

The Role of Human Mitochondrial Transcription Termination Factor in Mitochondrial Transcription Termination in Cultured Human Cells

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ABSTRACT

This extended essay investigates if mitochondrial transcription termination factor, mTERF protein, has a role in mitochondrial transcription termination *in vivo*. I studied if this protein regulates the transcription termination after ribosomal RNA (rRNA) genes in mitochondrial DNA (mtDNA) in cultured human embryonic cells.

mTERF is a protein which binds within tRNA^{Leu(UUR)} coding gene between 16S and ND1 coding genes in mtDNA. It has been suggested to regulate the transcription termination of rRNA since the transcription of rRNA is terminated before the binding site of mTERF. The transcription of another RNA transcript, messenger RNA (mRNA), continues after the binding site of mTERF.

Therefore 16S, which is located before mTERF binding site in mtDNA, is involved in both of the transcripts, rRNA and mRNA, whereas ND1, located after mTERF binding site, is transcribed only from the mRNA. By over-expressing or silencing the mTERF production in the cultured human cells its role in the transcription termination can be studied. The levels of the two transcripts, 16S and ND1, are detected using LightCycler which performs real-time quantitative polymerase chain reaction (qPCR).

In the assay no dependency between the amount of mTERF and the transcription termination of rRNA was found, since the relative amounts of the transcripts remained the same in both over-expression and silencing the production. The relative amounts of the transcript levels of 16S and ND1 were approximately the same in each of the compared cell lines thus indicating that mTEF alone does not regulate the amounts of different transcripts in mitochondria *in vivo*.

TIIVISTELMÄ

Tässä esseessä tutkitaan mitokondriaalisen transkription terminaatiotekijän, mTERF proteiinin, roolia mitokondrion transkription terminaatioissa. Tutkin, säätelekö kyseinen proteiini mitokondrion transkription terminaatiota ribosomaalisen RNA (rRNA) geenien jälkeen mitokondriaalisessa DNA:ssa viljellyissä ihmisen alkion munuaissoluissa.

mTERF on proteiini, joka sitoutuu tRNA^{Leu(UUR)} koodaavaan geeniin 16S ja ND1 koodaavien geenien väliin mitokondriaalisessa DNA:ssa. Sen on ehdotettu säätelevän rRNA:n transkription terminaatiota, sillä rRNA:n transkriptio päättyy mTERF proteiinin sitoutumiskohtaan tRNA^{Leu(UUR)} koodaavassa geenissä. Toisen RNA:n, lähetti-RNA:n (mRNA), transkriptio jatkuu mTERF proteiinin sitoutumiskohdan jälkeen.

Sen tähden 16S, joka sijoittuu ennen mTERF:ia mitokondriaalisessa DNA:ssa, on mukana molemmissa transkripteissa, rRNA:ssa ja mRNA:ssa kun taas ND1, joka on mTERF:n jälkeen, transkriptoidaan ainoastaan mRNA:sta. Tuottamalla ylimäärin tai vaientamalla mTERF:n tuotanto viljellyissä ihmissoluissa sen roolia transkription terminaatioissa voidaan tutkia. Näiden kahden transkriptin (16S ja ND1) tasot havaitaan käyttämällä LightCycleria, joka suorittaa reaaliaikaista kvantitatiivista polymeerasiketjureaktiota (qPCR).

Tässä kokeessa ei havaittu riippuvuutta mTERF:n määrän ja rRNA:n transkription terminaation välillä. 16S ja ND1 transkriptitasojen suhteelliset määrät olivat suurin piirtein samat jokaisessa verratussa solulinjassa osoittaen, että mTERF:n määrä yksin ei säätele eri transkriptien määriä mitokondriossa *in vivo*.

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1. INTRODUCTION

Mitochondria are organelles in a cell whose main function is to produce energy in the form of adenosine triphosphate. According to the evolution theory their current structure has evolved from symbiotic relationship of a protoeukaryote cell lacking mitochondria and a primitive prokaryote cell. Mitochondria are interesting since they contain their own maternally inherited 15-20 kb long double-stranded mitochondrial DNA (mtDNA) which codes 37 genes, and this genome is able to mutate. Mitochondrial DNA is under passionate investigation since some human diseases, such as MELAS syndrome¹, are caused by mutations in the mtDNA. Especially the transcription of mtDNA is an interesting factor in the development of the MELAS syndrome. A change of only one base in the gene coding for tRNA^{Leu(UUR)} causes most of all cases of MELAS. This mutation also affects the binding affinity of mitochondrial transcription termination factor protein (mTERF) on its target sequence. Based on *in vitro* studies mTERF has been suggested to regulate the termination of transcription of mtDNA after ribosomal RNA coding genes. Therefore, to understand the transcription termination is essential in order to find out effective treatment for this syndrome. The possible role of mtDNA in aging, apoptosis² and carcinogenesis³ is also worth more close investigating in the future.

In this extended essay the main focus is on the termination of transcription of the mtDNA. The aim is to establish whether mTERF really has a role in the termination of transcription since there have been contradictory results of the role of mTERF in it. Hence my research question will be: Does mTERF regulate the transcription termination after the ribosomal RNA genes in mitochondrial DNA in cultured human cells? It has been newly found that mTERF has a role in the replication of the mtDNA as a replication pause site (Hyvärinen *et al.*, 2007). Therefore, now it is highly interesting to study whether it has *in vivo* a role in the transcription termination of the mtDNA, too.

The role of mTERF in the termination of transcription is studied by comparing the ratio of two RNA transcripts when mTERF is either over-expressed or silenced in cultured human cells. If mTERF has a role in the termination, this should be seen in the results as variation of the ratio of

1 Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes

2 A form of programmed cell death.

3 A process where normal cells are transformed into cancer cells.

the two transcription products. The levels of these two transcripts are detected using LightCycler⁴. This is a real time process, and therefore the levels of the transcripts can be effectively measured after each cycle, thus detecting the initial concentration of the template.

I had the opportunity to join Professor Jacobs' research group in the Institute of Medical Technology at the University of Tampere for two weeks to work alongside PhD student Anne Hyvärinen. For the past few years Anne has studied the role of mTERF in transcription and replication and elaborated me into this field of research of biology. I got a chance to take part in Anne's research on mTERF. I carried out one subproject on a project focusing on studying whether mTERF has a role in mitochondrial transcription termination. Anne herself has studied this using different probes from different parts of 16S and ND1 coding genes than I did. She has also studied tRNA-Leu (UUR)/tRNA-Phe relationship and observed that the over-expression or silencing the mTERF production has no effect on the transcription termination. The experiment was done using the same material, cell lines and apparatus as Anne had used in her assays in order to gain reliable and comparable data.

4 Real-time polymerase chain reaction system

1.1. TRANSCRIPTION OF MITOCHONDRIAL DNA

Human mtDNA has two strands, heavy strand and light strand, which are compact and lack introns. The two strands are transcribed in opposite directions (see Figure 1 below).

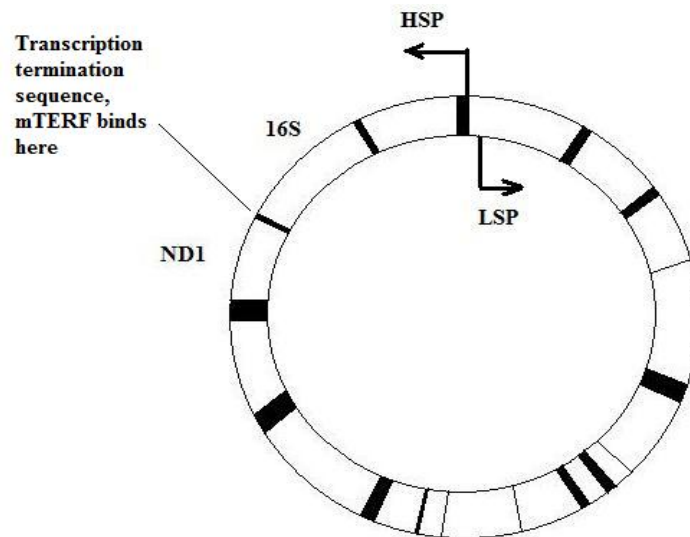


Figure 1. Mitochondrial DNA. Its two strands, heavy strand and light strand, are transcribed in opposite directions indicated by the arrows. HSP = heavy strand promoter; LSP = light strand promoter. These promoter sequences were discovered by Chang & Clayton 1984.

