Characterization of Delta-like 1 homolog (DLK1) overexpressing tumorigenic breast cells

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Verk- og náttúruvísindasvið
Háskóli Íslands

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15 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í Lífefna- og Sameindalíffræði

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Líf- og umhverfisvísindadeild
Verkfræði- og náttúruvísindasvið
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Skráningarupplýsingar:
Kristrún Ýr Holm, 2018, Characterization of Delta-like 1 homolog (DLK1)-overexpressing tumorigenic breast cells, BS ritgerð, Líf- og umhverfisvísindadeild, Háskóli Íslands, 27 bls.

Prentun: Háskólaprent
Reykjavík, maí 2018

Útdráttur

Delta-like 1 homolog (DLK1) er prótein sem inniheldur (EGF) endurtekningar. DLK1 próteinið er mikið tjáð í þroskun og endurnýjun spendýra og einnig er DLK1 próteinið mikið tjáð í ýmsum gerðum krabbameina, þar með talið brjóstakrabbameini. Hins vegar er hlutverk DLK1 í þróun krabbameina enn óljós.

DLK1 er ekki venjulegur (non-canonical) Notch bindill og hindrar Notch boðferilinn með beinni tenginu við NOTCH1 viðtakann. Óreguleg stjórnun á Notch boðferlinum er algengur atburður í þróun krabbameina. Nýlega hefur verið sýnt fram á að DLK1 stýrir NOTCH1 háðri frumufjölgun og þróun sortuæxla og brjóstakrabbameina, sem gefur til kynna að DLK1 gæti spilað mikilvægu stjórnunarhlutverki í framþróun krabbameinsfruma.

Í þessu rannsóknarverkefni var DLK1 yfirtjáð í meinvarpamyndandi brjóstakrabbameinsfrumum úr manni (MDA-MB-231) til þess að greina hvort yfirtjáning DLK1 hafi áhrif á svipgerð og virkni frumunnar, ásamt því að greina hvort mismunandi tjáningarstig DLK1 hafi áhrif á hæfni þeirra til að mynda krabbamein. Mismunandi tjáningarstig er byggt á tjánignu eGFP og frumurnar svo flokkaðar í $DLK1^{High}$, $DLK1^{Low}$ og Empty. Niðurstöður okkar rannsóknar sýna að mismunandi tjáningarstig DLK1 hafi ekki áhrif á frumufjölgun né tjáningu Notch downstream gena, Notch viðtaka, EMT gena eða Sox9. Hins vegar sýna okkar niðurstöður það að $DLK1^{High}$ hefur mest frumuskrið og að $DLK1^{Low}$ frumurnar eru með mestu innrásagetuna af frumulínunum þremur.

Abstract

Delta-like 1 homolog (DLK1) is a member of the epidermal growth factor (EGF)-like repeat-containing superfamily of proteins. The DLK1 protein is highly expressed during mammalian embryonic development and regeneration as well as in a wide range of tumor types, including breast cancer. However, the role of DLK1 in tumor development and progression is still unclear.

DLK1 is a non-canonical NOTCH ligand and inhibits NOTCH signaling through a direct interaction with NOTCH1 receptor. Dysregulation of the Notch signaling pathway is a common event in cancer. Recently, DLK1 was shown to modulate NOTCH1-dependent proliferation and oncogenic potential of melanoma and breast cancer cell, demonstrating that DLK1 may play an important role in the control of cancer cell proliferation.

In this project DLK1 was overexpressed in metastatic human breast cancer cells (MDA-MB-231) in order to characterize the phenotypic and functional effects of DLK1. Furthermore, we elucidated what role differential expression levels of DLK1 play for their oncogenic potential. The different expression levels of DLK1 was based on their eGFP expression and sorted into three cell lines, $DLK1^{High}$, $DLK1^{Low}$ and Empty. The results from our study show that different levels of DLK1 does not affect proliferation nor the expression of Notch downstream genes, Notch receptor, EMT markers or Sox9 gene. However, our results demonstrate that $DLK1^{High}$ migrates the fastest of the three cell lines and the $DLK1^{Low}$ cells are most invasive.

