Trypsin-induced VEGF expression in adipose-derived stem cells; optimization of assays

By Ditte Helene Lundsted

Master's Thesis

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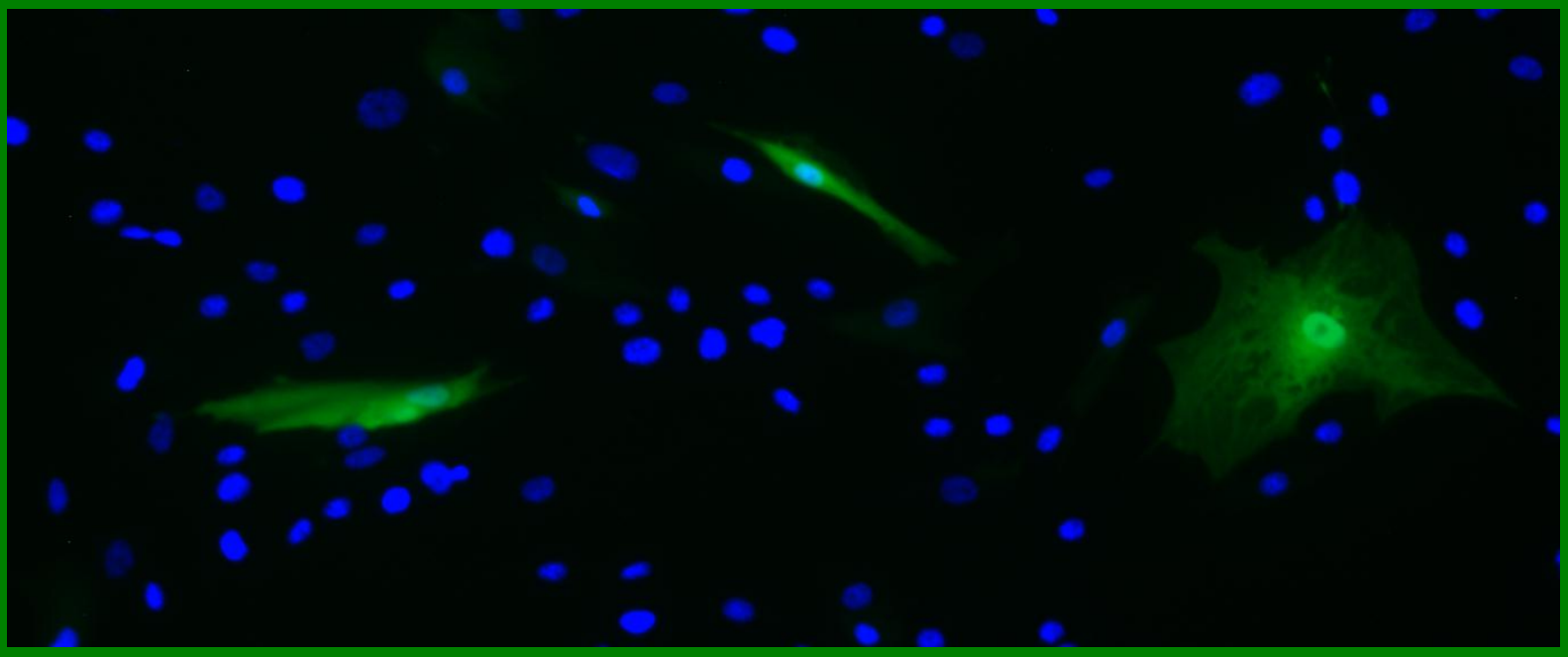

TITLE PAGE

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PREFACE

This thesis was written by Ditte Helene Lundsted during the 3rd and 4th semester of the Master of Science in Medicine with Industrial Specialization program, Aalborg University, Department of Health Science and Technology.

I have worked with adipose-derived stem cells in previous projects. Moreover, as a part of a research group investigating the potential wound healing properties of human adipose-derived stem cells, the objective of this project was to contribute to understanding the cellular mechanisms during cell preparation prior to clinical use. Therefore, the reader must have in mind that this master's thesis is a fragment in the process of fulfilling an overall aim to increase the therapeutic effect of stem cells in the treatment of chronic wounds.

Acknowledgement

The plasmids used were kindly provided by Dr. Lee M. Ellis, The University of Texas.

It is my pleasure to thank those who made this thesis possible. I would like to thank my fellow students and colleagues in the Laboratory of Stem Cell Research for their academically support and encouragement. Particularly Associate Professor Trine Fink and Professor Vladimir Zachar for their appreciated advice and discussions. I would also like to acknowledge Helle Skjødt Møller and Ole Jensen for their technical support in the laboratory. Furthermore, Simone Elkjær Riis and Maj Schneider Thomsen, fellow students and friends of mine, who have also been working within this focus area and I owe them appreciated thank for mutual professional sparring.

Without a doubt I am especially grateful for my family, whose love, guidance, and patience has supported me during the times I needed it the most.

ABSTRACT

Background:

Increasing evidence indicates that adipose-derived stem cells (ASCs) possess wound healing properties. The pro-angiogenic effect is mainly paracrine, exerted through cytokines, such as the vascular endothelial growth factor (VEGF). Hence, there has been great interest in the attempt to increase VEGF expression in order to optimize the effect of transplanted stem cells. One way to do this is to shortly expose the ASCs to trypsin, which induces the VEGF expression through activation of the protease-activated receptor 2. However, trypsin-induced response elements on the human VEGF promoter are yet to be elucidated.

Objective:

The current work will focus on determining the most optimal experimental setup and conditions for analyzing trypsin-induced VEGF-promoter activity.

Methods/Results:

Five deletion mutants of the human VEGF promoter cloned into the pGL3-Basic Vector were verified by restriction screening, polymerase chain reaction, and sequencing.

The ASCs were transfected with a green fluorescent protein reporter plasmid by the three different methods; lipofection, polyfection, and electroporation. For each method, the cell recovery and transfection efficiency were evaluated microscopically. Optimized electroporation conditions for the ASCs were found to be 300V, 1,000 µF, and 10 µg DNA per sample. With these conditions electroporation was found superior to both lipofection and polyfection.

In the optimization of the luciferase activity assay, the ASCs were electroporated with the vector containing the full-length VEGF promoter. When the transfected ASCs were shortly exposed to trypsin 18 h post electroporation, measurable level of luminescence was detected 36 h post electroporation.

Conclusion:

The VEGF promoter deletion mutants cloned into pGL3 vectors were verified. Furthermore, optimized electroporation conditions were found superior to both lipofection and polyfection. Finally, the luciferase assay was designed and found suitable for measure the trypsin-induced VEGF promoter activity in ASCs. In order to clarify promoter regions involved in the trypsin dependent VEGF expression, further investigations are necessary.

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1 INTRODUCTION

Chronic wounds, including pressure sores, leg ulcers, and diabetic foot ulcers, represent a serious medical and societal problem (Buchberger et al., 2010, O'Meara et al., 2000). Approximately 1 % of the European population suffers from these chronic and recurrent wounds, which can be caused by external pressure, trauma, venous insufficiency, diabetic neuropathy, arterial ischemic disease, or vasculitis (Bitsch et al., 2009). Chronic wounds lead to a significant reduction in quality of life due to pain and immobility, while the challenge for treatment and nursing is adequate care of patients, who often must be treated for months to years, resulting in high economic costs. (Buchberger et al., 2010) Diabetic foot ulcer is a type of chronic wound and is a serious problem with an enormous impact on the overall global disease burden due to the increasing prevalence of diabetes mellitus. Worldwide 366 million suffer from diabetes mellitus and approximately 2-10 % of these patients suffer from diabetic foot ulcers, which are difficult to treat and have a tendency to recur. (International Diabetes Federation, 2011, Buchberger et al., 2010, Sundhedsstyrelsen, 2011).

Normal wound healing is a process involving a well-orchestrated sequence of events in order to restore the barrier function and mechanical integrity of the skin (Schultz et al., 2011). It is a complex process including cell migration, cell proliferation, angiogenesis, and extracellular matrix depositions (Cherubino et al., 2011). Wound healing usually occurs in four intertwining phases; hemostasis, inflammation, proliferation, and remodeling (Schultz et al., 2011). In chronic wounds this sequence is disrupted leading to dysfunctional healing including delayed wound closure, prolonged inflammation, poor angiogenesis, and decreased matrix deposition in the wound bed (Sharma et al., 2006).

The current treatment of chronic wounds includes pressure relief, debridement of devitalized tissue, compression, percutaneous transluminal angioplasty, growth factor therapy, and amputation. Furthermore, prophylactic treatment involves usage of appropriate footwear and self care (Sundhedsstyrelsen, 2011). Nevertheless, as these treatments are often inadequate research into new treatment options are desirable. New approaches in the attempt to improve the treatment of chronic wounds include the clinical use of stem cells (Song et al., 2010). Moreover, transplantation of especially adipose-derived stem cells (ASCs) to induce angiogenesis is in an increasing extend being recognized as a potential treatment approach of chronic wounds (Cherubino et al., 2011, Lee et al., 2011). Therefore, this report will focus on the pro-angiogenic properties of ASCs through the expression of the vascular endothelial growth factor (VEGF).

1.1 ADIPOSE-DERIVED STEM CELLS

Stem cells are unspecialized cells characterized by their ability for self-renewal by mitosis, long-term viability, and the ability to differentiate into one or several mature cell types, which renders them suitable for regenerative medical applications (Gimble *et al.*, 2007). In general, adult stem cells are referred to by their tissue of origin such as mesenchymal stem cells. Previously bone-marrow derived stem cells (BSCs) have been the main focus of mesenchymal stem cell research (Kim *et al.*, 2009, Wu *et al.*, 2007). However, due to the clinical limitations of bone marrow biopsies, such as pain and low cell number yield,

alternative sources have been investigated. The discovery of related mesenchymal stem cells in adipose tissue (Zuk et al., 2001) has led to the perception of these as a promising candidate for therapeutic application due to several advantages (Cherubino *et al.*, 2011).

The ASCs are located in the perivascular space in subcutaneous adipose tissue, where they seem to function in the repair of injured tissue and interaction with other cells in response to stimuli (Lee *et al.*, 2009). Several animal studies have confirmed the wound healing effects of ASCs by demonstrating accelerated wound closure, angiogenesis, re-epithelialization, proliferation and migration of fibroblasts, and improved scar thickness (Lee et al., 2011, Song et al., 2010, Nakagami et al., 2005). The mechanism of action by which ASCs accelerate skin regeneration is not well characterized. In general, stem cells are assumed to home to injured tissues and organs, where they contribute to repair by differentiating into tissue specific cells (Chung *et al.*, 2009). However, the theory of repopulation appears unlikely, because the survival rate of engrafted cells is low and functional improvement often occurs too rapidly after transplantation. Therefore, it is suggested that the ASCs, instead of replacing the damaged tissue by differentiation, secrete soluble factors which affect the surrounding cells and the extracellular matrix to facilitate the regenerative effect (Song *et al.*, 2010). Hence, the observed wound healing effect can partially be explained by a complex paracrine mechanism (Figure 1). ASCs have been confirmed to secrete several growth factors including VEGF, platelet-derived growth factor, fibroblastic growth factor, insulin-like growth factor, hepatocyte growth factor, and transforming growth factor β, all promoting several aspects of the wound healing process (Rehman et al., 2004, Kim et al., 2007, Planat-Benard et al., 2004, Gimble et al., 2007). Nonetheless, these cytokines do not seem to function separately, but rather in an unidentified synergic mechanism or in interaction with other regulatory proteins (Kim et al., 2007, Rehman et al., 2004). Supporting this theory, cell-free

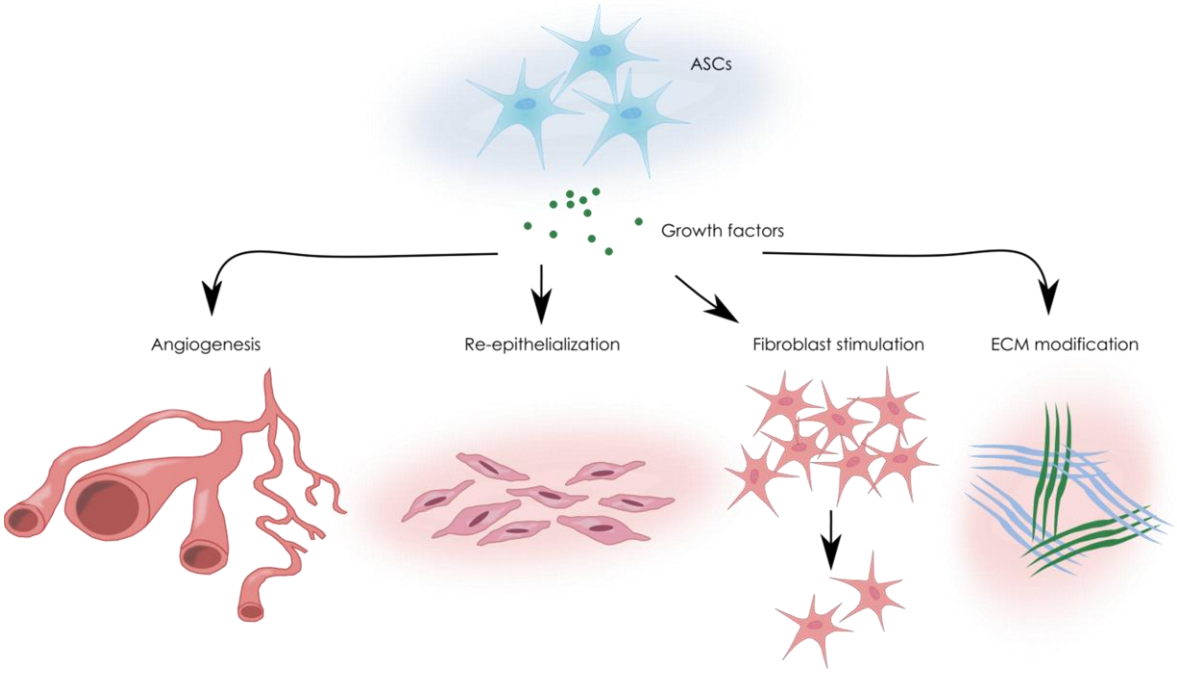

Figure 1. The paracrine mechanism of adipose-derived stem cells (ASCs) in wound healing. The ASCs secrete growth factors such as vascular endothelial growth factor and thereby stimulate angiogenesis, epithelial cells, fibroblasts, and extracellular matrix deposits (ECM). (Own graphic)

conditioned medium from ASCs has been observed to stimulate angiogenesis, collagen synthesis, and migration of cultured dermal fibroblasts (Figure 1)(Kim et al., 2009, Chung et al., 2009, Rasmussen et al., 2011). This report focuses on VEGF, as this growth factor plays a pivotal role in angiogenesis, as described in the following section.

1.2 VASCULAR ENDOTHELIAL GROWTH FACTOR

The VEGF is a growth factor, first characterized in 1983 (Senger et al., 1983). It is produced by cells, which stimulate vasculogenesis and angiogenesis. The formation of new blood vessels is required for embryonic development, placental development, cyclical changes within the endometrium, and muscle growth. Additionally, VEGF plays a role in many pathological conditions, such as wounds, diabetes, psoriasis, and cancer (Song et al., 2010, Nowak et al., 2008, Bhushan et al., 1999). In the complex regulation of angiogenesis, VEGF represents the most important molecule in this regulation (Adams and Alitalo, 2007, Josko and Mazurek, 2004). The crucial role of VEGF has been established by genetic studies in mice by demonstrating that loss of even a single VEGF allele leads to embryonic lethality because of failure in vasculature formation (Ferrara et al., 1996, Carmeliet et al., 1996). VEGF is required for the chemotaxis and differentiation of endothelial precursor cells, endothelial cell mitosis, and formation of endothelial cells into vascular structures (Adams and Alitalo, 2007). Furthermore, VEGF is also known as a vascular permeability factor inducing plasma protein leakage, leading to the formation of an extravascular fibrin gel, which serves as a substrate for endothelial and tumor cell growth (Ferrara et al., 2003).

VEGF is also referred to as VEGF-A in the literature. VEGF belongs to the platelet-derived growth factor family and besides VEGF-A, the VEGF sub-family includes placenta growth factor, VEGF-B, VEGF-C, and VEGF-D. These growth factors bind to and activate tyrosine kinase receptors on target cells, leading to receptor dimerization and autophosphorylation (Song et al., 2010). VEGF-A has affinity for VEGF receptor 1 and 2 (VEGFR-1 and VEGFR2). VEGFR-2 mediates all known VEGF-induced processes, whereas VEGFR-1 modulates VEGFR-2 activities (Ferrara et al., 2003).

