Constructing endothelial-specific hypoxia-regulated vectors

Jarkko Laitalainen
Master’s thesis
University of Jyväskylä
Department of Biological and Environmental Science
Cell and Molecular Biology
15.11.2013

Alkusanat

Taustalla soi Fleshgod Apocalypsen levy Labyrinth. Viimeiset korjaukset Pro Graduun on tehty. Onko labyrintin ulospääsy todellakin löytynyt vai onko se sittenkin vain uusi umpikuja? Minotauruksen äänet kuuluvat raskaana jostain kaukaa ja pakottavat jatkamaan. Tekstiä on synnyttävä, vaikka tulevaisuudesta ei ole tietoa. Onko tässä mitään järkeä vai onko viisaus juuri siinä? Kuka ajaa takaa ja ketä? Yksi vastaus synnyttää vähintään kaksi uutta kysymystä – sen tiede on opettanut. On pakko hengähtää, katsoa mieleeni ja löydettävä todellinen intohimoni. Labyrintin reunoilla näen eksyneiden jäänteitä ja järjen rajamailla kamppailevia kulkijoita. Ihmiset etsivät ulospääsyä vuosia ehkä koskaan löytämättä sitä - intohimosta puhumattakaan. Opin ulkoisista ärsykkeistä ja tiedän mitä haluan. Olen onnekas.

”Mikä se oli?”, ajattelen. Avaan silmäni ja palaan todellisuuteen. ”Vai olinko se minä?”, kuuluu epäilyksen ääni sisältäni. Sorkkien pahaenteiset kumahdukset kuuluvat jo paljon lähempää kaikuen labyrintin kivisillä seinillä ja tiedän olevani järjissäni. Peto on vaeltanut labyrintissään yli 10 kuukautta odottaen uhriansa. Pitkään piinattu liha on mureinta maustettuna ripauksella toivoa. Pedon verenhimo on kasvanut vuodenaikojen vaihtuessa. Nyt sitä ajaa raivonälkä, joka on täytettävä tai tukahdutettava. Murskaavan mylvinnän kuuluessa läheltä aistini terävöityvät ja tiedän mitä on tehtävä. ”Peto on tapettava”, kuuluu ääni sisältäni.

Astun varoen kohti käytävän kivistä nurkkaa ja herkistän kuuloani. Hivuttaudun kohti reunaa ja huomaan pedon olevan kulman takana. Noin 11 000 sanaa ja 40 sivua koristavat pedon ruumista, joka on kasattu useasta osasta. Kokoamisen on suorittanut tikkauksista päätellen päihtynyt kirurgi, vai onko kauneus katsojan silmässä? En anna pedon ulkokuorelle enempää huomiota vaan keskittymiseni on täysin viimeisessä siirrossa. Käteni kietoutuu miekan ympärille, jota jo uljas prinssi Thadeous aikoinaan käytti, ja tiukennan otettani. Päätöksen tehtyäni hyökkään kohti petoa pohjanmaalaisten ärräpäiden raikuessa kivisellä käytävällä. Peto luo viimeiset katseensa kaula lävistettynä kohti minua - kohti vapauttajaansa. Samalla huomaan tulleeni labyrintin uloskäynnille. Seikkailu on lopussa, mutta toinen on jo alkanut.

Haluan kiittää Seppo Ylä-Herttualaa erinomaisesta gradupaikasta ja apurahoista. Minna Kaikkoselle kuuluvat kiitokset hyvästä ohjauksesta, kommenteista ja työn ideoimisesta. Henri Niskaselle suuret kiitokset käytännön ohjauksesta, neuvoista ja avusta! Kiitokset myös muulle AIV-instituutin henkilöstölle hyvistä keskusteluista ja neuvoista. Vanhempiani tahdon sydämestäni kiittää kaikesta tuesta jota 26 vuoden aikana olen saanut. Ilman teitä en olisi päässyt tähän pisteeseen. Viimeisenä haluan kiittää avopuolisoani Raisaa, jonka psykologisetkin taidot ovat välillä tulleet tarpeeseen labyrintin nakertaessa miestä.

Jyväskylän yliopisto
Pro gradu –tutkielman tiivistelmä
Matemaattis-luonnontieteellinen tiedekunta

Tekijä:	Jarkko Laitalainen
Tutkielman nimi:	Endoteelispesifisten hypoksia-säädeltyjen vektoreiden rakentaminen
English title:	Constructing endothelial-specific hypoxia-regulated vectors
Päivämäärä:	15.11.2013
Sivumäärä:	40
Laitos:	Bio- ja ympäristötieteiden laitos
Oppiaine:	Solu- ja molekyylibiologia
Tutkielman ohjaajat:	Minna Kaikkonen, Henri Niskanen
Tutkielman valvoja:	Jari Ylänne

Tiivistelmä:

Soluspesifinen geenien ilmentyminen on molekyylibiologian yksi suurista tutkimusalueista. Geenit aktivoituvat eri solutyypeissä eri tavoin, vaikka soluissa on sama geneettinen perimä. Tutkimuksissa on löydetty useita tekijöitä mm. enhanserit ja enhanseri-RNA molekyylit, jotka vaikuttavat mm. solukohtaiseen geenin ilmentymiseen. Enhanserit ovat alueita genomissa, jotka sitovat proteiineja ja vuorovaikuttavat transkriptiokompleksin kanssa lisäten geenin traskriptiota. Enhanseri-RNA:t ovat enhaserialueilta tuotettuja RNA-molekyylejä. Geenin ilmentymiseen vaikuttaa todella moniulotteinen säätelyjärjestelmä, kun mukaan lasketaan kaikki transkriptioon ja translaatioon vaikuttavat tekijät.

Endoteelisolut ovat veri- ja imusuonten seinämien soluja, jotka muodostavat ohuen kerroksen suonten sisäpinnalle. Solukerroksen tehtävä on säädellä mm. nesteiden ja ravinteiden pääsyä suonesta ympäröivään kudokseen. Endoteelisolut ovat myös suuressa roolissa uusien verisuonten muodostumisessa niin normaalioloissa kuin syövissäkin. Hypoksia on eräs tekijä, joka aktivoi verisuonten muodostumista endoteelisoluista. Hypoksia on fysiologinen tila, jossa solu, kudos tai koko keho kärsii hapen puutteesta. Hypoksia johtaa vakavimmillaan hapenpuutteesta kärsivän kudoksen kuolemaan eli nekroosiin. Hypoksia voi muodostua, mikäli verenkierto häiriintyy esim. verisuonen tukkeuman tms. takia eikä veri kulkeudu normaalisti kudokseen.

Työn tarkoitus oli luoda endoteelispesifisiä hypoksia-aktivoituvia vektoreita. Genomista valittiin yksi promoottori, kolme enhanseria ja kolme 3'-UTR-aluetta. Alueet kloonattiin napanuoran verisuonten endoteelisoluista (HUVEC) eristetystä genomista ja liitettiin pGL4.10 pohjaisiin lusiferaasi-vektoreihin. Tuotetut konstruktit transfektoitiin viiteen solulinjaan, joista kaksi oli endoteelisolulinjoja. Transfektion jälkeen soluja kasvatettiin 24 tuntia normaalissa happipitoisuudessa tai 1%-hypoksiassa. Käsittelyiden jälkeen konstruktien lusiferaasi-aktiivisuus mitattiin soluista ja aktiivisuuksia vertailtiin keskenään.

Tulokset osoittavat soluspesifisyyden ja hypoksia-aktivoitumisen olevan monen eri tekijän summa. Geenin ilmentyminen oli suurempaa kahdella konstruktilla kahdessa solulinjassa verrattuna kontrolleihin. Konstruktien luciferaasi-geeni aktivoitui endoteelisoluissa, mutta myös muissa solulinjoissa. Hypoksia-aktivoitumisesta oli tilastollisesti merkittäviä tuloksia yhden konstruktin kohdalla, joskin rohkaisevia tuloksia saatiin myös muilla konstrukteilla.

Avainsanat: Hypoksia, endoteelisolut, promoottori, enhanseri, UTR, geeniterapia,

University of Jyväskylä
Abstract of Master´s Thesis
Faculty of Mathematics and Science

Author:	Jarkko Laitalainen
Title of thesis:	Constructing endothelial-specific hypoxia-regulated vectors
Finnish title:	Endoteelispesifisten hypoksia-säädeltyjen vektoreiden rakentaminen
Date:	15.11.2013
Pages:	40
Department:	Department of Biological and Environmental Science
Chair:	Cell and molecular biology
Mentors:	Minna Kaikkonen, Henri Niskanen
Supervisor:	Jari Ylänne

Abstract:

Cell specific gene expression is one of the most interesting fields in molecular biology. Genes are expressed differently in different cell types even the cells have same genetic material. Studies have found multiple factors affecting cell specific gene expression, enhancer regions and enhancer-RNA (eRNA) molecules among other things. Enhancers are DNA regions that can bind transcription factors and then interact with transcription complexes enhancing the expression. eRNAs are RNA-molecules transcribed from the enhancer regions. Cell specific gene expression is a complex system when all factors affecting transcription and translation are counted in.

Endothelial cells are a thin layer of cells in blood- and lymphatic vessel walls. Main functions of endothelial cells are to regulate access of fluids and nutrients from vessels to the surrounding tissues. Endothelial cells play also a critical role in angiogenesis in normal situations and also in cancers and in cardiovascular diseases. Hypoxia is one factor that can activate angiogenesis. Hypoxia is a physiological condition where a cell, a tissue or the whole body is lacking oxygen. It can lead to cell deaths or even tissue necrosis. Hypoxia can be formed when blood circulation is disturbed for example by partial blockage of a blood vessel so that blood cannot flow freely.

The aim of this study was to produce endothelial-specific hypoxia-regulated vectors. One promoter-, three enhancer- and three 3'UTR-regions were selected to be cloned from the genome of human umbilical vein endothelial cells (HUVEC). Cloned regions were inserted into pGL4.10 based vectors containing luciferase gene. The constructs were transfected into five cell lines where two of them were endothelial cells. After transfection cells were subjected to normoxia or 1%-hypoxia. After the treatments luciferase activities were measured and compared.

Results indicate that cell specific gene expression and enhancing is sum of a multiple factors. Gene expressions were higher with two of the constructs compared to the controls. All constructs expressed luciferase in endothelial cells but also in other cell types. One construct showed statistically significant hypoxia activation and the other constructs gave promising results of hypoxia activation.

Keywords: Hypoxia, endothelial cells, promoter, enhancer, UTR, gene therapy

Table of Contents

Alkusanat 2
Tiivistelmä 3
Abstract 4
Abbreviations 6
1. Introduction 8
1.1 CHROMATIN MODIFICATIONS 8
1.2 TRANSCRIPTIONAL REGULATION AND GLOBAL RUN-ON SEQUENCING 9
1.3 mRNA PROCESSING AND TRANSPORT CONTROL 13
1.4 TRANSLATIONAL CONTROL 13
1.5 REGULATION OF GENE EXPRESSION UNDER HYPOXIA 14
1.6 GENE THERAPY ON ENDOTHELIAL CELLS 15
2. The aim of the study 16
3. Materials and Methods 17
3.1. PCR 17
3.2. AGAROSE GEL ELECTROPHORESIS 18
3.3. PURIFICATION OF PCR AND DIGESTION PRODUCTS 19
3.4. MEASURING DNA PURITY AND CONSENTRATION 19
3.5. DIGESTION OF PCR PRODUCTS AND PLASMIDS 19
3.6. LIGATIONS AND TRANSFORMATIONS 21
3.7. SMALL SCALE DNA ISOLATION, RESTRICTION ANALYSIS AND SEQUENCING 21
3.8. LARGE SCALE DNA PRODUCTION 22
3.9. CELL CULTIVATION 22
3.10. TRANSFECTIONS 23
3.11. IMAGING THE CELLS 24
3.12. LUCIFERASE ASSAY AND ANALYSIS 24
3.13. STATISTICS 25
4. Results 25
4.1. CREATING THE CONSTRUCTS 25
4.2. TRANSFECTION EFFICIENCY 26
4.3. LUCIFERASE ASSAY 28
5. Discussion 31
5.1. ENDOTHELIAL SPECIFICITY 33
5.2. ENHANCING ABILITY 34

5.3. HYPOXIA RESPONSES 35
5.4. CONCLUSION AND FUTURE WORK 36
6. References 37

Abbreviations

AGE	agarose gel electrophoresis
ALL	acute lymphocytic leukemia
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
ChIP-seq	chromatin immunoprecipitation sequencing
CIP	calf intestinal alkaline phosphatase
CREB	cAMP response element binding
DMEM	Dulbecco’s Modified Eagle Medium
EMEM	Eagle’s Minimal Essential Medium
eRNA	enhancer RNA
FBS	fetal bovine serum
GFP	green fluorescent protein
GRO-seq	global run-on sequencing
HIF	hypoxia inducible factor
HPH	HIF-prolyl hydroxylase
HRE	hypoxia response element
LB	lysogeny broth
MAPK	mitogen activated protein kinase
miRNA	micro RNA
mRNP	messenger ribonucleoprotein
NPC	nuclear pore complex
NRO-seq	nuclear run-on sequencing
RISC	RNA-induced silencing complex
RPM	rounds per minute
SCID	severe combined immunodeficiency
SNP	single nucleotide polymorphism

SOC	super optimal broth
S/P	streptomycin/penicillin
SRC-1	steroid receptor coactivator-1
TBP	TATA-binding protein
TF	transcription factor
TIF2	transcription intermediary factor-2
UTR	untranslated region
VEGF	vascular endothelial growth factor

1. Introduction

Genes are certain DNA sequences that contain information needed to produce proteins or specific RNA-molecules. According to current knowledge most of human genes have been recognized and there seems to be approximately 21000 coding and 22500 non-coding genes in the human genome (Collins et al., 2004). The accurate amount of different proteins is unknown because different reading frames, transcriptional modifications and post translational modifications make it possible to produce many proteins from a single gene. Basically gene expression can be regulated on three levels: initiation, transcription and post transcriptional level. Initiation is the phase where cell receives stimulus exemplified by a change in the concentration of glucose in the blood and the cell reacts to the stimulus. Transcription is a process where RNA molecule is synthesized using DNA molecule as a template. Post transcriptional phase is where mRNA molecule has been synthesized and it is modified and taken into cytosol for translation.