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Abbreviation

cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
DLK1	Delta-like 1 homolog
EGF	Epidermal growth factor
eGFP	Enhanced Green Fluorescent Protein
EMT	Epithelial to mesenchymal transition
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
Rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute medium
T25	Tissue culture flask, 25 mL

Acknowledgements

I would like to thank everyone at the Stem Cell Research Unit (SCRU), which is a part of the Biomedical Center at University of Iceland, for their help and assistance. My sincerest thanks go to Gunnhildur Ásta Traustadóttir for giving me the opportunity to do this research project and her excellent guidance, patience and fruitful discussions. Additionally, I would like to thank Guðmundur Hrafn Guðmundsson and Þórarinn Guðjónsson for agreeing to take on this project.

A special thanks to my family and friends for their unconditional support, patience, encouragement and always believing in me.

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1 Introduction

1.1 Breast cancer

Breast cancer is the most common cancer in women in the world and the second most fatal (Ferlay et al., 2015). Instead of being a cancer of single origin, breast cancer can results from a collection of complex genetic and epigenetic events which results in a transformation of a normal epithelial cells (Buchholz & Wazer, 2002). The different types of breast cancer formation can be molecularly classified, which will then give an implication on how to treat a certain type of a breast cancer (Baldassarre & Belletti, 2016).

The Notch family of proteins can participate in forming a breast cancer and studies show that Notch signalling and its interaction with other signalling pathways play an important role in breast cancer proliferation, invasion angiogenesis and metastasis (Wang, Li, Ahmad, & Azmi, 2010). Notch signalling is overexpressed and activated in breast cancers, and has been suggested to play a role of the development of adenocarcinomas in the murine mammary gland (Zang et al., 2007).

1.2 Notch signaling pathway

Notch receptors and their ligands are transmembrane proteins and belong to the EGF-like superfamily of proteins. Notch signaling pathway is evolutionary conserved cell signaling pathway and mediates cell-cell communication. Notch is involved in a wide array of cellular processes during development including cell specialization and proliferation. In addition, Notch is required in many cellular processes in adult organism and the dysregulation of the Notch signaling pathway is a common event in diseases, including cancer (Chillakuri, Sheppard, Lea, & Handford, 2012). In particular, NOTCH receptors participate in cancer cell proliferation and survival, and activation of NOTCH receptors has been involved in many tumor types. However, it has been demonstrated that depending on the cellular context,

NOTCH receptors can act both as oncogenes and as tumor-suppressor genes (Nueda et al., 2017).

Notch is best known for its lateral inhibition in *Drosophila* neural cells, where progenitor cells express both NOTCH receptor and its ligand Delta. Eventually, one of the cells begins to express Delta more strongly than the others, hence interacting with NOTCH receptors on neighboring cells. In turn the cell that expresses Delta differentiates into a neural cell while inhibiting the differentiation of its neighbors that express NOTCH. When a Delta ligand binds to a Notch receptor the cytoplasmic tail of the receptor will be cleaved by protease complex called γ-secretase (Alberts et al., 2015. p867). The cleavage will cause the activation of the cytoplasmic tail or the NOTCH intracellular domain (NICD). NICD translocate into the nucleus. In the nucleus NCID binds to the CSL (CBF-1/SU(H)/LAG-1)/RBP-Jk complex ad Mastermind (MAML) protein and together it forms an activated transcription factor for a set of Notch response genes, including HES and Hey family (Falix, Aronson, Lamers, & Gaemers, 2012).