1.2.1 GENE AND PROMOTER STRUCTURE

The human *VEGF* gene is located on chromosome 6 at 6p21.3. It encompasses 14 kb and is organized in 8 exons (Figure 2A) separated by 7 introns. The bioavailability of VEGF is profoundly increased by several isoforms, which are generated by alternative splicing of the gene (Figure 2B)(Pages and Pouyssegur, 2005). These have different properties and are named according to their number of amino acids; $VEGF_{XXX}$. Of the different isoforms, the most abundant form is $VEGF_{165}$, which is a basic, heparin-binding, and homodimeric glycoprotein. The lack of exon 6 and 7a in $VEGF_{121}$ results in an acidic protein with no affinity for heparin. Since this isoform does not bind to heparin, it is soluble and diffusible. In contrast, $VEGF_{206}$ and $VEGF_{189}$ are more basic with high affinity for heparin, causing anchoring in the extracellular matrix (Ferrara et al., 2003). An alternate family of isoforms with anti-angiogenic properties has been identified and is referred to as $VEGF_{XXXb}$. These are generated by alternate 3' splice site selection in exon 8 and the consequence is six alternative amino acid

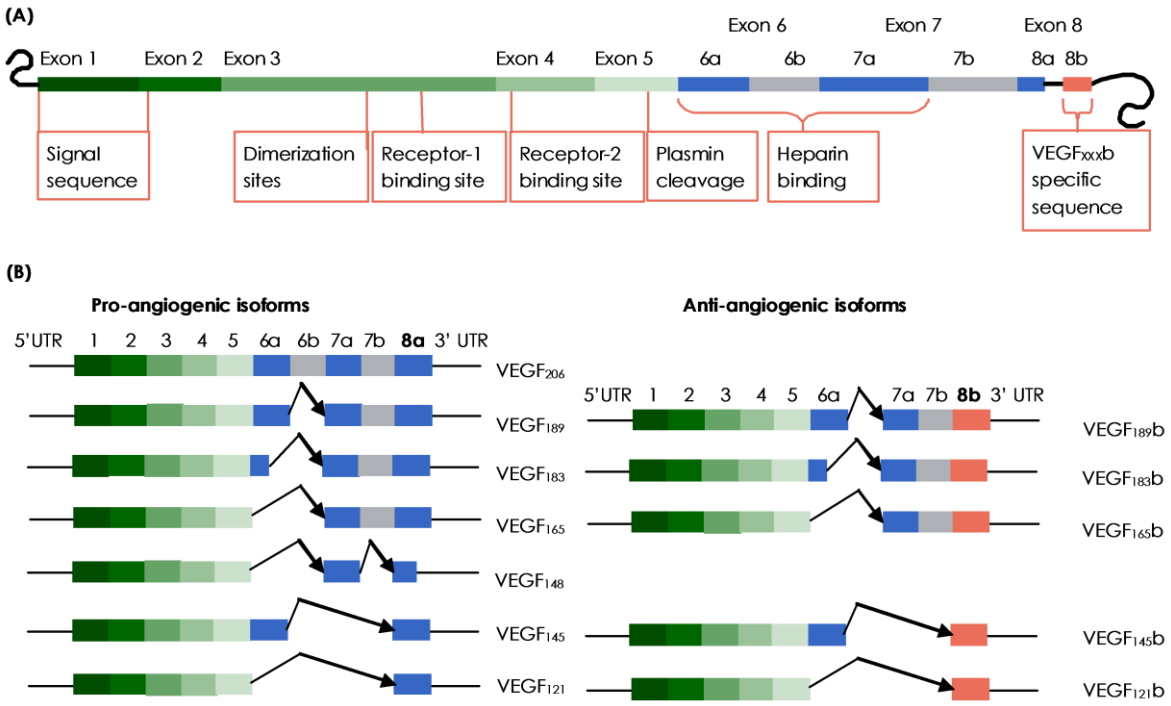

Figure 2. The vascular endothelial growth factor (VEGF) gene. (A) The exonic structure of the VEGF gene and the locations of regions coding for functional groups. (B) The structure of 12 VEGF splice variants generated from the VEGF gene by alternative splicing. Two families of isoforms, $VEGF_{xxx}$ and $VEGF_{xxx}b$, are presented. (Inspired by Nowak *et al.*, 2008)

residues in the C-terminus of the final protein (Nowak et al., 2008). The $VEGF_{XXXb}$ isoforms have unchanged receptor affinity, and thereby function as competitive inhibitors (Ladomery et al., 2007).

The synthesis of VEGF is regulated by a plethora of external stimuli. This regulation works at multiple levels and involves promoter activation, mRNA stabilization, and translational regulation. The tight regulation is in accordance with the essential role of VEGF in angiogenesis and in consequence the expression is up-regulated during different physiological conditions allowing adaptation to hypoxic stress, transient inflammation, and wound healing (Pages and Pouyssegur, 2005, Finkenzeller et al., 1997, Akiri et al., 1998).

The VEGF promoter has been sequenced and characterized and it contains all DNA sequences essential for initiation of transcription (Tischer et al., 1991). Several binding sites for transcription factors such as specificity protein 1 (Sp1), specificity protein 3 (Sp3), hypoxia-inducible factor (HIF-1), signal transducer and activator of transcription 3 (STAT3), activator protein 1 (AP-1), activator protein 2 (AP-2), early growth response protein 1 (Egr-1), and estrogen receptor have been identified (Figure 3 and Table 1). In contrast to many other promoters, the human VEGF promoter does not contain any TATA box. Sequences -88 upstream of the transcription initiation site contains high proportions of guanine and cytosine. These DNA motifs contain GC-boxes and have several overlapping binding sites for Sp1, Sp3, AP-2, and Egr-1(Pages and Pouyssegur, 2005, Josko and Mazurek, 2004).

Figure 3. The full-length vascular endothelial growth factor promoter and *trans*-acting factors. The arrow indicates the initiation site of transcription. Estrogen receptor (ER), activator protein-1 (AP-1), hypoxia inducible factor 1 (HIF-1), signal transducer and activator of transcription 3 (STAT3), liver X receptor (LXR), specificity protein 1 and 3 (Sp1,3), activator protein-2a (AP-2), early growth response protein 1 (Egr-1). (Inspired by Pages and Pouyssegur, 2005)

Table 1. List of transcription factors involved in VEGF transcriptional regulation. The exact positions and sequences of the *cis*-regulatory elements. Activator protein-1 (AP-1), activator protein-2a (AP-2), early growth response protein 1 (Egr-1), hypoxia inducible factor 1 (HIF-1), specificity protein 1 and 3 (Sp1,3), signal transducer and activator of transcription 3 (STAT3), 12-O-tetradecanoylphorbol 13-acetate-response element (TPA response element), liver X receptor response element (LXR response element). (Inspired by Pages and Pouyssegur, 2005)

Transcription factor	Response element	Recognition site	Location of binding sites
Ap-1	TPA response element	TGAATCA / TGAGTGA	-1168/-1015
Ap-2	-	GGCCGGGG	-79/-72
Egr-1	-	CCGGGGGC	-77/-70
Estrogen receptor	Estrogen response element	AATCAGACTGAC	-1525/-1514
HIF-1	Hypoxia response element	TACGTGGG	-975/-968
Liver X receptor	LXR response element	TGTCCGcacgTAACCT	-317/-302
Sp1, 3	GC-box	GGGCGG CCGCCC	-238/-233; -94/-89; -84/-79; -73/-68; -57/-52
STAT3	-	TTCCCAAA	-848/-840

An additional promoter region has been identified in the 5' Untranslated Region. This cryptic hypoxia-independent promoter regulates transcription by an alternative transcriptional initiation site located +650 downstream from the major initiation site (Akiri et al., 1998). It has been hypothesized, that this might result in recruitment of different splice factors, leading to a switch in the balance between the isoforms (Nowak et al., 2008).

1.2.2 TRANSCRIPTIONAL REGULATION

Transcriptional activators of the VEGF gene act through different intracellular signaling pathways and the presence of mutations in oncogenes and tumor suppressor genes can result in increased expression of VEGF (Josko and Mazurek, 2004). Many *trans*-activating factors and environmental conditions have been described in the literature and include growth factors (Nowak et al., 2008), cytokines (Kawaguchi et al., 2004), hormones (Mueller et al., 2000), hypoxia, and hypoglycemia (Shweiki et al., 1992). Some of the factors affecting the VEGF expression will be elaborated upon the following.

Up-regulation of the VEGF expression is mediated through activation of tyrosine kinase receptors, such as the epidermal growth factor receptor. VEGF expression is then induced through the two major signaling pathways; MAPK/Erk and PI3K/Akt (Figure 4)). The transcriptional regulation is in this case mediated through the proximal region of the VEGF

promoter, where transcription factors such as Sp1, Sp3, and AP-2 bind to and act through the GC-box (Pages and Pouyssegur, 2005). However, the signaling pathways by which VEGF is induced are cell type-specific (Berra et al., 2000, Kawaguchi et al., 2004). Sp1 and Sp3 are transcription factors belonging to the family of transcriptions factors with "zinc finger domains". Both Sp1 and Sp3 have consensus binding sites in the GC-box in the proximal part of the VEGF promoter. Sp1 plays a pivotal role in expression of many genes following induction by various stimuli. Several kinases can phosphorylate Sp1 and particularly Erk phosphorylation of Sp1 is a major determinant in VEGF expression through Ras activation. Sp3 is a transcription factor homologous to Sp1 and these two compete for the same DNA elements (Pages and Pouyssegur, 2005). AP-2 has been demonstrated to induce VEGF expression by affecting the AP-2 consensus element in the proximal region of the promoter in response to serum, UVA exposure, transforming growth factor α, hepatocyte growth factor, and epidermal growth factor (Pages and Pouyssegur, 2005).

Hypoxia and ischemia are well-known inducers of the VEGF expression with the aim of restoring the oxygen homeostasis (Josko and Mazurek, 2004). HIF-1α is constantly expressed, but during normoxic conditions enzyme-mediated hydroxylation leads to degradation by a proteasome complex (Figure 5). During hypoxic conditions no such hydroxylation occurs and HIF-1α escapes degradation. This leads to an increased level of stabilized HIF-1α in the

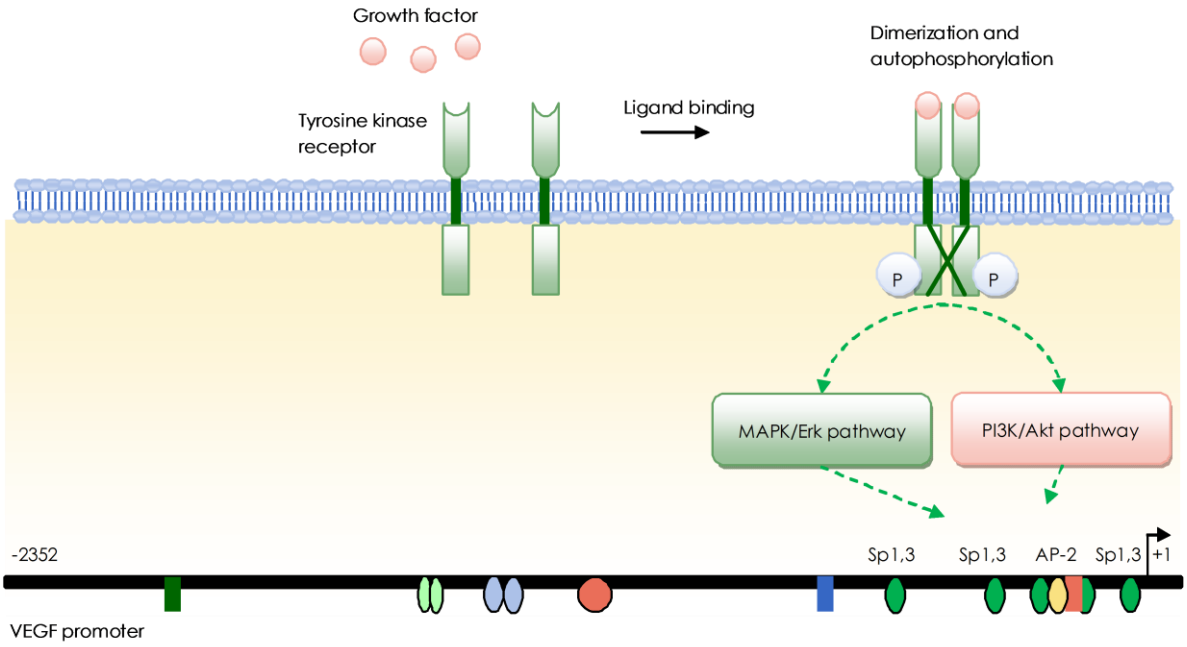

Figure 4. Vascular endothelial growth factor (VEGF) expression induced by growth factors. The growth factors bind to the exodomain of the tyrosine kinase receptor causing conformational changes, dimerization, and autophosphorylation. The stimulation of the receptor leads to activation of the intracellular signaling cascades; MAPK/Erk pathway and PI3K/Akt pathway. These pathways mediate the VEGF expression through the proximal region of the promoter which binds the transcription factors; specificity protein 1 and 3 (Sp1,3), and activator protein 2 (AP-2). Green dashed arrows indicate multiple steps in the signaling (Own graphic. See text for further details and references)

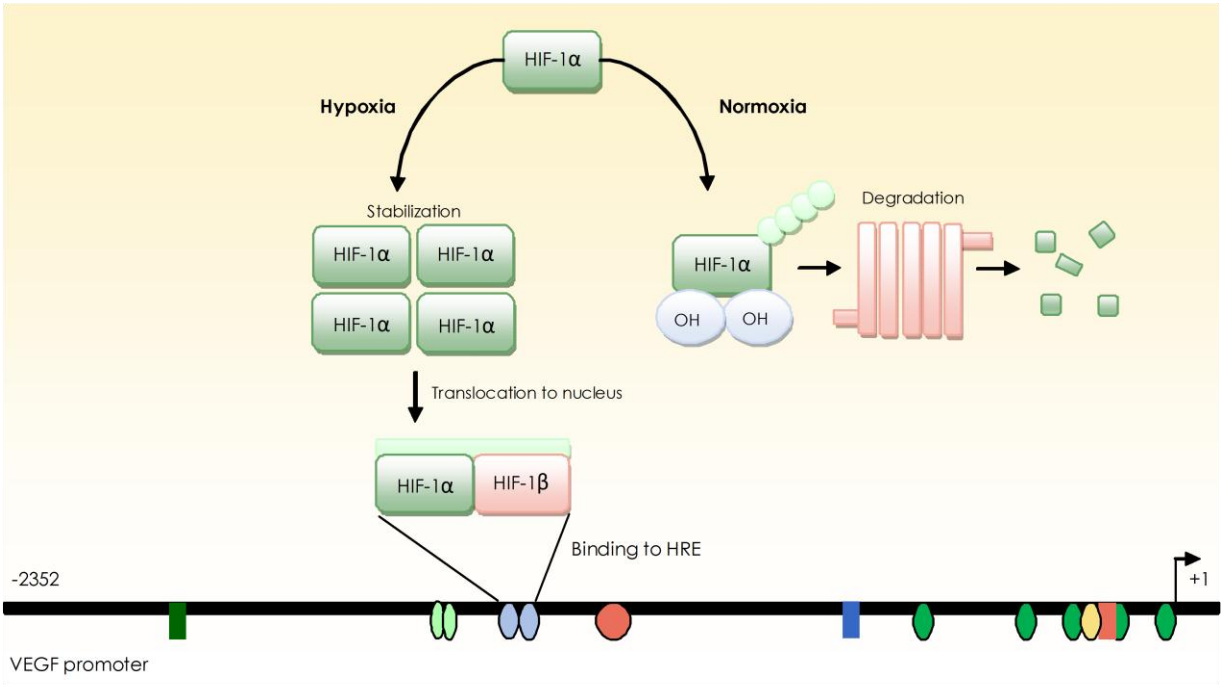

Figure 5. Hypoxia-induced vascular endothelial growth factor (VEGF) expression. Hypoxia inducible factor 1α (HIF-1α) is constantly expressed. During normoxia enzyme-mediated hydroxylation of HIF-1α leads to degradation by a proteasome complex. During hypoxia HIF-1α is stabilized and translocated to the nucleus. HIF-1α forms a transcription factor complex with hypoxia inducible factor 1β (HIF-1β). This complex recognizes and binds to hypoxia response element (HRE) located on the VEGF promoter and thereby stimulates VEGF gene expression. (Own graphics. See text for further details and references)

cytoplasm and translocation to the nucleus, where the HIF-1α dimerizes with HIF-1β subunits. The heterodimer functions as a transcription factor by binding to hypoxia response element and activate transcription of target genes. (Brahimi-Horn and Pouyssegur, 2009). Deletion analyzes of the human VEGF promoter have indicated the presence and significance of such response element on the human VEGF promoter (Forsythe et al., 1996).

1.2.3 ADIPOSE-DERIVED STEM CELLS EXPRESS VASCULAR ENDOTHELIAL GROWTH FACTOR

As mentioned, research indicates VEGF as a key paracrine mediator in ASC-induced wound healing. ASCs synthesize and secrete VEGF in response to external stimuli, such as hypoxia (Lee et al., 2009, Rehman et al., 2004, Song et al., 2010, Rasmussen et al., 2011). Nakagami *et al*. showed that ASCs secreted VEGF, which stimulated endothelial cell growth, migration, and tube formation. Moreover, transplantation of ASCs has been shown to promote angiogenesis through growth factor secretion in an ischemic hind limb model (Nakagami et al., 2005).

To optimize the therapeutic effect of ASCs prior to clinical use, researchers in our laboratory have identified trypsin as a potent activator of VEGF expression in ASCs. The following section will elucidate trypsin and its role in VEGF expression.

1.3 TRYPSIN AND ITS ROLE IN VASCULAR ENDOTHELIAL GROWTH FACTOR GENE EXPRESSION

Trypsin is a serine protease cable of catalyzing the hydrolysis of peptide bonds. (Di Cera, 2009) The zymogen precursor of trypsin is produced in pancreas and is secreted into duodenum. By cleavage trypsin is formed and breaks down nutritional polypeptides into shorter chains. (Guyton and Hall, 2006) The structure of the active site is termed the catalytic triad and contains the ionizable amino acids; serine, histidine, and aspartic acid. The polypeptide chains are cleaved at positively charged residues. (Di Cera, 2009, Polgar, 2005) Beside the proteolytic digestion of nutritional substrates, trypsin also regulates several physiological functions in the intestine and pancreas through activation of the protease-activated receptor 2 (PAR2). PAR2 is expressed on the cell surface in various tissues and function in embryonic development, inflammation, and angiogenesis. (Rothmeier and Ruf, 2012) This transmembrane receptor coupled to G proteins is activated by cleavage of the N-terminus exodomain, that in turn serves as a tethered ligand capable of intramolecular binding to the body of the receptor and thereby induces signal transduction (Figure 6A)(Coughlin, 2000). The activated G proteins interact with effectors to generate intracellular second messengers, which activate downstream signaling cascades depending on cell type (Coughlin, 2000, Dutra-Oliveira et al., 2012).