Even though most of the genes have been characterized and many regulatory mechanisms are well known, the work has just begun. A major question in the field is what the mechanisms behind cell-type-specific gene expression programs are. Human body has over 200 different cell types and many genes are expressed differently between cell types (Natarajan et al., 2012). Scientists around the world are trying to find out what the factors responsible for cell specific gene expression are and how they differ between cell types. Many answers have been found, but there are more questions to be answered. The next chapters will give short introduction to factors that have effects on cell specific gene expression.

1.1 CHROMATIN MODIFICATIONS

Chromatin is a dynamic entirety because tightly packed DNA strand needs to be opened for gene expression and packed again after transcription. Chromatin needs to be modified to reveal genes for transcription. Often the targets of these modifications are histones, the proteins that DNA is packed around. There are at least eight different modifications that can be done to histones: acetylation, methylation on lysines, methylation on arginines, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, deimination and proline

isomerization. All modifications play role in transcription but some have role also in DNA repair, replication and condensation (for the review see Kouzarides, 2007).

Chromatin immune precipitation sequencing (ChIP-seq) has been a great tool to indentify modification sites in the genome. ChIP-seq is based on protein-DNA interaction such as transcription factor (TF) binding. In a typical ChIP-seq protocol DNA is sheared with sonication and DNA-protein complexes are precipitated by using proper antibodies. The precipitated DNA is then sequenced. The sequences can be compared to the whole genome and the TF binding sites can be localized (Johnson et al., 2007).

Histones in the enhancer and promoter regions are often modified. Promoter regions of actively transcribed genes are commonly acetylated and trimethylated whereas monomethylation has been shown to be involved in many enhancer regions, as well as acetylation. Acetylation is the best modification to unfold chromatin because it removes the positive charge of lysine residue. The finding that acetylated residues are often present in promoter and enhancer regions supports the idea that transcription factors bind to open regions (Heintzman et al., 2007). In this study previously collected ChIP-seq data from different cell lines was used to find the enhancer and promoter regions that are acetylated and methylated mainly in endothelial cell lines like HUVEC.

1.2 TRANSCRIPTIONAL REGULATION AND GLOBAL RUN-ON SEQUENCING

Transcription is a process that can be regulated in several ways. The key players of transcriptional regulation are promoters and enhancers. Promoters are regions of DNA where RNA polymerase initiates transcription by binding with multiple other factors. Promoters are usually localized 20 – 250 bp upstream from the target gene and they have different sequence motifs with different functions including transcription start site (TSS), binding motifs for RNA polymerase and TFs. Most common feature of promoters is the TATA-box (sequence TATAAA), the binding site for TATA-binding protein (TBP). TBP is a subunit for TFIID which binds to the TATA-box via TBP. Further interactions are needed for transcription and TFIIB binds to TFIID/TBP complex. RNA polymerase is attached to this protein complex with TFIIE, TFIIH and other factors. TFs are proteins that can bind to DNA or directly to RNA polymerase and interact with the transcription complex boosting

or repressing transcription. After all the needed factors are bound to the promoter the actual transcription starts. When the elongation of RNA transcript has begun most of the general TFs are released. RNA polymerase glides along DNA strand making RNA molecule using DNA as a template (Alberts et al., 2008).

Other key players in transcriptional regulation are the enhancers. Enhancers are DNA regions that can bind transcription factors and then interact with the promoter regions enhancing the expression. Enhancers usually act with the nearest promoter but they can be located in a great distance from the target promoter, even on another chromosome (Geyer et al., 1990). Enhancer regions are usually located in a non-coding DNA in inter- and intragenic nucleosome free or “open” regions but some studies suggest that they can be located also in the coding DNA (Ritter et al., 2012). Because enhancers are typically located on open regions, an early method for their recognition was to use DNaseI to cut DNA and sequence regions close to the cut site (Shu et al., 2011). TFs bind to enhancer regions by interacting with the DNA strand through hydrogen bonds, electric charges and other weaker forces so that the reaction can be reversed. Enhancer-bound TFs are usually bound to some co-factors like chromatin remodelers that can bend or exclude nucleosomes to make DNA regions accessible for further interactions with other proteins (for review see Clapier and Cairns, 2009). After necessary components have bound to the enhancer region it will interact with the target promoter. Common model for enhancer-mediated transcription activation depicts enhancer region looping to the promoter region (de Laat et al., 2008). Factors bound to enhancer interact with the transcription complex at the promoter and stabilize and/or activate the transcription (for the review see Blackwood and Kadonaga, 1998). A diagram of enhancer-promoter-complex is shown in the figure 1.

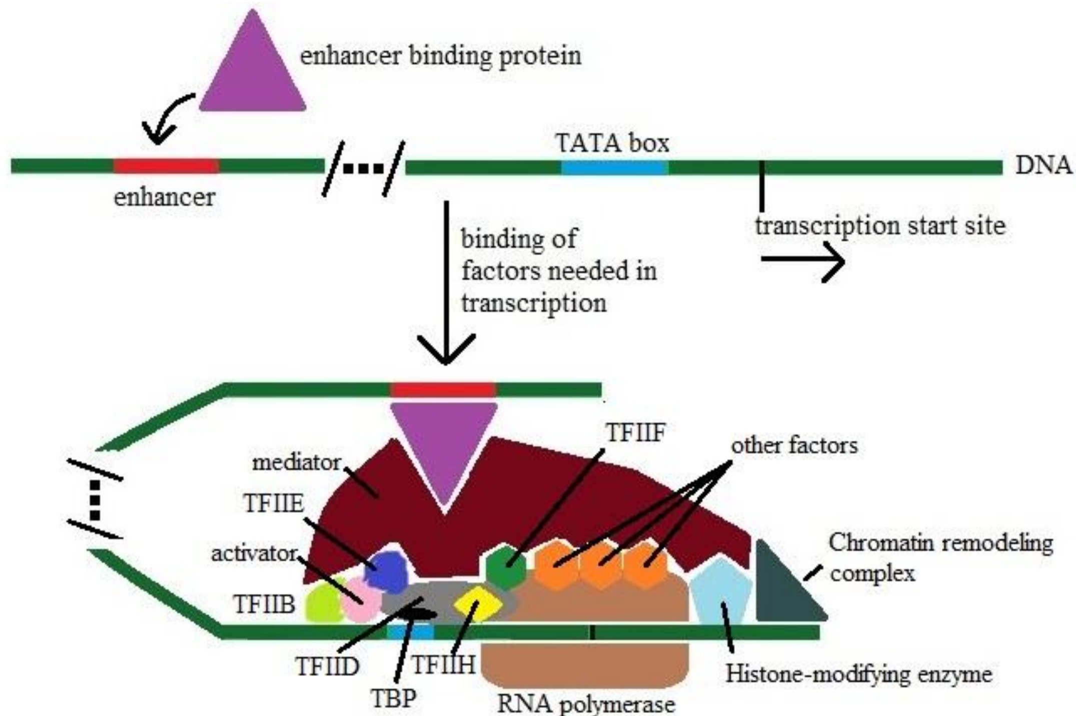

Figure 1. Diagram representing interaction between enhancer and transcription complex.

The non-coding RNAs originating from enhancer regions, called enhancer RNAs (eRNAs) were discovered in 2010 (de Santa et al., 2010; Kim et al., 2010). The eRNAs are transcribed from non-coding genome and they enhance the transcription of neighboring genes. Importance of the eRNAs was pointed out in a recent study which demonstrated that efficient transcription enhancement of p53 gene is dependent on the eRNA (Melo et al., 2013). The mechanism of eRNA-mediated enhancing is not known but there are speculations that eRNA forms a complex between the enhancer and the promoter via unknown factor.

Global Run-On Sequencing (GRO-seq) is a great tool to study global transcription. With GRO-seq it is possible to localize, orientate and quantify RNA polymerases in the genome and measure the transcription activity. An example of GRO- and ChIP-seq data is presented in the figure 2.

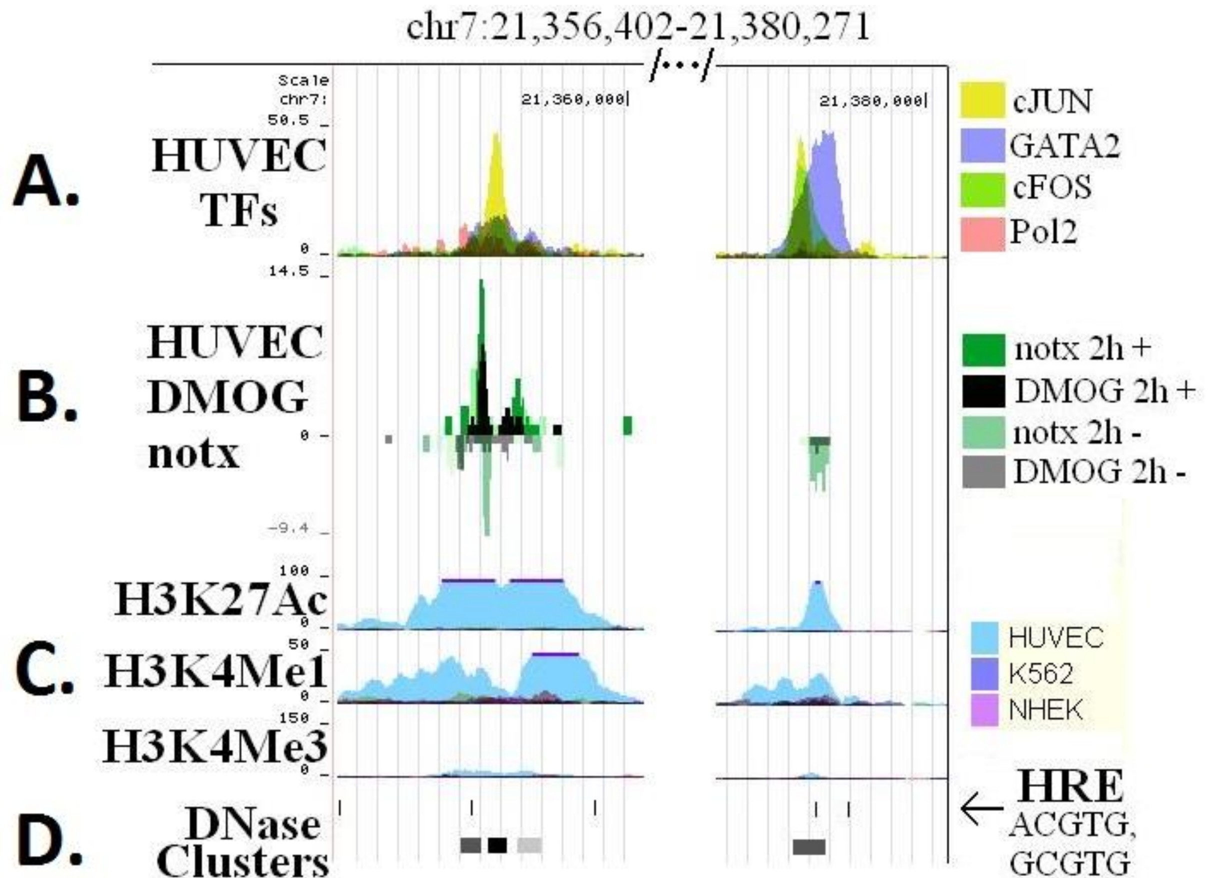

Figure 2. An example of ChIP- and GRO-seq data. Location in human genome is presented at the top of the figure and the color codes are on the right side. **A.** HUVEC TFs ChIP-seq data provide information about binding sites of different transcription factors in HUVEC genome. **B.** HUVEC GRO-seq data show synthesis of RNA molecules after 2h normoxia (notx) and 2h artificial hypoxia using dimethyloxaloylglycine (DMOG) to inhibit HIF-1 hydroxylase. Positive mark means that RNA molecule is synthesized from a coding strand and negative from a non-coding strand. **C.** Three ChIP-seq data provide information about histone modifications in different cell types. **D.** Hypoxia responsive elements that can bind HIFs and DNase sensitive regions. This data was used to choose Enhancer 1 and 2 regions. To visualize ChIP-seq and GRO-seq data UCSC genomebrowser was used (http://genome.ucsc.edu/).

