminal of the antisense strand; 4) no runs of more than 9 G/C residues.

The efficiency of gene silencing is influenced largely by the choice of the mRNA target site to which the complementary siRNA will bind. The accessibility for siRNA is impaired by secondary structures or proteins bound to the target RNA. The first 75-100nt of an mRNA is not recommended as a target site for RNA interference because they contain regulatory protein binding sequences. The target site of siRNA is chosen individually by a trial and error method in each case. The most common way is to synthesize three or four siRNAs complementary to different regions of the target mRNA and to experimentally select the most efficient.

The optimal size of the target site and, consequently, siRNA is thought to be 21nt. The target site must start with two adenines. RNA interference is most effective when synthetic double-stranded siRNA has 19 paired bases and two protruding uridines at the 3’ end of each strand. The sense strand of siRNA can differ from the target site in two 3’ terminal nucleotides. A perfect matching of them is not essential, because only the antisense strand is involved in the effector complex (RISC), which is responsible for mRNA degradation. Replacement of non-paired uridines with de-oxythymidines improves the siRNA stability in the cell. Although absolutely essential for the siRNA

function, phosphorylation of 5’ ends is not critical in designing siRNA, because its ends can be phosphorylated in the cell (Fig. 2.3) (Vilgelm *et al.* 2006).

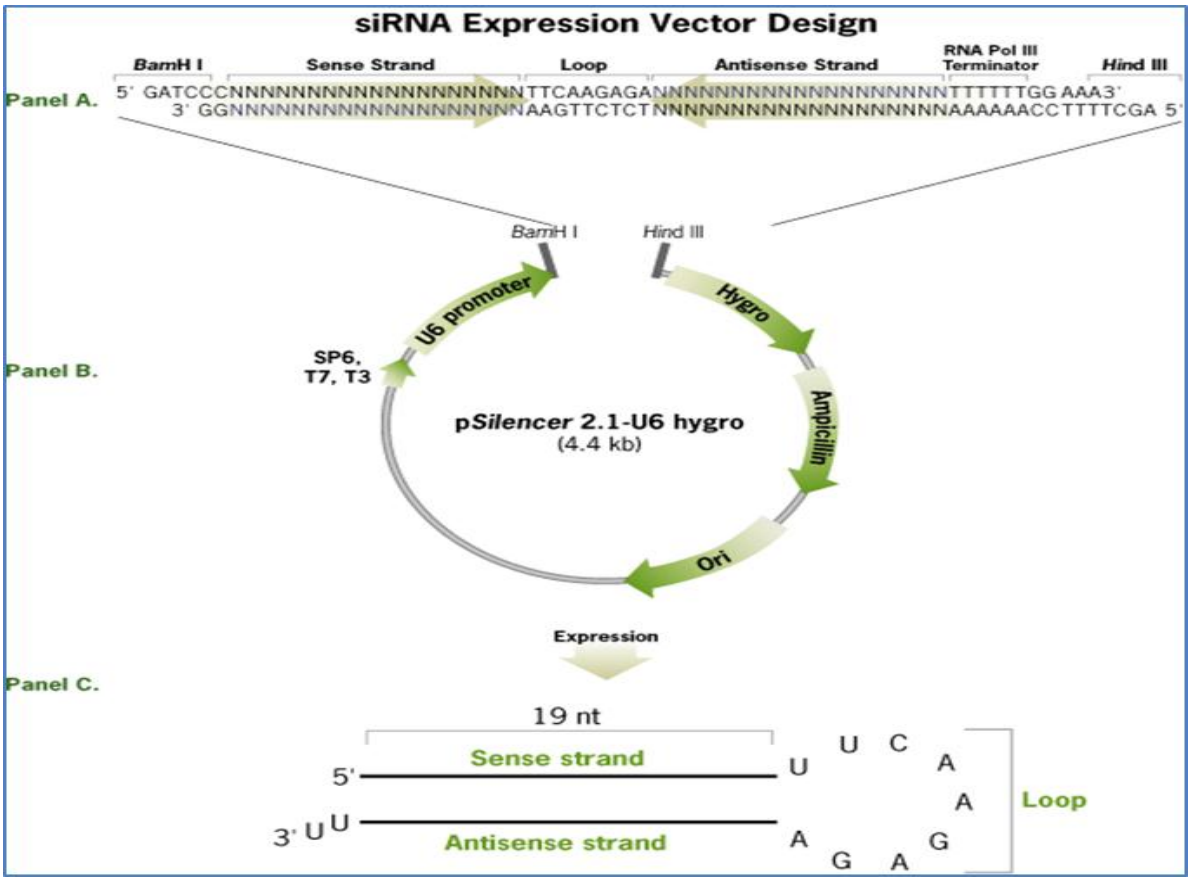

Figure 2.3: Design of synthetic siRNA (Life technologies. Invitrogen.com Ambion Tech Notes 10.1)

2.4.4. Generation of Short Interfering RNAs (siRNA)

siRNAs are the main effectors of the RNAi process. These molecules can be synthesized chemically or enzymatically in vitro (Micura 2002; Paddison *et al*. 2002; Betz 2003) or endogenously expressed inside the cells in the form of pshRNAs (Yu *et al*. 2002; McManus *et al*. 2002). Plasmid-based expression systems using RNA polymerase III U6 or H1, or RNA polymerase II U1, small nuclear RNA promoters, have been used for endogenous expression of pshRNAs (Brummelkamp *et al*. 2002; Sui *et al*. 2002, Novarino *et al*. 2004).

2.4.5. Delivery of siRNA

The efficient delivery of siRNAs is a vital step in RNAi-based gene silencing experiments. Synthetic siRNAs can be delivered by electroporation or by using lipophilic agents (McManus *et al*. 2002; Kishida *et al*. 2004).

siRNAs have been used successfully to silence target genes; however, these approaches are limited by the transient nature of the response. The use of plasmid systems to express small hairpin RNAs helps to overcome this limitation by allowing stable suppression of target genes (Dykxhoorn *et al*. 2003). Various viral delivery systems have also been developed to deliver pshRNA-expressing cassettes into cells that are difficult to transfect, creating new possibilities for RNAi usage (Brummelkamp *et al*. 2002; Rubinson *et al*. 2003). Successful delivery of siRNAs in live animals has also been reported (Hasuwa *et al*. 2002; Carmell *et al*. 2003; Kobayashi *et al*. 2004). To monitor siRNA delivery and to optimize transfection, a positive, verified cell must be transfected-to-silence control siRNA side by side with a non-targeting, negative control siRNA and then assessing delivery efficiency.