The transcripts of the two genes, 16S and ND1, are essential when the role of mTERF in the transcription termination is studied. According to one commonly accepted model about the mitochondrial heavy strand transcription, ribosomal RNA (rRNA) genes have their own transcription initiation site, I_{H1} , and the termination occurs at gene coding for tRNA^{Leu(UUR)}, the location where mTERF binds. The transcription of messenger RNAs (mRNA) initiates from I_{H2} , and produce a primary transcript of almost the whole heavy strand. See Figure 2. The first occurs more frequently than the second (Fernandez-Silva *et al.* 2008). 16S gene is located before gene coding for tRNA^{Leu(UUR)}, the binding site of mTERF and is involved in both of the transcripts, mRNA and rRNA. ND1 is produced only in mRNA transcription unit.

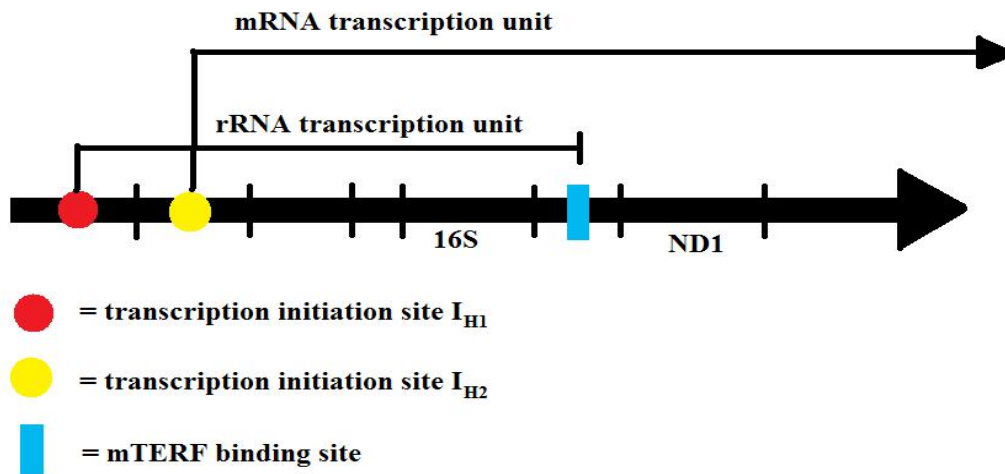


Figure 2. Transcription initiation sites of heavy strand in human mtDNA.

1.2. MITOCHONDRIAL TRANSCRIPTION TERMINATION FACTOR mTERF

Mitochondrial transcription termination factor, mTERF, a member of mTERF protein family, has been suggested to have a role in mtDNA transcription termination. mTERF coding gene is located on the chromosome 7, at locus 7q21-q22. When mature mTERF is a protein of 342 amino acids and causes the mtDNA to be bent (Shang & Clayton 1994). mTERF exists in 2-3 isoform sizes which vary from 31 to 34 kDa. This protein, which binds within tRNA^{Leu(UUR)} coding gene between 16S and ND1 coding genes (see Figure 3), has been shown *in vitro*⁵ to promote transcription termination when the transcription is started from the specific initiation site of ribosomal RNA, I_{H1} , of the heavy strand in the mtDNA (Kruse *et al.* 1989, Daga *et al.* 1993).

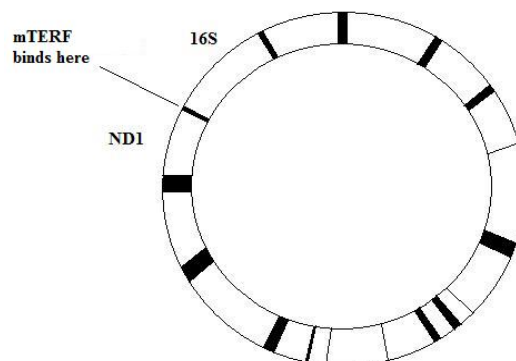


Figure 3. The circular genome of mtDNA. The binding site of mTERF is located between 16S, coding for 16S ribosomal RNA, and ND1, coding for NADH dehydrogenase 1, genes.

⁵ Experiment is carried out outside of a living organism i.e. in a test tube.

According to Falkenberg *et al.* 2007, Asin Cayuela *et al.* 2005 and Shang *et al.* 1994 have shown *in vitro* that mTERF-mediated transcription termination at its binding site occurs bidirectionally. Therefore the possible role of mTERF may also be in the termination of transcription of light strand unit.

One type of MELAS mutation, A3243G, is situated in the middle of the binding sequence of mTERF in mtDNA thus disturbing the binding affinity of mTERF and affecting the transcription termination efficiency after the rRNAs (Hess *et al.* 1991 according to Hyvärinen 2006, Chomyn *et al.* 1992). However, it does not change the ratio between I_{H1}/ I_{H2} transcripts (Chomyn *et al.* 1992 according to Falkenberg 2007) thus suggesting that the main role of mTERF is not to regulate the termination of the transcription.

2. INVESTIGATION

2.1 DEVELOPMENT OF THE RESEARCH METHOD

It has already been shown that mTERF promotes transcription termination *in vitro*. The experiment we performed was *in vivo*⁶ experiment where the role of human mTERF in mitochondrial transcription termination was studied.

The two initiation sites for the transcription of the heavy strand provide a method to investigate the effect of mTERF on the termination of transcription of mtDNA. The transcription of rRNA is terminated at the same location as mTERF binds within. It has been suggested that mTERF activity is vital in the regulation of RNA molecule levels, thus controlling the termination of rRNA. 16S gene is located before gene coding for tRNA^{Leu(UUR)}, the binding site of mTERF, and ND1 right after it (see Figure 2). 16S rRNA transcript is produced in both of the transcription units, mRNA and rRNA, whereas ND1 only in the mRNA transcription unit since the transcription of rRNA transcription unit is terminated before it (refer to Figure 2). Therefore the role of mTERF as mitochondrial transcription termination factor after rRNA genes can be examined by over-expressing or silencing the production of it in cultured human cells and hence comparing the relative amounts of the transcripts of these two genes in different kinds of situations.

⁶ Experiment is done using living cells.

When mTERF is over-expressed it presumably wants to bind within tRNA^{Leu(UUR)} gene between 16S and ND1 genes. If mTERF regulates the transcription termination, every time when the transcription is initiated from I_{H1}, it is most likely to be terminated at the binding location of mTERF, since it is assumed that the excessive amount of it provides a higher possibility for mTERF to be bound within the gene and thus the transcription of termination occurs more often. The transcription of mRNA will not be affected since it produces transcript of the whole heavy strand. The result is that the relative amount of transcripts of 16S gene should increase compared with the relative amount of transcript of ND1 gene.

When the production of mTERF is knocked down the transcription termination after the rRNA coding gene is decreased if mTERF has a role in the transcription termination. Thus nonsense transcript is produced since the transcription of the strand continues past the termination site. The nonsense transcript cannot be processed properly, resulting in a decrease in the relative amount of the 16S transcript compared with the ND1 transcript.