Nuclear Run-On (NRO) assay with conditions where new initiations are inhibited is the first step to be performed. Nascent RNA molecules can be isolated with immunopurification when ribonucleotide analog is used in NRO assay. Nascent RNA molecules are purified and cDNA library is made, sequenced and compared to genome. Locations, amounts and orientations of transcripts can be determined which gives valuable information about activities of RNA polymerases in specific regions (Core et al., 2008). In this study previously collected GRO-seq data from HUVEC cells together with the ChIP-seq data mentioned before, were used to find possible enhancer regions producing eRNAs to be cloned.

1.3 mRNA PROCESSING AND TRANSPORT CONTROL

The mRNA molecule undergoes different modifications in nucleus before it is transported to cytosol. There are three main types of mRNA modifications: capping, cleavage/splicing and polyadenylation. 5'- and 3'caps are made to the ends of mRNA molecule so that mRNA can be transported to cytosol through nucleopore complex (NPC) and to protect mRNA from ribonucleases that digest RNA molecules. During cleavage/splicing introns are spliced off from the mRNA molecule to produce mature mRNA that contains only information needed to make correct protein. Cleavage/splicing plays an important role in creating diversity among proteins as one mRNA can be spliced in many ways leading to multiple different mature mRNAs. Polyadenylation is needed for protein interactions and to protect mRNA 3'-end from ribonucleases. After transcription and modifications the mature mRNA is actively transported to cytosol through nucleopore complex. Complex of mature mRNA and proteins bound to it is called messenger ribonucleoproteins (mRNP). mRNP diffuses through chromatin free regions in nucleus to the NPC. Then mRNP interacts with NPC and NPC-associated proteins and the mRNP is pulled to cytoplasm 5'-end ahead (Cole and Scarcelli, 2006).

1.4 TRANSLATIONAL CONTROL

Translation is a process where mature mRNA is translated into a protein on ribosomes. Ribosomes are protein-RNA-complexes that catalyze the reaction where amino acids are linked together via peptide bonds. mRNA has untranslated regions (UTRs) that have an important role in regulating the translation. UTRs can be localized on both ends of the mRNA molecule, 5'- and 3'-ends. 5'UTR has different kinds of motifs: motifs that can bind proteins stabilizing mRNA, iron binding motif for iron activated translation, riboswitches to bind small molecules affecting translation and motifs which inhibit the translation. 3'UTR can contain microRNA (miRNA) binding sites, AU-rich sequences and a poly-A-tail which all have an important role in the translation. AU-rich sequence can bind multiple types of proteins that can activate translation, decay or stabilize mRNA structure. Poly-A-tail binding proteins are in a crucial role when 3'UTR is looped to the 5'-end of the mRNA to promote the translation initiation. miRNAs are small single stranded RNA molecules transcribed from non-coding regions. After transcription miRNA forms a hairpin loop which is spliced into mature miRNA molecule (Cai et al., 2004). miRNAs can

bind to complementary RNA strand by normal nucleotide pairing and this can activate the degradation of mRNA molecule via RNA induced silencing complex (RISC). RISC uses miRNAs or small interfering RNAs (siRNAs) to bind to single stranded RNA molecule through sequence complementarity and then degrades the formed double-stranded RNA molecule. miRNAs can bind to mRNA molecule and disturb translation also without degradation of the mRNA molecule (Ameres et al., 2007). According to current knowledge there are 1872 different hairpin precursor miRNA molecules expressing 2578 mature miRNAs in human. Many of them are expressed differently depending on a cell type and prevalent conditions (http://www.mirbase.org, 8.8.2013).

1.5 REGULATION OF GENE EXPRESSION UNDER HYPOXIA

Hypoxia is a physiological condition where cells, tissues, organs or a whole body have insufficient oxygen supply. Hypoxia has a big role in cell differentiation, in cardiovascular diseases and even in cancers. Lack of oxygen may lead to cell death and necrosis. Cell deaths would be desirable with cancer cells but in ischemic heart saving the cells is desirable. In cardiovascular diseases hypoxia is often formed when plaque attached to blood vessel disturbs blood flow in certain areas. Cells recognize drop of oxygen levels via three hypoxia inducible factor-prolyl hydroxylases (HPH) which in normoxia hydroxylate two proline residues (Pro402 and Pro564) of hypoxia inducible factor 1α (HIF-1α) (Masson et al., 2001). Hydroxylation of the proline residues leads to ubiquitinylation of HIF-1α and its proteasomal degradation (Huang et al., 1998). Under hypoxic conditions HPH lacks its co-substrate oxygen and cannot hydroxylate proline residues. HIF-1α is phosphorylated by p42/p44 mitogen activated protein kinases (MAPK) that is needed for the transcriptional activity (Richard et al., 1999). Phosphorylated HIF-1α is transported into nucleus through its nuclear translocation signal. In the nucleus HIF-1α forms a heterodimer with HIF-1β which enables subsequent DNA binding and transcriptional activation (Kallio et al., 1997). The heterodimer recruits more general co-transcriptional factors like CREB binding protein and p300 (CBP/p300), steroid receptor coactivator-1 (SRC-1), or transcription intermediary factor-2 (TIF2). CBP/p300 is a histone acetyltransferase that modulates histones so that chromatin reveals TF binding sites. The protein complex interacts with CREB, c-JUN and RNA polymerase II to bind hypoxia response elements (HRE) in the genome. HRE are sequences in DNA containing core

sequence A/GCGTG. After DNA binding the complex activates the target gene expression. HIF-1 activates gene expression of hundreds of genes and many of them have a great role in recovering from hypoxia. For example vascular endothelial growth factor (VEGF) and Flt-1 which is the receptor for VEGF (Forsythe et al., 1996; Gerber et al., 1997) are expressed in hypoxia. Both of these genes are important for angiogenesis, formation of new blood vessels. New forming vessels can provide oxygen rich blood to areas suffering from hypoxia.

1.6 GENE THERAPY ON ENDOTHELIAL CELLS

Gene therapy is a treatment where vectors are used to transport functional DNA into the cells. The most common vectors in gene therapy are lentivirus, adenovirus and adeno-associated virus. In future gene therapy might provide hope to otherwise incurable patients suffering from diseases like severe combined immunodeficiency (SCID), Parkinson's disease and acute lymphocytic leukemia (ALL) (Fischer et al., 2002; LeWitt et al., 2011; Grupp et al., 2013). One of the biggest problems in western world is the unhealthy lifestyle, including high fat diet, smoking and drinking which can lead to cardiovascular diseases. Gene therapy may also provide useful tools to treat patients suffering from cardiovascular diseases when vessel forming can be induced by using proper vectors (for review see Ylä-Herttuala, 2013).

Blood and lymphatic vessels are key components in the circulatory system which main function is to transport oxygen, nutrients, hormones etc. to all cells in the body. Another important function of the system is to maintain homeostasis by controlling for example pH balance and body temperature. Endothelial cells are located in an endothelium which is a thin layer of cells on the luminal side of blood and lymphatic vessels. Main functions of this layer are to act as a barrier for the exchange of fluids and macromolecules etc. between the intra-vessel space and surrounding tissues, to regulate leukocyte adhesion and platelet aggregation (for review see Evan and Kwak, 2013).

Endothelial cells are an interesting target from a therapeutic point of view because of their important role in angiogenesis. Endothelial cells release proteolytic enzymes that degrade surrounding basement membrane so that the cells can migrate, differentiate and form

sprouts and later proliferate to tube-like structures and finally to a new functional vessel (Liang et al., 2013). Angiogenesis is under heavy research as scientists around the world are trying to find effective treatments for cancer and cardiovascular diseases. In cancers tumors secrete growth factors to activate angiogenesis. New vessels are “feeding” the tumor so that it can continue to grow. Angiogenesis in this situation is not desirable and research is concentrating on discovering means to prevent the angiogenesis. In cardiovascular diseases the aim is the opposite as formation of new blood vessels is desirable in tissues that are suffering from ischemia.

2. The aim of the study

From previously collected ChIP- and GRO-seq data one promoter, three enhancer and three 3’UTR regions were selected to be cloned. ChIP- and GRO-seq data provided information about the binding sites of hypoxia activated transcription factors (HIF-1 etc.), activities and binding sites of RNA-polymerases under hypoxia in different cell types. Promoter of gene NDUFA4L2 was chosen because the gene had been shown to be activated under hypoxia (Tello et al., 2011). Enhancer 1 region was cloned from -100 kB and Enhancer 2 -90 kB before SP4 gene which codes transcription factor SP4. These enhancer regions bind TFs especially in HUVEC cells (endothelial cell line) as shown in the figure 2. Enhancer 3 was found -80 kB before MIR1246 gene coding miRNA1246. UTR1 and U1/GLUT3 regions are selected from 3’UTR region of gene GLUT3 (SLC2A3) coding glucose transporter 3. UTR1 has the complete 3’UTR-region of GLUT3 when 3’U1/GLUT3 has the first 146 bp of the 3’UTR region including specific binding sites to miRNA195. miRNA195 has been proven to suppress expression of GLUT3 gene in bladder cancer T24 cells by binding the miRNA binding site (Fei et al., 2012). 3’U2/ZNF704 has part of the 3’UTR region of gene ZNF704 coding zinc finger protein 704. 3’U2/ZNF704 contains 11 miRNA sites according to UCSC genome browser.

The aim of this study was to produce vectors that allow the regulation of the (therapeutic) transgene in endothelial cells under ischemic conditions i.e. hypoxia. There were three main questions within this study:

1. Do the constructs exhibit cell-type-specificity especially in endothelial cells?
2. Are the constructs able to enhance gene expression?
3. Are the constructs responding to hypoxia as they have been predicted?

3. Materials and Methods

3.1.PCR

Primers for the enhancers, 3'-UTRs and the promoter were designed with Primer3web (version 3.0.0) web tool and ordered from Integrated DNA Technologies Inc company (IDT, Coralville, Iowa 52241, USA). Primers are shown in the table I. Sizes and locations of the amplified regions are shown in the table II.

Table I. Primers used in cloning. Restriction enzyme sites are highlighted in yellow.

Name	Sequence	Restriction site
Promoter NDUFA4L2 FW	5'-TTGGTACCGCTCTCCCTCCACGAAACT-3'	KpnI
Promoter NDUFA4L2 REV	5'-TGATATCCCGTGCTTCTTTGGACTCAC-3'	EcoRV
Enhancer 1 FW	5'-TTGGATCCTGCAGTCTTGGCTTGATAATGT-3'	BamHI
Enhancer 1 REV	5'-AAAAGTCGACAAGCCTAGCTGGTTCCTTCC-3'	SalI
Enhancer 2 FW	5'-TTGGATCCTGCAATGTATCAAGCATCGTT-3'	BamHI
Enhancer 2 REV	5'-TTTTGTCGACGGTGCAGAAAACCACCATG-3'	SalI
Enhancer 3 FW	5'-TTGGATCCGCACCCATCCAAAATGAAAC-3'	BamHI
Enhancer 3 REV	5'-TTTTGTCGACAGGTGGGATCACAAGGTCAG-3'	SalI
3'UTR-1 FW	5'-TGCTAGCGTCGTGCCTCCTTCCACC-3'	NheI
3'UTR-1 REV	5'-TTGGCCGGCCCCATAATGTCGTGTACATTCAGA-3'	FseI
3'U1/GLUT3 FW	5'-TTTGGCCGGCCGTCGTGCCTCCTTCCACC-3'	FseI
3'U1/GLUT3 REV	5'-TTTGGCCGGCCTTGGGGTGCTCATGGAGT-3'	FseI
3'U2/ZNF704 FW	5'-TTTGGCCGGCCAGAGACCCCCAGGCAGAG-3'	FseI
3'U2/ZNF704 REV	5'-TTTGGCCGGCCTGGTGTTGCACTGTAGAACCTCC-3'	FseI

Table II. Locations in human genome and sizes of the regions amplified with PCR

Amplicon	Chromosome, location	Size	Restriction sites
Promoter NDUFA4L2	chr12:57630986 - 57635105	4120 bp	KpnI, EcoRV
Enhancer 1	chr7:21357433 - 21359505	2073 bp	BamHI, SalI
Enhancer 2	chr7:21378117 - 21379063	947 bp	BamHI, SalI
Enhancer 3	chr2:177382930 - 177384820	1891 bp	BamHI, SalI
3'UTR1	chr12:8071818 - 8074008	2191 bp	NheI, FseI
3'U1/GLUT3	chr12:8073863 - 8074008	146 bp	FseI
3'U2/ZNF704	chr8:81551905 - 81553601	1697 bp	FseI

Reaction mixtures for successful PCRs are shown in the table III and the reaction protocols in the table IV. Phusion Hot Start Flex DNA polymerase (New England Biolabs, NEB, Ipswich, USA) was used in all PCRs. Annealing temperatures and extension times varied due to different primers and lengths of the amplified regions. PCRs were performed with a thermal cycler (PTC-200 Peltier Thermal Cycler, MJ Research Inc, Quebec, Canada).

