BioResearch

Assessment of the Anti-angiogenic Effect of VEGFR2 siRNA in Clonetics™ HUVEC using the Lonza 4D-Nucleofector™ System

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1. Abstract

Angiogenesis is a hallmark of most cancers, and is thus an attractive target for the treatment of cancer. One of the easiest screening and target validation strategies for anti-angiogenic target identification involves knocking down targets in Human Umbilical Vein Endothelial Cells (HUVEC) and assessing subsequent effects on tube formation in Corning's Matrigel™ product.

The usage of small interfering RNA (siRNA) is one of the strategies to knock-down RNA, and thereby protein expression within cells. siRNA can be delivered within cells using either chemical transfection or electroporation-based strategies such as the one offered by the Lonza Nucleofector™ Technology.

In the current study we used the Lonza 4D-Nucleofector™ X-Unit to transfect single-donor Clonetics™ HUVEC cultured in EGM™ 2 Endothelial Cell Growth Media. Cells were transfected with siRNA directed against Vascular Endothelial Growth Factor Receptor 2 (VEGFR2). Transfection conditions were fine-tuned using pmaxGFP™ Vector, and it was shown that the Nucleofection process had no deleterious effect on the tube formation potential of HUVEC on Corning's Matrigel™ product.

Efficient knock-down of VEGFR2 protein levels was demonstrated after the transfection of VEGFR2-siRNA. Best knock-down efficiency was observed 24 hours after transfection. VEGFR2-siRNA transfected cells demonstrated significant inhibition of tube formation on Corning's Growth Factor Reduced Matrigel™ product in comparison to control samples. Since the efficient knock-down of the VEGFR2 protein in Clonetics™ HUVEC using the Nucleofector™ Technology can be demonstrated at time points as early as 24 hours after transfection, this is an efficient approach for target identification, validation and screening of siRNA based anti-angiogenesis therapeutics for cancer treatment.

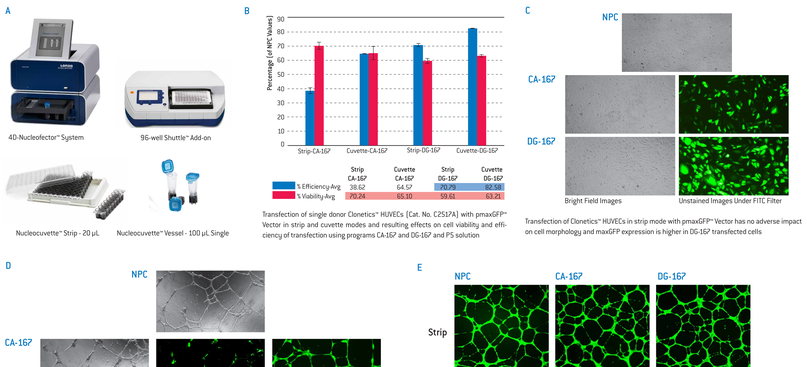
2. Introduction

The key role of angiogenesis in tumor development and cancer metastasis makes inhibition of angiogenesis an attractive strategy to target a number of cancer types (Timar *et. al.*, 2001). VEGF is a key cytokine involved in physiological and pathological angiogenesis. While VEGF mediates its effects through multiple cell surface receptors, VEGFR2 appears to be the major mediator of VEGF-induced proliferation and migration of endothelial cells (Murga *et. al.*, 2005); and endothelial morphogenesis into tube-like structures (Yang *et. al.*, 2001). Thus VEGFR2 based pathway inhibition becomes interesting from a standpoint of cancer therapeutic intervention.

siRNA and shRNA based gene silencing is being increasingly used to identify genes and pathways involved in various cellular processes within mammalian cells and to enable target identification and validation. siRNA based approaches are being utilized in the cancer area, for therapeutic approaches; and to identify and characterize cancer genes involved in specific pathways, disease etiology and the progression of cancer (Guo *et. al.*, 2013).