Thus the following experiment setting arises: the levels of 16S and ND1 transcripts are measured from each cell line using real-time qPCR. cDNA synthesis requires forward (For) and reverse (Rev) reactions, producing antisense and sense transcripts as shown in Figure 4 below. For reactions produce a complementary copy of sense strand producing antisense transcript and Rev reactions produce a complementary copy of antisense strand producing sense transcript. Hence four different series of qPCR runs are required to quantify all transcript levels: 16S For, 16S Rev, ND1 For and ND1 Rev.

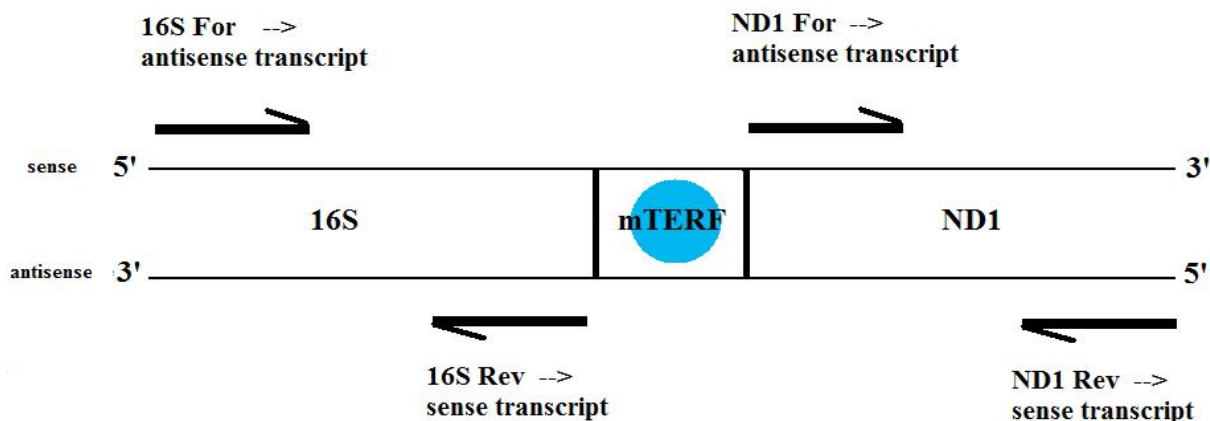


Figure 4. *qPCR For and Rev reactions.*

With this experiment method the possible role of mTERF as transcription terminator for both of the strands, antisense and sense, can be investigated.

2.2. METHOD

2.2.1. EXPERIMENT SETTING

Anne Hyvärinen had already done the first steps of the experiment when I arrived into the laboratory. The time I was able to spend there was only two weeks and an actual biological research may take up to several years.

Anne had nine human embryonic kidney cell lines. These lines are paired up in a way that the results are comparable: the other cell line of the pair is a positive sample where the production of mTERF is either over-expressed or silenced. The other cell line is a control sample meaning that it is treated exactly in the same way as the positive sample in order to eliminate possible background noise except the mTERF production is not over-expressed or silenced.

- 1 and 2 are cell lines where the production of mTERF is stably over-expressed using an expression vector (pcDNA 3.1 hygro (+/-)).
- Cell line 3 contains the expression vector pcDNA 3.1 hygro (+/-) coding only hygromycin resistance thus being an empty vector and not over-expressing mTERF.
- The production of mTERF is silenced in the cell line 4 by RNA interference using mTERF specific siRNA⁷ expressing retrovirus vector (pGIPZ vector construct).
- Cell line 5 expresses nonsense siRNA (i.e. non-silencing) in pGIPZ expression vector.
- In cell line 6 mTERF is over-expressed in inducible⁸ T-REx cells using pcDNA5/FRT/TO (Invitrogen) expression vector. The expression of mTERF can be activated with Doxycyclin which then controls the transcriptional activation.
- In cell line 7 the cells contain the same plasmid construct as in 6 but the cells are not induced with doxycyclin and therefore mTERF is not over-expressed.

⁷ Short interfering RNA

⁸ Not in action all the time

- In the cell line 8 mTERF has been knocked down by transfecting the cells with mTERF specific siRNA.
- In the line 9 control cells have been transfected with nonsense siRNA, i.e. mTERF is not silenced.

2.2.2. TREATMENT OF THE CULTURED CELLS

This process Anne had performed already for her assay: first, RNA is extracted from these cultured cell lines using TRIzol reagent (Invitrogen) where RNA is maintained and everything else in the cell is broken down. DNase I⁹ (Fermentas) treatment is then done to get rid of any contaminating DNA. The purity of RNA is checked by means of PCR: RNA is treated with RNA degrading RNase and DNA is precipitated and then used as template¹⁰ for PCR. Nothing was amplified which means that RNA is DNA free. From that, complementary DNA strand (cDNA) is synthesized using reverse transcriptase¹¹ where RNA free from DNA is used as template. The quality of cDNA is then checked by means of polymerase chain reaction (PCR) using Taq polymerase¹². Thus cDNAs, sense and antisense, from different kinds of cells are produced and hence the RNA transcript levels of sense and antisense of 16S and ND1 coding genes can be determined.

From each cell line there are three independent RNA samples (independent samples meaning that the cells are from separate bowls) for every reaction (16S For, 16S Rev, ND1 For and ND1 Rev) making it total of $9 \times 3 = 27$ samples of cDNA for all of them. From all of these samples three different dilutions are made: 1:10, 1:20 and 1:50, thus producing $3 \times 27 = 81$ cDNA samples for each reaction (16S For, 16S Rev, ND1 For and ND1 Rev). In all of these cDNA samples the levels of 16S and ND1 transcripts are quantified using LightCycler for 16S and ND1 For and Rev reactions in order to detect the sense and antisense transcripts separately.

9 An endonuclease that digests single and double stranded DNA by hydrolyzing phosphodiester bonds, the backbone of the DNA strand.

10 A strand which sets the genetic sequence of new strands.

11 DNA polymerase enzyme that transcribes single stranded RNA into double stranded DNA.

12 A thermostable DNA polymerase which greatly amplifies short segments of DNA

2.2.3. DETECTING THE LEVELS OF THE TWO TRANSCRIPTS

The levels of 16S and ND1 transcripts are quantified using cDNA as template in real-time qPCR using LightCycler. In order to find out the initial amount of the transcript, in each cycle the amount of it must be observed. In LightCycler the levels of the transcripts are detected by measuring the amount of fluorescence signals emitted by two probes¹³ hybridized to the amplified cDNA in a head-to-tail arrangement. At one end of the other probe, a donor dye probe, there is a fluorescein label which is excited by the light source of LightCycler and thus the label emits green fluorescence light which excites the acceptor dye probe, LightCycler Red label 640# in this case, by fluorescence energy transfer (FRET). At the end of each annealing step – a step where happens the hybridization of the probes to their target sequences – the red fluorescence emitted by the acceptor probe is measured and thus the relative amount of the amplified fragment is determined.

The two probes have a specific target sequence in the cDNA where they bind to, and are in close contact with each other, thus enabling a highly accurate detection of the amount of the sequence present by FRET. After annealing the temperature raises so much that it leads to the formation of the double-strand, leaving the probes free and too far apart so that FRET cannot occur (see Figure 5).

The sequences of the probes we use in our assay can be found from the Appendix I. Different probes are needed for ND1 and 16S since their base sequences are not similar.

¹³ A fragment of DNA which is used to detect the presence of a certain DNA sequence that is complementary to the sequence of the probe.