Table III. Reaction mixtures for the PCRs.

Component	EN1, EN2, EN3, U2/ZNF704, U1/GLUT3	UTR1, PRO
H_2O	26,5 µl	24 µl
5xPhusion HF buffer	10 µl	-
5xPhusion GC buffer	-	10 µl
10 nM dNTPs	1 µl	1 µl
10 pmol/µl FW-primer	2,5 µl	2,5 µl
10 pmol/µl REV-primer	2,5 µl	2,5 µl
HUVEC gDNA	1 µl	2 µl
Betaine	5 µl	5 µl
100 % DMSO	-	1,5 µl
Phusion Hot Start DNA polymerase	0,5 µl	0,5 µl

Table IV. Protocols of the successful PCRs.

Cycle	Temperature	Time	Number of cycles
Initial denaturation	98 °C	30 s	1
Deanturation	98 °C	10 s	28
Annealing	57-63 °C	30 s	
Extension	72 °C	30-120 s	
Hold	4 °C	∞	1

3.2.AGAROSE GEL ELECTROPHORESIS

PCR and other products from different phases were confirmed with agarose gel electrophoresis (AGE) using 1 %-agarose gel (Sigma, St. Louis, USA) made in buffer containing Tris base, acetic acid and ethylenediaminetetraacetic acid (TAE-buffer), Horizon 11·14 electrophoresis machine (Life Technologies, California, USA) and Standard Power Pack P25 (Biometra, Goettingen, Germany) power supply. GeneRuler 1kb DNA Ladder Plus 0,1 µg/µl (Fermentas, Thermo Fisher Scientific, Massachusetts, USA) was used as a molecule marker. Bands were visualized using SYBR Safe DNA gel stain (Invitrogen, Life Technologies, California, USA). Samples were run in the gel at 80 – 110 V for 30 – 90 minutes.

3.3.PURIFICATION OF PCR AND DIGESTION PRODUCTS

PCR and digestion products were purified using ChIP DNA Clean & Concentrator kit (Zymo Research, California, USA). The samples were eluted into 17 µl at kit's elution buffer.

3.4.MEASURING DNA PURITY AND CONSENTRATION

DNA concentrations of the purified PCR- and digestion products and the purified plasmids were measured with NanoDrop ND-1000 spectrophotometer using NanoDrop 3.1.2 software (Thermo Fisher Scientific, Massachusetts, USA)

3.5.DIGESTION OF PCR PRODUCTS AND PLASMIDS

PCR products were digested with restriction enzymes (New England Biolabs, NEB, Ipswich, USA) listed in the table II. pGL4.10[luc2] plasmid (Promega, Wisconsin, USA) was digested for the NDUFA4L2 amplicon and the pGL4.10[luc2]-TAL plasmid for the rest of the amplicons. Plasmid maps for these two plasmids are shown in the figures 3 and 4. Plasmids were digested with the enzymes shown in the table V.

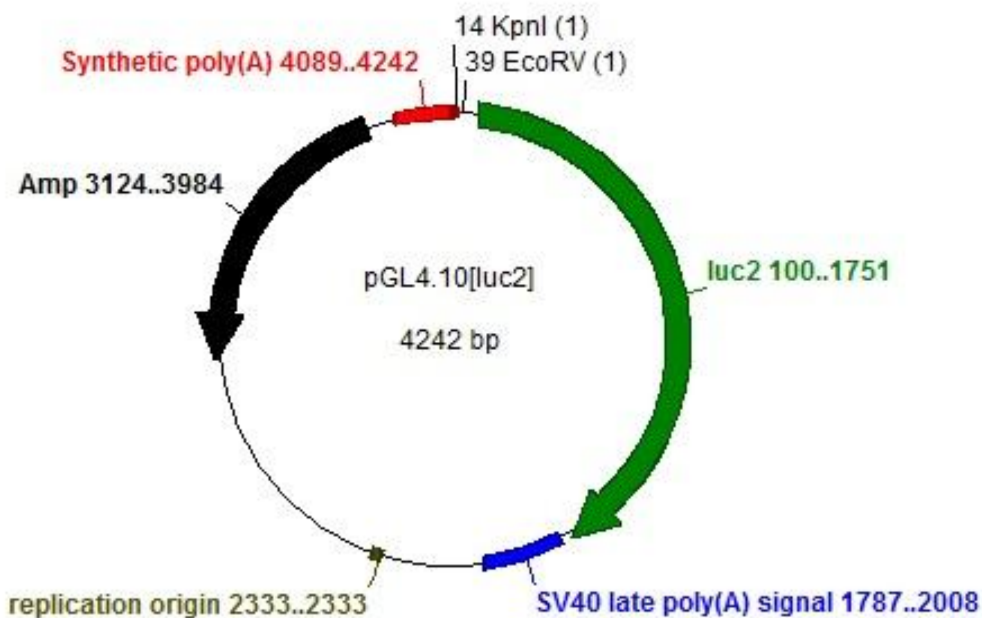

Figure 3. Plasmid map for pGL4.10[luc2] plasmid Restriction sites KpnI and EcoRV for the promoter NDUFA4L2 are marked to the map. **Luc2**: firefly luciferase gene which acts as a marker gene in the study. **SV40 late poly(A) signal:** Simian Virus 40 late polyadenylation signal. **Amp:** synthetic β-lactamase coding region. **Synthetic poly(A):** transcriptional pause site to reduce spurious expression of the reporter gene.

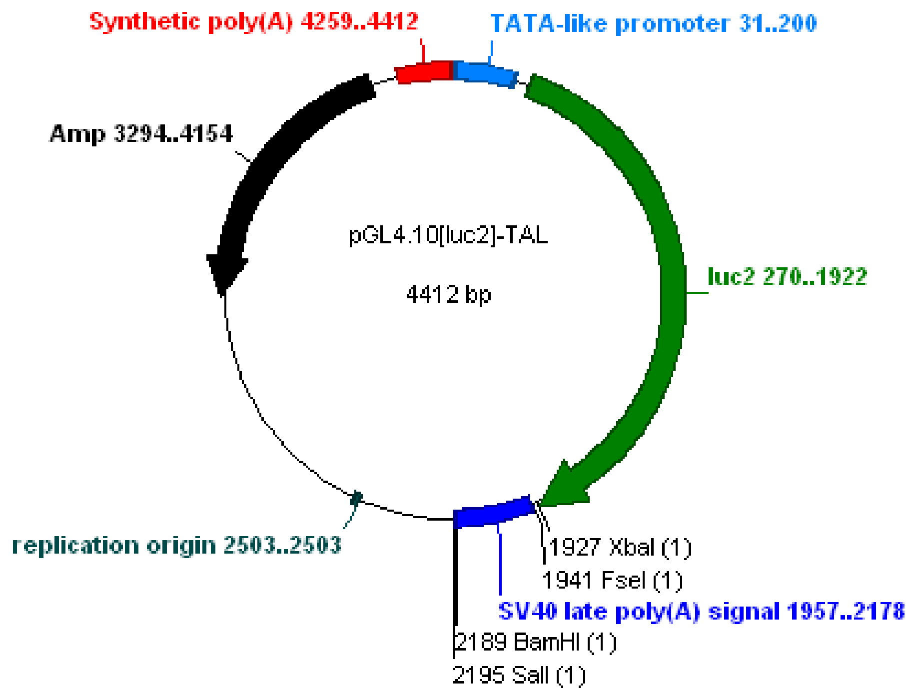

Figure 4. Plasmid map for pGL4.10[luc2]-TAL plasmid. Restriction sites for the enhancers (BamHI and SalI) and 3'UTRs (XbaI and FseI) are marked to the map. **Luc2**: firefly luciferase gene which acts as a marker gene in the study. **SV40 late poly(A) signal:** Simian Virus 40 late polyadenylation signal. **Amp:** synthetic β-lactamase coding region. **Synthetic poly(A):** transcriptional pause site to reduce spurious expression of the reporter gene. **TATA-like promoter:** small promoter region.

Table V. List of restriction enzymes used to digest vectors for ligations.

Plasmid	Restriction enzymes
pGL4.10[luc2]	KpnI, EcoRV
pGL4.10[luc2]-TAL for enhancers	BamHI, SalI
pGL4.10[luc2]-TAL for 3'-UTR1	XbaI, FseI
pGL4.10[luc2]-TAL for U1/GLUT3 and U2/ZNF704	FseI

PCR products were first digested for an hour at +37 ^{o}C and after that both restriction enzymes were added and samples were digested for additional 1,5 hours. After that the digestion products were purified as described on the section 3.3.

Plasmids were digested for 30 minutes at +37 ^{o}C with the enzymes listed in the table IV. Calf-intestinal alkaline phosphatase (CIP) (New England Biolabs, NEB, Ipswich, USA) was then added and samples were incubated for additional 30 minutes. Restriction enzymes were added again and incubation was continued for an hour. Digested plasmids were then purified as described on the section 3.3.

3.6.LIGATIONS AND TRANSFORMATIONS

The digested and purified inserts and plasmids were ligated for 30 min at + 23 °C using T4 buffer, T4 ligase (New England Biolabs, NEB, Ipswich, USA), ~100 ng insert and 100 – 200 ng plasmid in one ligation reaction. Constructs were transformed into *Escherichia coli* (*E.coli*) DH5α -strain using a heat shock method. Transformed bacteria were cultivated in 500 µl Super Optimal Broth with Catabolite repression-medium (SOC) (Life Technologies, California, USA) for an hour at +37 °C 220 RPM. 100 or 200 µl of cultures were then plated on LB 100 µg/ml ampicillin plates. Plates were incubated at +37 °C overnight.

3.7.SMALL SCALE DNA ISOLATION, RESTRICTION ANALYSIS AND SEQUENCING

The right clones were screened by cultivating bacteria in 5 ml Lysogeny Broth-medium (LB-medium) (Life Technologies, California, USA) with ampicillin 100 µg/ml overnight started from colonies on transformation plates. Bacteria stocks were made from the cultures using 200 µl bacteria and 60 µl 100 % glycerol and stored in a -70 °C freezer. Plasmids were then purified using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) as guided in the user's manual. Portions from isolated plasmids were digested for 30 minutes at +37 °C using Fast Digest green buffer and Fast Digest enzymes (Thermo Fisher Scientific, Massachusetts, USA) listed in the table VI. Digested plasmids were analyzed with AGE as described in the section 3.2.

Table VI. Restriction enzymes used in restriction analysis to characterize right clones.

Construct	Restriction enzymes	Size of fragments
pGL4.10[luc]	BamHI	4242 bp
pGL4.10[luc2]-TAL	BamHI	4412 bp
Promoter NDUFA4L2	PvuII	6790 bp, 1553 bp
Enhancer 1	HindIII	3547 bp, 2938 bp
Enhancer 2	SacI	3066 bp, 2293 bp
Enhancer 3	ClaI	4019 bp, 2284 bp
3'UTR1	EcoRI	3515 bp, 3080 bp
3'U1/GLUT3	BamHI	4558 bp
3'U2/ZNF704	NcoI	3834 bp, 2280 bp

Plasmids that were digested correctly were sent to Haartman-institute, University of Helsinki, for the sequencing. Sequencing primers were designed with Primer3web (version 3.0.0) web tool and ordered from Integrated DNA Technologies (IDT, Coralville, Iowa 52241, USA). company. Primers are shown in the table VII.

Table VII. Primers used in sequencing.