In laboratories, trypsin is commonly used in the method of subcultivating cells by detaching the adherent cells from their underlying solid substratum. Cell attachment occurs due to the expression of extracellular proteins, glycoproteins, and proteoglycans that bind to the substratum. The proteolytic activity of trypsin results in cleavage of these proteins and thereby leaving the cells floating in the solution. (Adams, 1990)

It has been demonstrated that trypsin induces up-regulated VEGF expression through activation of the PAR2 followed by activation of MAPK/Erk signaling pathways in breast cancer cell lines and endothelial cell lines (Zhu et al., 2006, Liu and Mueller, 2006) In line with these observations Milia et al. found trypsin to increase angiogenesis through PAR2 in an ischemic hindlimb mouse model (Milia et al., 2002). Moreover, researchers in our laboratory have confirmed the trypsin-dependent VEGF expression in human ASCs. They found the expression of VEGF to be up-regulated 1 day after trypsinization when compared to 4 days after trypsinization (Rasmussen et al., 2011). Additional data from the laboratory indicates, that the expression of the VEGF gene in ASCs peaks 6 h after trypsin exposure. Furthermore, the presence and the trypsin-dependent activation of PAR2 have been confirmed in ASCs as well. By using selective kinase inhibitors, MAPK/Erk and PI3K/Akt signaling pathways were demonstrated to be involved in the signaling (Figure 6B and C) (Data not published).

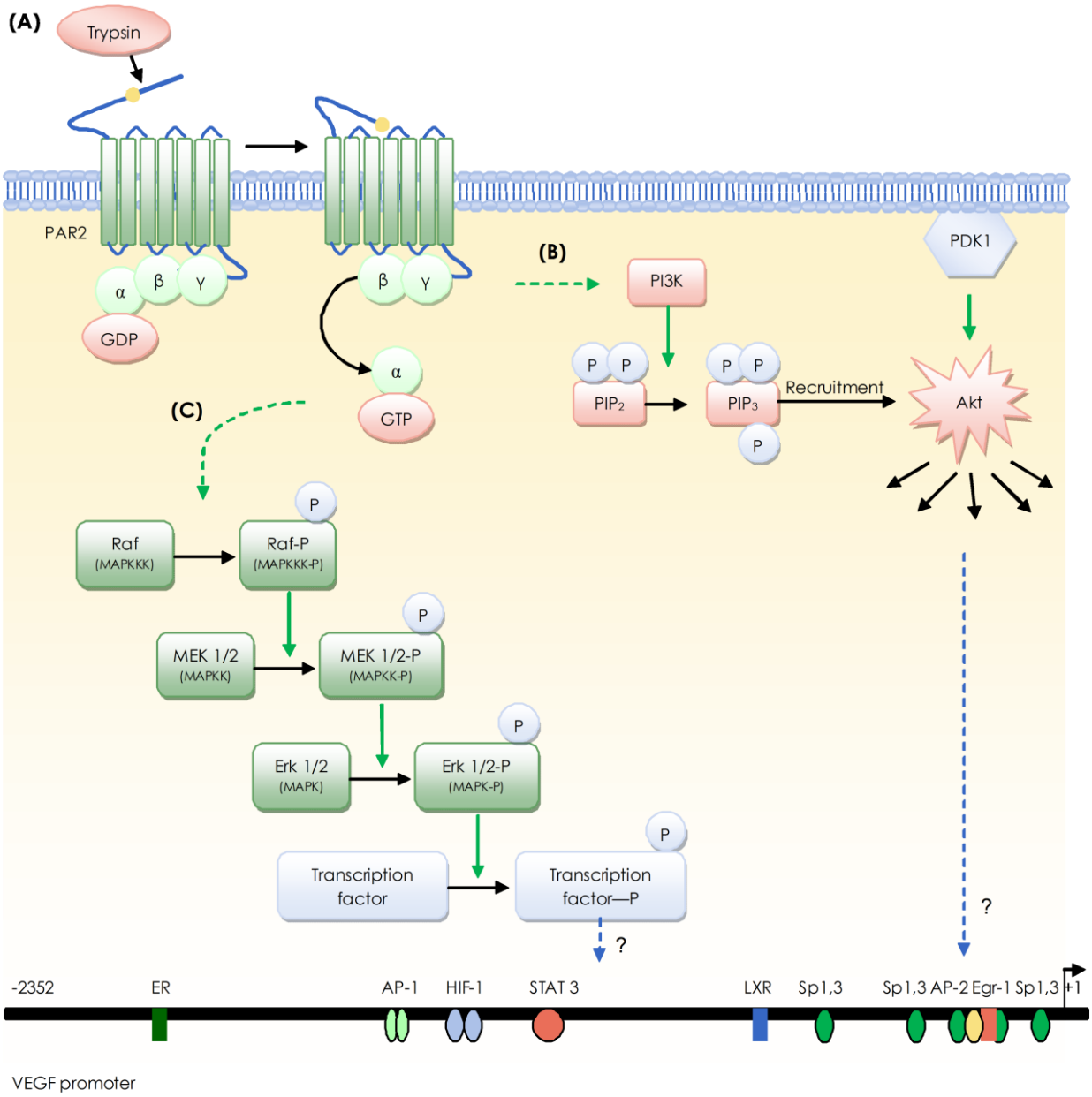

Figure 6. Trypsin-induced signaling pathways leading to increased vascular endothelial growth factor (VEGF) gene expression in adipose-derived stem cells. (A) Trypsin cleaves the extracellular domain of the protease-activated receptor 2 (PAR2) causing conformational changes and activation of the receptor. Guanosine diphosphate (GDP) is exchanged for guanosine triphosphate (GTP) causing the α-subunit to dissociate from the receptor and the βγ-subunits. The activated α-subunit and βγ-subunits interact with effectors to stimulate downstream signaling cascades. (B) Phosphoinositide 3 kinase (PI3K) catalyzes the phosphorylation of phosphatidylinositol 4,5-biphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3). Akt is recruited to the membrane, where it is activated by phosphoinositide-dependent kinase-1 (PDK1). Akt activation results in phosphorylation of numerous other downstream proteins. (C) A cascade of phosphorylations involving mitogen-activated kinase kinase kinase (MAPKKK/Raf), mitogen-activated kinase kinase (MAPKK / MEK 1/2), and mitogen-activated kinase (MAPK / Erk 1/2). The activated Erk1/2 translocate from the cytosol to the nucleus, where they regulate the activity of various transcription factors. Green dashed arrows indicate multiple steps. Blue dashed arrows indicate steps in the signaling not yet fully elucidated. Green arrows indicate kinase activity. (Own graphic. See text for references)

2 THESIS RATIONALE

In order to optimize the therapeutic effect of ASCs prior to clinical use, enzymatic pretreatment with trypsin is a potential candidate. Trypsin induces increased VEGF expression in ASCs through the activation of the PAR2. However, multiple signaling intermediates have been shown to be involved in the VEGF induction. The current work will focus on the determination of the most optimal setup and conditions for analyzing trypsin-induced VEGF-promoter activity in ASCs.

The objectives of this project are to:

- Verify the deletion mutants of the VEGF promoter cloned into vector
- Determine the most optimal transient transfection method and conditions for ASCs
- Verify the experimental setup for luciferase activity assay

3 MATERIALS & METHODS

3.1 CELL LINES AND CULTURE

Human adipose derived stem cells (ASCs), line 21 and 23, were previously isolated in our laboratory (Pilgaard et al., 2008) from lipoaspirate obtained from Grymer Private Hospital, Skejby, Denmark. In brief, the lipoaspirate was harvested from subcutaneous adipose tissue by elective liposuctions approved by the regional committee on biomedical research ethics of Northern Jutland, Denmark. Patients (age 58 and 52 year) had given written informed consent and were not taking any medication.

The ASCs were maintained under standard cell culture conditions at 37 °C and 5 % CO_2 humidified air in a Steri-Cycle CO_2 incubator (Thermo Scientific). The cells were cultured in α - Minimum Essential Medium with GlutaMAX™ (Invitrogen, cat. no. 32561) with 10 % fetal calf serum (Invitrogen, cat. no. 10106-169), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen, cat. no. 15140).

Once the cells reached 70-80 % confluence they were subcultivated to new tissue culture plates. The media were aspirated from the cell culture plate, and the cells were gently washed with Dulbecco's phosphate buffered saline (PBS, Invitrogen, cat. no. 14200-067) to remove excess media. The cells were incubated with a mixture of 0.125 % trypsin (Invitrogen, cat. no. 15090-046) and 0.01 % ethylenediaminetetraacetic acid (EDTA, VWR & Bie & Berntsen, cat. no. 1.08418.0250) at 37 °C for ~5-6 min until the cells detached. Once removed from the incubator, trypsin was inactivated with the serum-containing growth medium.

To calculate the cell concentration, the ASCs were counted manually. 10 µL well mixed cell solution were transferred to a counting chamber of a Bürker-Türk hemocytometer. The cells were counted using ≥ four large corner squares and the cell concentration was calculated.

3.2 SEQUENCE ANALYSIS AND PRIMER DESIGN

The full length VEGF promoter sequence was retrieved from The Eukaryotic Promoter Database (http://epd.vital-it.ch/). The pGL3-Basic Vector sequence was retrieved from Promega. Both sequences are listed in Appendix A. Sequencing results were compared to reference sequence by pairwise sequence alignment using European Molecular Biology Open Software Suite (EMBOSS) Needle tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

Basic information of each primer can be found in Table 2 and Table 3. Furthermore, an overview of annealing sites on the VEGF promoter and pGL3 Vector is presented on Figure 7. All primers have been produced by DNA Technology A/S, Aarhus, Denmark. Primers used for cloning and verification of the VEGF promoter and plasmid constructions were designed using NCBI Primer-BLAST Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequences for the pGL3 Basic forward and reverse primers were provided by Promega. The primers used for real-time reverse transcriptase polymerase chain reaction (PCR) were kindly provided from other researchers in the laboratory.

Table 2. Primers used for vascular endothelial growth factor promoter amplification and sequencing.

Primer name	Sequence	Annealing temp.
VEGF promoter forward primer A	5′ -GGT TGT TGT AAC ACA CCT TGC TGG- 3′	50 °C
VEGF promoter reverse NheI	5′ -CAG AGC GCT GGT GCT AGC CC- 3′	
pGL3 Basic forward primer	5′ -CTA GCA AAA TAG GCT GTC CC- 3′	54 °C
pGL3 Basic reverse primer	5′ -CTT TAT GTT TTT GGC GTC TTC CA- 3′	
pGL3 forward primer D1	5′ -GGT ACG GGA GGT ACT TGG AGC GG- 3′	55 °C
Insert-2352 reverse primer	5′ -CAT GGT GGT ACC CAG CAA GGT GT- 3′	
Insert proximal forward primer	5′ -GGG CCG GGG AGG AAG AGT AGC- 3′	55 °C
pGL3 reverse primer D1	5′ -TGC TCT CCA GCG GTT CCA TCT T- 3′	
Insert distal forward primer	5′ -GGT GGG AGC CAG CCC TTT TCC- 3′	Only used for sequencing
Insert distal reverse primer	5′ -ACC TCC GAG CTA CCC GGC TG- 3′	

Table 3. Primers used for real-time reverse transcriptase polymerase chain reaction. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), cyclophilin A (PPIA), forward (fw), reverse (rv).

Target gene	Primer sequence	Annealing temp.	Product length
hVEGF	Fw: 5′ -CGA TTC AAG TGG GGA- 3′	61 °C	107 bp
	Rv: 5′ -CAT TGA TCC GGG TTT- 3′		
PPIA	Fw: 5′ -TCC TGG CAT CTT GTC- 3′	63 °C	90 bp
	Rv: 5′ -CCA TCC AAC CAC TCA- 3′		
YWHAZ	Fw: 5′ -ACT TTT GGT ACA TTG- 3′	63 °C	94 bp
	Rv: 5′ -CCG CCA GGA CAA ACC- 3′		

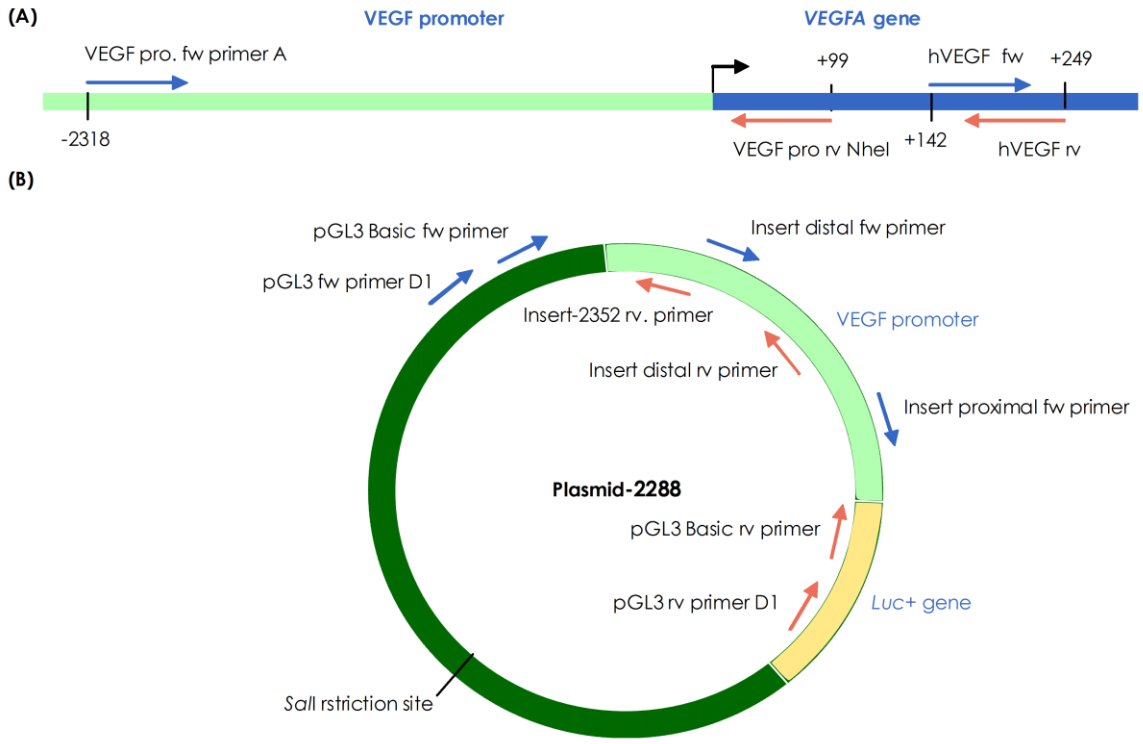

Figure 7. Schematic illustration for primer annealing sites located on (A) the vascular endothelial growth factor (VEGF) promoter, VEGF gene and (B) the Plasmid-2288 (B). Promoter (pro.), forward (fw), reverse (rv).

3.3 AGAROSE GEL ELECTROPHORESIS

1 or 2 % agarose was dissolved in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) by heating. After complete heat dissolving, Ethidium Bromide (Sigma, cat. no. E-1385) was added to reach a final concentration of 0.05 µg/mL and poured into a tray with wells. After solidification the gel was used immediately or stored at 4 °C. The gel was placed in a RunOne™ Electrophoresis Cell (Embi Tec) containing cold 1x TAE buffer. PCR products and restriction reaction mixtures were prepared by mixing with 6x loading dye (Fermentas, cat. no. R0611) and loaded in appropriate volumes according to well size. For each gel, an appropriate DNA ladder was selected and loaded along with the samples. All gels were run at 100 V for approximately 15-20 min. Amplified VEGF promoter fragments for further processing were visualized and excised on a TF-20M UV fluorescent table (Vilber Lourmat). Otherwise visualization was performed on a Kodak Image Station 4000MM PRO with an excitation filter of 530 nm and an emission filter of 600 nm.

3.4 PLASMID CONSTRUCTION

3.4.1 GENOMIC DNA ISOLATION FROM ADIPOSE-DERIVED STEM CELLS

Human ASCs (cell line 23, passage 1) were thawed in a 37 °C water bath for 90 sec. The tube was disinfected with ethanol and the cells transferred to a tube containing 5 mL PBS. The suspension was centrifuged for 5 min at 300 x g and the cell pellet wad resuspended in 200 µL fresh PBS. Genomic DNA was extracted with a DNeasy Blood & Tissue kit (QIAGEN, cat. no. 69504), using the protocol for Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol). Shortly, proteinase K and buffer were added to the cells and incubated at 56 °C for 10 min to allow sufficient cell lysis. Then ethanol was added and mixed before the sample was transferred to a silica-based membrane binding DNA. After two washing steps DNA was eluted. The final DNA concentration and purity were determined spectrophotometrically using Nanodrop® ND-1000 Spectrophotometer (Thermo Science). The final DNA solution was stored at -20 °C for further use.

3.4.2 VEGF PROMOTER AMPLIFICATION BY STANDARD POLYMERASE CHAIN REACTION

Using genomic DNA as template, the human VEGF promoter was amplified by standard PCR. A 50 µL reaction mix containing 1X cloned *Pfu* DNA polymerase reaction buffer (Stratagene, cat. no. 600153-82), 0.4-0.8 mM deoxyribonucleotide mix (dNTPs, Sigma, cat. no. DNTP100A), 100 ng template, 0.2 µM VEGF promoter forward primer A, 0.2 µM VEGF promoter reverse NheI, and 2.5 U *Pfu*Turbo DNA polymerase (Stratagene, cat. no. 600252-52) were mixed. The PCR reaction were completed in a thermo cycler (Perkin-Elmer, GeneAmp PCR system 2400) at: 1 cycle of 2 min at 94 °C, 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C, and 2½ min at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were separated by gel electrophoresis using 1 % agarose gel. The bands corresponding to the length of the VEGF promoter fragment (2.4 kb) were excised from the gel and the DNA extracted using

either QIAquick Gel Extraction Kit (QIAGEN, cat. no. 28706) or GFX™ PCR DNA or Gel Band Purification Kit (Amersham Pharmacia biotech, cat. no. XY-071-00). Both methods were based upon dissolving the DNA-containing gel. Then the solution was transferred to a membrane capable of binding DNA. After a washing step the DNA were eluted. The final DNA concentration and purity were determined spectrophotometrically and stored at -20 °C for further use.