Screening with siRNA or shRNA involves delivery into cells of interest using various approaches. The most commonly used of these is the lipid-mediated transfection methodology; however many of these reagents are cytotoxic, alter gene expression profiles and induce immunogenic responses. In addition, they demonstrate low efficiency of transfection in primary cells, suspension cell lines, and do not work for non-dividing cells like neurons, due to their dependence on cell division (Zumbansen *et. al.*, 2009). Thus for applications where high transfection efficiencies are essential (e.g. in quantitative evaluation of knock-down efficiencies post siRNA transfection), Nucleofection is the method of choice. (Zeitelhofer *et. al.*, 2007). The 4D-Nucleofector™ System from Lonza (Figure 1) is a transfection system which enables efficient transfection of primary cells and cell lines using a variety of substrates like DNA and siRNA.

In the current study we have used siRNA targeting VEGFR2 as an example to study knockdown of VEGFR2 and subsequent inhibition of tube formation by HUVECs on Growth Factor Reduced Matrigel™ in a 96-well plate format. The same strategy can be used for screening and validating siRNA based inhibitors of the angiogenic process *in vitro* and thus could be of utility in anti-cancer screening strategies.



Transfection of single donor Clonetics™ HUVECs (Cat. No. C2517A) with pmaxGFP™ Vector in strip and cuvette modes and resulting effects on cell viability and efficiency of transfection using programs CA-167 and DG-167 and P5 solution

Transfection of Clonetics™ HUVECs in strip mode with pmaxGFP™ Vector has no adverse impact on cell morphology and maxGFP expression is higher in DG-167 transfected cells

Transfection of Clonetics™ HUVECs with pmaxGFP™ Vector in strip and cuvette modes demonstrates similar degree of tube formation on Matrigel™ suggesting assay scalability

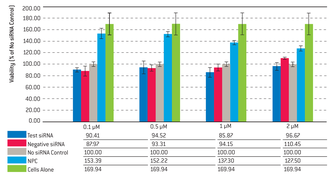
Figure 1. pmaxGFP™ Transfection studies using the Lonza 4D-Nucleofector™ System.
(**A**) The 4D-Nucleofector™ System along with accessories – 20 µL Nucleocuvette™ Strip, 100 µL Nucleocuvette™ Vessel, and the 96-well Shuttle™ Add-On.
(**B**) Fine-tuning of transfection conditions for single donor Clonetics™ HUVECs (Lonza, Cat. No. C2517A) grown in complete EGM™ 2 Media (Lonza, CC-3162) suggests that program DG-167 is more optimal for donor. Briefly, cells (1 x 10^5 cells) were transfected with 0.4 µg pmaxGFP™ Vector using the P5 Nucleofector™ Solution. 2.5 x 10^4 transfected cells were seeded and transfection efficiency was analyzed 24 hours post transfection using flow cytometry. Viability was determined in parallel using the ViaLight™ Plus BioAssay Kit (Lonza, LT07-221). Transfection efficiencies and viabilities were expressed as percentages of untransfected negative control (No Program Control NPC) values. Experiments were run once in duplicate. Percentage viabilities were greater than 60% using both programs but DG-167 demonstrated better transfection efficiency.
(**C**) Transfection of pmaxGFP™ Vector has no significant effect on cell morphology. Expression of maxGFP™ is higher in HUVECs transfected using program DG-167 at 24 hours post-transfection. Images were taken under 5X magnification. Experiments were run once in duplicate.
(**D**) Transfection of pmaxGFP™ Vector has no deleterious effect on tube formation capability of HUVECs on Phenol-Red Free Matrigel™ (Corning, 356237) in EGM™ 2 Media at a 24 hour assay point. Images were taken under bright field and FITC filter and again after staining with Calcein AM (Life Technologies, C3100MP). Tube formation assay was set up in a 96-well plate format and with a seeding density of 25,000 cells per well. All images were taken under 5X magnification. Experiments were run once in duplicate.
(**E**) Tube formation capability of transfected HUVEC cells on Matrigel™ is similar in 20 µL Nucleocuvette™ Strip and 100 µL Nucleocuvette™ Vessel formats, underlining scalability of the assay. Assay set up was similar to Figure 1C and cells were observed after Calcein AM staining. Experiments were run once in duplicate.