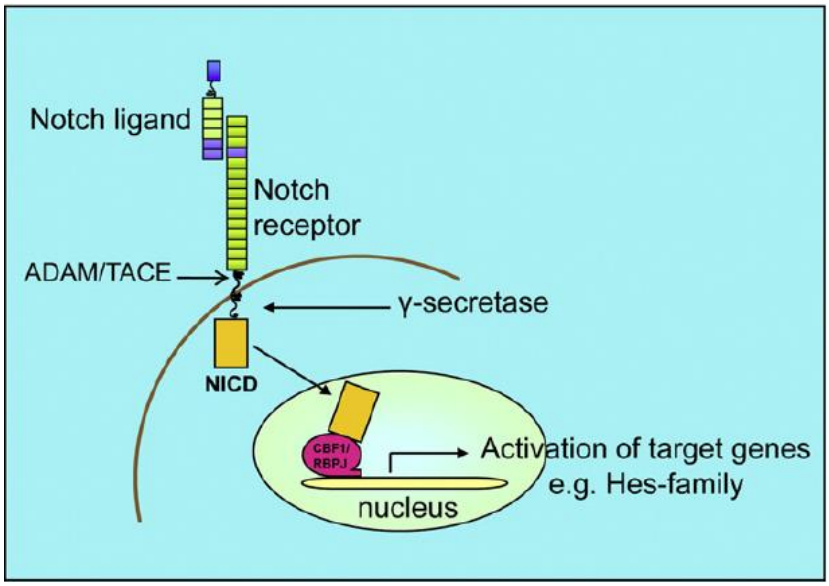

Figure 1: Notch signaling pathway. *Notch ligand (such as Delta) will bind to the Notch receptor, causing the γ-secretase to cleave the cytoplasmic tail of the Notch receptor. The cytoplasmic tail will travel into the nucleus as a transcription factor and activate target genes such as HES genes* (Falix et al., 2012).

1.2.1 Notch receptors and their ligands.

In mammals there are four Notch receptors (NOTCH1- 4) (Falix et al., 2012) which allows diversity in the function of Notch signaling, it depends on which family member is active in

a particular situation. Notch signaling can therefore be mediated by NOTCH1 in one tissue and NOTCH4 in another tissue (Alberts et al., 2015. p1150).

Five canonical NOTCH ligands have been determined; JAGGED1, JAGGED2 and Delta-like 1, 3 and 4 (DLL1, DLL3 and DLL4). These canonical ligands all have three related structural motifs: An N-terminal Delta-Serrate-LAG-2 (DSL) domain, DOS domain and variable number of EGF-like repeats. There are few non-canonical Notch ligands and the best studied among those is Delta-like 1 homolog. Delta-like 1 homolog (DLK1) is homologous to DLL but it is lacking the DSL motif. Evidence exists that DLK1 directly binds to the NOTCH1 receptor and inhibits the Notch signaling pathway (Baladrón et al., 2005; Traustadóttir et al., 2016).

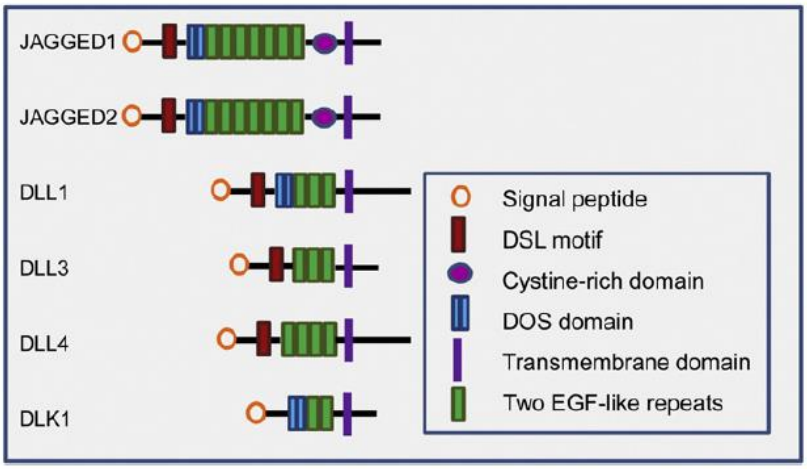

Figure 2: Structural motifs of Notch ligands. *Canonical Notch ligands have three structural motifs: DSL, DOS and EGF domains. However, the non-canonical Notch ligand DLK1 does not contain the DSL domain* (Falix et al., 2012).

1.3 The DLK1 gene and protein

The Delta-like 1 homolog gene is located on human chromosome 14 and mouse chromosome 12 (Gubina, Ruiz-Hidalgo, Baladron, & Laborda, 2000). The gene encodes a protein that is highly expressed during mammalian embryonic development. Even though, the Delta-like homolog protein has a well-established role in differentiation and proliferation, the molecular function of Dlk1 protein is not well established (Gunnhildur Ásta Traustadóttir, 2015).