3.4.3 CLONING

The restriction enzyme cleavages and ligation is illustrated on Figure 8. To obtain compatible ends, the VEGF promoter fragment and pGL3-Basic Vector (Promega, cat. no. E1751) were restricted separately in a double digestion at 37 °C for 1 h with a 50 µL reaction mix containing 1X NEBuffer 1 (NEB, cat. no. B7001S), 0.1 µg/µL Bovine Serum Albumin (BSA, NEB, cat. no. B9001S), 10 U *Kpn*I (NEB, cat. no. R0142S) and 10 U *Nhe*I (Amersham Pharmacia Biotech, cat. no. E1162Y). The enzymes were removed using GFX™ PCR DNA and Gel Band Purification Kit. Then 40 ng digested VEGF promoter fragment were cloned into the 50 ng digested plasmid by 1 U T4 DNA ligase (NEB, cat. no. M0202S) in a 20 µL reaction mix with 1X Ligation Buffer (NEB, cat. no. B0202S). The plasmid construct is referred to as plasmid-2288.

Figure 8. Digestion and cloning of the vascular endothelial growth factor (VEGF) promoter fragment and pGL3-Basic Vector.

One Shot® Stbl3™ Chemically Competent *E. coli* (Invitrogen, cat. no. C7373-03) were transformed with the ligation mixture according to the manufacture's protocols. Briefly, the bacteria were thawed on ice, gently mixed with the ligation mixture (14 ng DNA), and incubated on ice for 30 min. The tubes were heat shocked in 42 °C water bath for 45 sec without shaking, and incubated on ice for 2 min. After the last incubation, 250 µL room temperature S.O.C. medium was added to each tube and the tubes were then incubated at 35 °C for 1 hour at 225 rpm on a KS 501 Digital Laboratory Shaker (IKA). The transformed bacteria suspension were plated on room temperature agar plates containing 100 µg/mL ampicillin (Sigma, cat. no. A-0166) and incubated at 35 °C overnight. Both negative and positive controls were included.

3.4.4 COLONY POLYMERASE CHAIN REACTION

Six colonies were screened for transformants using colony PCR. The 20 µL master mix consisted of 1X Taq DNA polymerase BUFFER with $MgCl_2$ (Promega, cat. no. M1881), 0.8 mM dNTPs, 1 µM of pGL3 Basic forward primer, 1 µM of pGL3 Basic reverse primer, 1 U TAQ DNA

Polymerase (Ampliqon, cat. no. 112103). The side of each selected colony was touched with a pippette tip and transferred to the master mix. The PCR reactions were completed in a thermo cycler (Perkin-Elmer, GeneAmp PCR system 2400) at : 1 cycle of 5 min at 95 °C, 30 cycles of 30 sec at 95 °C, 30 sec at 54 °C, and 2½ min at 72 °C, and a final extension at 72 °C for 5 min. Following amplification, DNA was visualized by 1 % agarose gel electrophoresis.

3.5 VERIFICATION OF PLASMIDS

Plasmid-2352, -1800, -780, -120, +599, and pGL3-Basic Vector were kindly received from Dr. Lee M. Ellis, The University of Texas. To ensure the presence of DNA in the samples, the DNA concentrations and purities were determined spectrophotometrically. The samples were stored at -20 °C for further use. The plasmids constructs were propagated in competent bacteria, as described in section 3.4.3.

To confirm the presence of correct insert, four colonies for each plasmid construct were screened using colony PCR. The procedure for colony PCR is described in section 3.4.4.

The colonies were transferred to 5 mL Lysogeny Broth medium with 100 µg/mL ampicillin and incubated on a KS 501 digital Laboratory Shaker set to 225 rpm at 35 °C overnight. The plasmids were extracted from the bacteria using a GenElute™ Plasmid Miniprep Kit (Sigma, cat. no. PLN-350). In brief, the recombinant bacteria grown overnight were harvest by centrifugation and subjected to cell lysis. The lysate were loaded on a silica membrane in the presecence of high salts. Contaminants were removed by a spin-wash step before the plasmids were eluted. The final DNA concentration and purity were determined spectrophotometrically. The final DNA solution was stored at -20 °C for further use.

DNA isolated by miniprep were digested with 60 U *Sal*I (NEB, cat. no. R0156S) in a reaction mix with 1X NEBuffer 3 (NEB, cat. no. B7003) at 37 °C for 1 hour and visualized by agarose gel electrophoresis (Figure 7).

In the end, two clones of plasmid-2352, -1800, -780, -120 and +599 were sent to sequencing at Beckman Coulter Genomics, UK. Primers used for sequencing were pGL3 forward and reverse primer D1. Additionally, the Insert distal forward and reverse primers were used for plasmid-2352 and -1800.

3.6 REAL-TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

The ASCs were seeded in a 6 well plate (Costar®, cat. no. 3506) with a cell density of 10,000 cells/cm^2. After three days the cells were subcultivated. First the growth medium was aspirated and the cells were washed twice with S-PBS to remove excess media. The cells were incubated at 37 °C with either 3.2 U/mL Dispase II (Sigma, cat. no. D4693-1G) and 0.01 % EDTA for 2 hours, Cell Dissociation Solution (Sigma, cat. no. C5914) for 1 hour, or 0.125 % trypsin and 0.01 % EDTA for 6 min until cells detached. The cells in suspension were pelleted by centrifugation and transferred to new wells with fresh growth media. Six hours after subcultivation the cells were analyzed for their VEGF expression level as described in the following. The experiment was run in biological duplicates and non-subcultivated cells were included as negative control.

Cell lysis and RNA extraction were performed using an Aurum Total RNA Mini Kit according to manufactures instructions (Bio-Rad, cat. no. 732-6820). First the cells were washed with S-PBS and lysed in 350 µL lysis solution. Then 350 µL 70 % ethanol were added and the sample was loaded on a silica membrane. Following a wash step, the sample was treated with DNase I for 15 min at room temperature. Additional washing steps were performed before the RNA was eluted and the quantity and quality was determined spectrophotometrically.

cDNA was synthesized using a iScript™ cDNA Synthesis Kit (Bio-Rad, cat. no. 170-8891). A mixture of iScript reaction mix, iScript reverse transcriptase, and diluted RNA was run in the thermo cycler at: 5 min at 25 °C, 30 min at 42 °C, and finally 5 min at 85 °C.

To generate a standard curve, aliquots of cDNA from each sample were pooled and diluted in a four-fold serial dilution. The PCR reactions were performed in a final volume of 25 µL containing 1X iQ™ SYBR® Green Supermix (Bio-Rad, cat. no. 170-8882), 5 µM of each primer, and diluted cDNA. Thermal cycling (Bio-Rad, MyiQ™ Single-color Real-Time PCR Detective System) was initiated with 5 min at 95 °C, 50 cycles of 10 sec at 95 °C and 30 sec at annealing temperature (see Table 3). Finally, a melt curve was created. Expression of the VEGF gene was normalized by geometric mean to the respective levels of the housekeeping genes cyclophilin A (PPIA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) Table 3. All samples were run in duplicates.

3.7 TRANSFECTION

To find the optimal transfection method for the ASCs, the cells were transfected with Vivid Colors™ pLenti6.2-GW/EmGFP Expression Control Vector (Invitrogen, cat. no. V369-20) by three different methods; lipofection, polyfection, and electroporation. This vector expresses green fluorescent protein (GFP), which allows visualization of successfully transfected cells microscopically. The following experiments were carried out using ASC cell line 21 below passage 12.

The methodologies of the different transfection techniques are presented on Figure 9 and will be described in the following sections.

3.7.1 LIPOFECTAMINE 2000

Lipofectamine® 2000 Tranfection Reagent (Invitrogen, cat. no. 11668-027) is a commercial available transfection kit that employs cationic lipids to introduce DNA into cells.

One day prior to transfection, ASCs were seeded with a cell density of $15.6*10^3$ cells/cm^2 in growth medium without antibiotics in a 96-well plate (Costar®, cat. no. 3596). At the day of transfection, DNA and the Lipofectamine 2000 Reagent were diluted in Opti-MEM® I Reduced Serum Medium (Invitrogen, cat. no 31985), separately. 15 min after dilution of Lipofectamine 2000, the diluted DNA and diluted lipid were combined and incubated at room temperature for 30 min to allow liposome-DNA complexes to form. The solution were dropwise added to the wells containing cells and 100 µL growth medium and mixed gently by rocking the plate back and forth. The cells were incubated at 37 °C with 5 % CO_2 for 48 hours. The media were aspirated and replaced with fresh growth medium 24 h post

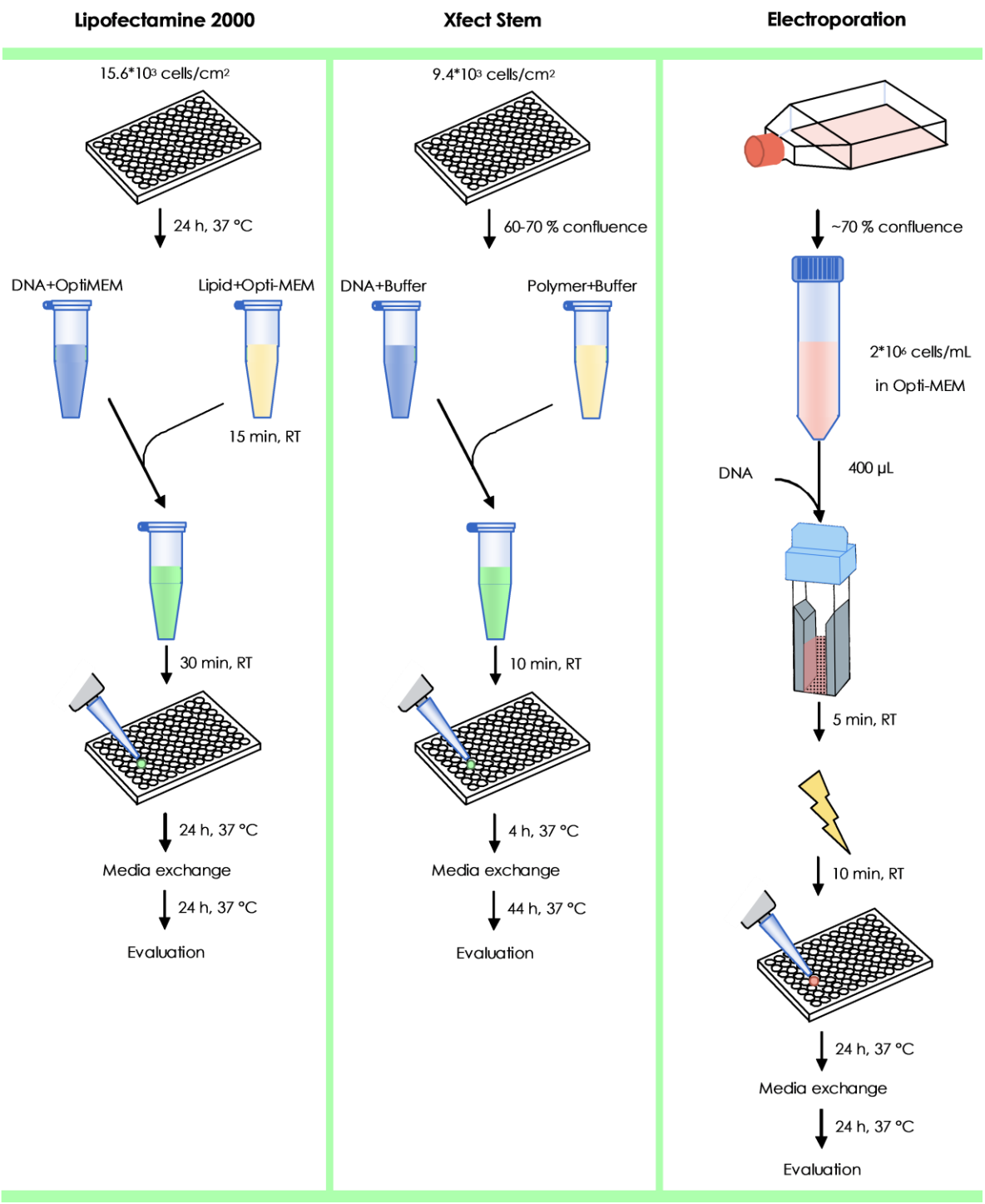

Figure 9. Transfection overview. Room temperature (RT)(See text for further details)

transfection. For an experiment involving suspension cells, the solution containing liposome-DNA complexes were added to the wells immediately before seeding cells with a density of $40*10^3$ cells/cm^2.

3.7.2 XFECT STEM

Xfect™ Adult Stem Cell Transfection Reagent (Clontech, cat. no. 631329) is a commercial available transfection kit that employs polymers to introduce DNA into adult stem cells.

The cells were seeded with a density of $9.4*10^3$ cells/cm^2 in 96 well plates and grown until they reached 60-70 % confluence. DNA and the polymer were diluted in Xfect Reaction Buffer, separately. The polymer solution were added to the DNA solution, vortexed thoroughly for ~ 10 sec, and incubated at room temperature for 10 min to allow nanoparticles to form. The solution were dropwise added to the wells containing cells and 100 µL growth medium and mixed gently by rocking the plate back and forth. The cells were incubated at 37 °C with 5 % CO_2 for 48 hours. The media were aspirated and replaced with fresh growth medium 4 h post transfection.

For an experiment involving suspension cells, the solution containing nanoparticle complexes were added to the wells immediately before seeding cells with a density of $40*10^3$ cells/cm^2.

3.7.3 ELECTROPORATION

The ASCs were grown until they reached 70 % confluence. Then the cells were detached and diluted in Opti-MEM® I Reduced Serum Medium to reach a final concentration of $2*10^6$ cells/mL. DNA solution and 400 µL cell suspension were loaded into a Gene Pulser® Cuvette with a 0.4 cm electrode gap (Bio-Rad, cat. no. 165-2088). The cells were allowed to equilibrate for 5 min before exposed to exponential wave pulse with predetermined voltage and capacitance using Gene Pulser® II Electroporation System (Bio-Rad). The time constant for each pulse were recorded. The cells were allowed to recover at room temperature for 10 min before seeded with fresh growth medium in culture plates. The cells were incubated at 37 °C with 5 % CO_2 for 48 hours. The media were aspirated and replaced with fresh growth medium 24 h post transfection.

3.7.4 EVALUATION OF TRANSFECTION

Phase contrast and fluorescent images were obtained with an Olympus CKX41 or an Axiovert 200 M (Carl Zeiss) microscope. The software AxioVision rel. 4.7 (Carl Zeiss) were used for image analysis. Cell morphology was rated 24 h post transfection. 48 h post electroporation, the nuclei were stained with 0.5 µg/mL Hoechst 33342 (Invirogen, cat. no. H3570) for 25 min. Cell recovery was measured by comparing the number of nuclei in the transfected sample with the number of nuclei in a non-transfected control sample. Transfection efficiency was calculated from the number of green cells on the GFP channel representing the transfected cells divided by the number of nuclei present on the Hoechst

channel representing the total number of cells. Furthermore, the mean intensity of GFP expression per transfected cell was analyzed using ImageJ software (National Institutes of Health).

3.8 LUCIFERASE ACIVITY ASSAY

Electroporation were performed on the ASCs, as described in Section 3.7.3. The program settings were 300 V and 1,000 µF. After electroporation the cells were seeded in triplicates in a 96-well plate with cell density factors; 1, 2, 4, 8, and 12. The density factor 1 corresponded to $5*10^3$ cells/cm^2 calculated from the cell concentration prior to electroporation, knowing that a fraction of the cells would not survive the treatment. Following electroporation, the cells were incubated at 37 °C with 5 % CO_2.

To measure the luciferase activity in each well a Steady-Glo® Luciferase Assay System (Promega, cat. no. E2520) were used. The culture plates and reagent were equilibrated to room temperature. Then 100 µL of reagent was added to cells grown in 100 µL of medium. To allow sufficient cell lysis, the samples were incubated at room temperature for 15 min before luminescence measurement on Wallac VICTOR$^{2}{}_{TM}$ 1420 Multilabel Counter (PerkinElmer) for 5 sec.

Three different luciferase assays were conducted. The first experiment involved electroporation with pGL3-Control Vector (Promega, cat. no. E1741), which constantly expresses the firefly luciferase. The luciferase activity was assessed both 24 h and 48 h post electroporation. Furthermore, it was determined whether a transparent or white plate was the most appropriate type of plate to retrieve clear luminescence signals.

In the second experiment, the ASCs were electroporated with plasmid-2288. As illustrated on Figure 10, the time point of luciferase measurement was 36 h post electroporation. 18 h before measurement a group of cells was exposed to 0.125 % trypsin for 5 min at 37 °C. 5 min was time enough for the cells to become round in shape, but not enough time for detachment, if the plates were handled with care. As positive control a group of cells were incubated in Galaxy Incubator with CO_2 and O_2 control (O_2 = 1 %)(New Brunswick) 24 h prior to measurement. Moreover, a group of cells did not receive any treatment and served as a negative control.