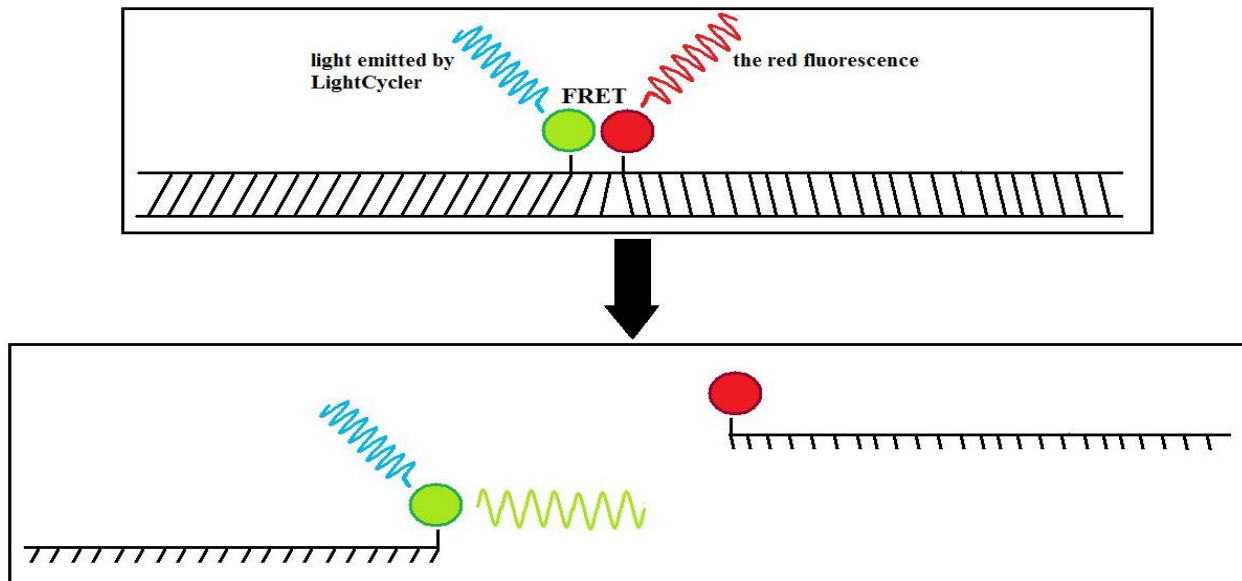


Figure 5. *FRET between two probes which are in close contact. When they are far apart FRET cannot occur.*

3. DATA COLLECTION

3.1. TREATMENT OF THE SAMPLES

In order to gain comparable data four standard runs are performed: 16S Rev, 16S For, ND1 Rev and ND1 For separately. In these runs cDNA templates from the cell line 9, which is a control cell line, are diluted with a regular pattern (1:2, 1:10, 1:50, 1:250, 1:1250 and 1:6250), run using LightCycler and thus a standard curve is generated. Standard runs also include six constant samples with a dilution 1:20 and one water sample for negative control. Constant samples are present in all the other runs so that it is possible to plot the unknown samples to the standard curve. With this standard curve the concentrations of the 16S and ND1 transcripts can be determined from the other runs.

Four standard runs are performed to produce standard curves for ND1 For, ND1 Rev, 16S For and 16S Rev. For all of them there are 81 cDNA samples from which the levels of 16S and ND1 Rev and For transcripts are detected. This makes a total of 4x81 sample to be run using LightCycler.

The work I perform with the cDNA templates is the following: first, magnesium chloride, water, vials 1a and 1b (from the kit¹⁴, exact content: see Appendix II), probes and primers are thawed on ice. The storage of them in cold is essential for their correct maintenance and functioning, especially for the enzyme in the vial 1a. Vial 1b is mixed by flicking and enzyme in the vial 1a and the vial 1b are briefly centrifuged. 60 µl from vial 1b is transferred to the vial 1a which then contains LightCycler Fast-Start DNA Master HybProbe and mixed by pipetting up and down or by flicking. The mixture is then centrifuged shortly and put back on ice in order to maintain the enzyme activity.

The next step is to prepare Master mix. Depending on the number of samples, different amount of substances are used. For standard runs x20 is enough and for the actual runs x32. One extra reaction eases the pipetting.

Master mix pipetting instructions:

Substance	Amount x 1/ µl	Amount x 20/ µl	Amount x 32/ µl
Water, H ₂ O (ROCHE)	5.6	112	179.2
Magnesium chloride, MgCl ₂ (ROCHE)	2.4	48	76.8
Primer F (TIB MOLBIOL, concentration 5.0 µM (pmol/ µl))	2	40	64
Primer R (TIB MOLBIOL, concentration 5.0 µM (pmol/ µl))	2	40	64
Probe 1 (TIB MOLBIOL, concentration 2.0 µM (pmol/ µl))	2	40	64
Probe 2 (TIB MOLBIOL, concentration 2.0 µM (pmol/ µl))	2	40	64
Fast Start (ROCHE) (Kit: LightCycler Fast Start DNA Master HybProbe)	2	40	64

Master mix is prepared in 1.5 ml Eppendorf tube. Master mix is prepared fresh for every run in order to guarantee the functionality of the content and the best result possible. By centrifuging each of the vials the reaction mix is harvested the bottom and the traces of content on the pipette tip,

¹⁴ The kit used is LightCycler FastStart DNA Master HybProbe from Roche.

which could disturb the assay, are avoided.

Then enough glass capillaries (volume = 20 μ l) are placed into a cooling block in order to maintain the cold temperature. Into each of the capillaries is pipetted 18 μ l of Master mix and 2 μ l of the cDNA dilution keeping an extremely cautious care that each of the capillaries gets only once the cDNA dilution portion. Each of the capillaries is closed with a cap. Then the capillaries are spun in a special centrifuge (LightCycler Carousel Centrifuge) in order to get the template and Master mix mixed and also all of the content of the capillary into the bottom of it.

3.2 PERFORMING THE qPCR RUNS

ND1 and 16S Rev and For runs were performed separately. All of these run series included the standard run from which the concentration scale was produced and the relative concentrations of the following samples of the three following runs were determined by observing the fluorescence emitted in every cycle.

The run programs can be found from Appendix III.