The DLK1 protein is transmembrane but can also be cleaved, generating a biologically active soluble form (Hudak & Sul, 2013). Besides interacting with NOTCH1, DLK1 interacts with several other proteins such as itself, cysteine-rich FGF receptor (CFR) and FIBRONECTIN and thus acts in both Notch-dependent and -independent manner (Gunnhildur Ásta Traustadóttir, 2015).

1.3.1 DLK1 protein structure

The delta like homolog protein is a EGF-like membrane bound glycoprotein that contains six extracellular EGF-like repeats. Hence, DLK1 is a member of the EGF-like superfamily of proteins, which is a family of signaling molecules involved in proliferation and differentiation through their interactions of EGF-like repeats. It has a juxtamembrane region with a ADAM17 (TACE) cleavage site, a transmembrane domain and a short cytoplasmic tail. When DLK1 is cleaved at the TACE cleavage site, it forms a biologically active soluble form, which is released into the circulation.

1.3.2 Role of Dlk1 in development, differentiation and regeneration

The expression of *Dlk1* has been detected in many stages of mammalian development, where it is considered to play a role in differentiation and organogenesis, and branching morphogenesis in the lung and pancreas. However, in most adult tissues the expression of *Dlk1* is abolished, and DLK1 expression is limited to cells of neuroendocrine origin and preadipocytes within the stromal vascular fraction (SVF) of adipose tissue. The relatively mild phenotype of the *Dlk1*-knockout mouse led to the discovery of another gene, *Dlk2*, highly homologous to DLK1 (Nueda et al. 2006). It is speculated that when *Dlk1* is absent the DLK2 protein functionally makes up for the absence of DLK1, even though the *Dlk1* and *Dlk2* gene play different roles during developing tissues.

In adult tissues the *Dlk1* gene is sometimes re-expressed at sites of regeneration and thus Dlk1 is thought to play a role in adult-tissue regeneration. For example, in healthy adult muscle tissue there is usually no expression of Dlk1, but the expression has been identified in diseased and damage muscle, where there is an active regeneration. In addition, *Dlk1* expression has been detected in a liver regeneration and fibrosis.

Several lines of evidence have demonstrated that DLK1 plays a role in maintaining undifferentiated cells, as Dlk1 has been shown to negatively regulate several differentiation processes. Indeed, the role of Dlk1 in adipocyte differentiation has been extensively studied, where DLK1 is expressed in progenitor cells and needs to be downregulated in order for the cells to differentiate (Garcés, Ruiz-Hidalgo, Bonvini, Goldstein, & Laborda, 1999; Smas & Sul, 1993).

1.4 Role of DLK1 in Cancer

Expression of Dlk1 has been found to be upregulated in several types of human cancer including neuroblastoma, breast cancer, small-cell lung carcinoma and leukaemia (Khoury, Suarez-Saiz, Wu, & Minden, 2010; Laborda, 1993). However, Dlk1 has been shown to either promote tumor growth or inhibit it, depending on the context and type of cancer (Gunnhildur Ásta Traustadóttir, 2015). Hence, the role of DLK1 in tumour development and progression is still unclear. In a recent study, DLK1 was shown to regulate the levels of NOTCH activation and signaling and thus the proliferation rate and invasion capabilities of the metastatic MDA-MB-231 breast cancer cell line. In particular, high levels of DLK1 led to lower levels of NOTCH signaling, associated with decrease in proliferation and invasion. On the other hand, lower levels of DLK1 expression led to lower inhibition of NOTCH signaling, associated with increase in proliferation and invasion (Nueda et al., 2017).

2 Objective

The aim of this project is to characterize phenotypic and functional effects of DLK1 overexpression in MDA-MB-231 cells, and elucidate what role differential expression levels of DLK1 play for their oncogenic potential.

In order to characterize the oncogenic potential of Delta-like 1 homolog we overexpressed DLK1 in metastatic human breast cancer cells (MDA-MB-231) and sorted the cells into $DLK1^{High}$ and $DLK1^{Low}$ populations.