2.4.6. Assaying plasmid delivery into cell lines

Cell viability should also be measured in negative control transfected cells versus non-transfected cells. There are four possible ways to monitor siRNA transfection efficiency using control siRNAs:

1. Monitor target mRNA knockdown

2. Monitor target protein knockdown

3. Monitor an induced phenotype

4. Label the siRNA and monitor uptake by fluorescence microscopy or flow cytometry.

Because siRNAs exert their effects at the mRNA level, monitoring target mRNA levels by real-time PCR is an excellent method for assessing siRNA delivery efficiency (RNA Interference research guide. www.ambion.com/RNAi).

2.4.7. Expression of small interfering RNA in the cell

Vectors that express siRNAs within mammalian cells typically use an RNA polymerase III promoter to drive expression of a short hairpin RNA that mimics the structure of a siRNA. The insert that encodes this hairpin is designed to have two inverted repeats separated by a short spacer sequence (Fig.2.3). One inverted repeat is complementary to the mRNA to which the siRNA is targeted. A string of thymidine's added to the 3' end serves as a pol III transcription termination site. Once inside the cell, the vector constitutively expresses the hairpin RNA, which induces silencing of the target gene (RNA Interference research guide. www.ambion.com/RNAi).

2.5. Aims and objectives

2.5.1. Aim

To design and determine the efficiency of three pshRNAs in silencing the GALT gene in mammalian cell line using RNA interference techniques.

2.5.2. Study objectives

The objectives identified for this study were:

- To design a shorthairpin RNA (pshRNA) and target different regions of the coding sequence of our target GALT gene.
- To propagate the pshRNAs in *Escherichia coli* and subsequently isolate the respective plasmids for transfection.
- To transfect HeLa cells and test the efficiency of relevant pshRNAs in knocking down the GALT gene expression.
- To quantify the expression of GALT against a house-keeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) and evaluate knockdown efficiency using real time PCR.

CHAPTER 3
Methodology

3.1. Design of pshRNAs

For this study three complementary shorthairpins were used to bind with the target mRNA site for effective knockdown of the GALT gene. Promega website http://www.promega.com/siRNAdesigner/program was used to design the two pshRNA used in this study. That is pshRNA1 and pshRNA2. The other two sequences were purchased from Sigma Biosciences via the Mission pshRNA programme and were named pshRNA3 and pshRNA4, respectively.

3.2. Transformation of *E.coli* JM109 with pshRNA vectors

The design combining hygromycin for Promega designed vectors and puromycin for Sigma Mission RNA designed vectors with Ampicillin resistant genes, respectively, was used to select for the bacterial and later on mammalian cells that contained the desired vector. The actual vector maps of each are shown in (Fig. 2.3) for the Promega vector and Fig 3.1 for the Sigma mission RNA vector. These plasmids were cloned into competent cells of *E. coli* JM109 of the strain BL21 (DE3) pLysS purchased from Promega (Madison, WI, USA). The procedure was as follows;

LB/Ampicillin/IPTG/X-Gal plates were prepared using standard laboratory procedures. Competent cells (50 µl) and 100 ng of each plasmid were transferred into 1.5 ml eppendorf tubes, separately. The tubes were then placed on ice for 20 min. The cells were heat shocked for 45 sec in a water bath for 42 °C and quickly transferred to ice for 2 min. SOC broth (950 µl) was added to each tube. The tubes were incubated at 37 °C in a shaker for 1, 5 hours. Hundred (100) µl of the solution from each tube was plated on the LB/Ampicillin/IPTG/X-Gal plates using the spread-plate technique and incubated at 37°C for 16-24 hrs. The plates were screened for white and blue colonies.

White colonies were streaked on LB/Ampicillin/IPTG/X-Gal plates and incubated at 37 °C to isolate pure culture. The single colonies containing the desired plasmid as shown

by absence of blue colour were subsequently inoculated in 5 ml LB broth containing 0.5 % ampicillin and were incubated at 37 ^{o}C in a shaker for 16 hours.

The five different plasmids used included two controls, first a positive control with 80 % knockdown efficiency (pshRNA1). Secondly, a negative control with 0 % knockdown efficiency (control). The other three plasmids were pshRNA2 designed using the Promega website, while pshRNA3 and pshRNA4 were ordered directly via the mission RNA initiative from Sigma with catalogue numbers 35534 and 35535, respectively.

To upscale the propagation of the plasmids, the inocula were transferred to a 500 ml sterilized erlenmeyer flask containing 200 ml LB broth containing 0.5 % ampicillin and incubated in a shaker at 37 °C for a further 12-16 hours. The inocula were poured off into 100 ml centrifuge tubes for subsequent isolation and purification of plasmids.