3. Conclusion

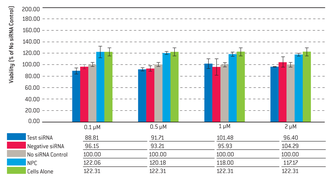
Transfection of HUVEC cells with small interfering RNA against VEGFR2 caused a significant decrease in VEGFR2 protein levels, and discernible impairment of tube formation by HUVECs on Growth Factor Reduced Matrigel™. Optimization was needed to see the effects on tube formation, probably because angiogenesis is an involved process with many pathways acting in concert. However, the advantage of using the 4D-Nucleofector™ System X-Unit, in combination with the 96-well Shuttle™ Device, was the good cell viability post transfection, knockdown at early time points (24 hours), easy screening in a 96-well format and a significant inhibition on tube formation post VEGFR2 siRNA transfection. These factors make the Nucleofector™ Technology an attractive methodology for the anti-angiogenic screening of a large number of siRNAs in a single screen.

A Transfection of Clonetics™ HUVECs with ON-TARGETplus™ siRNA Pools with the 4D-Nucleofector™ X-Unit in combination with the 96-well Shuttle™ Add-on Demonstrates no Cytotoxicity of VEGFR2 siRNA over no siRNA Controls at the siRNA Concentrations and Time Points used in this Assay

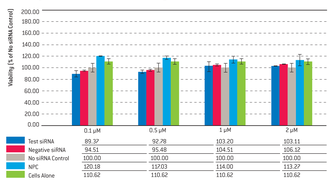
%Viability in Clonetics™ HUVECs Following siRNA Transfection - 24 hour Data



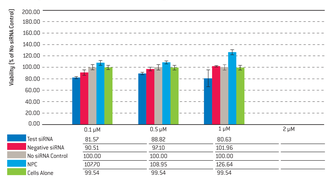
%Viability in Clonetics™ HUVECs Following siRNA Transfection - 48 hour Data



%Viability in Clonetics™ HUVECs Following siRNA Transfection - 72 hour data

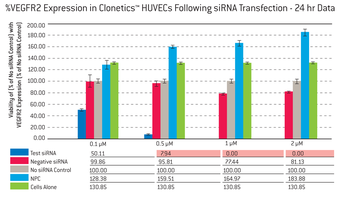


%Viability in Clonetics™ HUVECs Following siRNA Transfection - 96 hour Data

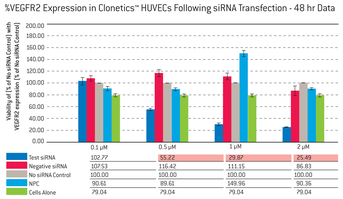


B Transfection of Clonetics™ HUVECs with ON-TARGETplus™ siRNA Pools with the 4D-Nucleofector™ X-Unit in Combination with the 96-well Shuttle™ Add-on Demonstrates Efficient Knock-down of VEGFR2 24 Hours and 48 Hours After Transfection

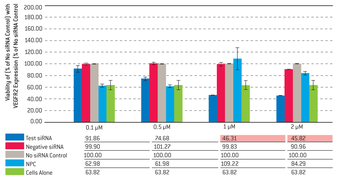
%VEGFR2 Expression in Clonetics™ HUVECs Following siRNA Transfection - 24 hr Data



%VEGFR2 Expression in Clonetics™ HUVECs Following siRNA Transfection - 48 hr Data



%VEGFR2 Expression in Clonetics™ HUVECs Following siRNA Transfection - 72 hour Data



%VEGFR2 Expression in Clonetics™ HUVECs Following siRNA Transfection - 96 hour Data