Name	Sequence
Promoter NDUFA4L2 seq FW 1	5'-CTAGCAAAATAGGCTGTCCC-3'
Promoter NDUFA4L2 seq FW 2	5'-TCTCAGTGACAGTGCGTAGG-3'
Promoter NDUFA4L2 seq FW 3	5'-AGAGGGACAAAGCCAGGTAG-3'
Promoter NDUFA4L2 seq REV 1	5'-CGATATGTGCGTCGGTAAAG-3'
Promoter NDUFA4L2 seq REV 2	5'-GAGAGACTTGCTTGCTCTGC-3'
Promoter NDUFA4L2 seq REV 3	5'-CGGCGAGAACAAAGAGACAG-3'
Enhancer 1 seq FW 1	5'-TCAGGTTCAGGGGGAGGT-3'
Enhancer 1 seq FW 2	5'-AAAGAAGCCTGAAAGCCTCA-3'
Enhancer 1 seq REV	5'-CATAAGTGCGGCGACGATAG-3'
Enhancer 2 seq FW	5'-TCAGGTTCAGGGGGAGGT-3'
Enhancer 2 seq REV	5'-CATAAGTGCGGCGACGATAG-3'
Enhancer 3 seq FW	5'-TCAGGTTCAGGGGGAGGT-3'
Enhancer 3 seq REV	5'-CATAAGTGCGGCGACGATAG-3'
3'UTR-1 seq FW 1	5'-GACTGACCGGCAAGTTGGA-3'
3'UTR-1 seq FW 2	5'-AGCACCTTCCTCACTTCCAT-3'
3'UTR-1 seq REV 1	5'-AAAACCTCCCACACCTCCC-3'
3'UTR-1 seq REV 2	5'-ACTTCCACCCAGAGCAAAGT-3'
3'U1/GLUT3 seq FW	5'-GACTGACCGGCAAGTTGGA-3'
3'U1/GLUT3 seq REV	5'-AAAACCTCCCACACCTCCC-3'
3'U2/ZNF704 seq FW 1	5'-GACTGACCGGCAAGTTGGA-3'
3'U2/ZNF704 seq FW 2	5'-AGCTCTTCCCCTAAAGGCAG-3'
3'U2/ZNF704 seq REV	5'-AAAACCTCCCACACCTCCC-3'

3.8.LARGE SCALE DNA PRODUCTION

Overnight cultures were started from the frozen stock cultures described in the section 3.7: 5 µl of bacteria stocks were grown in 1ml of on LB 100 µg/ml ampicillin medium at +37 ^{o}C 220 RPM for 7 hours. After 7 hours 200 µl from the cultures were transferred into 200 ml on LB 100 µg/ml ampicillin medium in 2 liter flasks each and incubated at +37 ^{o}C 220 RPM for 16 hours. Plasmids were then purified using Endofree Plasmid Maxi Kit (Qiagen, Hilden, Germany) as guided in the kit's manual. Constructs were dissolved in to 400 µl kit's TE-buffer. Purities and concentrations of the samples were then measured as described in the section 3.4. Dilutions of 500 µg/µl were made into ultra pure water and concentrations were confirmed as above.

3.9.CELL CULTIVATION

Five different cell lines were cultivated in 100 mm dishes: C-166, MS-1, 293T, A549 and HepG2. Cells were ordered from American Type Culture Collection (ATCC, Virginia, USA). More information about the cells can be seen in the table VII. Dulbecco's Modified Eagle Medium (DMEM) with 10 % (v/v) Fetal bovine serum (FBS) and 100 µg/ml

streptomycin/penicillin (S/P) was used as a medium for C-166, 293T and A549 cells. MS-1 medium contained DMEM with 5 % (v/v) FBS and 100 µg/ml S/P. Eagle's Minimal Essential Medium (EMEM) with 10 % (v/v) FBS, 1 % (v/v) Na-pyruvate, 1 % (v/v) non-essential amino acids and 100 µg/ml S/P was used as a growth medium for HepG2. Cells were cultivated in incubator at +37 ^{o}C with 5 % CO_2.

Transfected cells were subjected to hypoxia for 2 or 16 hours after transfection. Cells were incubated in hypoxia cabinet (Ruskinn InVivo$_2$ 400, Ruskinn Gas mixer Q) at +37 ^{o}C with 1 % O_2 and 5 % CO_2. Cells were kept in hypoxic conditions for 22 or 8 hours.

3.10. TRANSFECTIONS

Constructs were transfected to cells to measure luciferase activity in different cell types. The cells were divided to 96-well plates into wells with 100 µl/well of proper growth medium one day before transfections. The cell counts are shown in the table VIII below. The cells were counted using Countess Automated Cell Counter (Invitrogen, Life Technologies, California, USA). Lipofectamine 2000 (Invitrogen, Life Technologies, California, USA) was used to transfect MS-1, A549 and 293T cell lines. ExGEN 500 reagent (Thermo Fisher Scientific, Massachusetts, USA) was used for C-166 and HepG2 cells.

Table VIII. Amount of the cells plated on 96-well plate for transfections.

Cell line	Organism	Tissue	Cell type	Cell count/well
C-166	mouse	yolk sac	endothelial	12000
MS-1	mouse	pancreas	endothelial	12000
A549	human	lung	epithelial	12000
HepG2	human	liver	epithelial	15000
293T	human	kidney	epithelial	6000

Transfection with lipofectamine 2000 was performed as guided in the user manual. Following mixture was used for one well on the 96-well plate: 100 ng of reporter plasmid was diluted into 5 µl OptiMEM with 6,67 ng pGL4.75 plasmid (Promega, Wisconsin, USA). OptiMEM with plasmids was then mixed with 5 µl OptiMEM containing 0,4 µl Lipofectamine 2000 reagent by vortexing. The mix was then incubated for 5 minutes at +23 ^{o}C and was then added on the cells.

Transfection with ExGEN 500 was performed as guided in the user manual. Following mixture was used for one well on the 96-well plate: 300 ng of reporter plasmid and 20 ng of pGL4.75 were diluted into 10 µl 150 mM NaCl and 1 µl of ExGEN 500 reagent was added to the solution. The mixture was then mixed by vortexing and incubated at + 23 ^{o}C for 10 minutes and then added on cells.

Different sizes of the constructs were taken account by adding relatively more bigger constructs than control plasmid pGL4.10. Calculations above are done for pGL4.10 plasmid. Transfections were also made with green fluorescent protein (GFP) (manufacturer unknown) plasmid to visualize transfection efficiency. The protocols were the same as above.

3.11. IMAGING THE CELLS

GFP transfected cells were visualized with Olympus 1x-71/MT_10D fluorescence microscope (Olympus, Pennsylvania, USA) using 10X object and fluorescein isothiocyanate (FITC) filter with 100 % excitation. Shutter speed varied from 250 ms to 1 s when capturing GFP emission.

3.12. LUCIFERASE ASSAY AND ANALYSIS

Luciferase activity was measured 24 hours after transfections using Dual-Glo Luciferase Assay System (Promega, Wisconsin, USA) and POLARstar Optima (BMG Labtech, Ortenberg, Germany, software version 2.20R2) fluorimeter with sim. dual emission luminescence optics. Assay was performed as guided in the user manual: 75 µl of Dual-Glo reagent was added to one well and incubated for 10 minutes at + 23 ^{o}C before measuring the activity of the firefly luciferase. After the measurement 75 µl Stop&Glo buffer with the substrate was added to the well and incubated for 10 minutes and the activity of the renilla luciferase was measured. Luminescences were read three times per well in both measurements.

The firefly luciferase signals from the constructs were compared to the renilla luciferase signals from the pGL4.75 plasmids. Background signals (untransfected cells) were

subtracted from the actual signals in both measurements. Firefly signals were then divided by renilla signals and average quotient of pGL4.10 plasmid was counted. Quotients of all plasmids were then divided by the average quotient of pGL4.10 plasmid to gain the fold changes. In the experiments 6 to 9 replicants/construct/cell line/treatment were used.

3.13. STATISTICS

Statistical analysis for luciferase assay results were made with GraphPad Prism 5 using one-way ANOVA (and non-parametric) parameters, one-way analysis of variance test and Dunnet post test comparing all constructs (except NDUFA4L2 and pGL4.10) to pGL4.10-TAL plasmid. NDUFA4L2 was compared to pGL4.10 plasmid using t Test (and non-parametric) parameters and unpaired test with two-tailed P values. The significances were calculated for each treatment separately.

4. Results

4.1.CREATING THE CONSTRUCTS

Single PCR products were obtained with all cloning primers except with the 3'UTR-1 primers. PCR with the 3'UTR-1 primers gave 4 products where 2 of them were amplified strongly including the product with right size, which was cloned in the plasmid as the others. Right clones from the small scale DNA isolations were confirmed with a restriction analysis. The restriction enzymes cut the constructs as predicted. Agarose gel from the restriction analysis is shown in the figure 5. below.

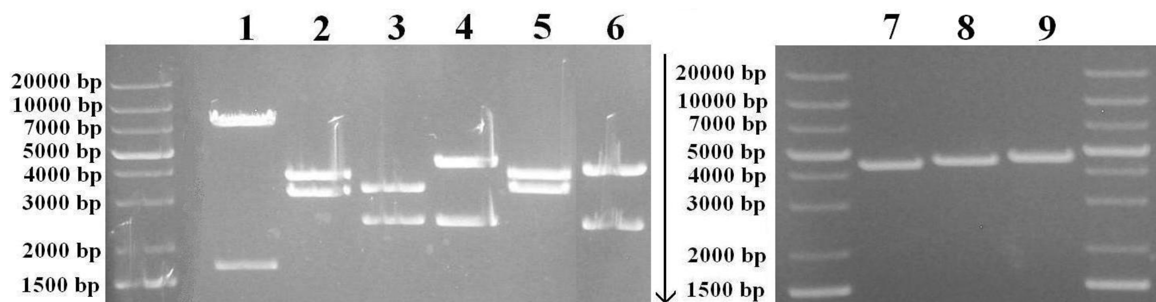

Figure 5. Restriction analysis of the constructs. The samples used restriction enzymes and correct bands are followings: **1.** Promoter NDUFA4L2 (PvuII, 6790 and 1553 bp) **2.** Enhancer 1 (HindIII, 3547 and 2938 bp) **3.** Enhancer 2 (SacI, 3066 and 2293 bp) **4.** Enhancer 3 (ClaI, 4019 and 2284 bp) **5.** 3'UTR-1 (EcoRI, 3515 and 3080 bp) **6.** U2/ZNF704 (NcoI, 3834 and 2280 bp), **7.** pGL4.10 (BamHI, 4242 bp), **8.** pGL4.10-TAL (BamHI, 4412 bp), **9.** U1/GLUT3 (BamHI, 4558 bp). GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) was used as a molecule marker and bands were visualized using SYBR Safe DNA gel Stain (Invitrogen, Life Technologies, California, USA).

The sequencing analysis confirmed the right clones and revealed single nucleotide polymorphisms (SNP) in some inserts. Enhancer 1 had two SNPs in the insert where one G and one C nucleotide were transformed into T nucleotides. Both SNPs were found in the database (UCSC genome bioinformatics, California, USA). According to ChIP- and GRO-seq data the SNPs were not on the TF binding site. Enhancer 2 had three SNPs (two G→A, one A→C) and one additional TT residue in the insert. Two of the SNPs were found in the database and they were on the TF binding site. Enhancer 3 had two SNPs (A→C and C→G) where one was partly on the TF binding site. In 3'UTR1 following SNPs were found in the database: deletion A → - and T → C. The next SNPs were unidentified: T → - and C → A. 3'U1/GLUT3, 3'U2/ZNF704 or NDUFA4L2 did not have any SNPs or deletions.

After large scale DNA isolation dilutions were made to be used in the experiments. Concentrations of the constructs used in the experiment are shown in the table IX.

Table IX. Concentrations of the construct dilutions.

Construct	C µg/µl	A260	A260/A230	A260/A280
Promoter NDUFA4L2	502,77	10,056	2,16	1,89
Enhancer 1	498,27	9,966	2,25	1,90
Enhancer 2	502,18	10,044	2,24	1,90
Enhancer 3	500,08	10,002	2,25	1,90
3’UTR1	496,05	9,921	2,28	1,89
3’U1/GLUT3	501,85	10,037	2,22	1,90
3’U2/ZNF704	498,06	9,962	2,27	1,90

4.2.TRANSFECTION EFFICIENCY

Transfection efficiency was confirmed by transfecting the cells with GFP plasmid. Tranfections with lipofectamine 2000 reagent achieved approximately 40 – 90 % and with ExGEN 500 reagent 10 – 40 % efficiencies Images of GFP transfected cells are shown in the figures 6 and 7.

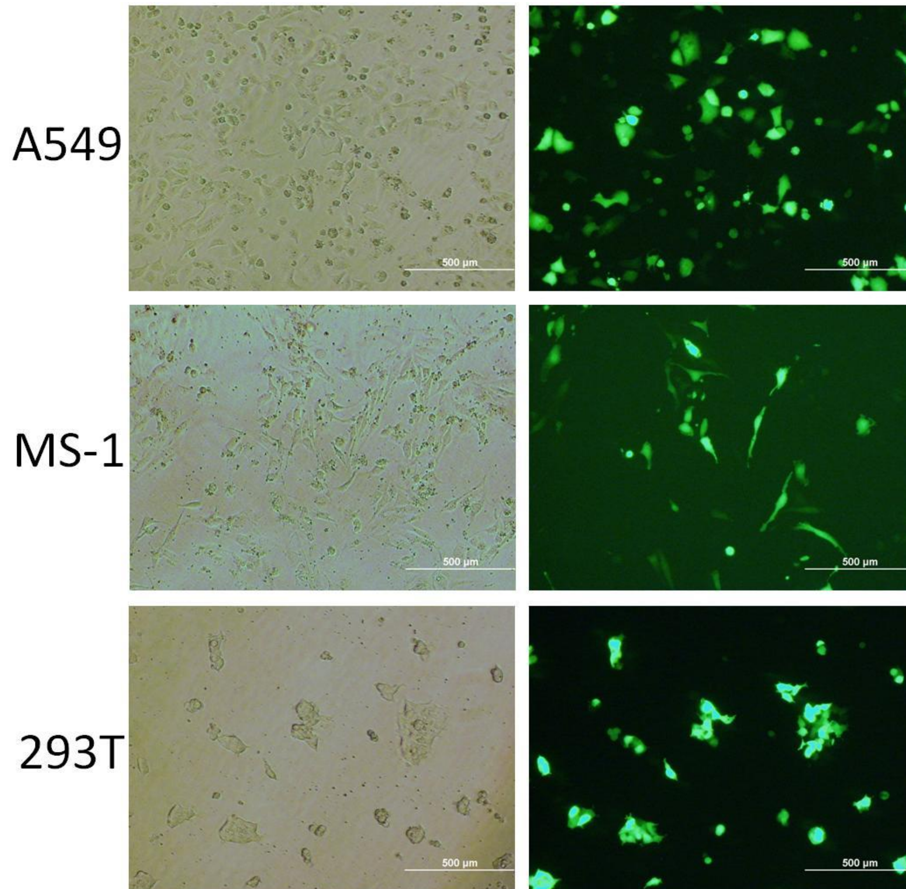

Figure 6. Cells transfected with GFP using Lipofectamine 2000 reagent. Shutter times in GFP images from top to bottom: 400 ms, 1 s and 250 ms. The code of the cell line is shown on the left. The left image is normal and the right image is GFP fluorescence.