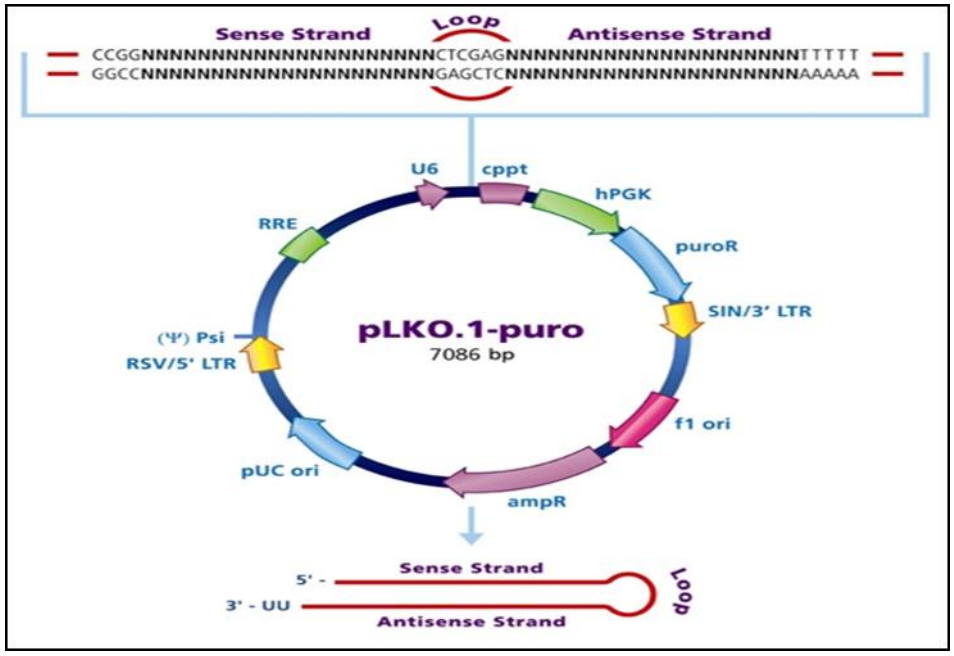

Figure 3.1: The vector map of the mission pshRNA from Sigma

3.3 Plasmid extraction using the Qiagen midikit

Plasmid DNA was isolated from the transformed cells using the Qiagen midikit (Cape Town, South Africa). The bacterial cells were harvested by centrifugation at 6000×g for 15 min at 4 °C. The cells were then lysed by resuspending them in 1 ml P1 buffer and were thoroughly mixed by pipetting up and down. Four millilitres of buffer P2 was added to the tube and was thoroughly mixed by inverting tubes at least 6 times. The tubes were incubated at room temperature for 5 min. The bacterial cells were neutralized by adding 4 ml of chilled buffer P3 and were immediately and thoroughly mixed by inverting the tubes at least 6 times. The tubes were incubated on ice for 15 min. The cells were then centrifuged at 20 000×g for 30 min at 4 °C and the supernatant was retained. Centrifugation was repeated to remove all the residuals and supernatant was retained.

The Qiagen tip was equilibrated by applying 4 ml of buffer QBT and was allowed to empty by gravity flow. The supernant was applied to the Qiagen tip and was allowed to enter the resin by gravity flow. For removal of contaminants the Qiagen tip was washed with 10 ml buffer QC and this step was repeated at least twice. The DNA was eluted with 5 ml buffer QC. The eluted DNA was precipitated by adding 3.5 ml of isopropanol to it and was immediately centrifuged at 15000×g for 30 min at 4 °C and the supernatant was discarded. The DNA pellet was washed with 70 % ethanol and centrifuged at 15000×g for 10 min. The supernatant was discarded without disturbing the pellet. The pellet was air dried for approximately 10min and the plasmid DNA was redissolved in TE buffer. The DNA plasmid concentration and purity was determined using the Nanodrop spectrometer 2000c (Thermo Scientific, Waban, USA).

3.4. Transfection of mammalian HeLa cells

3.4.1. Seeding of HeLa cells

Transfection was prepared at the following ratio: Dulbecco's modified Eagles medium (DMEM from Sigma Aldrich, St. Louis, US) at 44.5 ml with 5 ml fetal bovine serum (FBS from Sigma Aldrich), 0.5 ml Penicillin/Streptomycin (Penstrep from Life Technologies,

Victoria, Australia) and 200 mM L-glutamine in a cellstar tissue culture flask (T25) from Greiner (Bio-one,Solingen, Germany). The medium was filtered with a Durcapore 0.22 µm filter from Millipore (Darmstadt, Germany). The mammalian cell culture, namely HeLA cells were seeded in 5 ml of the medium in T25 cellstar flask.

The seeded cells were observed under the microscope to check for structural conformity and subsequently incubated at 37 °C, 5 % CO_2, and 100 % humidity for 24 hr**.** After 24 hr the cells were taken out of the incubator and observed under a light microscope for confluence, attachment and possibility of contamination. The cells were then washed three times with 10 ml of phosphate buffered saline (PBS from Sigma Aldrich) in T25 flasks under sterile conditions. Five milliliters of trypsin EDTA 0.25 % solution (Sigma Aldrich) were added to the flask to detach the cells. The flask was then incubated for a total of 2 min in the incubator at 37 °C and was checked at intervals of 45 sec to check if all the cells were detached. Then 15 ml of 20 % Fetal Bovine Serum (FBS) in DMEM was added to the flask to neutralize the effect of trypsin. Cells and media were decanted into a 50 ml centrifuge tube and centrifuged at 3000×g for 2 min at room temperature.

The supernatant was poured off and the rehabilitated cells were resuspended in 1 ml of DMEM containing 20 % FBS culture medium.

3.4.2. Enumeration of the viable HeLa cells:

To seed a required amount of 15000 cells per 96 well plate, enumeration of the viable cells is critical. The cells have to be at least 70 – 80 % confluent before transfection. The enumeration was performed as follows:

Fifty (50) µl of the rehabilitated cells were added to 400 µl of culture medium. Then 50 µl of pipette trypan blue was added to the cells. Twenty (20) µl of the above solution was pipetted to a hemocytometer and cells in four outer squares were counted. The final count was divided by 4 to obtain the average cell count. Cell number was expressed as 5000 cells/µl. The seeded cells were then taken out of the incubator. The cells were then counted using a haemacytometer under a light microscope and the percentage of live cells recorded.

3.4.3. Transfection of HeLa cells with relevant plasmids

The seeding culture medium was removed and cells washed three times with PBS. Each well was filled with 100 µl of Opti-Mem I medium just before transfection.

On transfection day (24 hours after seeding), for each well to be transfected, 330 ng plasmid DNA was diluted into 33 µl of Opti-Mem I medium without serum to accommodate triplicates and 10 % surplus reagent. A separate tube with 1.65 µl lipofectamine reagent (Life Technologies cat# 15338-100) with 33µl Opti-Mem I was prepared per triplicate reaction. The contents of the latter tube were added directly into the diluted plasmid DNA tube and allowed to stand for 10 min after brief gentle mixing by pipetting up and down three times. A total of 20 µl of the mixed transfection medium containing plasmid DNA and lipofectamine reagent was added to each triplicate well. The cells were allowed to stand in the incubator for six hours and the transfection medium removed from the wells. Fresh warm culture medium was added to the cells and left to stand for a further 36 hours.

3.5 Total RNA isolation

The transfected cell cultures were rinsed with 1 ml PBS and the washing liquid aspirated. Using the Biorad Aurum RNA isolation kit (cat# 732-6820) (Hercules, USA) the cells were lysed with 350 µl of lysis solution and mixed by pipetting up and down 12 times. For reducing the mixture viscosity, 350 µl of 70 % ethanol was added to each well and mixed by pipetting up and down. The elution buffer was placed in a 70 °C waterbath to facilitate dissolution of its components. The RNA binding column was inserted into a 2 ml capless wash tube and the lysate was decanted into the RNA binding column and centrifuged for 30 sec at full speed (14000 rpm).