3 Materials and methods

3.1 DLK1 overexpression and cell lines

DLK1 was overexpressed in metastatic human breast cancer cells (MDA-MB-231). The open reading frame of DLK1 was cloned into the pWPI bicistronic lentiviral vector which also contains an eGFP marker. The DLK1 containing lentiviral plasmids were transfected into HEK293T cells and the MDA-MB-231 cells infected with viral supernatant supplemented with polybrene. This allows simultaneous expression of DLK1 and GFP. Likewise, MDA-MB-231 cells were infected with viral supernatant generated by transfecting HEK293T cells with an empty pWPI lentiviral plasmid, that is without the open reading frame of DLK1. This population of cells serves as a control and will be referred to as Empty. The DLK1 overexpressing MDA-MB-231 cells were sorted into $DLK1^{High}$ and $DLK1^{Low}$ populations based on eGFP expression levels in FACSaria (BD), which is available at the Department of Immunology. Control cells were transduced with empty vector backbone. Therefore, three cell lines were cultured; $DLK1^{High}$, $DLK1^{Low}$ and Empty. DLK1-overexpression was verified with gene expression analysis and immunocytochemistry.

3.2 Cell culture

The three cell lines were cultured in T25 flasks on RPMI medium with 10% of Fetal Bovine Serum (FBS) and 0.5% of Penicillin-Streptomycin Solution. The cell lines were kept in incubator at 37°C and 5% CO_2 humidity. Cell splitting was performed twice a week and once a week, between the splitting, the medium was changed to maintain cell growth.

When the splitting was performed the old medium was suctioned away and the cells washed with 5 ml of Phosphate-buffered saline (PBS). After the rinsing, PBS was removed and 1 ml of Trypsin was added to the cells. The cells were then moved into the incubator for 1 minute. Trypsin is used to detach adherent cells from the bottom of the flask and incubation speeds up the progress.

When the cells were detached 4 ml of PBS with 10% FBS was added to the 1 ml of Trypsin, which inhibits further tryptic activity that might damage the cells. The cells were then added to centrifuge tube and spun down at 1750 rpm for 3 minutes. After the centrifuge the supernatant was suctioned away leaving the cells on the bottom of the centrifuge tube.
When setting up an experiment the cells were resolved with 2 ml of a new heated medium and counted with hemocytometer before splitting, this promotes similar number of cell between the cell lines. After counting the cells, it was often assumed to be 250,000-300,000 cells/flask, therefore possible to calculate the µl of cells needed for splitting. The calculated µl were taken from the 2 ml and moved to another centrifuge tube, then 5 ml of a new medium added, mixed well and added to T25 flask. The flasks were then put in the incubator.

If the cells did not need to be counted the cells were resolved with 1 ml of new heated medium. The cells were most often splitted 1:8, which means 125 µl of the cells were moved to another centrifuge tube. The rest of the splitting procedure was thereafter performed the same way as mentioned here above.

3.3 Immunocytochemistry

Immunocytochemistry is a technique where the proteins of interest are marked using an antibody which is linked to a label that gives a signal. This signal can be visualized in a microscope. With this technique the DLK1 proteins and the nucleus were labeled. Immunocytochemistry was performed both on permeabilized and non-permeabilized cells.
The cells from the three cell lines were grown on RMPI medium (containing 10% FBS and 0.5% P/S) on chamber slide for five days. In the upper row the cells were permeabilized and in the lower the cells were non-permeabilized. After five days the cells were fixed and then stained.

Table 1: Shows the design of the chamber slide used immunocytochemistry

Permeabilized cells	$DLK1^{High}$	$DLK1^{Low}$	Empty
Non-Permeabilized cells	$DLK1^{High}$	$DLK1^{Low}$	Empty

3.3.1 Fixing MDA-MB-231 cells on chamber slides

Before the protein can be labeled with antibodies the cells need to be fixed. Fixation locks the cells in its place which allows better targeting of the protein of interest. After four days the cells were confluent and the fixation could be performed.

The medium was removed with a suction and 1 mL of 4% (w/v) formaldehyde solution were added on the cells and led incubate for 15 minutes at room temperature. Formaldehyde is most commonly used to fix cells; it creates a crosslink between protein that makes the cell locked in its place. The formaldehyde solution was then removed using suction and the cells were washed using PBS three times.