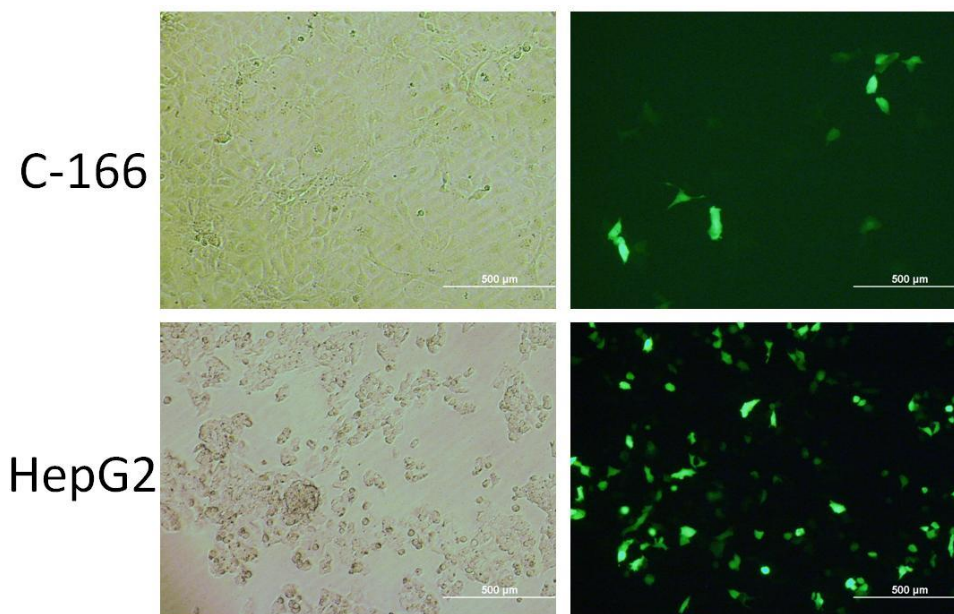

Figure 7. Cells transfected with GFP using ExGEN 500 reagent. Shutter times in GFP images from top to bottom: 1 s and 400 ms. The code of the cell line is shown on the left. The left image is normal and the right image is GFP fluorescence.

4.3.LUCIFERASE ASSAY

Luciferase activities of the constructs were compared to the control plasmid pGL4.10 to get the fold changes. The luciferase activities of different constructs in different conditions and cell lines are represented in the figures 8, 9, 10, 11 and 12. Statistically significant enhancing effects are marked with "*" in the figures.

In C-166 cells the only statistically significant enhancing property was observed with the 3'U1/GLUT3 construct when it gave in normoxia treatment ~1,6 times higher response than the control plasmid pGL4.10-TAL. 3'U1/GLUT3 had also the highest activity with 22 hours hypoxia treatment but the difference to pGL4.10-TAL activity was not significant. Other notable finding was that the NDUFA4L2 construct had a weak expression response as well as the 3'UTR1. Complete C-166 luciferase activity graph is shown in the figure 8.

In MS-1 cell line the highest activities were observed with the Enhancer 1 construct even though differences to the control plasmid pGL4.10-TAL were not significant. The NDUFA4L2 and the 3'UTR1 constructs had the same weak expression response as in C-166 cells. Complete MS-1 luciferase activity graph is shown in the figure 9.

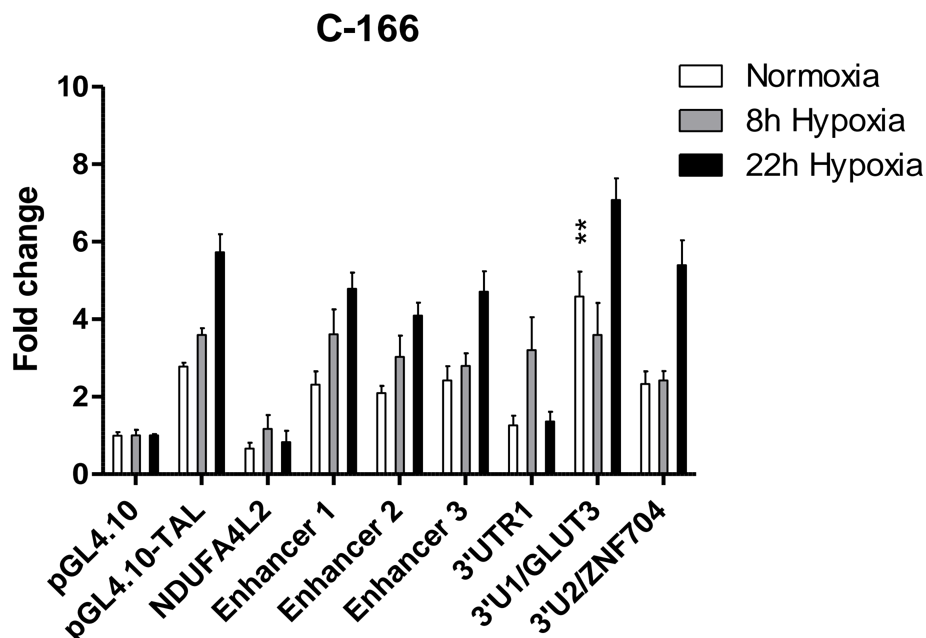

Figure 8. Luciferase activities of the constructs in the C-166 cell line. All constructs are compared to the pGL4.10 plasmid. Calculating significances all constructs (except NDUFA4L2) were compared to pGL4.10-TAL plasmid using one-way ANOVA (and non-parametric) parameters, one-way analysis of variance test and Dunnet post test. ** = 0,001 < P < 0,01

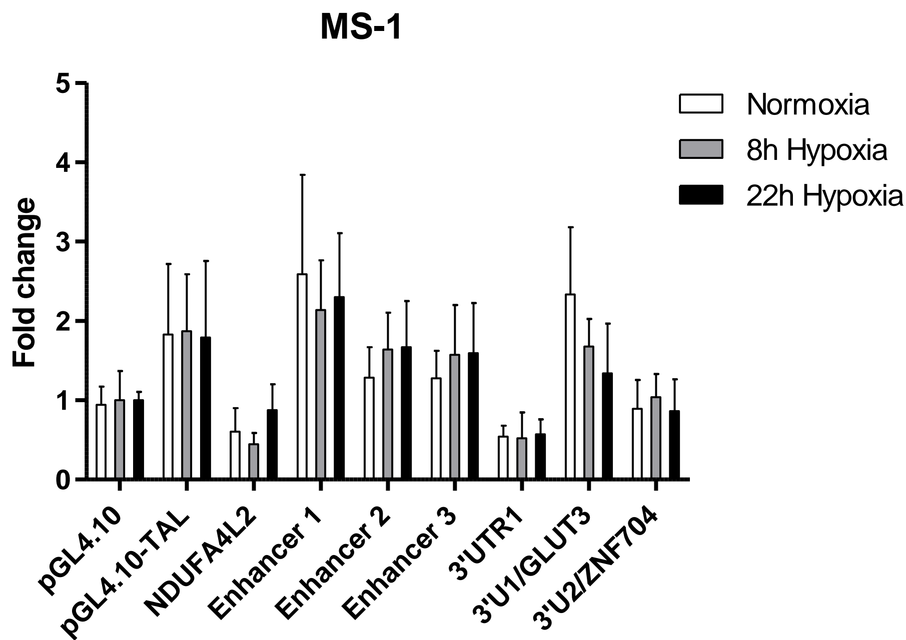

Figure 9. Luciferase activities of the constructs in the MS-1 cell line. All constructs are compared to the pGL4.10 plasmid. No statistically significant enhancing properties were found.

In the A549 cells the Enhancer 1 construct gave ~1,5 times higher expression than the control plasmid pGL4.10-TAL in all treatments. The differences were also statistically significant. NDUFA4L2 and UTR1 constructs responded as in the C-166 and MS-1 cells. Complete A549 luciferase activity graph is shown in the figure 10 below.

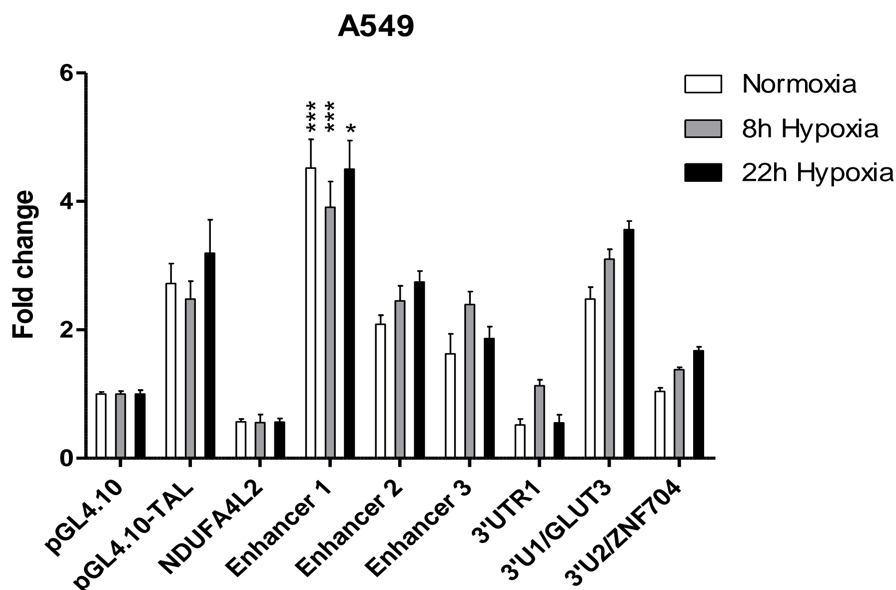

Figure 10. Luciferase activities of the constructs in the A549 cell line. All constructs are compared to the pGL4.10 plasmid. Calculating significances all constructs (except NDUFA4L2) were compared to pGL4.10-TAL plasmid using one-way ANOVA (and non-parametric) parameters, one-way analysis of variance test and Dunnet post test. *** = 0,001 < P, * = 0,01 < P < 0,05

In the HepG2 cells only statistically significant enhancement was observed with the 3’U1/GLUT3 construct in 8 hours hypoxia treatment. The expression level was ~1,7 times higher than with the control plasmid pGL4.10-TAL. Other constructs gave lower responses in every treatment than the control plasmid, including NDUFA4L2 construct. Complete HepG2 luciferase activity graph is shown in the figure 11.

The highest luciferase responses and fold changes were observed in 293T cells although the expression patterns were similar compared to the other cell lines. Any significant enhancing results were not achieved even though 3’U1/GLUT3 and 3’U2/ZNF704 constructs gave slightly higher responses than the control plasmid in 8 hours hypoxia treatment. Complete 293T luciferase activity graph is shown in the figure 12.