The RNA binding column was removed from the wash tube, the filtrate discarded and the column was replaced into the same tube. A low stringency wash solution (700 µl) was added to the RNA binding column that was centrifuged for 30sec at 14 000 rpm. The low stringency wash solution was discarded and the column was replaced into the same tube. For the removal of genomic DNA contaminants 80 µl of diluted DNAse 1 (digestion buffer) was added to the membrane stack at the bottom of each column and

incubated for 15 min at room temperature and then centrifuged for 30 sec at full speed. The digestion buffer was discarded; the column was replaced into the same tube and centrifuged for 30 sec at full speed. A high stringency wash solution (700 µl) was added to the RNA binding column and centrifuged for 30 sec at full speed.

The high stringency wash solution was discarded and the column was replaced into the same tube. Finally, 700 µl of the low stringency wash solution was added to the RNA binding column and centrifuged for 1 min at full speed. After discarding the low stringency wash solution the column was replaced into the same tube and centrifuged for 2 min at full speed.

The RNA binding column was transferred to a 1.5 ml capped microcentrifuge tube. For elution of total RNA a volume of 80 µl of warmed elution solution was pipetted onto the membrane stack at the bottom of RNA binding column, the solution was allowed to stand for 1 min for saturation, and centrifuged for 2 min to elute the total RNA. The concentration of the RNA was determined with a Nanodrop 2000c (Life Technologies, Victoria, Australia).

3.6. Synthesis of complementary DNA (cDNAs) from total RNA

The cDNA synthesis was performed according to the cDNA transcriptor, cat # 043790122001 (Roche, Mannheim, Germany). In short, the total RNA, random primers and water PCR grade were mixed together as shown in Appendix A. The template-primer mixture was denatured by heating the tube for 10min at 65 °C then cooled on ice for 10 min. Then, 7 µl master mix was added to the template-primer mixture. The reagents were thoroughly mixed, then incubated at 25 °C for 10 min and for further 30 min at 55 °C. For inactivation of transcriptor reverse transcriptase the reagents were incubated at 85 °C for 5 min.

3.7. Real time PCR relative gene expression

Real time PCR was performed as on the Bio-Rad CFX96 touch detection instrument. The realtime PCR was performed using the Bio-Rad iQ green supermix. The primers specific for human GALT gene (Quantitech primer assay cat# QT00082782) and human

GAPDH (Quantitech primer assay cat# QT00079247) were purchased from Qiagen, Cape Town.

Fifteen microliters of iQ SYBR Green supermix (cat# 170-88880) from Bio-Radmaster and 5 µl of cDNA were mixed as shown for each PCR reaction tube. For control reaction, 15 µl master mix and 5 µl of PCR grade water were mixed in PCR tube. For positive control 15 µl master mix and 5µl of pooled sH3b were mixed in PCR tubes.

For standard, 15 µl master mix and 5 µl of 5 variations of 10x dilutions of the control pshRNA sample were mixed in different PCR tubes. The PCR protocol included 5 min deactivation at 95 °C, followed by cycles of three steps, 10 sec at 95 °C denaturation, 30 sec at 55 °C annealing and 30 sec at 72 °C elongation. This cycle was repeated 29 more times with reading captured at 530 nm at every elongation step. A melt curve analysis step was performed from 60 °C – 95 °C at increments of 0.5 °C for identification of amplified products.

The relative quantification was determined by using the correlation between the standard curve values and the sample values of both pooled GAPDH and that of each respective GALT sample. The data was then plotted graphically and percentage knockdown determined.

CHAPTER 4
Results and Discussion

4.1. Design of pshRNAs

The Promega website http://www.promega.com/siRNAdesigner/program was useful in designing the 3 of the pshRNAs used in this study. The programme uses the Flexi Vector System which is a directional cloning method for protein-coding sequences. This programme is based on two rare-cutting restriction enzymes, *Sgf*I and *Pme*I. The programme provides a rapid, efficient and high-fidelity way to transfer protein-coding regions without the need to re-sequence and does not require an archival entry vector, unlike site-specific recombination vector systems. This programme was chosen because of its features and benefits which are versatility, time saving, enhanced productivity and easy access. The sequences that were ultimately used for this study are shown in Table 4.1 below:

Table 4.1: Name and sequences of primers designed by using the Promega software

Name	**Sequence**
pshRNA 1	5'-GCCATCAGCATACCGCTACAACTCGAGTTGTAGCGGATATGCTGATGGTTT-3'
pshRNA 2	5'-GCCGGAAATTCATGGTTGGCTACTCGATAGCCAACCATGAATTTCCGTTT-3'

4.2. Transformation of *E.coli* JM109 with pshRNA vectors

The transformation of *E.coli* was successfully performed with the final concentrations of each of the DNA plasmids pshRNA1, 2 and 3 determined to be 400, 435 and 417 ng/µl, respectively, on the Nanodrop 2000c.

4.3 Real time PCR relative gene expression

The isolation of total RNA was performed using the Bio-Rad Aurum total RNA isolation kit. To lessen the possibility of contamination, the RNA was not run on the gel to determine integrity nor was it quantified on the Nanodrop. Instead it was assumed that, if the PCR reaction amplifies well with the standard curve as well as the mock dilution samples, it would be safe to assume that the total RNA isolation as well as the cDNA synthesis has been successful. Based on the successful standard curve determination and the satisfactory linear regression fit of 0.999, both isolation and cDNA synthesis were successfully performed. A dilution series of total cDNA obtained from untreated cells was made and used to determine the efficiency of the realtime PCR protocol specifically for the GALT gene amplification from the synthesized cDNA. Five dilutions were made as well as triplicate samples of both GALT and GAPDH as a test run. The mock samples were 10^4 dilutions of the untreated samples. The efficiency of the PCR protocol was found to be closer to 2 and the regression value of 0.999. This means that the PCR cycling conditions and chemical composition allowed for proper doubling of amplification with every completed cycle.