3.3.2 Staining MDA-MB-231 cells on chamber slides

After fixing the cells the staining could be performed. The PBS was removed using suction from the upper chambers but not from the lower chambers. 1 mL of 0.5% Triton® X-100 (v/v) in PBS was added to the cells in the upper chambers for permeabilization and led incubate for 15 minutes at room temperature. The permeabilization solution was then removed with suction and washed three times with PBS in the upper chambers. PBS was then removed both from the upper and lower chambers. Two mL of 3% bovine serum albumin in PBS (blocking solution) was added to the cells on the chamber slide and incubated for 60 minutes. The blocking solution prevents nonspecific binding of the primary antibodies.

The blocking solution was removed and a primary antibody solution was made. The primary antibody solution contained 3 µL of rabbit anti-human DLK1 primary antibody mixed with 1500 µL of 1% BSA. 250 µL of the primary solution was added to each chamber. This was incubated for 60 minutes at room temperature. The primary antibody solution was removed and the cells washed 3 times with PBS.

A secondary antibody solution was made. The secondary antibody solution contained 1.5 µL of goat anti-rabbit IgG-546 secondary antibody and 0.15 µL of DAPI mixed with 1500 µL of 1% BSA. 250 µL of the secondary antibody solution was added to each well and incubated for 30-60 minutes at room temperature. The secondary antibody solution was then removed and the cells washed three times with PBS.

After two days the PBS on the chamber slide was removed and 150 µL of 0.1% sodium azide added to each chamber. This was done to store the chamber slide.

3.4 Proliferation

Growth properties of the cells was assessed using the Incucyte® ZOOM Live-Cell Analysis System. 48,000 cells/well were seeded on a 6 well plate and cultured on RPMI medium containing 10% FBS and 0.5% P/S. The plate was put directly to the Incucyte® ZOOM Live-Cell Analysis System which automatically images the cells on the plate every two hours. In this way, it is possible to analyze the proliferation of the cell lines which makes it possible to determine if any difference in proliferation is between them.

3.5 Relative Expression of Genes

3.5.1 RNA isolation

The cell lines were seeded in a two 6 well plate and cultured on RPMI medium containing 10% FBS and 0,5% P/S. Three wells for each cell line. After the cells were confluent the medium was removed and 1 mL of Trizol reagent added to the cells for 5 minutes at room temperature. To loosen the cells from the well the sample was pipetted up and down and 1.5 total volume was collected and transferred to an RNase free Eppendorf tube. 0.2 mL chloroform was added to the tube, shaken and led stand for 5 minutes at room temperature. The sample was then centrifuged at 12000 g for 15 minutes at 4°C. Three phases are then formed, clear, pink and white phase. The clear phase contains the RNA while the pink phase contains DNA and the white protein. The clear phase was collected and transferred to a new RNase free Eppendorf tube. To the clear phase 0.5 mL of isopropanol were added, mixed gently and the allowed to stand for 10 minutes at room temperature. The sample was then centrifuged at 12000 g for 10 minutes at 4°C, where the RNA will precipitate and form a pellet. After the centrifuge the liquid was poured off immediately, remaining a little pellet. In order to wash the pellet 800 µl of 96% ethanol were added and centrifuged at 7500 g for 5 minutes at 4°C, this part was then repeated. The ethanol was poured off and allowed to dry before resuspended in 30 µl of RNase-free H_2O. The RNA concertation was measured using the Nanodrop and stored at -80°C.

3.5.2 cDNA synthesis

The RNA concertation measured from the Nanodrop was used to calculate how much of the isolated RNA were needed to synthesize cDNA. The SuperScript® IV (SSIV) kit was used for the cDNA synthesis. For each sample, 1 µg of RNA was mixed with 1 µL of random primers, 1 µl of dNTP and RNase free water up to 13 µL. The samples were then heated at 65°C for 5 minutes in a PCR (Polymerase Chain Reaction) machine. After incubation the samples were cooled down on ice. The samples were then mixed with a 4 µL of 5xSSIV buffer, 1 µL 0.1M DTT, 1 µL RNase OUT, and 1 µL SuperScript IV reverse transcriptase and incubated with the following PCR program.

1. 23°C for 10 minutes.
2. 50-55°C for 10 minutes.
3. 80°C for 10 minutes.