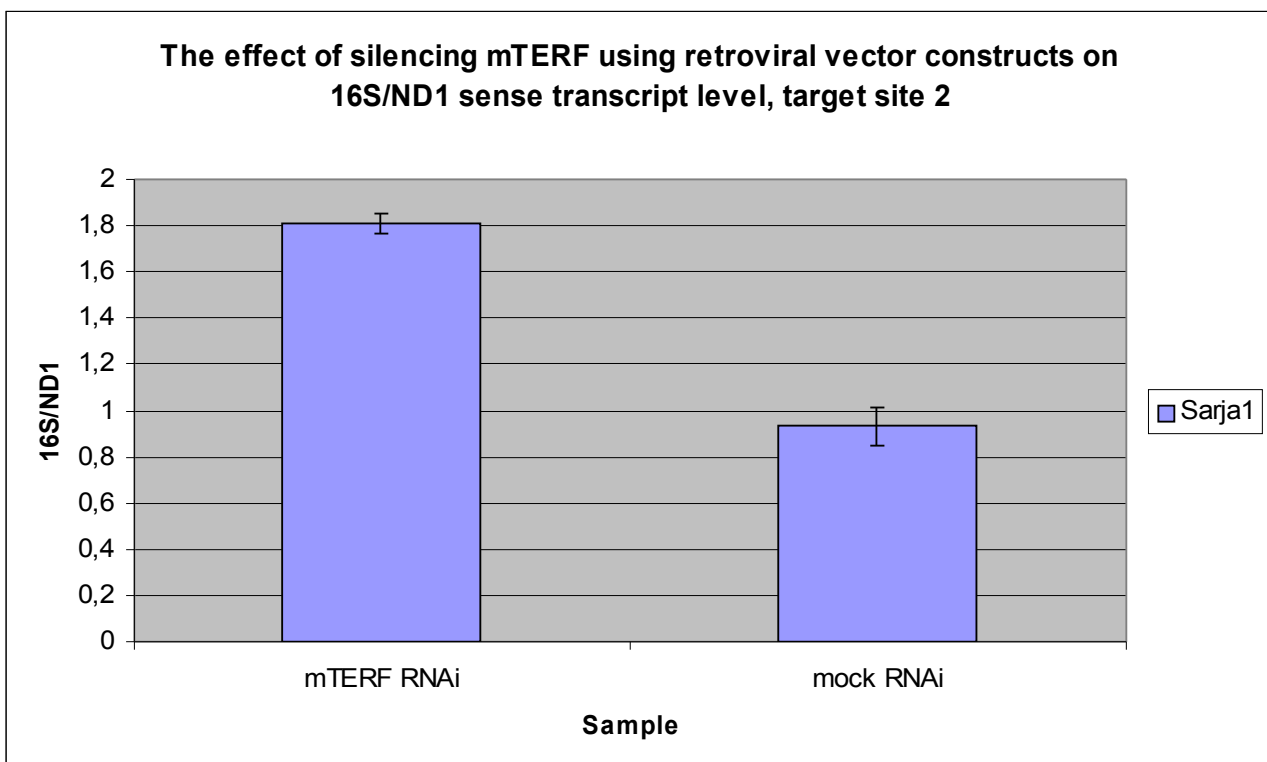
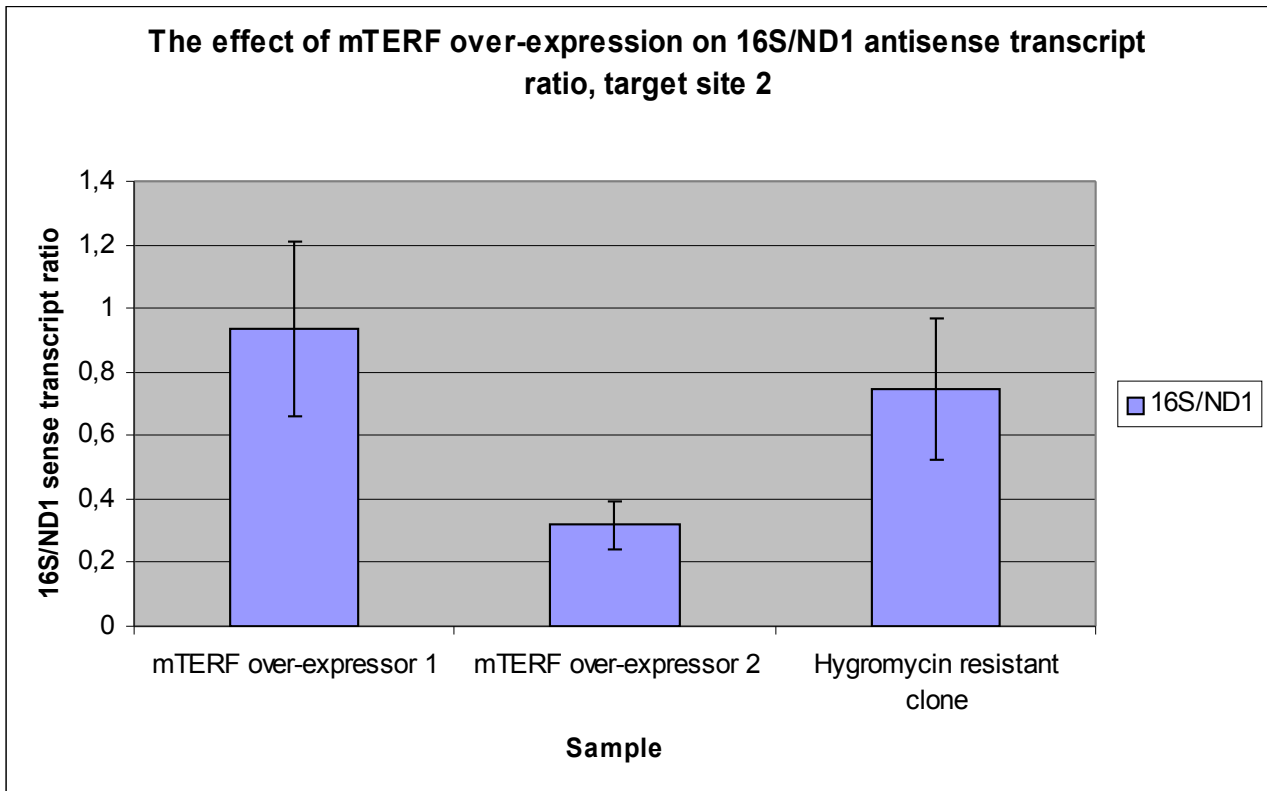
4. DATA PROCESSING

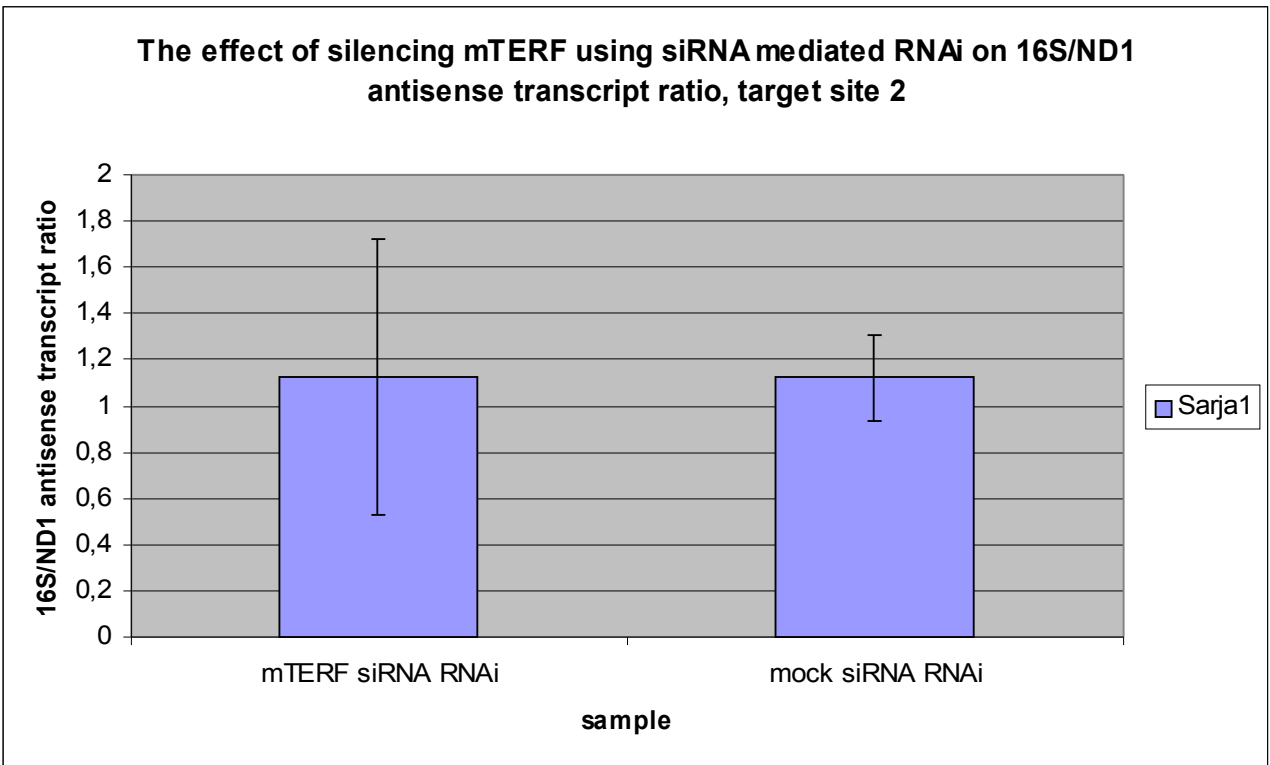
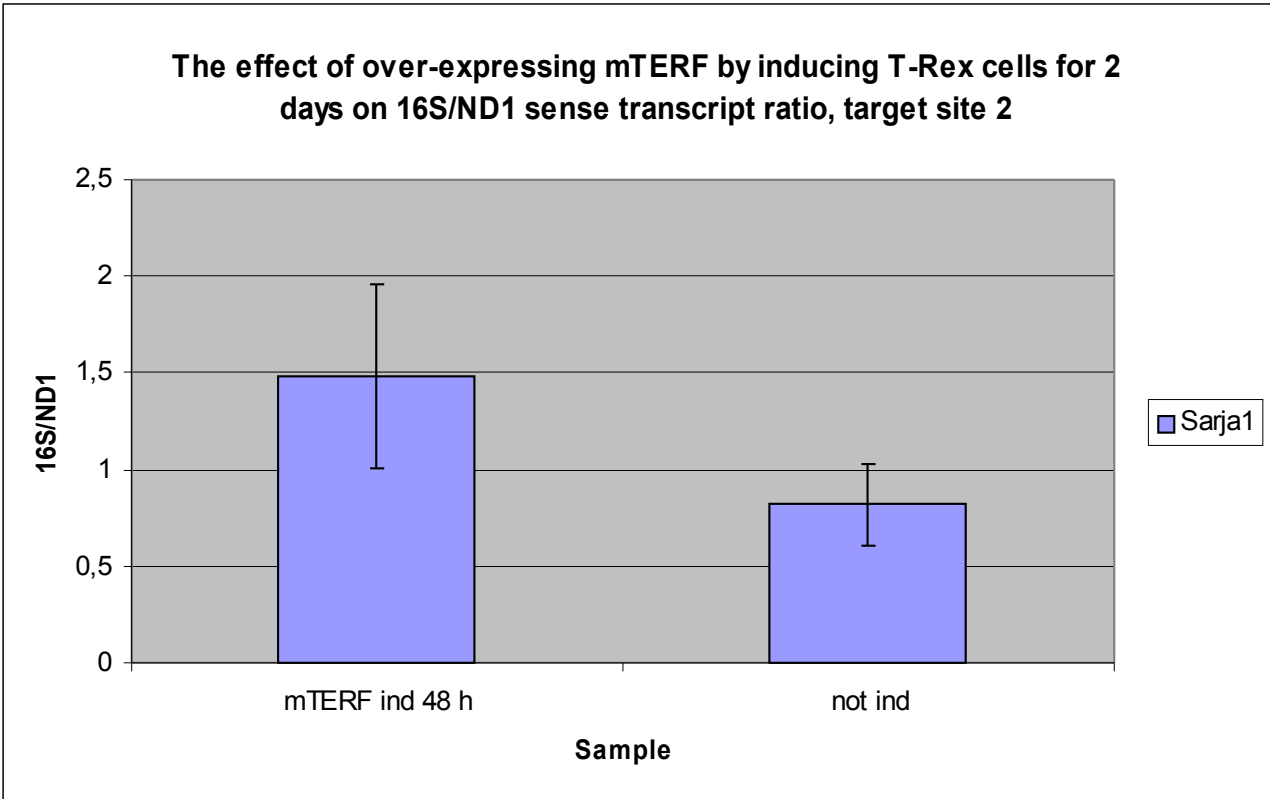
4.1 RESULTS