The linear regression fit of closer to 1, that is 0.999, shows that the actual dilution process was done appropriately and the pipetting error was negligible. The standard curve threshold cycles are shown in Fig 4.1, while the efficiency and regression square value are illustrated in Fig 4.2. Subsequently, the GALT and GAPDH graphs for the dilution triplicates (mock samples) are shown in Fig 4.3 and 4.4, respectively. The proximity of the threshold cycles as illustrated in both figures does show the superior accuracy and precision within which the experimental data were collected. All three samples were within the 24,5 -24.7 cycle range for GALT gene and 25.0 – 25.1 for GAPDH amplification curves. These can be considered to virtually be the same cycle number for qualitative expression level values. Importantly, the negative control also came out negative, which suggests that the amplification is only that of the desired specific fragment and not contamination.

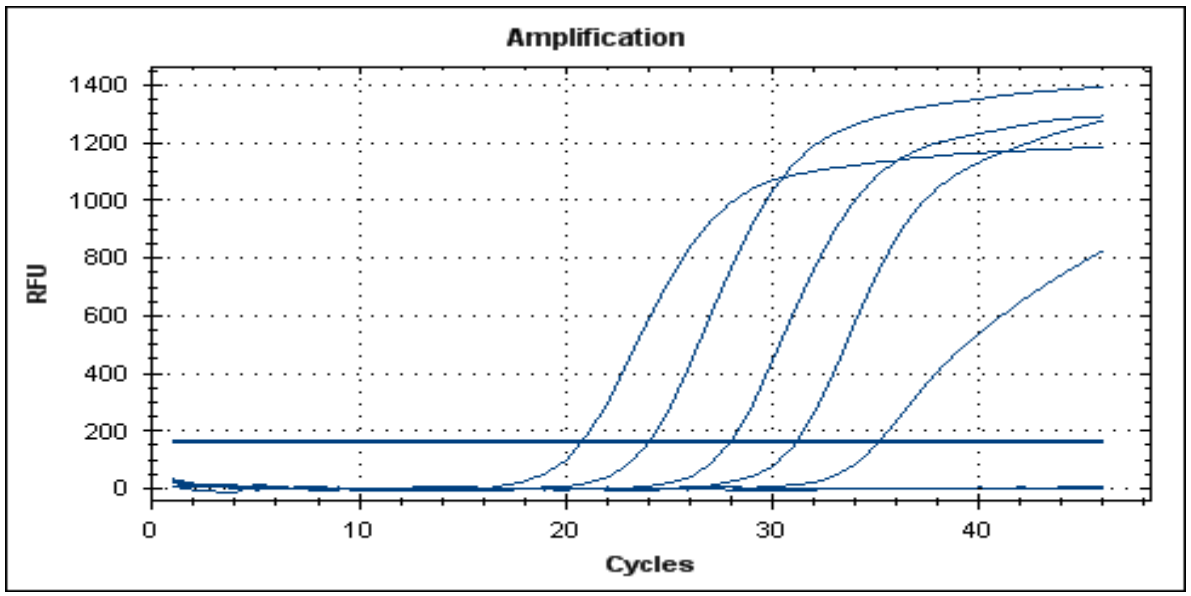

Figure 4.1: Standard curve determination for GALT gene realtime PCR using serial dilutions of cDNA from untreated HeLa cells. The curves show progressive dilution series from left to right with the negative control being the flat blue line at the bottom of the curves.

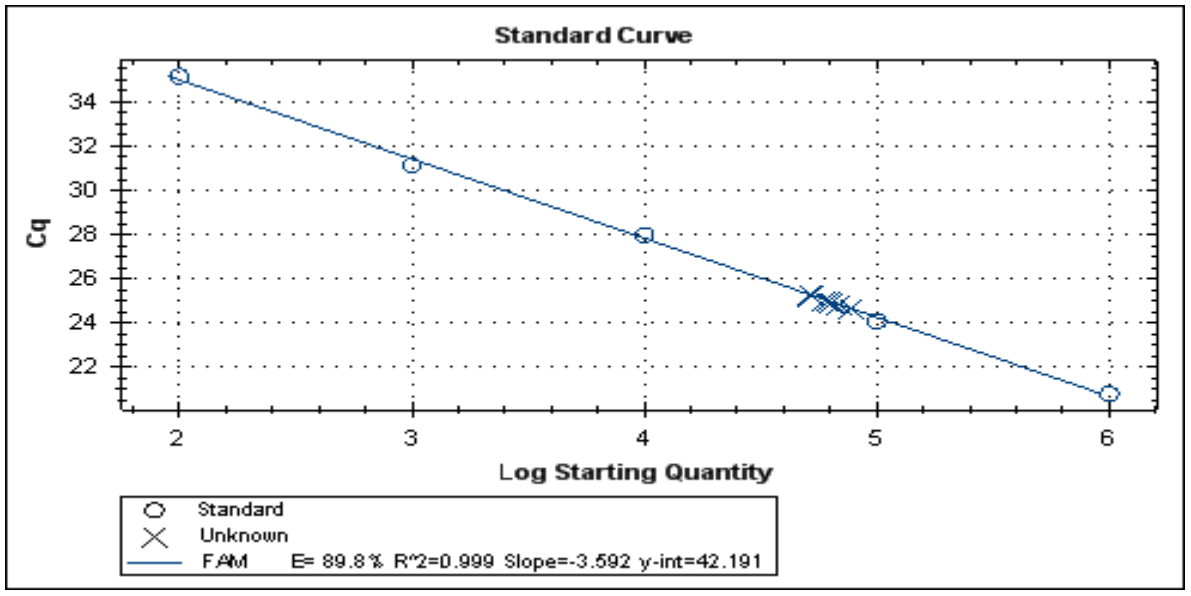

Figure 4.2: Standard curve plot for GALT gene untreated samples. The circular points show the dilution series, while the cross points show the position of the GALT and GAPDH concentration correlation relative to the dilution points.