Thereafter, the cDNA samples were cooled down and diluted in water in the ration 1:10. The samples were then stored at -20°C.

3.5.3 Real-Time qPCR

Quantitative PCR was used to estimate relative expression of genes. A master mix was made that was added to a 96 well plate. For one well, the master mix contained 10 µL of SYBR green, 8 µL H_2O and 1 µL of forward and reverse primers. 19 µL of the master mix were put in each well and 1 µL of cDNA was then added to the master mix. The wells were closed with adhesive cover and centrifuged. The plates were transferred into a Fast 7500 real-time qPCR system from Applied Biosystems 7500 where the samples were run and analyzed. The housekeeping gene that was used was GAPDH. The organization of the plates are shown in table 2-5.

Table 2: The plate organization, with the genes DLK1 and HES1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			DLK1
B	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
C	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
D	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			HES1
E	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
F	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
G	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			GAPDH
H	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				

Table 3: The plate organization, with the genes HEY1 and HEY2.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			Hey1
B	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
C	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3		Empty 1		
D	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC	Empty 2		Hey2
E	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3		Empty 3		
F	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3		Low 1	High 1	
G	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC	Low 2	High 2	GAPDH
H	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3		Low 3	High 3	

Table 4: The plate organization, with the genes NOTCH2, NOTCH3 and Sox9.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			NOTCH2
B	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
C	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			NOTCH3
D	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
E	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			Sox9
F	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
G	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			GAPDH
H	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				

Table 5: The plate organization with the genes CDH1, CDH2 and NOTCH1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			CDH1
B	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
C	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			CDH2
D	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
E	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			Notch 1
F	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				
G	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3	NTC			GAPDH
H	Empty 1	Empty 2	Empty 3	Low 1	Low 2	Low 3	High 1	High 2	High 3				

3.6 Migration and Invasion

3.6.1 Migration scratch assay

After trypsinizing the cells and counting, 250,000 cells were seeded on to each well on a 96 ImageLock™ plate with RPMI medium (10% FBS and 0.5% P/S). The plate was allowed to stand at room temperature for 5 minutes, then placed into a 37°C incubator until the plate is 90% confluent. When the plate was confluent the WoundMaker™ and wounding procedure to create a precise wound in all 96 well on the ImageLock™ plate. After the wound making the wells are washed two times with the RPMI medium (10% FBS and 0.5% P/S) to prevent reposition of the cells. The plate is than place into the Incucyte ZOOM® were the wound was measured for 48 hours.

3.6.2 Migration transwell assay

Nine transwell filters, with the pore size 8 µm, were placed in 24 well plate. Three for each cell line. After trypsinizing the cells and counting, 40,000 cells were seeded in 250 µL RPMI (only containing 0.5% P/S) on top of each filter. 500 µL of RPMI containing 10% FBS and 0.5% P/S were added below each filter and incubated at 37°C for 24 hours.

Table 6: The organization of the 24 well plate.

High		Low		Empty	
High		Low		Empty	
High		Low		Empty	

The medium was removed from the transwell filters and PBS was placed in the wells next to the filters. The filters were washed two times with PBS and while washing the non-migratory cells were removed inside the filter with cotton swabs. After washing the filters, 4% PFA were added to the required number of wells and the filters were transferred from the PBS and placed in the 4% PFA for 5 minutes at room temperature. The filters were then again washed three times. To count the migrating cells, the cells were stained with DAPI. Staining solution contained 7500 µL of PBS and 1.5 µL of DAPI. In each well 750 µL of the staining solution were added to the required number of wells. The filters containing the

migrating cells were then placed in the staining solution for 30-60 minutes. The filters were then washed with PBS two times.

3.6.3 Invasion transwell assay

Invasion transwell assay was performed the same way as the migration transwell assay. However, before placing the cells straight to the filter a matrigel was first placed on the filters. 900 µL of cold RPMI was mixed with 100 µL of matrigel and 100 µL of this matrigel solution was placed to the filters and incubated at 37°C for 20 minutes. 40,000 cells were then seeded in 250 µL serum-free RPMI (only containing 0.5% P/S) on top of each filter. 500 µL of RPMI containing 10% FBS and 0.5% P/S were added below each filter and led incubate at 37°C for 48 hours.