The initial concentrations of the transcripts in the samples can be detected by computer when the fluorescence emitted by the sample is detected on every cycle. From the detected values the relative concentrations of 16S and ND1 can be processed taking into account the different dilutions of the cDNAs. From each of the nine cell lines there were three independent samples and from these three dilution series (1:10, 1:20 and 1:50). The detected values for the 1:20 samples are about half of the 1:10 values and 1:50 about a fifth. Thus 1:20 values are doubled and 1:50 multiplied by five in order to gain comparability and same magnitude. Then the average of these three is taken and hence three concentration values gained from one cell line for 16S Rev/ For and ND1 Rev/ For. Thus three different 16S / ND1 Rev and For relations are calculated from one cell line. From these three values a bar chart is drawn for antisense and sense transcripts separately. The bar charts are shown

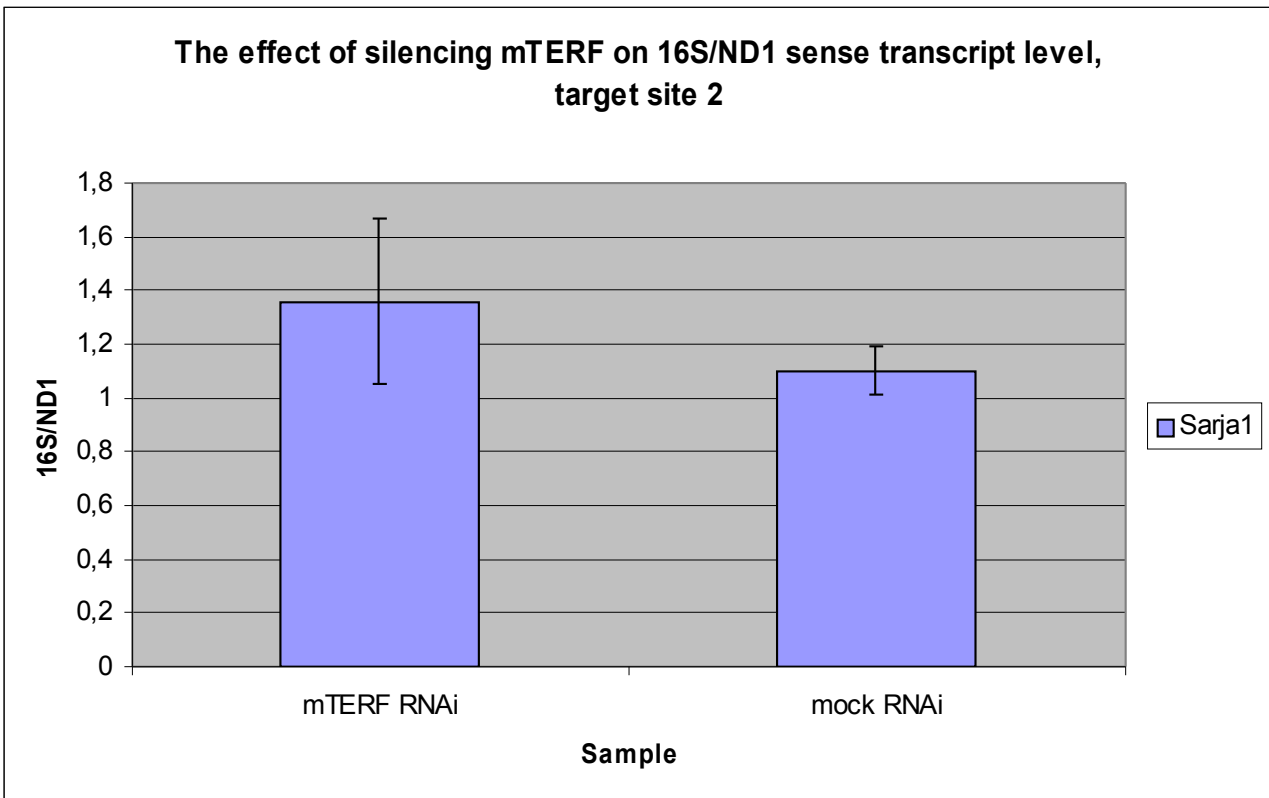
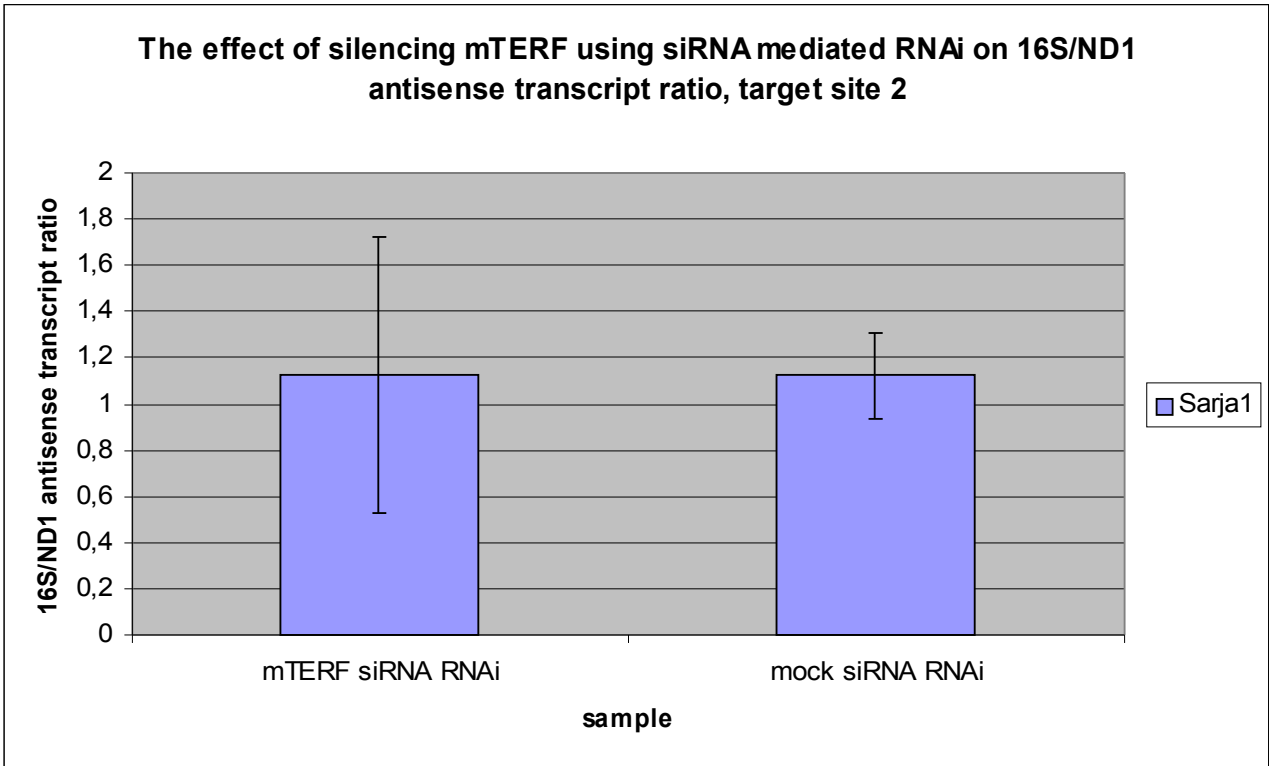
here below, title indicating from which of the cell lines they are.