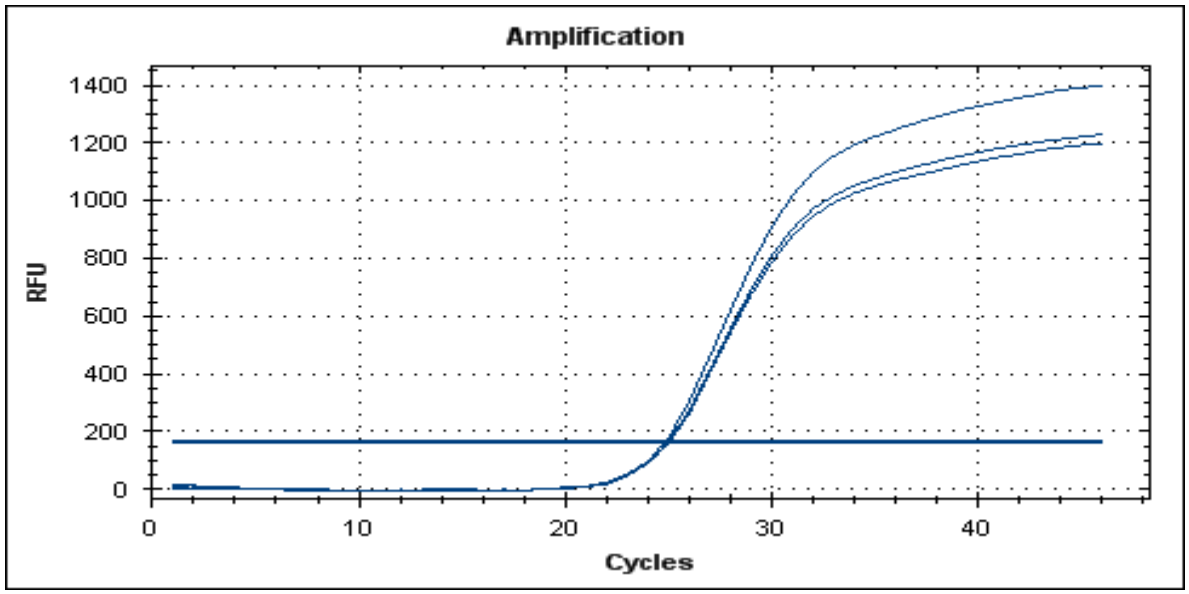

Figure 4.3: GALT gene threshold cycle determination using triplicates from untreated samples.

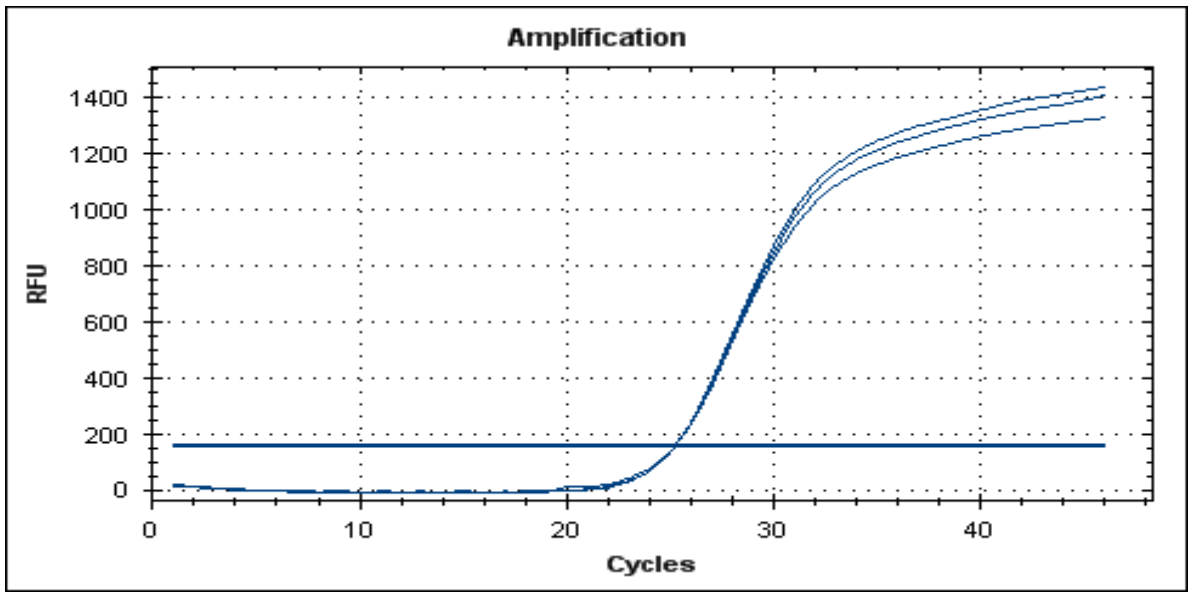

Figure 4.4: GAPDH gene threshold cycle determination using triplicates from untreated samples

4.4. Transfection of Hela cells with relevant plasmids

Enumeration of the viable Hela cells was performed using the heamacytometer and a light microscope. The cell viability was found to be over 98 %. It is expected that some cells would perish due to overdigestion of their cell membrane during trypsinisation. From the two flasks that were seeded, the final combined concentration of cells was 5.3 x 10^6 cells per ml. The appropriate cell number of 15000 cells per well were seeded in a 96 well plate with suitable media and subsequent transfection reagents and plasmid DNA as explained in the methods section. To control for the experiment, a known pshRNA1 that has been determined previously to knock down GALT gene efficiently up to 80 % knockdown was included. A mock plasmid, without a knock down insert was also included and 0 % knockdown was expected from the control plasmid. Fig 4.5 shows the realtime amplification of averaged triplicates of pshRNA1, 2, 3, 4, control and pooled GAPDH. The GAPDH samples were pooled to compensate for any variations that may be the cause of external factors other than the effect of transfection. There are two ways in which one can use the GAPDH to correlate the actual knockdown efficiency of the GALT gene. Either run a GAPDH reaction for each sample and average later, or pool all samples and determine the GAPDH threshold cycle and therefore the expression capacity of GAPDH. In this case we followed the later example.