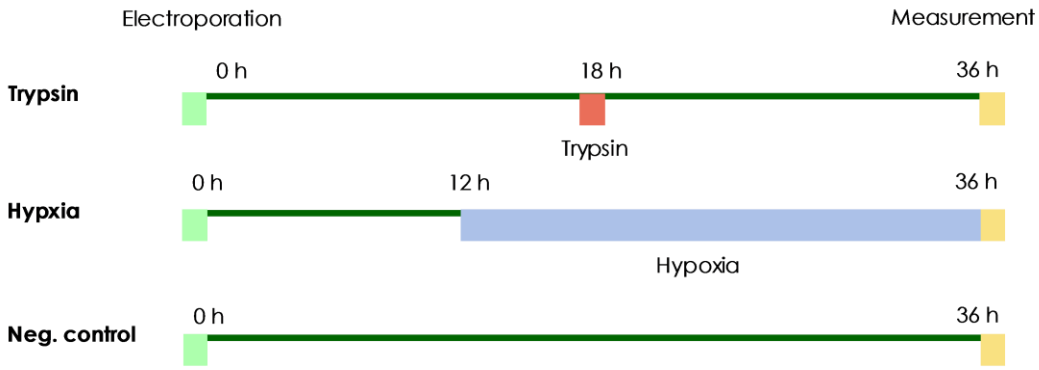

Figure 10. Time line for luciferase assay.

In the third experiment, the luciferase kit used so far (lot: 184771, expiration date: July 2005) were compared with a newly purchased kit (lot: 25409, expiration date: Jun 2014). The ASCs were electroporated with the pGL3-Control Vector and luciferase activity was measured 24 h, 48 h, and 72 h post transfection.

3.9 DATA ANALYSIS

Data were analyzed using SigmaPlot version 12 (Systat Software). When comparing more than two groups, a one-way analysis of variance (ANOVA) test was used, and when comparing experiments with several variables, a two-way ANOVA or tree-way ANOVA test were used. When data failed normality test (Shapiro-Wilk) or equal variance test, Kruskal Wallis or Holm-Sidak methods were used. Data are presented as mean ± standard error (S.E.). A *p*-value of < 0.05 was considered statistically significant.

4 RESULTS

4.1 PLASMID CONSTRUCTION

Genomic DNA was isolated from human ASCs at low passage. The final concentrations and purity were determined spectrophotometrically to be 73 ng DNA/µL and an A_{260}/A_{280} ratio of 1.96.

VEGF promoter fragments were amplified by PCR followed by gel electrophoresis. Despite visual confirmation of positive bands with expected length compared to DNA marker, no measureable DNA were present in the samples after gel extraction using a commercial kit from Qiagen. This step was repeated 5 times with the same result. To optimize the set-up, the following were executed: increasing the numbers of samples, increasing the concentration of dNTP from 0.4 mM to 0.8 mM, primer optimization, reamplification, and touchdown PCR. In a directly comparison experiment, the gel extraction kit from Amersham Pharmacia Biotech resulted in ~10 times higher DNA concentration compared to the kit from Qiagen.

The VEGF promoter was digested with appropriate restriction enzymes and cloned into pGL3-Basic Vector. The plasmid construct was referred to as plasmid-2288. Then, competent *E. coli* bacteria were transformed with the ligation mixture. Extensive bacteria growth were observed on the agar plates containing bacteria transformed with plasmid-2288 and undigested pGL3-Basic Vector. The control with digested pGL3-Basic Vector resulted in several colonies on the selective plate. No bacteria growth was observed on the agar plate seeded with untransformed bacteria.

Six colonies were selected and screened for vectors containing insert by colony PCR with the commercial designed primers. Four out of six colonies were tested positive for inserts. The bands appear to be located at ~ 1 kb and not the expected 2.4 kb position (Figure 11). Thus, we proceeded the work using plasmids received as a generous gift from Dr. Lee M. Ellis.

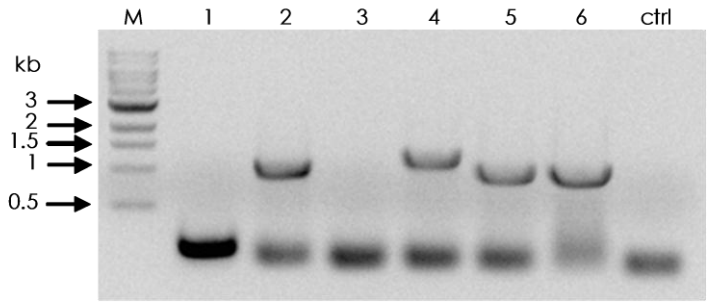

Figure 11. 1 % agarose gel from colony polymerase chain reaction with plasmid-2288 clones. Lane 1-6: Colonies containing putative plasmid-2288 constructs. DNA ladder (M), negative control (ctrl).

4.2 VERIFICATION OF PLASMIDS

In order to carry out valid analysis of VEGF promoter activity, it was crucial that the plasmid constructs contained the inserts of interest in proper orientation upstream from the *luc*+ gene and did not contain mutations in putative response elements. Therefore, verifications of plasmid-2532, plasmid-1800, plasmid-780, plasmid-120, plasmid+599, and pGL3-Basic Vector were performed. Initially, it was confirmed that each sample contained measurable amounts of DNA. Subsequently, several efforts were made to screen plasmids for correct insert before the plasmids were sent away for sequencing.

To retrieve higher DNA amounts, each plasmid construct were propagated in *E. coli*. Extensive bacteria growth was observed on the selective agar plates containing transformed bacteria, indicating successful transformation. No bacteria growth was observed on the agar plate seeded with untransformed bacteria.

4.2.1 COLONY POLYMERASE CHAIN REACTION

Three to four colonies for each plasmid construct were selected and screened for vectors containing insert by colony PCR with the commercial designed primers. As seen on Figure 12, three of the six constructs was found positive as indicated by the arrows. The samples containing the putative plasmid-2352 showed single strong bands at ~1 kb, as determined by comparison with size markers, instead of the expected 3.5 kb. The samples containing the putative plasmid-1800, plasmid-780, and plasmid-120 showed no visual bands on the agarose gel. The samples containing the putative plasmid+599 and pGL3-Basic Vector showed single strong bands at the expected size. The PCR was repeated twice for each colony with the same results (Appendix B).

Selecelted colonies were harvest and miniprep performed to isolate putative plasmids from the bacteria. Following plasmid purification it was possible to measure reasonable amounts

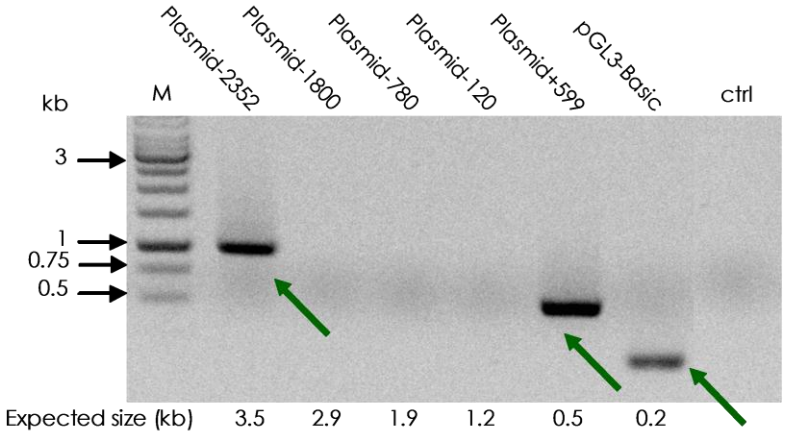

Figure 12. 1 % agarose gel from colony polymerase chain reaction with putative plasmid-2352, -1800, -780, -120, +599, and pGL3-Basic Vector representative clones. DNA ladder (M), negative control (ctrl).

of DNA in each sample spectrophotometrically with values ranging from 53 to 163 ng/µL and A_{260}/A_{280} ratios from 1.77 to 1.96. Therefore, further attempts were made to screen plasmids for insert.

4.2.2 RESTRICTION SCREENING

Since the colony PCR did not detect insert in all clones, screening with restriction enzymes was executed. The pGL3-Basic Vector contains a restriction site for *SalI* (Figure 7). First, a preliminary setup using plasmid-2532 was conducted to confirm the effect of the restriction enzyme. Figure 13A illustrates the result; Lane 1 represents the digested sample and appeared as a total smear on the gel, lane 2 represents the uncut sample, which showed multiple bands. Therefore, it was suspected that one or more of the solutions in the restriction reaction were contaminated with nucleases. Thus, this was tested on pGL3-Basic Vector and it revealed that the buffer was the scapegoat, as seen in lane 1 on Figure 13B (green arrow). With the optimized restriction reaction mix, a new preliminary setup using pGL3-Basic Vector confirmed that *SalI* was able cut the vector efficient at one site, as seen in lane 1 on Figure 13C.

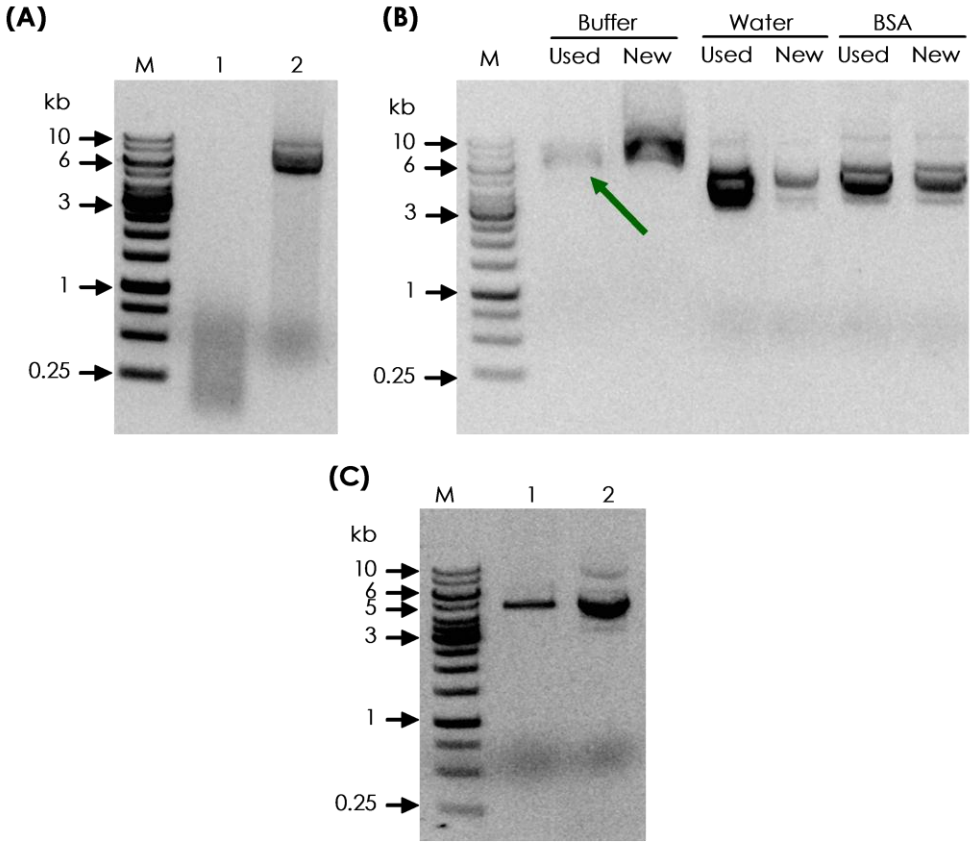

Figure 13. Optimization of restriction reaction on 1 % agarose gel. (A) Lane 1: Plasmid-2352 digested with *SalI*. Lane 2: plasmid-2352. (B) Results for screening for possible nuclease contamination in buffer, water, and Bovine Serum Albumin (BSA). pGL3-Basic Vector was incubated with solutions used in previously reactions (used) or new solutions (new). (C) Lane 1: pGL3-Basic Vector digested with *SalI* using new buffer. Lane 2: pGL3-Basic Vector.

After finding *SalI* suitable for cutting the plasmids, all samples were digested and run on an agarose gel (Figure 14). The results confirm that all samples contained the plasmids with the correct length.

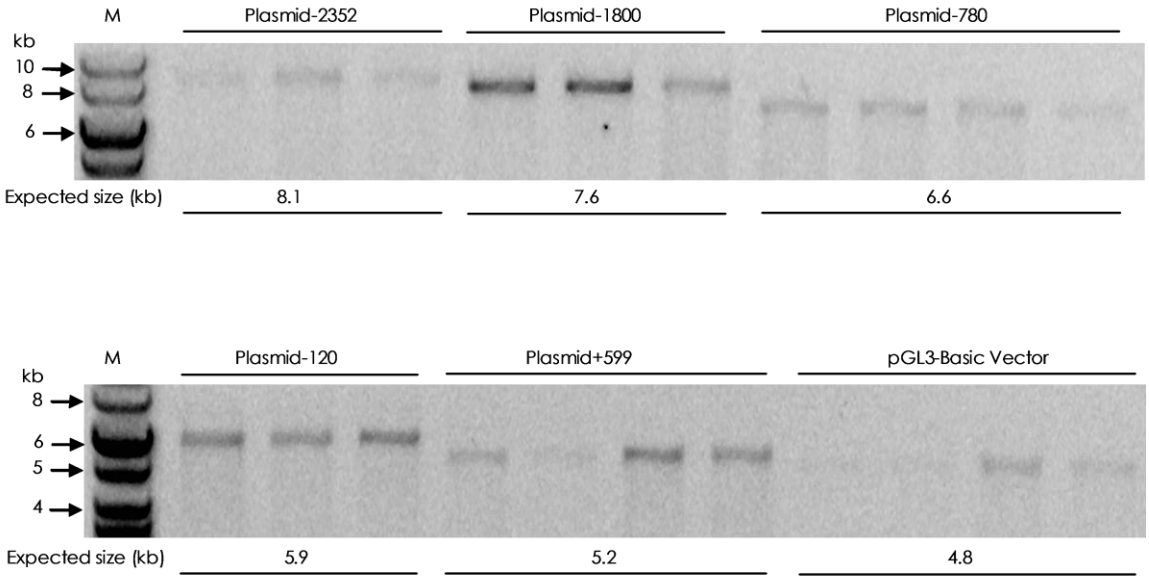

Figure 14. Restriction screening. 1 % agarose gel with plasmid-2352, plasmid-1800, plasmid-780, plasmid-120, plasmid+599, and pGL3-Basic Vector clones digested with *SalI*. DNA ladder (M).

4.2.3 SCREENING BY STANDARD POLYMERASE CHAIN REACTION

At this point only the sizes of the plasmids had been verified. Yet, it was crucial to determine the presence of correct inserts and the orientation of these. Due to repeated negative results using the commercial designed primers, four new vector- and insert-specific primers were designed and used in standard PCR (Section 3.2 for primer design). As seen on Figure 15 and Appendix C it was possible with these new primers to verify the presence and proper orientation of the inserts in all the isolated plasmid samples. Both a distal and a proximal region of the VEGF promoter were amplified from plasmid-2352. For plasmid-1800, plasmid-780, plasmid-120, and plasmid+599 the proximal part of VEGF promoter was amplified. A fragment of pGL3-Basic Vector was amplified using the vector-specific primers. All samples were successfully amplified and the sizes of the fragments had the expected length, as determined by comparison with size markers, confirming the presence of interest in proper orientation.

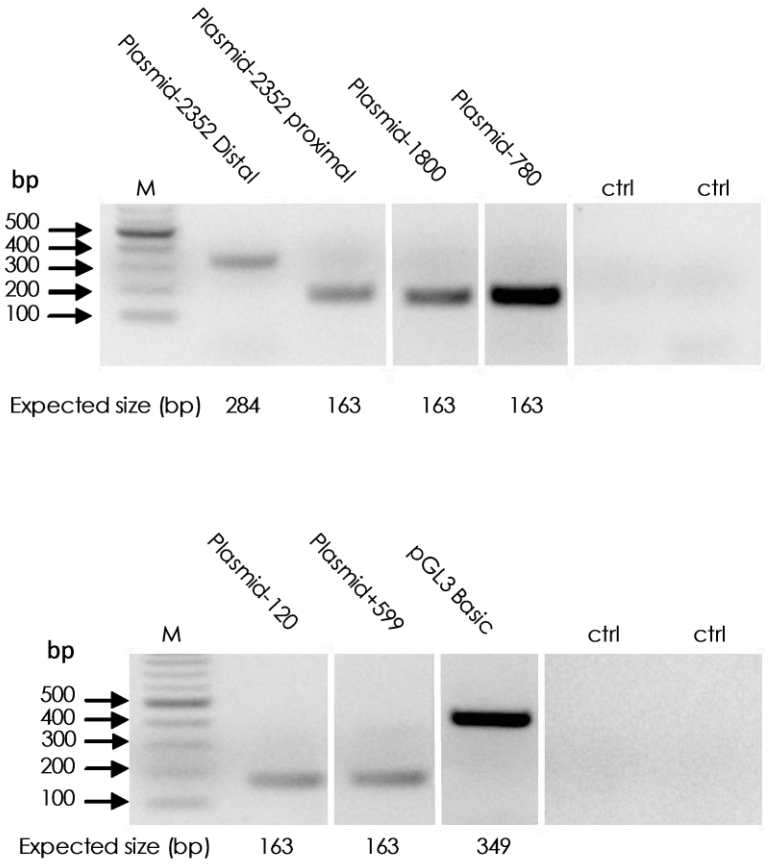

Figure 15. Screening for insert by standard polymerase chain reaction. Representative 2 % agarose gels for inserts in plasmid-2352, plasmid-1800, plasmid-780, plasmid-120, plasmid+599, and pGL3-Basic Vector. To view the complete gels, see Appendix C. DNA ladder (M), negative control (ctrl).