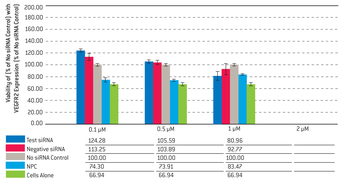


Figure 2: siRNA transfection studies using the 4D-Nucleofector™ System in combination with the 96-well Shuttle™ Add-On. (**A**) Cell viability and (**B**) VEGFR2 levels in Clonetics™ HUVECs following transfection with ON-TARGETplus™ siRNA pools (Dharmacon, Thermo Fisher Scientific Biosciences) and fine-tuned Nucleofection program (DG-167). Briefly, Clonetics™ HUVECs were transfected with various concentrations (0.1-2 µM) of Test (ON-TARGETplus™ Human KDR(3791) siRNA SMARTpool - L-003148-00-0005), Neg (ON-TARGETplus™ Non-targeting Pool - D-001810-10-05) and SC (no siRNA control). NPC (No program controls and cells only) were also run in this assay. Cells were harvested at various time points following transfection (24 – 96 hrs). Cell viability was determined using the ViaLight™ Plus BioAssay Kit (Lonza, LT07-221). Total VEGFR2 assessment, following cell lysis, was done using ELISA (R&D Systems, DYC1780-5, Detection limit - 62 pg/mL). VEGFR2 data was normalized to cell numbers obtained from the ViaLight™ standard curve and calculated as pg/10^6 cells. Viabilities and VEGFR2 protein levels were then expressed as percentages of no siRNA control (SC). Results are mean of 1 – 2 experiments performed in duplicate.
No adverse effects on cell viability were observed following transfection with siRNA pools over SC values at all the concentrations (0.1 – 2 µM) and time points used in the study; however, the DG-167 program alone led to a 40% reduction in cell viability in SC set (over NPC controls). Best knockdown in VEGFR2 protein levels within cells following transfection was observed at 24 hours, with levels coming back to up to normal by 72-96 hours. The 1 µM test siRNA concentration evidenced maximum knockdown (65-100% at 24 – 48 hours time point in comparison to no siRNA control), and was thus chosen for all further tube formation studies.

Media Composition for Tube Formation on Growth Factor Reduced Matrigel™ Clonetics™ EGM™ 2 BulletKit™ (Lonza, CC-3162)

Media Additives (to EBM™ 2 Basal Media)	Complete Media	Cytokine Media	No Cytokine
Fetal Bovine Serum (FBS)	2%	0.5%	0.5 - 1% (as indicated)
Ascorbic Acid	■	■	■
Hydrocortisone	■	■	■
Gentamicin/Amphotericin-B (GA)	■	■	■
Heparin	■	■	■
hEGF (Human Epidermal Growth Factor)	■	■	
hFGF-β (Human Fibroblast Growth Factor-Beta)	■	■	
R3-IGF-1 (R3-Insulin-like Growth Factor-1)	■	■	
VEGF (Vascular Endothelial Growth Factor)	■	Added externally (as indicated)	Added externally (as indicated)

Figure 4: Media composition for tube formation on Growth Factor Reduced Matrigel™

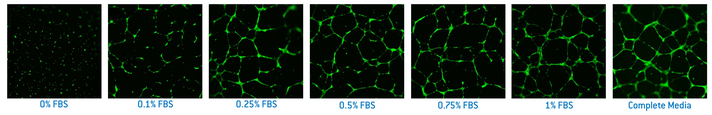
A Optimization of siRNA Tube Formation Assay – Workflow

Choose optimal program for Nucleofection of cells using pmaxGFP™ Vector
↓
Check that chosen program does not impact adversely tube formation capacity of cells
↓
Transfect cells with siRNA and determine best time point and most effective concentration for protein knockdown
Choose a concentration which has minimal effect on cell viability and minimal non-specificity
↓
Assess effect of siRNA transfection on tube formation

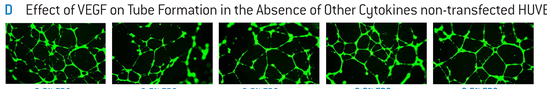
B Parameters Affecting Tube Formation on Matrigel™

1. Matrigel™ Type – Normal versus growth factor reduced Matrigel™
2. siRNA concentration – Identify non-toxic concentration that demonstrates an efficient knockdown of target protein
3. Time point of tube formation assessment – 24 hours versus 48 hours
4. Media composition – Complete media versus serum reduced media,
   a. Serum and cytokine starvation of cells post Nucleofection but prior to experimental set up
   b. Reduced serum during tube formation on Corning's Matrigel™ Product
   c. Removal of cytokines from the media both prior to and during tube formation assay
5. Optimal VEGF concentration assessment