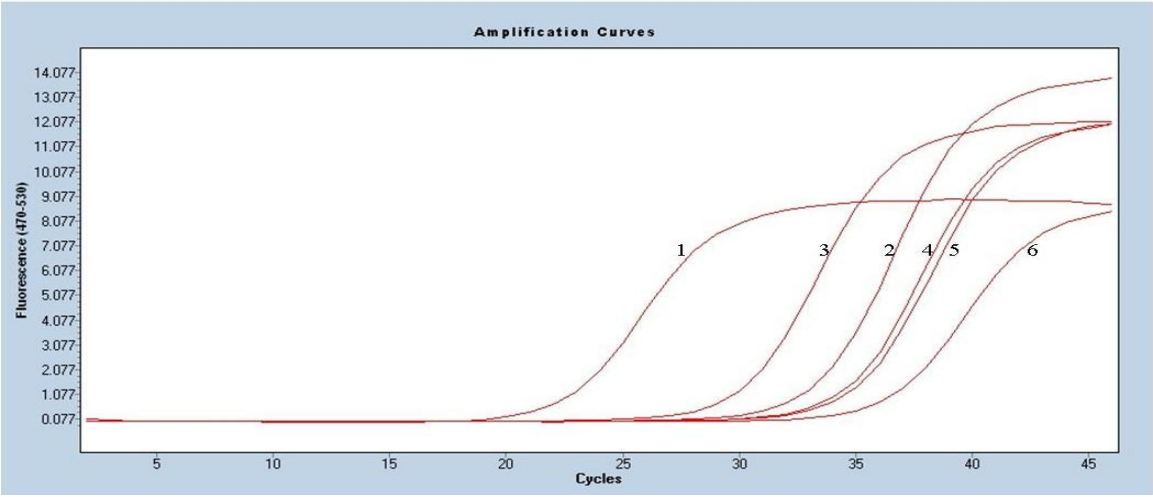

Figure 4.5: Amplification curve of all GALT samples including GAPDH. The plasmids corresponding to each number are shown in Table 2.

The calculation for the actual efficiency is shown in Table (4.2) below. The calculation is done using expression efficiency rather than knockdown efficiency. The figures are then normalized using the 100 % expression efficiency as expected from a control plasmid. To determine the knockdown efficiency, one has to subtract the expression efficiency percentage from 100 % since that is the amount of RNA expression reduction due to RNA interference. In this case the highest knockdown was observed with pshRNA1 at 24 % RNA expression hence 76 % knockdown efficiency. This was followed by the newly determined pshRNA3 with 48 % expression efficiency that equates to 52 % knockdown efficiency.

Table 4.2: Calculation of knockdown efficiency for the pshRNAs

Column ratio	**Actual values**	**Expression data**	**Normalised data**	**x-axis**
A5/A1	-4.15741	0.240534549	0.220105461	pshRNA1
A5/A2	-0.53432	1.871541832	1.712587979	pshRNA2
A5/A3	-1.89219	0.528487006	0.48360153	pshRNA3
A5/A4	-0.43791	2.283578564	2.089629593	pshRNA4
A5/A6	-0.91507	1.092814598	0.999999632	Control

The total knockdown efficiency is better illustrated in the bar graph in Fig 4.6. One can clearly see the efficiency of knockdown when comparing the GAPDH values to that of GALT expression.

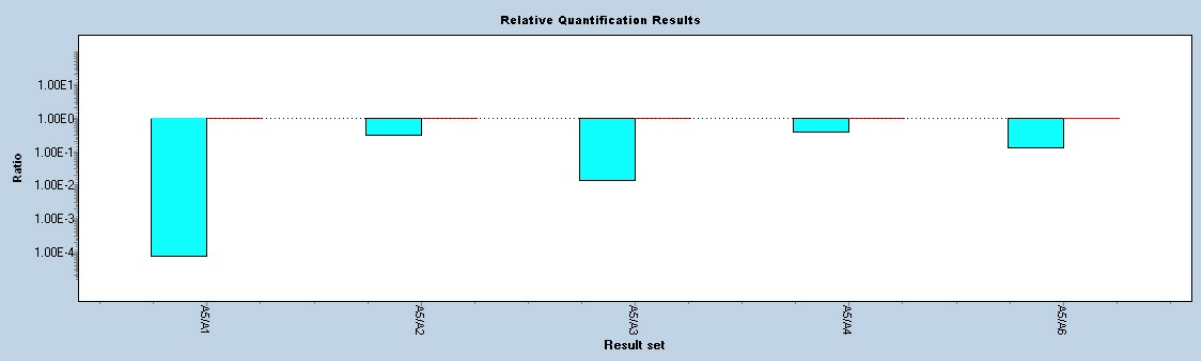

Figure 4.6: Relative quantification bar graph showing the knockdown efficiency of all transfected plasmids including the control vector.

Illustrated differently, one can plot the actual values from above graph Fig 4.6 into the graph below in Fig 4.7 to show the expression efficiency without having to show the GAPDH expression since it will already have been normalised.

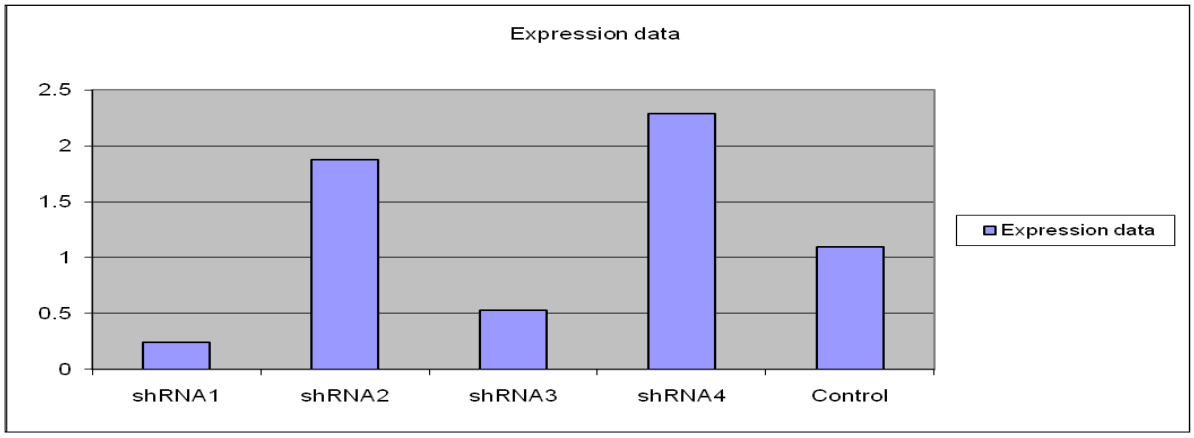

Figure 4.7: Expression data of GALT gene after transfection.

Often in experiments such as the qualitative expression of GALT gene, the unambiguous expression of the specified gene without primer-dimers and nonspecific product is important to form a proper conclusion that the effective knock down is not a result of inefficiencies in the PCR reaction itself. Therefore running either an agarose gel of the PCR products or running a melt curve of the amplified products is necessary to show absence of bias in the threshold cycles produced.