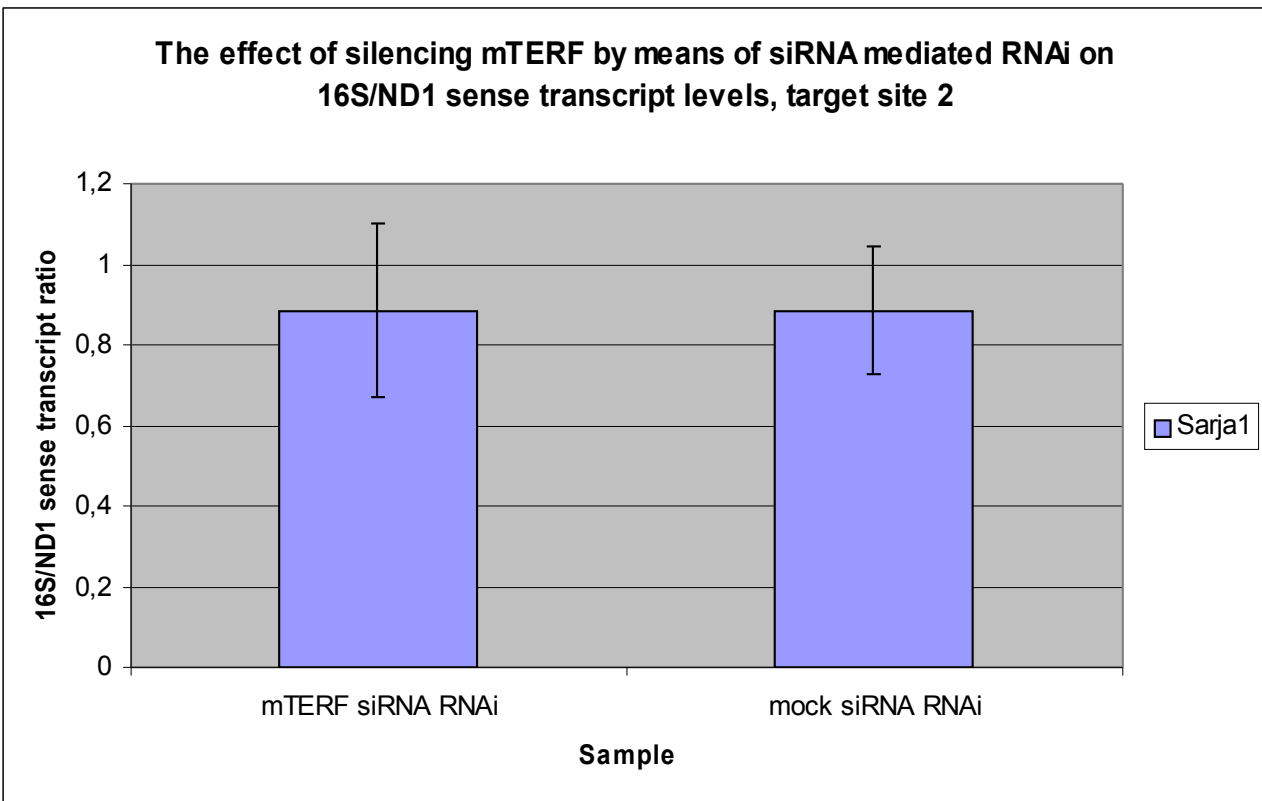
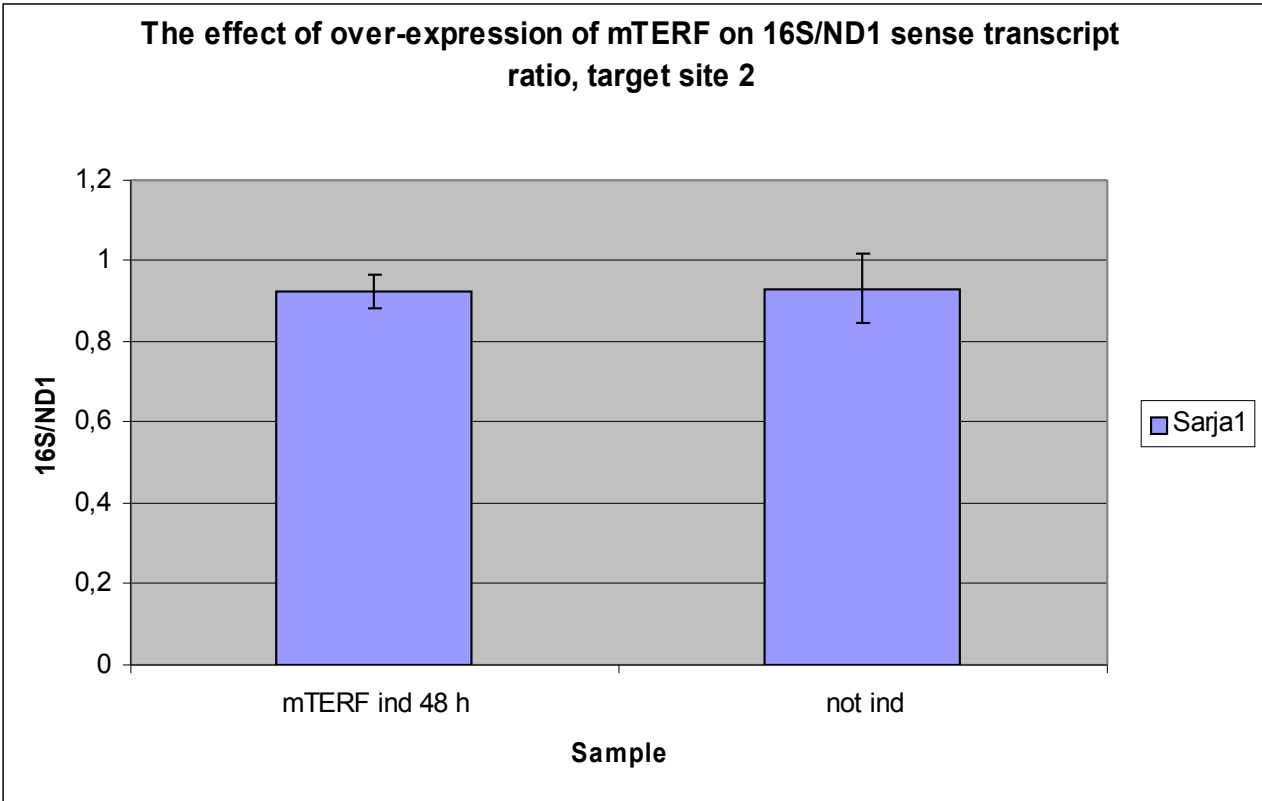
Antisense