4.2.4 SEQUENCING

Two clones of each plasmid construct were sent away for sequencing with the new designed vector-specific primers. The sequencing results confirmed that the inserts were the VEGF promoter sequence (Figure 16). At position -114, -150, and -458 base-pair substitutions have occurred. Based upon the chromatogram quality and the fact that these mutations were present in all samples supported the hypothesis that these are actual alterations and not errors in sequencing. All three single-base substitutions occurred in plasmid-2352, plasmid-1800, and plasmid-780. In plasmid-120 the adenine for guanine substitution occurred at absolute proximal part of the sequence.

In plasmid-1800 additional mutations have occurred near the estrogen response element; at position -1448 and -1555 single-base substitutions and a deletion mutation of 18 base pairs at position -1510 to -1528. There are no signs in the chromatogram of errors at these locations and likewise these mutations were presents in both samples sequenced (Figure 16B).

Please notice that none of the mutations found during sequencing were located directly in the evidenced response elements.

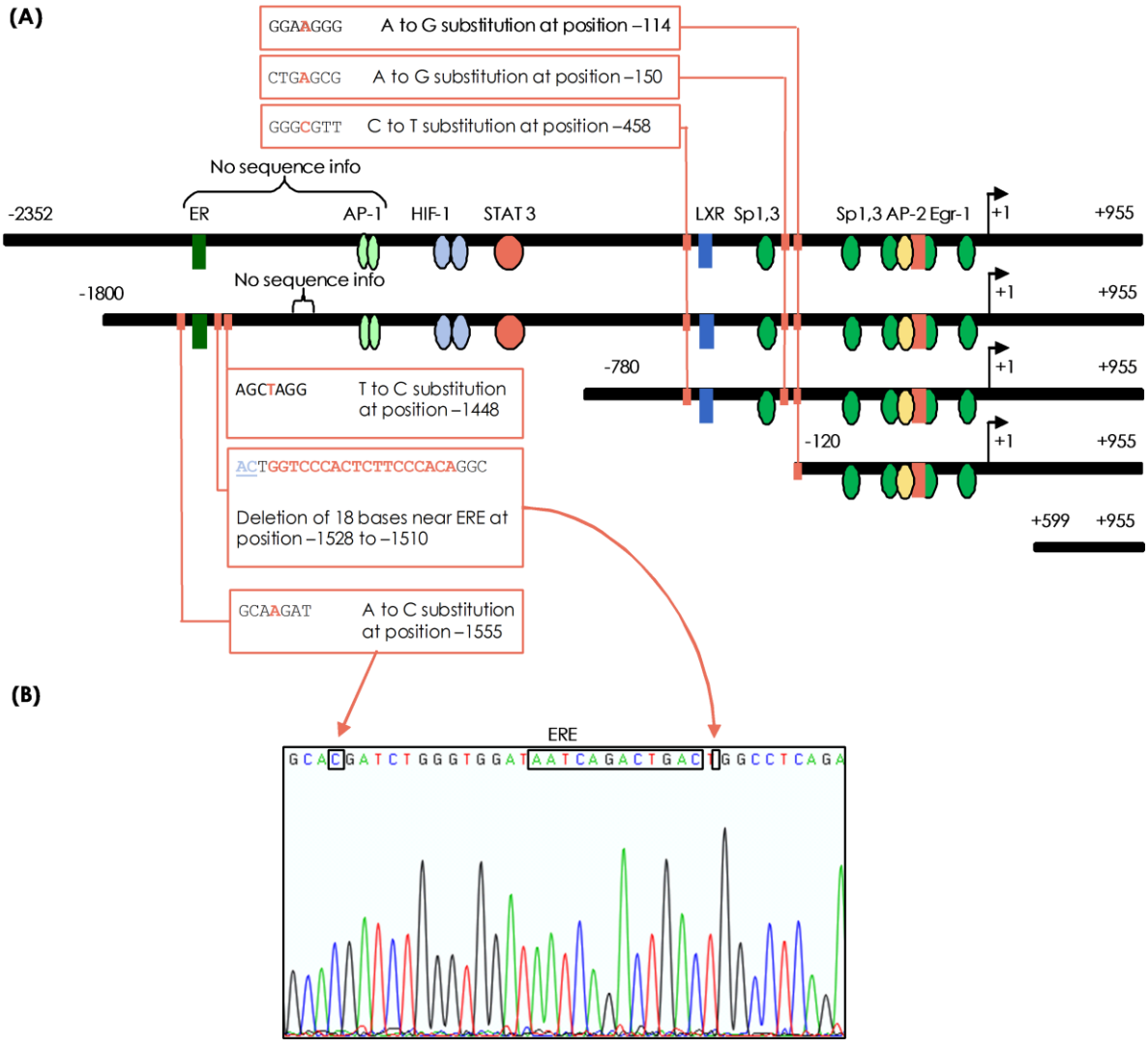

Figure 16. Schematic illustration of sequencing results. (A) The five deletion mutants were sequenced and several mutations were detected (red boxes). The location of each mutation (red) is indicated on the promoter in comparison to the location of putative binding sites for transcription factors (yellow). A fragment of plasmid-2352 and plasmid-1800 were not sequenced due to errors in sequencing reaction. (B) A representative segment of the sequencing data, showing the quality of chromatograms. Estrogen response element (ERE), Estrogen receptor (ER), activator protein-1 (AP-1), hypoxia inducible factor 1 (HIF-1), signal transducer and activator of transcription 3 (STAT3), liver X receptor (LXR), specificity protein 1 and 3 (Sp1,3), activator protein-2a (AP-2), early growth response protein 1 (Egr-1).

4.3 OPTIMIZATION OF TRANSIENT TRANSFECTION OF ADIPOSE-DERIVED STEM CELLS

One of the objectives was to determine the most suitable transient transfection method for the ASCs for the subsequent analysis of the VEGF promoter activity. This was achieved by testing three different transfection techniques; lipofection, polyfection, and electroporation.

4.3.1 TRYPSIN AND ALTERNATIVES

Prior to each transfection it was necessary to detach the ASCs from the culture flask to achieve cells in suspension. The normally procedure involves trypsin and EDTA, as described in section 3.1. However, due to the objective to assess the effect of trypsin, it was desirable to find alternatives to trypsin. Therefore, a preliminary cell detachment experiment with trypsin, Dispase II, and a non-enzymatic Cell Dissociation Solution was conducted. Following detachment the relative expression of VEGF was measured by real-time reverse transcriptase PCR. Trypsin increased the VEGF expression 2.4 ± 0.00 fold compared to ASCs not exposed to trypsin. Likewise, both Dispase II and Cell Dissociation Solution affected the VEGF expression 2.5 ± 0.02 and 4.3 ± 1.31 fold, respectively (Figure 17). Based on these observations the following experiments were carried out using normal trypsinization procedure for cell detachment prior to transfection.

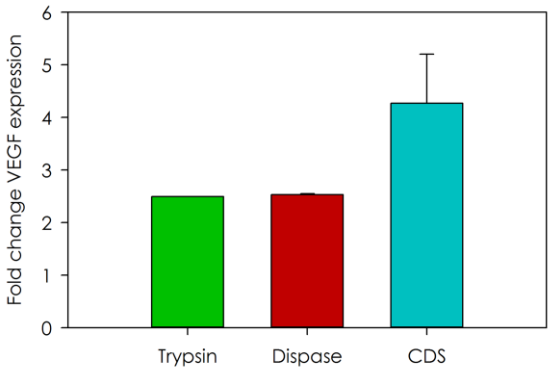

Figure 17. Fold change of vascular endothelial growth factor (VEGF) in adipose-derived stem cells 6 h after detachment by either trypsin, dispase, or Cell Dissociation Solution (CDS). Determined by real-time reverse transcriptase polymerase chain reaction and normalized to the expression of the reference genes; PPIA and YWHAZ. Data are shown as the mean ratio of a control group + S.E. of duplicates.

4.3.2 LIPOFECTAMINE 2000

Lipofectamine® 2000 Tranfection Reagent is a commercial available transfection kit that employs cationic lipids to introduce DNA into cells.

In a preliminary experiment different cell densities and evaluation time points were compared. The use of $15.6*10^3$ cells/cm^2 seemed preferable and assessment of transfection efficiency was best 48 h post transfection rather than 24 h post transfection (data not shown). Other experiments were conducted comparing the transfection efficiency of adherent ASCs and ASCs in suspension. In general, the transfection efficiencies were < 1 %,

when the ASCs were adherent during transfection. In comparison, the transfection efficiency was 7 % when transfecting the cells in suspension. However, as seen on the images on Figure 18, this method resulted in high toxicity and changed morphology of the ASCs compared to the control. Thus, the final optimization experiment was executed on adherent ASCs. To determine the optimal DNA:Lipid ratio, a range of 0.2 – 0.8 µg DNA/rxn and 0.4-1.6 µL Lipofactamine/rxn were tested. None of these conditions were optimal for transfecting ACS, as only few cells were transfected.

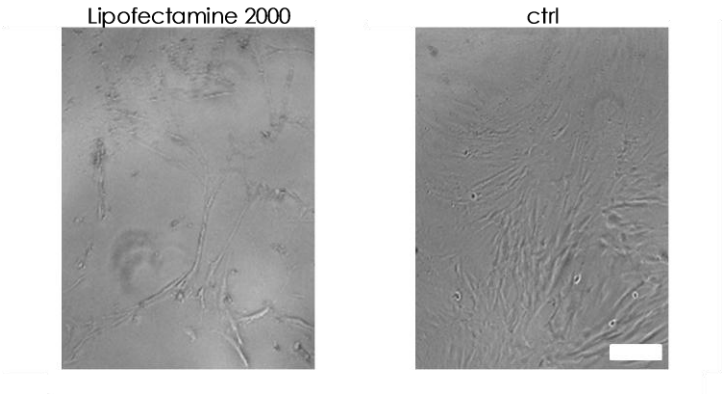

Figure 18. Lipofection of adipose-derived stem cells. Representative phase contrast micrographs 48 h following transfection of cells in suspension using Lipofectamine 2000 and their corresponding control (ctrl). Scale bar denotes 200 µm.

4.3.3 XFECT STEM

Xfect™ Adult Stem Cell Transfection Reagent is a commercial available transfection kit that employs polymers to introduce DNA into adult stem cells.

A comparison of transfecting ASCs in suspension and adherent ASCs was conducted. With a transfection efficiency of approximately 10 %, differences in either transfection efficiency or cell recovery were observed between methods (data not showed). Therefore, the following experiment was conducted using adherent cells.

To determine the most optimal DNA:Polymer ratio, a range of 0.2 – 0.8 µg DNA/rxn and 0.08 - 0.32 µL Xfect polymer/rxn were tested. The lower the amount of transfection mixture the cells were exposed to, the higher was the cell recovery (Figure 19A). In general, extensive exposure of ASCs to the polymer resulted in a significant lower cell recovery ($p < 0.001$). When using 0.32 µL polymer, the number of cells were reduced to 52.7 ± 3.8 % compared to the group of untreated cells. Furthermore, the amount of polymer used per reaction significantly affected the transfection efficiency ($p < 0.001$) (Figure 19B). The highest transfection efficiency was 10.3 ± 0.2 % and was reached using 0.32 µL polymer and 0.8 µg DNA per reaction mixture. However, variations in the amounts of DNA per reaction did not result in any significant difference in the mean values among cell recovery or transfection efficiency.

In summary, the amount of polymer added to the reaction affected both cell recovery and transfection efficiency, whereas the amount of DNA had no effect on these.

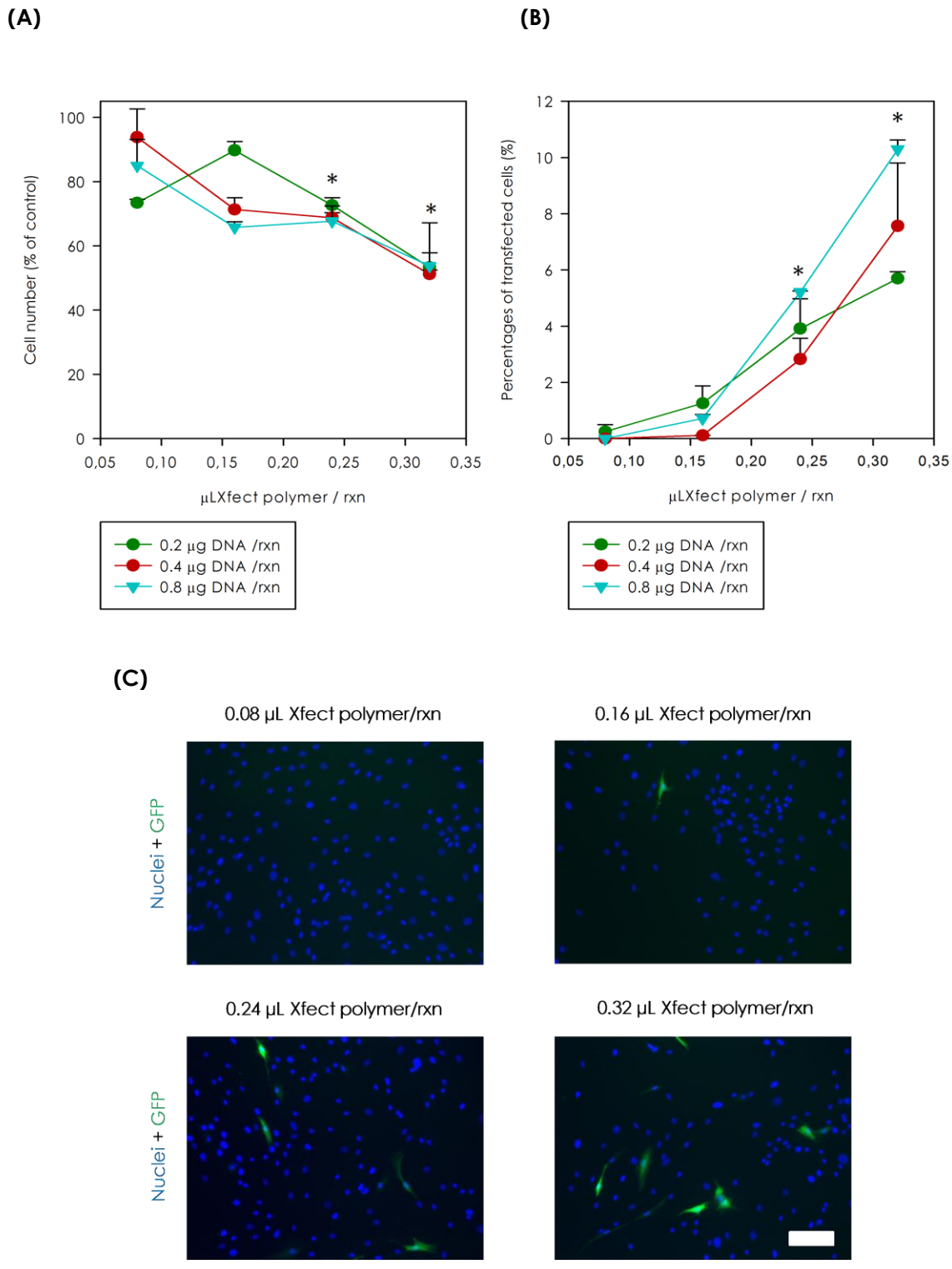

Figure 19. Xfect transfection optimization. (A) Cell recovery 48 h post transfection. (B) Transfection efficiency. (C) Representative fluorescent micrographs of adipose-derived stem cells following transfection. Successesfully transfected cells expressed green fluorescent protein (GFP, green). Cell nuclei were stained with Hoechst 33342 nuclear stain (blue). Scale bar denotes 200 µm. Data are shown as mean + S.E. of duplicates. *$p < 0.05$.

4.3.4 ELECTROPORATION

Electroporation is a transfection method where the cells are exposed to electric pulse causing membrane pores to open and DNA to enter.

By changing several variables the most optimal electroporation condition was determined. In the first experiment, the ASCs were electroporated with different electric parameters. Combinations of voltage between 200 V and 400 V and capacitance between 500 µF and 1,000 µF were used. The time constant varied between 14 msec and 32 msec, while the resistance was maintained at 20 Ω. The density of nuclei declined when the voltage were increased, which indicated lower cell recovery. Furthermore, the duration of the pulse affected cell recovery prominently (Figure 20A and 21). As a result, the combination of 400 V and 1,000 µF led to < 1 % survival, whereas the more gentle treatment with 200 V and 500 µF resulted in 78 % survival. Nevertheless, the transfection efficiency increased with higher voltage and capacitance (Figure 20B and 21)). Application of 300 V and 1,000 µF was determined, as the most optimal parameters because it resulted in the best combination of 9 % transfection efficiency and 55 % cell recovery.