The full details of the threshold cycles for each of the plasmid effect are shown in Appendix B. There were negligible amounts of primer dimers as shown in Fig 4.8 below. The primer dimers were present and melted at about 77 °C, while the product melted at 83 °C. All GALT products were of the same length and quality such that the melting profile irrespective of the threshold cycle was virtually the same. We can therefore conclude that there was no nonspecific binding by the Qiagen primers specified to be validated against the human GALT gene.

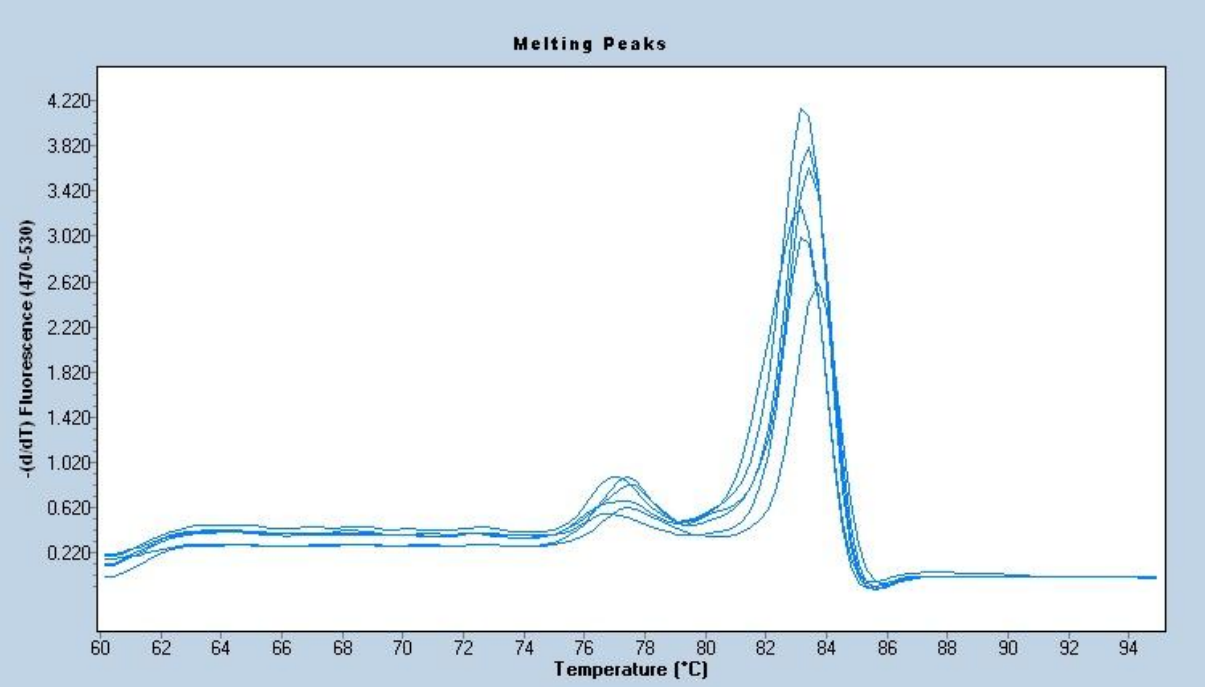

Figure 4.8: The melting curve profile of GALT gene amplified product.

One of the improvements that could have been implemented in this study would have been determining the expression stability of other housekeeping genes over and above the GAPDH. This would have strengthened the cellular model for classic galactosemia since it would properly show that the GALT knock down is not affected by other external factors but by the transfection protocol only. This would have included treating different cell types with lipofectamine and subsequent measurement of the specific housekeeping gene to determine their stability in terms of gene expression. However, since GAPDH has been used before in other studies in classic galactosemia study

modeling, it makes sense to use GAPDH even though it was not rigorously tested against other housekeeping genes in this study specifically.

However, the pooling of the housekeeping gene samples to use against all samples does minimize any effect that any nonspecific knock down of GAPDH could have exerted on any single sample. Therefore, the propensity to knock down GALT gene expression can be considered authentic based on the amount of knock down produced using the techniques developed in this study. Future studies for disease modeling using variable knock down can be reproduced using this study as a model.

CHAPTER 5
Conclusion

Knock down of gene expression of a specific chosen gene using RNA interference is one method that can be applied if variable reduction of gene expression is a requirement. Classic galactosemia is mainly a result of a malfunction in galactose metabolism caused by mutations in the GALT gene. However, since the mutations do not always result in the same severity on the function of the GALT enzyme, it is difficult to recreate the physiological conditions with the current models. The mouse model for instance shows the biochemical phenotype but not the clinical phenotype expected of severe functionally limited GALT enzyme. This means that a suitable and proper model in human immortalized cells would at least mimic what is expected from human tissue. This model is not possible without the variable gene expression to mimic the different mutations within the GALT gene whose cumulative effect is the reduced enzyme activity.

One of the ways to achieve this model is to use the same cell line to variably knock down GALT gene expression and compare the biochemical profiles of such cell lines. Comparisons could include galactose-1-phosphate levels, galactosylation of membrane lipids and proteins as well as comparing the ratios of UDP-galactose to UDP glucose. Such measurements would go a long way in elucidating the potential mechanisms with which poor long term prognosis of classical galactosemia occurs.

This study was an attempt at standardizing the protocol for creating variable gene expression for GALT gene to help create a cellular model for studying this disorder in specific cells in the future. For this reason, the study design and execution was a success in that it added one more pshRNA with much lower knock down efficiency of 52 % compared to the already determined efficiency of 80 % produced by pshRNA2. The pshRNA4 together with pshRNA2 have shown knockdown of GALT gene expression in different capacities and therefore used together can be considered a good model for classic galactosemia. More pshRNAs with different knock down efficiency would be important for this role.

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Appendix A

Complementary DNA (cDNA) synthesis using Roche Transcriptor Reverse Transcriptase

Calculation for template primer mix (for 1 reaction)

Component	**Volume**
Total RNA	Variable
Random primer	2 µl
Water PCR grade	Variable (add up to 13 µl)

Appendix B :Threshold cycles for relative gene expression of GALT gene

Selected Filter: 530 (470-530)				
Include	Color	Pos	Name	Cp
TRUE	255	A1	Target 1	21.48
TRUE	255	A2	Target 2	33.52
TRUE	255	A3	Target 3	29.01
TRUE	255	A4	Target 4	33.84
TRUE	255	A5	GAPDH	35.29
TRUE	255	A6	Control	32.25