Sense





5. CONCLUSION & DISCUSSION

5.1 THE ROLE OF mTERF IN THE TRANSCRIPTION TERMINATION

The levels of the two transcription units, rRNA and mRNA were detected by LightCycler which measured their levels by the emitted fluorescence. As can be seen from the bar charts in 4.1. in all of them the error bars overlap and there is no significant difference between the bars. This indicates that 16S/ ND1 relationship is not changed even though the production of mTERF is over-expressed or silenced in cultured human cells. It means that the relative concentrations of rRNA and mRNA remain the same regardless of how much mTERF there is in the cell. My results are coherent for both sense and antisense transcripts. Therefore the results suggest – contrary to the existing belief – that mTERF does not have a role in the transcription termination of mitochondrial DNA. The results obtained in this study support Anne Hyvärinen's previous results, when the relative levels of 1) tRNA-LEu(UUR) and tRNA-Phe or 2) 16S and ND1 using another pair of probes, were studied mTERF being either over-expressed or silenced (unpublished data, data not shown here). The coherence of our results indicates that the experiment setting and method were reliable for investigating the role of mTERF in the termination. However, there still exists the possibility that mTERF terminates the transcription by functioning together with some other protein(s).

Even though *in vitro* assays have shown mTERF terminating the transcription this was not it in our case. There are few possible reasons for the diverging between these two. The role of mTERF was studied by over-expressing or silencing its production in the cell. According to Micol et al. 1997 about 80% of the possible binding sites of mTERF are occupied all the time. Thus the over-expression of mTERF might not have so significant effect on the levels of the two RNA transcripts since almost all the time there already is mTERF present. The silencing of the mTERF production might have been imperfect thus leaving enough mTERF in the cell so that the transcription termination can take place.

5.2. LAB WORKING

When an experiment is done using as small amounts as micro liters an accurate pipetting and pipettes are essential in order to gain reliable and unbiased data. Especially when this process is performed manually there is always a possibility for human error. Therefore, before the actual assay was started the pipettes were checked and calibrated by pipetting a small volume of water (10 μ l) on a highly sensitive scale measuring tenth of milligrams, and this small experiment was extremely instructive: even a tiny drop left on the pipette tip might change the mass of the fixed volume by \pm 0.6 mg. This taught me to pipette accurately every tiny drop out of the pipette tip since every drop, for example the cDNA template, is essential so that the right concentration values are detected and the results are solid. Also the vertical position of the pipette and slow and steady pressing are important so that all of the content comes out of the pipette tip, especially when the solution inside the pipette tip is viscous. When calibrating the pipettes were adjusted so that the certain volume of water corresponded to the right mass reading on the scale and the same pipettes were used during the whole experiment in order to maintain the same standard error. Hence accurate pipetting was effectively learned and this resulted standard curves with really small error values and thus a better accuracy in the final results. Since the error bars were low, the pipetting can be considered reliable and the assay successful.

New master mix for each of the runs was carefully prepared using the same amounts of probes and primers in each of the them, so that it provided same kinds of conditions for the polymerase chain reaction to take place.

Centrifuging the vials also increases accuracy. It assures that no substance is left on the walls of the vial which could then end up on the pipette tip and increase the actual amount of the substance needed for the experiment part. Flicking mixes the content of the vial and makes sure that the concentration of each of the substances is uniform throughout the mixture. Keeping the reaction mix cold during the pipetting process is important especially for the enzyme so that it remains as efficient and active in all of the runs and thus enables the qPCR to happen.

In the standard runs cells from the cell line 9, which included nonsense siRNA transfected cells, were used since they were control cells neither over-expressing nor silencing mTERF, thus giving a

good indication of the normal production of mTERF in human cells and are thus suitable for the standard curve. The same constant sample in all of the runs of one series is essential in order to gain comparable data.

Overall, the experiment was successful. My results were coherent with Anne's, and also the standard curves had small error values thus indicating accurate and careful pipetting. All of the substances needed in the pipetting were treated carefully and in the same way so that the conditions for all of the reactions were as similar as possible. To conclude, mTERF was not found to terminate the transcription termination after rRNA in mtDNA. The exact function of this interesting protein, whether it terminates the transcription with some other protein or has a completely different role in mtDNA in addition to its role in mtDNA replication, remains to be established in the future.

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APPENDICES

Appendix I: The probes used in the experiment

Probe	Sequence in human mtDNA	Melting temperature
16S F	AgAgAgTAAAAAATTTAACACCCAT	53.1 °C
16S A	TTCTATAgggTgATAgATTggTCC	53.1 °C
16S FL	AAgCTCAACACCCACTACCTAAAAAA-FL	59.9 °C
16S LC	640-CCCAAACATATAACTgAACTCCTCACACC-p	62.8 °C

Probe	Sequence in human mtDNA	Melting temperature
ND1_F	CCTACATTgTACCCATTCTAATC	50.9 °C
ND1_R	CgTAgTTTgAgTTTgATgCT	50.1 °C
ND1_FL	CgCCACATCTACCATCACCCCTCTACA-FL	64.5 °C
ND1_LC	LC-CACCgCCCCgACCTTAgCTCT-p	66.3 °C

Appendix II: The content of the Lightcycler FastStart DNA Master HybProbe kit

Vial	Label
1a (red cap)	LightCycler FastStart Enzyme
1b (colourless cap)	LightCycler FastStart Reaction Mix HybProbe
2 (blue cap)	MgCl ₂ , stock solution, 25 mM
3 (colourless cap)	H ₂ O, PCR-grade

Appendix III: The run programs of the qPCR runs

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time	Acquisition Mode
PRE-INCUBATION					
None	1		95 °C	10 min	none
AMPLIFICATION					
		Denaturation	95 °C	10 s	none
Quantification	40	Annealing	16S: 47°C ND1: 45°C	10 s 10 s	single
		Extension	72 °C	16S: 10 s ND1: 20 s	none
MELTING CURVE					
		Denaturation	95 °C	0 s	none
Melting Curves	1	Annealing	16S: 55 °C ND1: 60 °C	30 s	none
		Melting	95 °C slope = 0.1 °C/ sec	0 s	continuous
COOLING					
None	1		40 °C	30 s	none