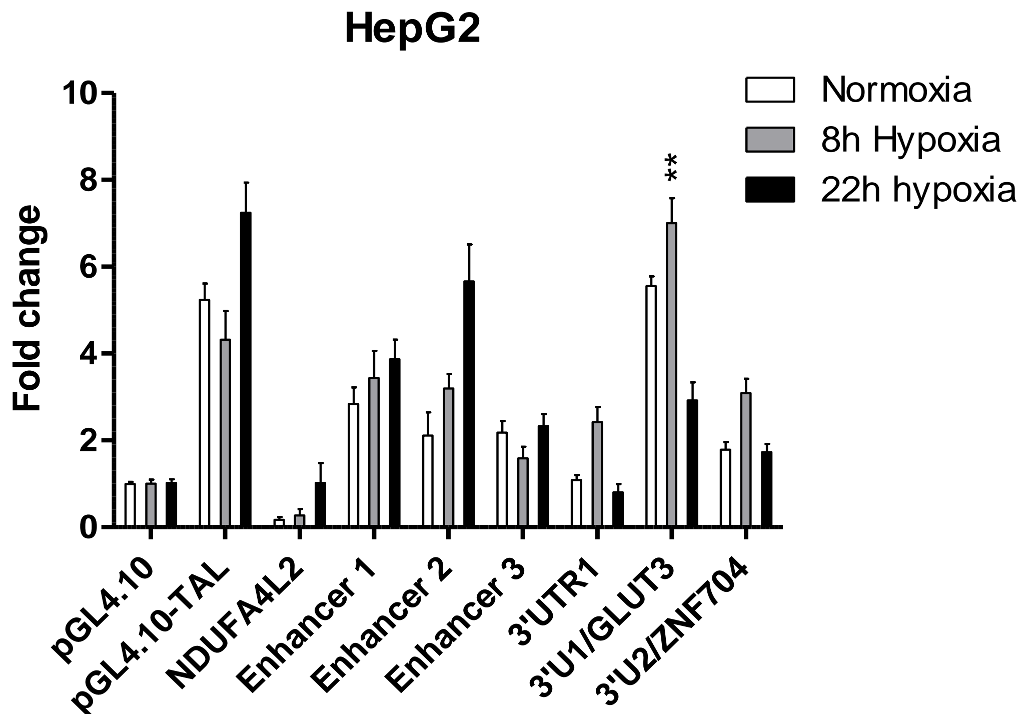

Figure 11. Luciferase activities of the constructs in the HepG2 cell line. All constructs are compared to the pGL4.10 plasmid. Calculating significances constructs (except NDUFA4L2) were compared to pGL4.10-TAL plasmid using one-way ANOVA (and non-parametric) parameters, one-way analysis of variance test and Dunnet post test. ** = $0{,}001 < P < 0{,}01$

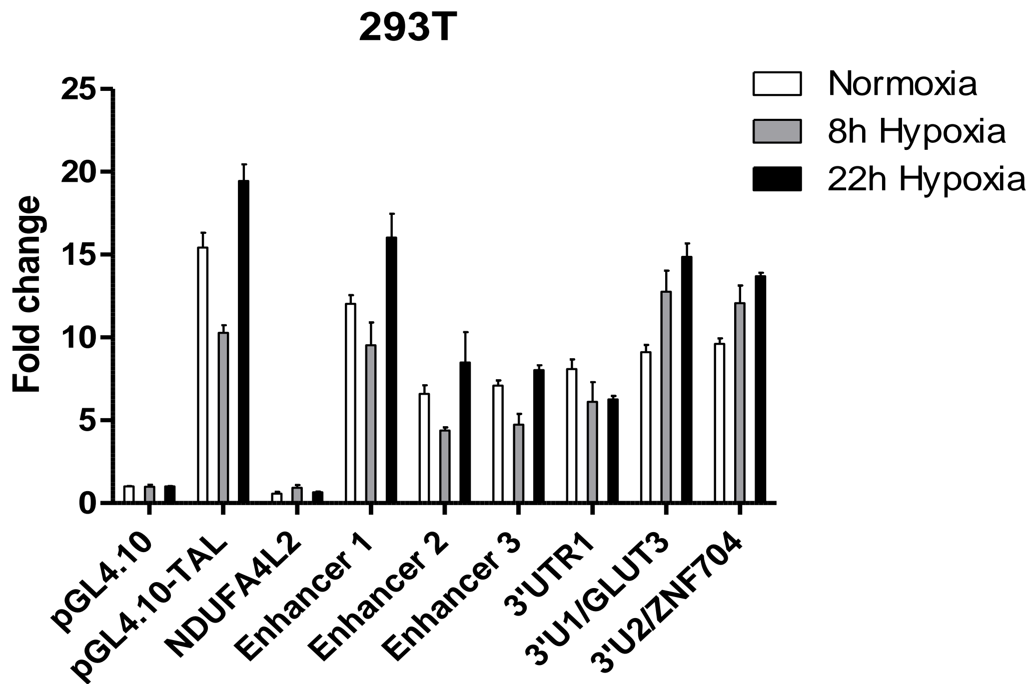

Figure 12. Luciferase activities of the constructs in the 293T cell line. All constructs are compared to the pGL4.10 plasmid.

5. Discussion

The transcription regulatory system is under heavy research and occasionally something new and interesting is found. Research has concentrated to find the factors explaining differences between cell types. It has been found that cell-specific gene expression is a complex biological system involving multiple different factors. Most of the transcription factors bind to promoter and enhancer regions where enhancers have majority of the TF binding sites. This emphasis the great role of enhancers in the cell specific gene expression as *cis*-regulatory regions are different across the cell types (Heintzman et al., 2009). These findings have been used to develop therapeutic vectors to be more controllable and cell specific. Human promoters have been cloned into therapeutic vectors and used successfully to promote gene expression in the target cells. In one study promoters of the human genes CD11b and CD18 were successfully used to promote expression of canine CD18 (Hunter et al., 2011). Enhancers have also been used in vectors raising expression levels greatly. One of the most known high level enhancers is the enhancer of Simian Virus 40 (SV40) which have been shown to enhance transcription levels up to 400-fold (Zenke et al., 1986). UTRs and their potentiality in gene expression regulation via miRNAs is known but number of attempts to use them in gene therapy vectors is unknown.

Aim of this study was to produce endothelial specific vectors that would be activated under hypoxic conditions. Previously collected ChIP- and GRO-seq data were used to choose one promoter and three enhancer regions due to their hypoxia respond properties especially in the endothelial cell line (HUVEC). Three 3'UTR regions were selected for their specific miRNA binding sites. The regions were cloned into a plasmid containing firefly luciferase gene as a marker gene. Activities of the constructs were observed by measuring the luciferase activity. The constructs and inserted regions are shown in the table X below.

Table X. Constructs and their properties

Construct	Inserted region
Promoter NDUFA4L2	Promoter of gene NDUFA4L2
Enhancer 1	Enhancer region near gene SP4
Enhancer 2	Enhancer region near gene SP4
Enhancer 3	Enhancer regions near gene MIR1246
3'UTR1	3'UTR of gene GLUT3
3'U1/GLUT3	Shortened 3'UTR of gene GLUT3
3'U2/ZNF704	Part of 3'UTR of gene ZNF704

There were three main questions: 1. Are the constructs endothelial specific? 2. Are the constructs enhancing the gene expression? 3. Do the constructs respond to hypoxia? The construct preparation was successful and primers etc. worked well. Concentrations and purities of the constructs after the large scale purification were great and the dilutions were pure to use with the cells. Transfections with the Lipofectamine 2000 and the ExGEN 500 gave the best results. Transfection efficiencies varied between cell types. The weakest efficiencies were achieved with the endothelial cell lines C-166 and MS-1 and the best with the 293T cells. The 293T cell line is known for its high transfectability and it is widely used in experiments (http://www.lgcstandards-atcc.org, 26.8.2013). Still the efficiencies were good enough for successful transfections and luciferase signals were gained from every cell line. One misfortune in the study was that HUVEC cells could not be used in the luciferase assay. Transfections did not succeed even though several different reagents were tested. It would have been relevant to study how the constructs would have worked in the HUVEC cells when the cloned regions were selected based on the data gained from the HUVEC cells.

5.1.ENDOTHELIAL SPECIFICITY

Cell type specificity and especially endothelial specificity was one of the features wanted from the constructs. Concentrating first to the endothelial cell lines C-166 (yolk sac, endothelial) and MS-1 (pancreas, endothelial), all constructs gave luciferase signal in every treatment. Expression patterns of the constructs were exceedingly similar within the cell types compared to the control plasmid pGL4.10-TAL with the slight differences in hypoxia response. Plasmids were expressed more constantly in the MS-1 cells where changes were lower compared to the C-166 cells. Comparing results from the endothelial cell lines to the other cell lines, a notable fact is that the constructs were working at least as well in the other cell lines as in the endothelial cells. The best overall response was seen in the 293T cells (kidney, epithelial) when the expression pattern remained the same compared for example to the patterns in MS-1 cells. The lowest changes were observed in MS-1 cells and statistically best results were observed in A549 (lung, epithelial) cell line with the Enhancer 1 construct. The HUVEC cells would have provided more data about the cell specificity when cloned regions were selected from the HUVEC GRO- and ChIP-seq data. Sadly proper transfection reagent for HUVEC cells was not found.

Endothelial specificity was not achieved with these constructs even though data from ChIP- and GRO-seq supports the idea of endothelial specificity of the promoter, UTRs and enhancers chosen. It is always a big question how it will work out when certain DNA regions are taken out from the natural environment and inserted into plasmid. There are studies suggesting that the key factors in cell-specific enhancer regions are histone modifications. Trimethylation of the H3K9 near the enhancer region has been shown to determine the cell-type-specific enhancement in many cell lines. In cells where gene expression is active H3K9 is not methylated when trimethylated H3K9 is repressing the gene expression in other cell types (Zhu et al., 2012). Demethylase JMJD2D has a key role in the cell-specific gene expression of the genes Mdc and IIb2 in dendritic cells and macrophages when the enzyme is responsible of demethylation of H3K9. JMJD2D is required for cell specific gene expression in this case (Zhu et al., 2012). Plasmids can bind histones to some extent if they have correct histone-binding sequences (Reeves et al., 1985). Histones can limit TFs binding to their binding sites and suppress gene expression (Kamiya et al., 2010). Present of histones in the constructs was not studied in this study,

but results indicate absence of histones as luciferase was expressed in all cell lines. Cell specific gene expression is a complex biological system where multiple different factors are involved and it is hard to achieve with only one specific DNA region.

5.2.ENHANCING ABILITY

The second feature demanded from the constructs was the enhancing ability. Statistically significant higher enhancing properties than with the control plasmid were found only in two constructs: Enhancer 1 in the A549 cells and 3'U1/GLUT3 in the HepG2 and the C-166 cells. The same constructs gave nice responses also in other cell lines but the results were not statistically significant. Most of the constructs followed the expression pattern of pGL4.10-TAL plasmid which is evident when the amplicons were inserted to pGL4.10-TAL plasmid except the NDUFA4L2. Still slight differences in expression patterns were noticed especially in 3'UTR constructs. Enhancing ability of the Enhancer 1 in A549 cells was clear but it did not work as well in other cell types. Results in MS-1 cells were quite the same, but the results were not statistically significant. Some enhancers have great impact to gene expression. In one study enhancer of cytomegalovirus (CMV) raised transcript production 8-90 times compared to transcription without the CMV enhancer (Liu et al., 2008). Enhancers in this experiment did not have great enhancing properties even some effects to gene expressions were observed. Cell specific enhancing system might be so sensitive that only slight rise in expression can do the difference between the cell types.

NDUFA4L2 construct had low activity in all treatments, even lower than the activity of the control plasmid pGL4.10 in most cases. NDUFA4L2 construct contained the entire promoter region of NDUFA4L2 gene but it did not promote gene expression in the pGL4.10 plasmid. There might be multiple reasons why the promotion failed. Size of the construct affects to transfection efficiency and it is possible that all NDUFA4L2 constructs did not get in the cells. It is the biggest construct with 8343 bp when others are around 4000 – 6000 bp. Structurally plasmids are different than normal chromosomes because the plasmid DNA is circular and the presence of the histones is different as described before. These factors may have affected to the function of the NDUFA4L2 promoter in the construct. The results indicate that the promoter alone might not promote the gene expression. The enhancer regions could be essential for the gene expression and without

proper enhancers the transcription will not start or it is insufficient (Xiaorong et al., 2010; Zhang et al., 2004). Enhancing ability of the 3'UTR constructs were poor except the 3'U1/GLUT3 construct in HepG2 cell line with 8 hour hypoxia treatment. 3'UTR regions have been noticed to enhance gene expression in certain cases up to 3-fold due to increased transcript level (Zeyenko et al., 1994; Li et al, 2012).

5.3.HYPOXIA RESPONSES

Hypoxia activation was the third desirable feature from the constructs. To ensure this ability the primers were designed so that the enhancer and the promoter constructs would include as many HREs (motif NCGTG) as possible. Good hypoxia responses were achieved with the Enhancer 1 construct in both hypoxia treatments in the A549 cells compared to the control. Also the 3'U1/GLUT3 construct gave great response in HepG2 cells in 8 hour hypoxia treatment. Comparing the Enhancer 1 expression pattern in A549 cells to the pGL4.10-TAL pattern similarity is obvious. Enhancer 1 did not respond better to hypoxia than the control plasmid but the expression level was higher due to enhancing property of the insert. 3'U1/GLUT3 instead had a different expression pattern than the control plasmid pGL4.10-TAL in HepG2 cells. The 3'U1/GLUT3 construct's expression level was higher in 8 hour hypoxia treatment than in normoxia when expression level was decreased with the control plasmid from normoxia to 8 hour hypoxia. This indicates that the 3'U1/GLUT3 construct responded better to short hypoxia than the control. In longer hypoxia expression level had lowered dramatically below the control values.

Over all, longer hypoxia activated all constructs more than the shorter hypoxia treatment, including the control plasmid pGL4.10-TAL. Hypoxia activates many genes as mentioned in the introduction and the same activation pathways may have activated the constructs in this study. Additional inserts had only small effect to hypoxia response excluding 3'U1/GLUT3 in the HepG2 cells. The 3'U1/GLUT3 construct gave good hypoxia responses also in the A549 and the 293T cells in 8 hour hypoxia treatments but the differences were not statistically significant. Otherwise the hypoxia responses of the constructs were close to the control responses or lower.