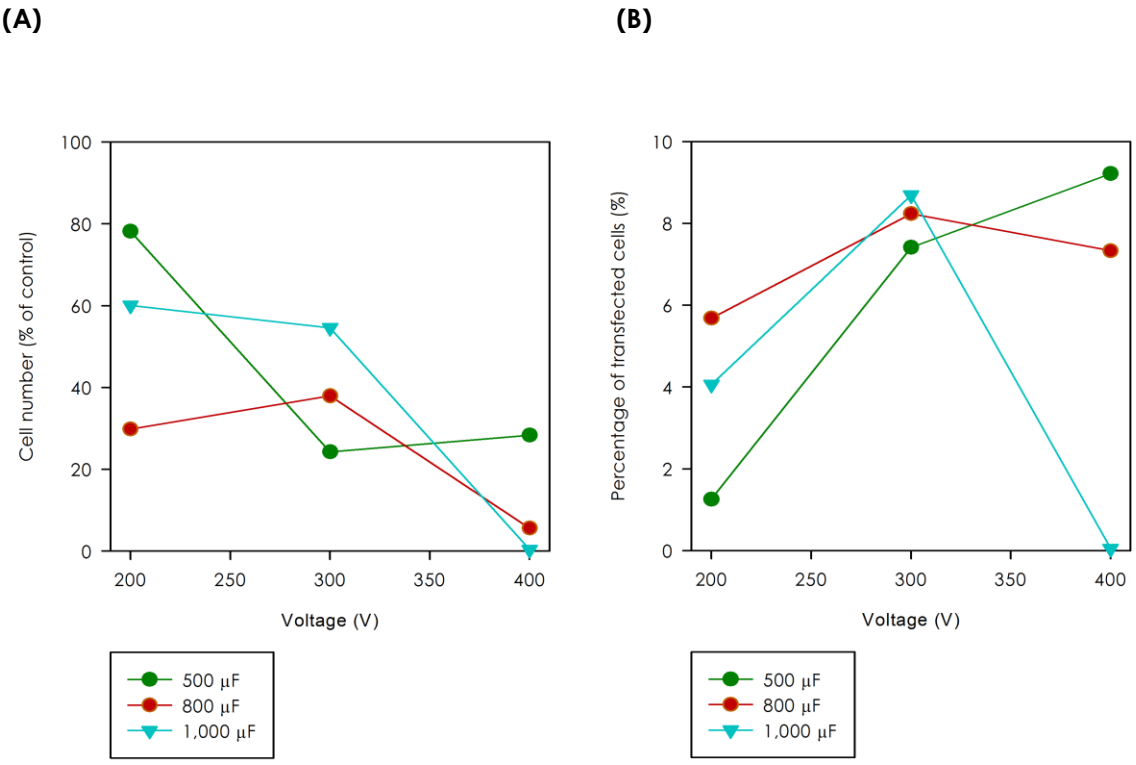

Figure 20. Optimization of electric settings. (A) Cell recovery and (B) transfection efficiency.

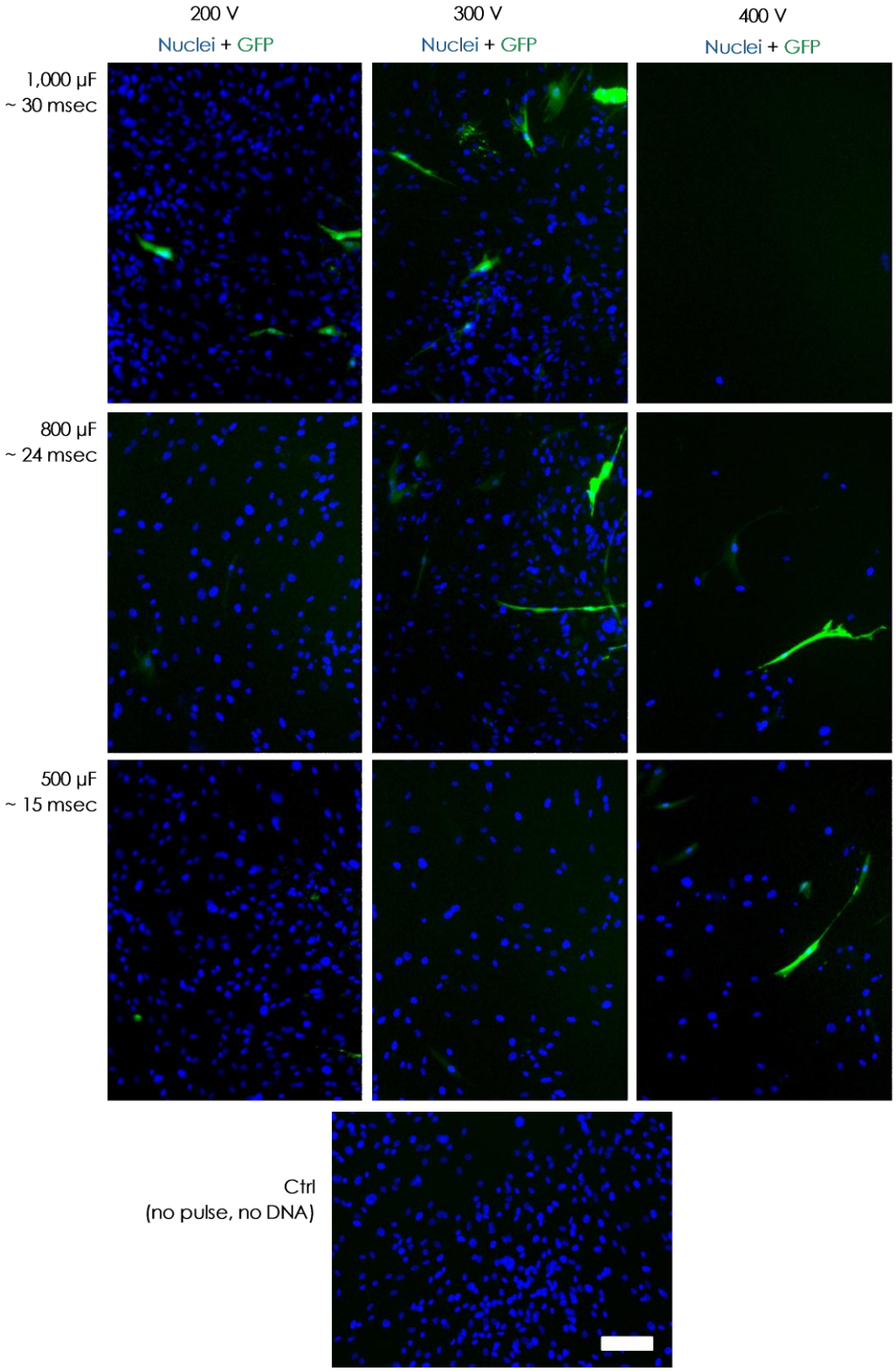

Figure 21. Representative fluorescent micrographs of adipose-derived stem cells following electroporation. Successfully transfected cells express green fluorescent protein (GFP, green). Cells are stained Hoechst 33342 nuclear stain (blue). Negative control (ctrl). Scale bar denotes 200 µm.

Next, the most optimal amount of DNA used for transfection was determined. While maintaining the most optimal electric parameters, the DNA content in the electroporation cuvette was increased from 10 µg up to 60 µg. When increasing the DNA concentration, the cell recovery was observed to decrease while the transfection efficiency increased (Figure 22A). Additionally, the mean intensity of fluorescence of the transfected cells was significantly higher when using 60 µg DNA than the lower amounts of DNA ($p < 0.001$) (Figure 22B). Since there could not be detected a significant difference in transfection efficiencies using 10, 20, or 30 µg DNA, 10 µg were used in the following experiments. Combining these result provided the optimal electroporation conditions of 300 V, 1,000 µF, and 10 µg DNA.

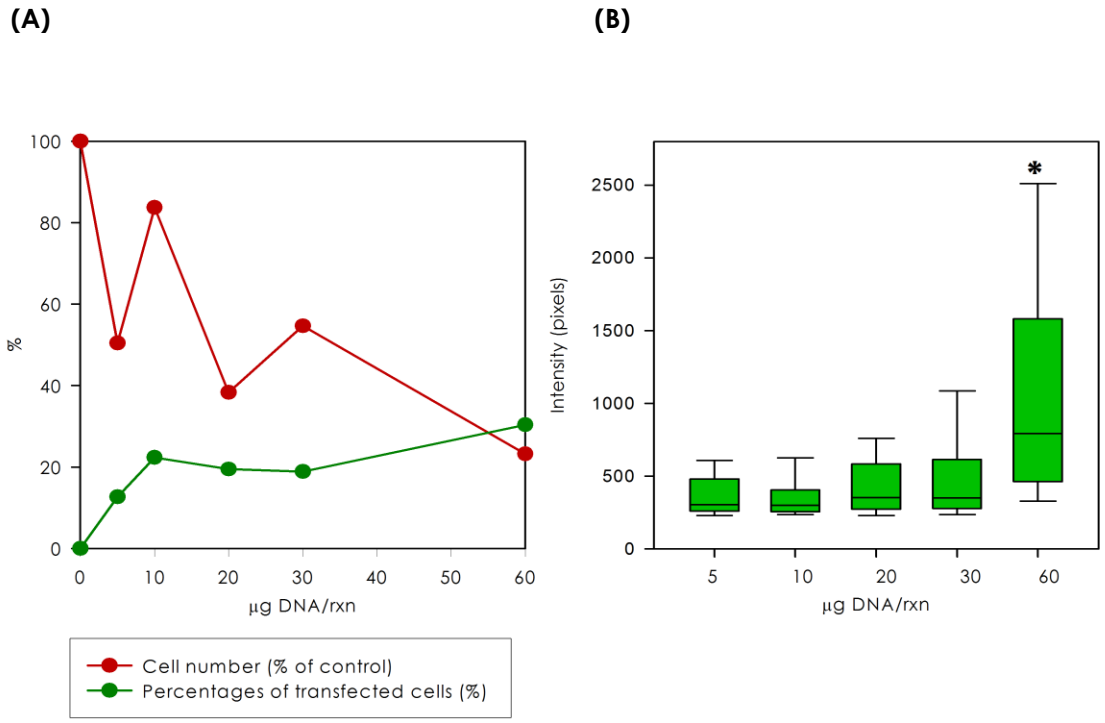

Figure 22. The effect of various amounts of DNA in electroporation reaction. (A) Cell recovery and transfection efficiency. (B) Box plot of the mean intensity of green fluorescent protein expression per cell; 5 µg (n = 47), 10 µg (n = 58), 20 µg (n = 36), 30 µg (n = 41), 60 µg (n = 78). *$p < 0.05$.

4.4 OPTIMIZATION OF LUCIFERASE ACTIVITY ASSAY

Prior to analyzing trypsin-induced VEGF promoter activity, the experimental setup was verified by several independent experiments.

4.4.1 DETERMINATION OF PLATE AND TIME OF MEASUREMENT

The ASCs were electroporated with pGL3-Control Vector and seeded with different densities. The luciferase activity were assessed both 24 h and 48 h post electroporation (Figure 23). The levels of luciferase activity were significantly higher at 24 h compared to 48 h ($p < 0.001$).

To determine the most appropriate type of plate to use during the assessment of luminescence levels with the luminometer, a comparison of a transparent plate and a white plate were conducted (Figure 23). The transparent plates were the plates used for cell culture. Therefore, luminescence was assessed directly in these culture plates. When using the white plate, the lysate were transferred from the culture plate prior to assessment. The white plate resulted in significant higher mean values when compared to transparent plate ($p < 0.001$). Consequently, all the following experiments were conducted using white plates.

There was a tendency for the level of luciferase activity to increase with higher cell densities. This observation was general for all the luciferase activity assays.

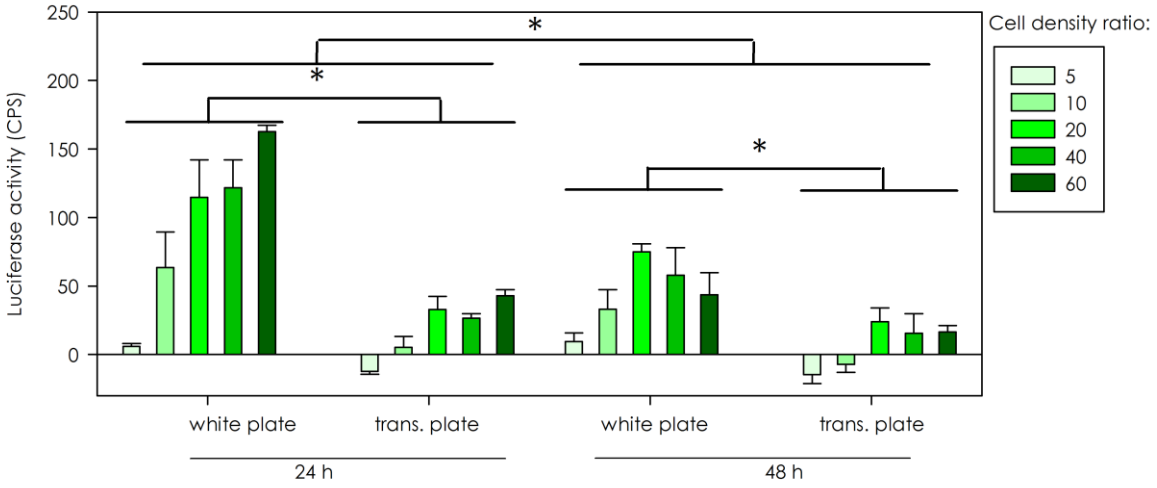

Figure 23. Optimization of luciferase activity assay. The luciferase activity was assessed in adipose-derived stem cells electroporated with pGL3-Control Vector and compared in white versus transparent (trans.) plates. Counts per second (CPS). Data are shown as mean ± S.E. of triplicates. * $p < 0.05$.

4.4.2 TRYPSIN INDUCED LUCIFERASE ACTIVITY

In order to control whether or not exposure of trypsin could induce measurable levels of luciferase activity, ASCs were electroporated with plasmid-2352. 18 h before assessment of luciferase activity, the cells were shortly exposed to trypsin. As positive control, a group of electroporated cells were incubated under hypoxic conditions 24 h prior to assessment. Furthermore, a group functioned as negative control and was not exposed to any known activators of the VEGF promoter.

Comparing the mean values, the trypsin exposed groups were significant higher than both the hypoxia groups ($p = 0.017$) and the negative control groups ($p = 0.007$) (Figure 24). There were not detected any significant increases in luciferase activity, when the cells were exposed to hypoxia.

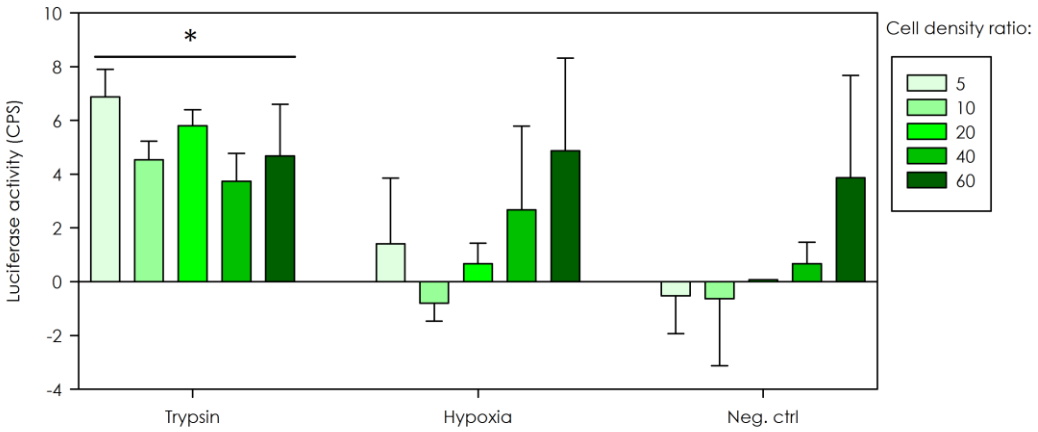

Figure 24. Optimization of luciferase activity assay. The luciferase activity was assessed in adipose-derived stem cells electroporated with plasmid-2532 and treated with trypsin, hypoxia, or no treatment. Counts per second (CPS). Data are shown as mean ± S.E. of triplicates. *$p < 0.05$.

4.4.3 COMPARISON WITH NEW LUCIFERASE REAGENTS

The Steady Glo reagents used so far belonged to a batch, which had passed the expiration date. Therefore, a new batch was purchased and compared to the old batch. Furthermore, the variables; time of measurement and cell density, were included in the same experiment. The results revealed that the luciferase activities of the new batch were not significant different from those of the old batch (Figure 25). There was significant difference (p=0.003) between the mean values of the different time points of measurements. The luciferase

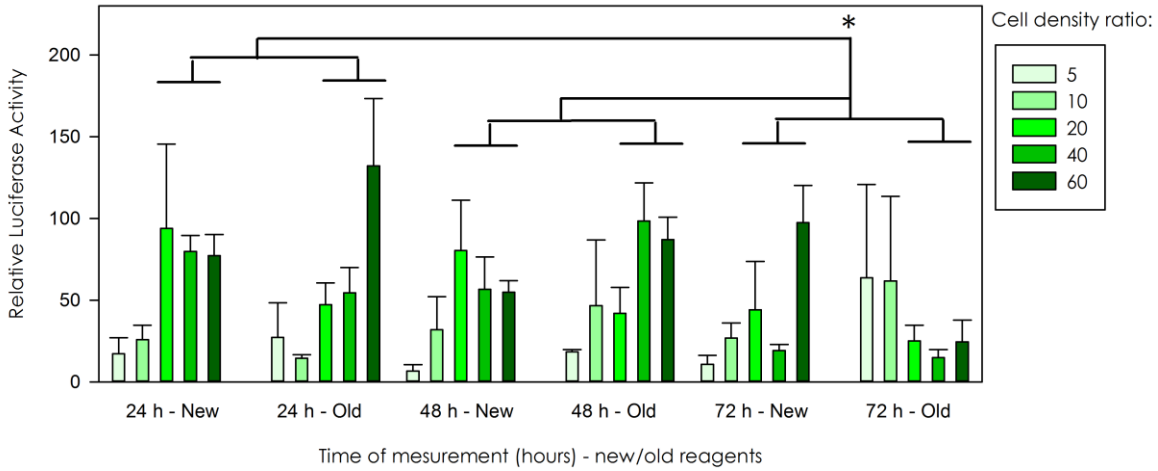

Figure 25. Optimization of luciferase activity assay. The luciferase activity was assessed in adipose-derived stem cells electroporated with pGL3 Control Vector and the luciferase activity were assessed using two different Steady Glo batches (old versus new). Counts per second (CPS). Data are shown as mean ± S.E. of triplicates. *$p < 0.05$.

activity was significantly decreased when the assessments were performed 72 h post electroporation compared to 24 h (p=0.039) and 48 h (p = 0.028), respectively. No significant difference was detected between 24 h and 48 h. The two groups with the lowest cell densities showed relatively low values and no significant differences between time levels or batches could be detected. In general, higher cell densities seemed to result in most reproducible outcomes.