C Effect of FBS on Tube Formation (non-transfected HUVEC)



D Effect of VEGF on Tube Formation in the Absence of Other Cytokines non-transfected HUVEC



E Effect of VEGF on Tube Formation in the Presence of Other Cytokines

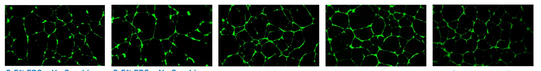
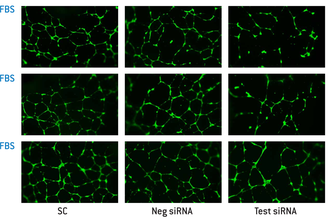
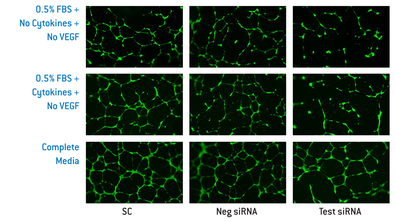


Figure 3: Workflow of the assay and the optimization steps undertaken for observation of tube formation on Phenol-Red free, Growth Factor Reduced Matrigel™ (Corning, 356231). (**A**) Optimization of Nucleofection conditions. (**B**) Optimization of miscellaneous parameters. (**C**) Optimization of FBS starvation conditions. (**D**) Optimization of VEGF dose for the study with non-transfected cells. (**E**). Concerted effect of cytokines, EGM™ 2 Bulletkit™ factors and VEGF on tube formation in cells transfected with no siRNA (SC set). Tube formation increased when cytokines were added to the media and further increased in the presence of the complete media containg 2% FBS.
The optimized protocol involved plating of transfected HUVECs on cell culture surfaces and incubation for 4 hours in complete EGM™ 2 Media to recover from the Nucleofection process. Media was changed to starvation media (0.5% FBS, no cytokines, no VEGF) and cells were incubated for another 18 hours at 37°C. After incubation, cells were trypsinized, counted and plated onto Phenol-Red Free Growth Factor Reduced Matrigel™ for tube formation assays in 96-well format in various media conditions using the previously standardized tube formation assay protocol (Kapoor *et. al.*, 2014). Tube formation was observed after 18 hours of incubation and images captured at 5X magnification post staining with Calcein AM under the FITC fluorescence filter.

A Effect of VEGFR2 siRNA on Tube Formation in the Presence of Different Concentrations of FBS



B Effect of VEGFR2 siRNA on Tube Formation in the Presence or Absence of EGM™ 2 Cytokines



C Effect of VEGFR2 siRNA on Tube Formation in the Presence or Absence of Recombinant VEGF

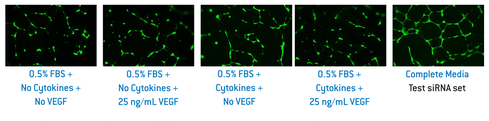


Figure 5: Transfection of Clonetics™ HUVECs with siRNAs and subsequent effect on tube formation on Growth Factor Reduced Matrigel™. Tube formation was set up under different media conditions (refer to Figure 4) post starvation as per the optimized protocol above and responses to FBS, cytokines and VEGF were measured. (**A**) Response to FBS - All the three samples demonstrated increased tube formation in response to increasing FBS concentrations, including Test, suggesting that VEGFR2 siRNA transfection does not impact response to FBS. (**B**) Response to cytokines - A similar increase in tube formation was seen when cytokines were added to the cells, and a still further increase was observed in complete media with all three samples, including test, demonstrating that VEGFR2 siRNA transfection does not impact response to other cytokines (other than VEGF). (**C**) Response to VEGF – VEGFR2 siRNA transfected test cells did not appear to respond to VEGF stimulation, either in the absence or presence of cytokines, suggesting that knockdown was maintained at this assay time point.

References

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Zeitelhofer M, Vessey, JP, Xie Y, Tubing F, Thomas S, Kiebler M, Dahm R (2007) High-efficiency transfection of mammalian neurons via Nucleofection. Nat. Protocols 2: 1692 – 1704.
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