These findings demonstrate that hypoxia response is not certain even if HREs are present. In this study the actual binding of the HIF-1α to the HRE elements were not studied so the functionality of the HRE elements cannot be commented. It has been studied that every HRE sequences do not bind HIFs even though it is 20 times more likely to have functional HRE sequences near DNase sensitive regions (Schödel et al., 2011). The cloned enhancers regions in this study were DNase sensitive according to genome browser so HRE elements in enhancers should be functional. Possible effects of SNPs should be counted out when none of the SNPs were on the HRE regions and most of the SNPs were found in the UCSC database. Three dimensional structure of the plasmid/polymerase/TF complex was not studied so possible steric hindrances which may affect to the gene expression are not known. Studies have shown that HREs might need other regions to give proper hypoxia response. Stress-response TFs are one example of the factors affecting HIF transcriptional response (Villar et al., 2012).

5.4.CONCLUSION AND FUTURE WORK

Cloning regions from knowledge based on ChIP- and GRO-seq data and trying to get them function in vectors is “gambling” like one wise man said. One might work as predicted or none of twelve may work. In this study the situation was somewhere between. One enhancer and one 3’UTR region gave promising results even the differences are minor, but still significant. These were interesting results considering future work. Combination of two or even three different regions in the same vector would be interesting to study. Based on results from this study the Enhancer 1 and the 3’U1/GLUT3 regions would be exciting to clone into same vector. Real effects can only be speculated but from the results some predictions can be done: hypoxia response could be better and some enhancement could be observed. HUVEC cells should be taken to experiments after finding a proper transfection reagent.

Constantly developing research field of the cell-specific gene expression will provide answers to many open questions and tools to make better gene therapy vectors. People are different and so should be the treatments given to them. After genome sequencing becomes cheap enough it is most likely that every person will be sequenced so that personal treatments can be easily planned. This could include personal gene therapy including

optimized cell targeting and gene expression. Science is taking small steps towards future, but tomorrow the future is here.

6. References

Alberts, B, Johnson, A, Lewis, J, Raff, M, Roberts, K, Walter, P. 2008. Molecular biology of the cell 5th edition, Garland Science, New York. 341 pp.

Ameres, S.L., J. Martinez, and R. Schroeder. 2007. Molecular basis for target RNA recognition and cleavage by human RISC. *Cell.* 130:101-112.

Blackwood, E.M., and J.T. Kadonaga. 1998. Going the distance: A current view of enhancer action. *Science.* 281:60-63.

Cai, X., C.H. Hagedorn, and B.R. Cullen. 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *Rna.* 10:1957-1966.

Clapier, C.R., and B.R. Cairns. 2009. The biology of chromatin remodeling complexes. *Annual Review of Biochemistry.* 78:273-304.

Cole, C.N., and J.J. Scarcelli. 2006. Transport of messenger RNA from the nucleus to the cytoplasm. *Curr.Opin.Cell Biol.* 18:299-306.

Collins, F.S., E.S. Lander, J. Rogers, and R.H. Waterson. 2004. Finishing the euchromatic sequence of the human genome. *Nature.* 431:931-945.

Core, L.J., J.J. Waterfall, and J.T. Lis. 2008. Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. *Science.* 322:1845-1848.

de Laat, W., P. Klous, J. Kooren, D. Noordermeer, R. Palstra, M. Simonis, E. Splinter, and F. Grosveld. 2008. Three-dimensional organization of gene expression in erythroid cells. *Current Topics in Developmental Biology.* 82:117-139.

de Santa, F., I. Barozzi, F. Mietton, S. Ghisletti, S. Polletti, B.K. Tusi, H. Muller, J. Ragoussis, C.-. Wei, and G. Natoli. 2010. A large fraction of extragenic RNA Pol II transcription sites overlap enhancers. *PLoS Biology.* 8.

Evans, P.C., and B.R. Kwak. 2013. Biomechanical factors in cardiovascular disease. *Cardiovasc.Res.* 99:229-231.

Fei, X., M. Qi, B. Wu, Y. Song, Y. Wang, and T. Li. 2012. MicroRNA-195-5p suppresses glucose uptake and proliferation of human bladder cancer T24 cells by regulating GLUT3 expression. *FEBS Lett.* 586:392-397.

Fischer, A., S. Hacein-Bey, and M. Cavazzana-Calvo. 2002. Gene therapy of severe combined immunodeficiencies. *Nature Reviews Immunology.* 2:615-621.

Forsythe, J.A., B.-. Jiang, N.V. Iyer, F. Agani, S.W. Leung, R.D. Koos, and G.L. Semenza. 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol.Cell.Biol.* 16:4604-4613.

Gerber, H.-., F. Condorelli, J. Park, and N. Ferrara. 1997. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J.Biol.Chem.* 272:23659-23667.

Geyer, P.K., M.M. Green, and V.G. Corces. 1990. Tissue specific transcriptional enhancers may act in trans on the gene located in the homologous chromosome: the molecular basis of transvection in Drosophila. *Embo j.* 9:2247-2256.

Grupp, S.A., M. Kalos, D. Barrett, R. Aplenc, D.L. Porter, S.R. Rheingold, D.T. Teachey, A. Chew, B. Hauck, J.F. Wright, M.C. Milone, B.L. Levine, and C.H. June. 2013. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N.Engl.J.Med.* 368:1509-1518.

Heintzman, N.D., G.C. Hon, R.D. Hawkins, P. Kheradpour, A. Stark, L.F. Harp, Z. Ye, L.K. Lee, R.K. Stuart, C.W. Ching, K.A. Ching, J.E. Antosiewicz-Bourget, H. Liu, X. Zhang, R.D. Green, V.V. Lobanenkov, R. Stewart, J.A. Thomson, G.E. Crawford, M. Kellis, and B. Ren. 2009. Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature.* 459:108-112.

Heintzman, N.D., R.K. Stuart, G. Hon, Y. Fu, C.W. Ching, R.D. Hawkins, L.O. Barrera, S. Van Calcar, C. Qu, K.A. Ching, W. Wang, Z. Weng, R.D. Green, G.E. Crawford, and B. Ren. 2007. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat.Genet.* 39:311-318.

Huang, L.E., J. Gu, M. Schau, and H.F. Bunn. 1998. Regulation of hypoxia-inducible factor 1a is mediated by an O2- dependent degradation domain via the ubiquitin-proteasome pathway. *Proc.Natl.Acad.Sci.U.S.A.* 95:7987-7992.

Hunter, M.J., L.M. Tuschong, C.J. Fowler, T.R. Bauer Jr., T.H. Burkholder, and D.D. Hickstein. 2011. Gene therapy of canine leukocyte adhesion deficiency using lentiviral vectors with human CD11b and CD18 promoters driving canine CD18 expression. *Molecular Therapy.* 19:113-121.

Johnson, D.S., A. Mortazavi, R.M. Myers, and B. Wold. 2007. Genome-wide mapping of in vivo protein-DNA interactions. *Science.* 316:1497-1502.

Kallio, P.J., I. Pongratz, K. Gradin, J. McGuire, and L. Poellinger. 1997. Activation of hypoxia-inducible factor 1a: Posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc.Natl.Acad.Sci.U.S.A.* 94:5667-5672.

Kamiya, H., H. Goto, G. Kanda, Y. Yamada, and H. Harashima. 2010. Transgene expression efficiency from plasmid DNA delivered as a complex with histone H3. *Int.J.Pharm.* 392:249-253.

Kim, T.-., M. Hemberg, J.M. Gray, A.M. Costa, D.M. Bear, J. Wu, D.A. Harmin, M. Laptewicz, K. Barbara-Haley, S. Kuersten, E. Markenscoff-Papadimitriou, D. Kuhl, H. Bito, P.F. Worley, G. Kreiman, and M.E. Greenberg. 2010. Widespread transcription at neuronal activity-regulated enhancers. *Nature.* 465:182-187.

Kouzarides, T. 2007. Chromatin modifications and their function. *Cell.* 128:693-705.

LeWitt, P.A., A.R. Rezai, M.A. Leehey, S.G. Ojemann, A.W. Flaherty, E.N. Eskandar, S.K. Kostyk, K. Thomas, A. Sarkar, M.S. Siddiqui, S.B. Tatter, J.M. Schwalb, K.L. Poston, J.M. Henderson, R.M. Kurlan, I.H. Richard, L. Van Meter, C.V. Sapan, M.J. During, M.G. Kaplitt, and A. Feigin. 2011. AAV2-GAD gene therapy for advanced Parkinson's disease: A double-blind, sham-surgery controlled, randomised trial. *The Lancet Neurology.* 10:309-319.

Li, W.J., L.L. Dai, Z.J. Chai, Z.J. Yin, and L.Q. Qu. 2012. Evaluation of seed storage protein gene 3'-untranslated regions in enhancing gene expression in transgenic rice seed. *Transgenic Res.* 21:545-553.

Liang, P., B. Jiang, C. Lv, X. Huang, L. Sun, P. Zhang, and X. Huang. 2013. The expression and proangiogenic effect of nucleolin during the recovery of heat-denatured HUVECs. *Biochimica Et Biophysica Acta - General Subjects.* 1830:4500-4512.

Masson, N., C. Willam, P.H. Maxwell, C.W. Pugh, and P.J. Ratcliffe. 2001. Independent function of two destruction domains in hypoxia-inducible factor-a chains activated by prolyl hydroxylation. *Embo j.* 20:5197-5206.

Melo, C.A., J. Drost, P.J. Wijchers, H. van de Werken, E. de Wit, J.A.F.O. Vrielink, R. Elkon, S.A. Melo, N. Léveillé, R. Kalluri, W. de Laat, and R. Agami. 2013. ERNAs are required for p53-dependent enhancer activity and gene transcription. *Mol.Cell.* 49:524-535.

Natarajan, A., G.G. Yardimci, N.C. Sheffield, G.E. Crawford, and U. Ohler. 2012. Predicting cell-type-specific gene expression from regions of open chromatin. *Genome Res.* 22:1711-1722.

Reeves, R., C.M. Gorman, and B. Howard. 1985. Minichromosome assembly of non-integrated plasmid DNA transfected into mammalian cells. *Nucleic Acids Res.* 13:3599-3615.

Richard, D.E., E. Berra, E. Gothié, D. Roux, and J. Pouysségur. 1999. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia- reducible factor (HIF-1a) and enhance the transcriptional activity of HIF-1. *J.Biol.Chem.* 274:32631-32637.

Ritter, D.I., Z. Dong, S. Guo, and J.H. Chuang. 2012. Transcriptional enhancers in protein-coding exons of vertebrate developmental genes. *Plos One.* 7.

Schödel, J., S. Oikonomopoulos, J. Ragoussis, C.W. Pugh, P.J. Ratcliffe, and D.R. Mole. 2011. High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. *Blood.* 117:e207-e217.

Shu, W., H. Chen, X. Bo, and S. Wang. 2011. Genome-wide analysis of the relationships between DNaseI HS, histone modifications and gene expression reveals distinct modes of chromatin domains. *Nucleic Acids Res.* 39:7428-7443.

Tello, D., E. Balsa, B. Acosta-Iborra, E. Fuertes-Yebra, A. Elorza, A. Ordóñez, M. Corral-Escariz, I. Soro, E. López-Bernardo, E. Perales-Clemente, A. Martínez-Ruiz, J.A. Enríquez, J. Aragonés, S. Cadenas, and M.O. Landázuri. 2011. Induction of the mitochondrial NDUFA4L2 protein by HIF-1a decreases oxygen consumption by inhibiting complex i activity. *Cell Metabolism.* 14:768-779.

Villar, D., A. Ortiz-Barahona, L. Gómez-Maldonado, N. Pescador, F. Sánchez-Cabo, H. Hackl, B.A.T. Rodriguez, Z. Trajanoski, A. Dopazo, T.H.M. Huang, P.S. Yan, and L. del Peso. 2012. Cooperativity of stress-responsive transcription factors in core hypoxia-inducible factor binding regions. *Plos One.* 7.

Xiaorong, Z., X. Yougui, and W.T. Garrard. 2010. The Igκ gene enhancers, E3' and ed, are essential for triggering transcription. *Journal of Immunology.* 185:7544-7552.

Ylä-Herttuala, S. 2013. Cardiovascular gene therapy with vascular endothelial growth factors. *Gene.* 525:217-219.

Zenke, M., T. Grundström, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon. 1986. Multiple sequence motifs are involved in SV40 enhancer function. *Embo j.* 5:387-397.

Zeyenko, V.V., L.A. Ryabova, D.R. Gallie, and A.S. Spirin. 1994. Enhancing effect of the 3′-untranslated region of tobacco mosaic virus RNA on protein synthesis in vitro. *FEBS Lett.* 354:271-273.

Zhang, J., N. Gao, S. Kasper, K. Reid, C. Nelson, and R.J. Matusik. 2004. An androgen-dependent upstream enhancer is essential for high levels of probasin gene expression. *Endocrinology.* 145:134-148.

Zhu, Y., D. van Essen, and S. Saccani. 2012. Cell-type-specific control of enhancer activity by H3K9 trimethylation. *Mol.Cell.* 46:408-423.