5 DISCUSSION

5.1 PLASMID CONSTRUCTION AND VERIFICATION

To conduct a valid analysis of the transcriptional response of trypsin, the experimental setup involved isolation of the human VEGF promoter and cloning into a proper vector. The vector of choice was pGL3 Luciferase Vectors, which contains a modified coding region for firefly luciferase allowing detection of the promoter activity. However, before proceeding it was crucial to verify the exact VEGF promoter sequence and correct insertion into the vector.

Genomic DNA was successfully extracted from the ASCs at low passage with measurable DNA yield and acceptable purity in the final elution. Using promoter specific primers, the full-length promoter were amplified and cloned into the vector. However, the length of the inserts was not as expected when amplified during colony PCR. Furthermore, restriction screening could not confirm the presence of the VEGF promoter (results not shown). Why that was not possible, we cannot explain.

Thus, we proceeded with the plasmid constructs from USA. Though, before continuing, it was essential to verify the presence and proper orientation of the VEGF promoter fragments. With restriction screening and the use of both insert- and vector-specific primers this verification was completed. Finally, the exact sequence was determined of each deletion mutant. Several mutations were detected. However, none of these were located within the evidenced binding sites for transcription factors (Pages and Pouyssegur, 2005). A segment of plasmid-2352 and plasmid-1800 were not sequenced due to error in the sequence reaction with Insert distal forward primer. Despite the observed alteration in the sequence, it was decided to continue the process with these plasmid construct, as no mutations were present in regions hypothesized to be important for trypsin-induced promoter activity.

5.2 DETACHMENT OF CELLS PRIOR TO TRANSFECTION

Prior to transfection of the ASCs with plasmids, it was necessary to detach the cells from the culture flask to achieve cells in suspension. The standard procedure involves trypsin and EDTA. However, due to the objective to assess the transcriptional effect of trypsin after transient transfection, it was desirable to find an alternative way for cell detachment prior to the transfection. Hence, Dispase II and Cell Dissociation Solution were tested for their effect on the VEGF expression in ASCs. However, none of these were found more suitable than trypsin and it was decided to carry on using the normal trypsinization procedure prior to transfection. In addition, later it was tested whether or not trypsinization prior to electroporation would conflict with the trypsin-induced luciferase activity. The results indicated that measurement of the luciferase activity 36 h post electroporation was not influenced by the foregoing trypsinization. Based on these observations, the subsequent experiments were carried out using normal trypsinization procedure for cell detachment prior to transfection.

5.3 OPTIMIZATION OF TRANSFECTION METHOD

Some cell lines appear to be more difficult to transfect than others (Zhang et al., 2007). Mesenchymal stem cells seem to be a challenging cell type to transfect efficiently (Helledie et al., 2008). Therefore, one might wonder why this study used the ASCs for transfection rather than another and more easily transfected cell type. However, the intracellular signaling pathways by which VEGF expression is induced are cell type-specific (Berra et al., 2000, Kawaguchi et al., 2004, Coughlin, 2000, Dutra-Oliveira et al., 2012) and consequently it was necessary to use the ASCs to conduct a valid experiment for analyzing the trypsin response in the ASCs. Therefore, to find the most suitable method for transient transfection to introduce the VEGF promoter-containing vectors into ASCs, three different techniques were tested. Lipofectamine 2000 is a cationic liposome-based reagent which introduces plasmid DNA into cells with a simple protocol (Dalby et al., 2004). Xfect is a system that uses polymers to transfect cells. It has been designed to meet requirements of stem cell transfection and has low toxicity (Clontech). Finally, electroporation is a simple method where the cells are exposed to an electric pulse causing membrane pores to open and entry of DNA (Heiser, 2000). To optimize the transfection efficiency and subsequent cell recovery for each transfection method, several parameters were tested.

A transfection efficiency below 1 % and high toxicity was observed when using Lipofectamine 2000. According to the literature the outcome depends on experimental variables such as cell density, liposome and DNA concentrations, and complex formation time (Dalby et al., 2004). All of these parameters were tested in this project, but no improvement was observed. However, the transfection efficiency was significantly increased, when the transfection was performed with cells in suspension compared to adherent cells. Yet, during this treatment most of the ASCs were killed and the morphology was significantly changed. Other researchers have observed similar outcomes using Lipofectamine 2000 for mesenchymal stem cells (Helledie et al., 2008).

The use Xfect Stem reagents led to a relatively higher transfection efficiency and caused less cytotoxicity than lipofection. According to the manufactures, the transfection efficiency in ASCs could reach 99 %. However, after several optimization experiment this level were far from reached.

Placing cells in an electric field causes cell lethality, but the harsh treatment also destabilizes the cell membrane and drives higher levels of DNA into the cells. According to the literature the most optimal electroporation condition is the one that kill approximately 40-80 % of the cells (Heiser, 2000). Based on this assumption, the most optimal electric settings were observed to be 300 V and 1,000 µF. Additionally, extensive DNA exposure was observed to be toxic, despite increased mean transgene expression per cell and transfection efficiency. Hence, the most optimal amount of DNA was found to be 10 µg DNA per sample. In accordance with these observations, other studies have demonstrated comparable transfection efficiencies with similar parameters in mesenchymal stem cells (Helledie et al., 2008, Ferreira et al., 2008).

Various techniques have been described for transfection of mammalian cells (Kim and Eberwine, 2010). In general, the requirements for a useful transfection are that it is applied using a reproducible and reliable protocol resulting in high transfection efficiency, without

extensive cytotoxicity. Based upon the obtained results, neither lipid-based nor polymer-based methods were found satisfying for transient transfection of ASCs. Therefore, the electroporation was found superior and the method of choice for further proceeding.

5.4 LUCIFERASE ACTIVITY ASSAY

The cloned pGL3 vectors were constructed in such a manner that the measurable luciferase expression was dependent of the VEGF promoter activity. Thereby, it was possible to assess the promoter activity by the luciferase activity. To design the best experimental setup for analyzing trypsin-induced promoter activity, it was crucial to optimize several parameters of the luciferase activity assay. Parameters such as cell density, plate type, time point of measurement, and time point of trypsin exposure were evaluated in the current work.

The primary difference between the white and transparent well-plates is their reflective properties and the negative results of the transparent well-plate might be caused by background and crosstalk. In contrast, the white plates reflect light and maximize light signal output. Thus, the white well-plate rather than the transparent well-plate were found most appropriate for the luminescence measurements.

Two independent experiments indicated that the luminescence signal declined over time. To determine the optimal time point of luciferase activity, it was a balance between having time enough for sufficient effect of trypsin exposure (Rasmussen et al., 2011) and still reach measurable signals. Therefore, 36 h was selected as the time point of luminescence measurements and trypsin exposure 18 h prior to measurement.

The experiment with plasmid-2352 containing the full-length VEGF promoter indicated that trypsin exposure for 5 min at 37 °C 18 h prior to measurement was enough to detect a significant increase in the luciferase activity compared to the control group. Furthermore, exposing a group of transfected cells to hypoxia 24 h prior to luminescence measurement did not affect the signal compared to the control group. Thereby, the hypoxia group, which was thought to function as a positive control based on evidence of hypoxia-induced VEGF expression (Forsythe et al., 1996, Josko and Mazurek, 2004, Rasmussen et al., 2011), did not meet the hypothesized expectations.

In summary, the luciferase activity assay using Steady-Glo was suitable for measuring the trypsin-induced VEGF promoter activity in ASCs.

5.5 SUMMERY AND PERSPECTIVES

Based upon the optimized parameters determined in this project, a valid analysis of the trypsin-induced VEGF promoter activity could be performed. The experimental setup should include electroporation (300 V and 1,000 µF) of ASCs with 10 µg of each plasmid construct. The pGL3-Basic Vector would function as a negative control and pGL3-Control Vector as positive control. The electroporated cells should be seeded in a 96-well plate in a minimum of triplicates. 18 h post electroporation, the cells should shortly be exposed to trypsin. In this process media containing debris and dead cells would be exchanged with fresh media. For each plasmid construct, it is crucial to include a group of non-trypsin exposed cells serving as

control. 18 h after trypsin exposure, the luciferase activity could be measured as an effect of trypsin-induced promoter activity. It is hypothesized that such an experiment could reveal the localization of *cis*-acting elements in the promoter required for transcriptional response of trypsin. Moreover, site-directed mutagenesis PCR and electrophoretic mobility shift assays would be proper methods for investigating specific transcription factor binding sites and detection of involved transcription factors.

6 CONCLUSION

The current work has determined the most optimal setup and conditions for analyzing trypsin-induced VEGF-promoter activity in ASCs. VEGF promoter deletion mutants cloned into pGL3 vectors were verified. Furthermore, optimized electroporation conditions for the ASCs were found to be 300V, 1,000 µF, and 10 µg DNA per sample. With these conditions electroporation was found superior to both lipofection and polyfection. Finally, the luciferase activity assay was designed and found suitable for measuring the trypsin-induced VEGF promoter activity in ASCs. However, to identify transcription factors and promoter regions involved in the trypsin-dependent VEGF expression, further investigations are needed.

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8 APPENDIX A

8.1 VEGF PROMOTER SEQUENCE

Explanations:
G: Transcription initiation site
Primer binding sites:
- VEGF promoter forward primer
- VEGF promoter revarse primer
- Insert-2352 reverse primer
- Insert proximal forward primer
- Insert distal forward primer
- Insert distal reverse primer

Restriction enzyme recognition site
- *KpnI*
- *NheI*

VEGF promoter (-2352 - +955)

>gi|224589818:43735594-43738901 Homo sapiens chromosome 6, GRCh37.p5 Primary Assembly
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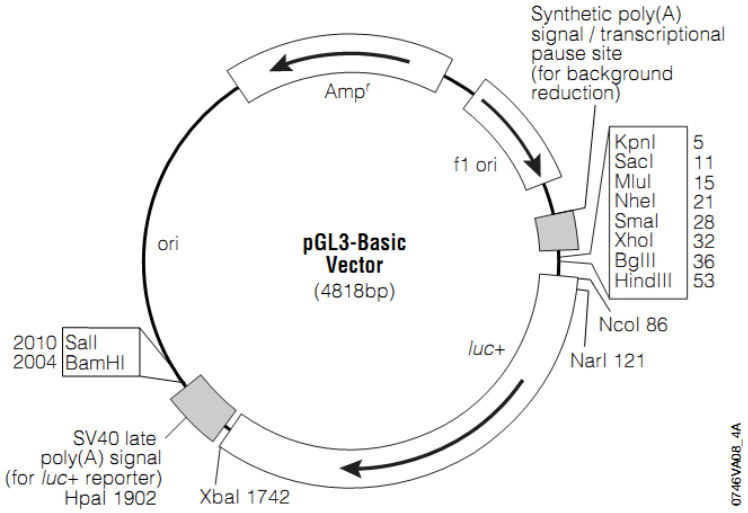
8.2 PGL3-BASIC VECTOR INFORMATION



pGL3-Basic Vector circle map

luc+, cDNA encoding the modified firefly luciferase; Ampr, gene conferring ampicillin resistance in E. coli; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in E. coli. Arrows within luc+ and the Ampr gene indicate the direction of transcription; the arrow in the f1 ori indicates the direction of ssDNA strand synthesis.

Manufactor: Promega

Explanations:
Primer binding sites:
pGL3 Basic forward primer binding site
pGL3 Basic reverse primer binding site
pGL3 forward primer D1
pGL3 reverse primer D1
Restriction enzyme recognition site:
SalI

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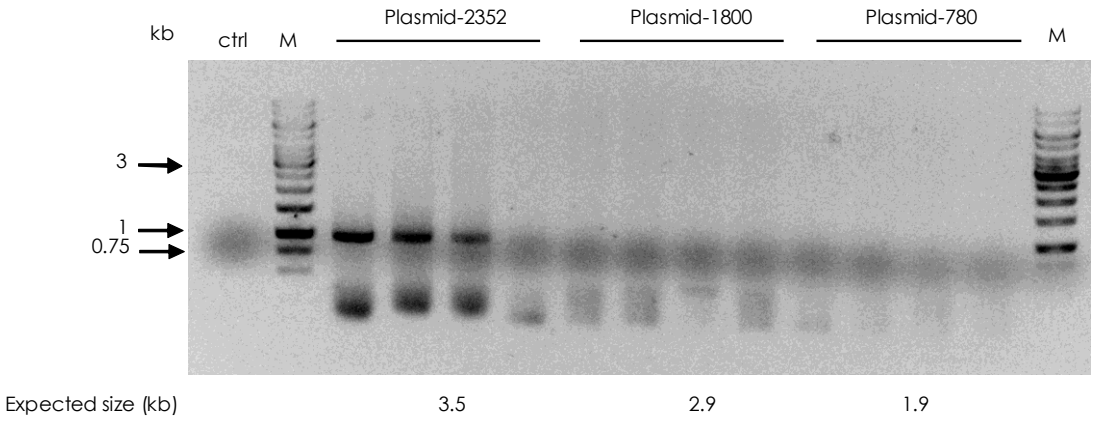
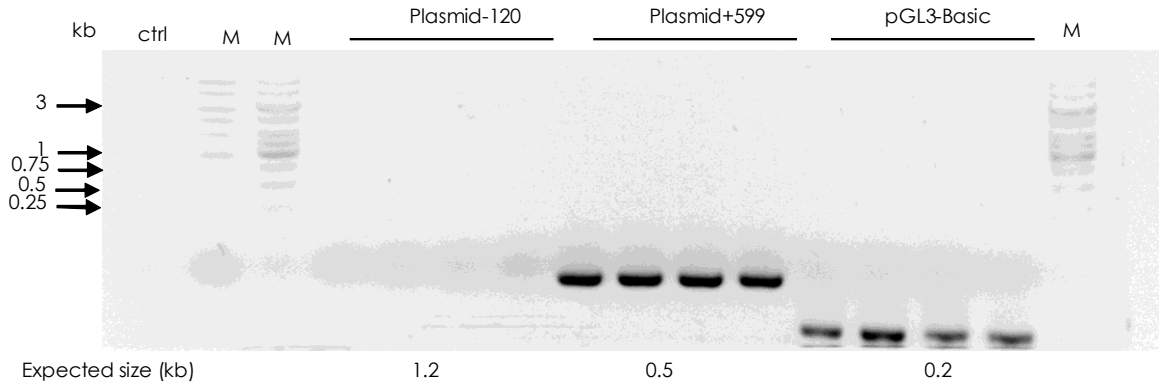
9 APPENDIX B

1 % agarose gel from colony polymerase chain reaction with putative plasmid-2352, -1800, -780, -120, +599, and pGL3-Basic Vector all clones. DNA ladder (M), negative control (ctrl).

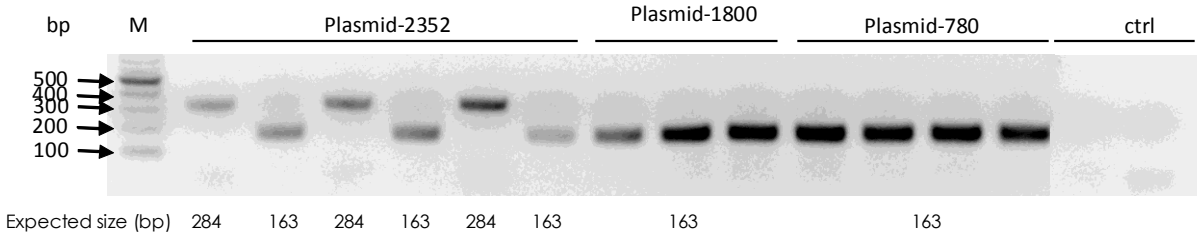




10 APPENDIX C

Screening for insert by standard polymerase chain reaction: 2 % agarose gels for inserts in plasmid-2352, plasmid-1800, plasmid-780, plasmid-120, plasmid+599, and pGL3-Basic Vector. DNA ladder (M), negative control (ctrl).

Due to the presence of an extra band in the samples for plasmid-120 and plasmid-+559, it was suspected that contamination from neighbor well had occur. Therefore, these samples were repeated and led to satisfying results, as seen below.



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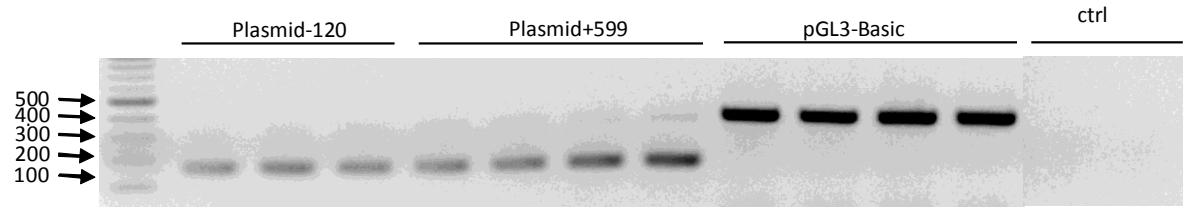

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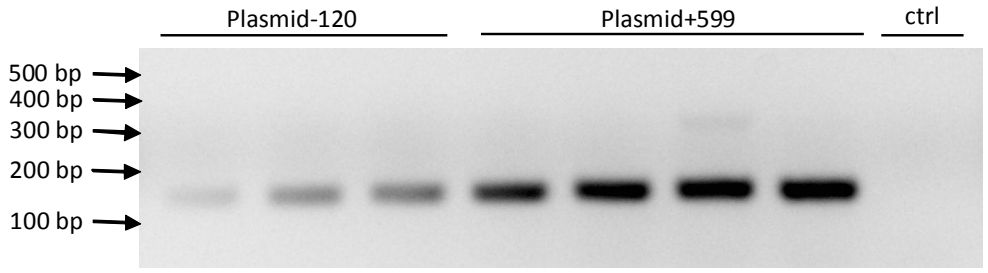

Expected size (bp)	163	163