4 Results

4.1 DLK1 overexpression sorting based on eGFP expression levels.

The sorting of the MDA-MB-231 cells was based on GFP expression using FACSaria (BD), available at the Department of Immunology. The DLK1 overexpressing MDA-MB-231 cells were sorted into $DLK1^{High}$ and $DLK1^{Low}$ based on eGFP expression levels.

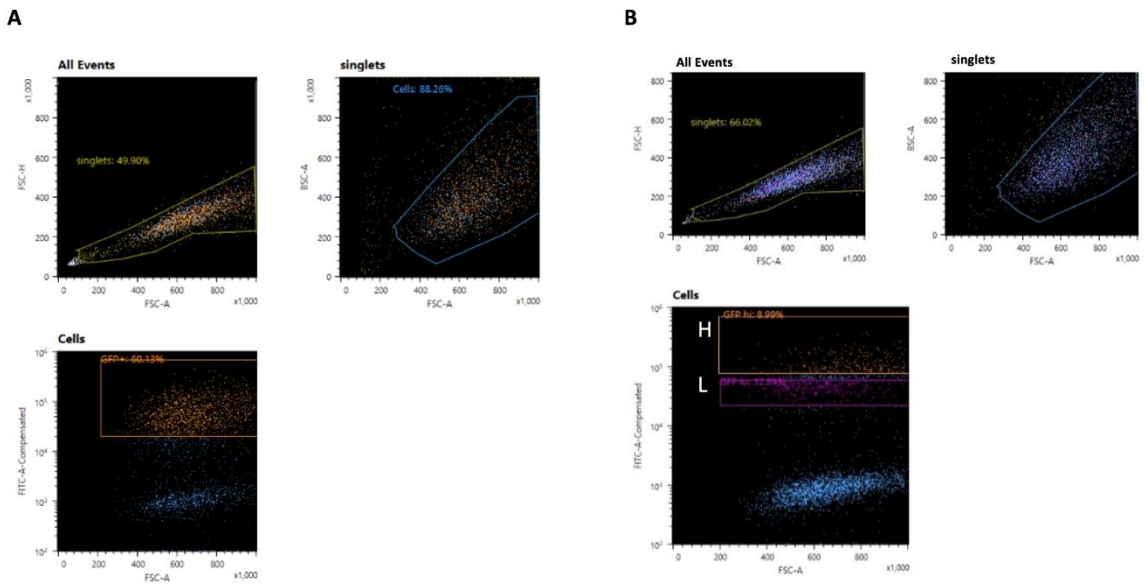

Figure 3: FACS analysis of eGFP expression levels. *A) Sorting control cells that have been transduced with empty vector backbone. B) Sorting DLK1 overexpressing MDA-MB-231 cells based on eGDP expression levels. The MDA-MB-231 cells expressing high levels of eGFP are labled with H and the cells expressing low levels of eGFP labled with L.*

4.2 Verification of the DLK1 overexpression.

To verify if the overexpression of DLK1 can be based on eGFP expression levels, gene expression analysis and immunocytochemistry was performed.

4.2.1 Immunocytochemistry.

To visualize the expression of the DLK1 proteins immunocytochemistry is used. The immunocytochemistry was both performed on permeabilized and non-permeabilized MDA-MB-231 cells. The staining for DLK1 in permeabilized cells was to visualize if the production of DLK1 would increase in the cells that expressed high levels of eGFP. The staining in non-permeabilized cells was to verify the increase or decrease in DLK1 signaling in the cell membrane based on the eGFP levels.

The results show that there is definite difference in eGFP signals between $DLK1^{High}$ and $DLK1^{Low}$ in both permeabilized and non-permeabilized cells. The results show as well that the ones that express high levels of eGFP also express higher levels of DLK1. Both permeabilized and non-permeabilized cells show a difference between the florescence signal of $DLK1^{High}$ and $DLK1^{Low}$. However, there is more difference between $DLK1^{High}$ and $DLK1^{Low}$ in the non-permeabilized cells compared to the permeabilized cells (see figure 4). This indicates that the expression levels of DLK1 can be based on the expression levels of eGFP.

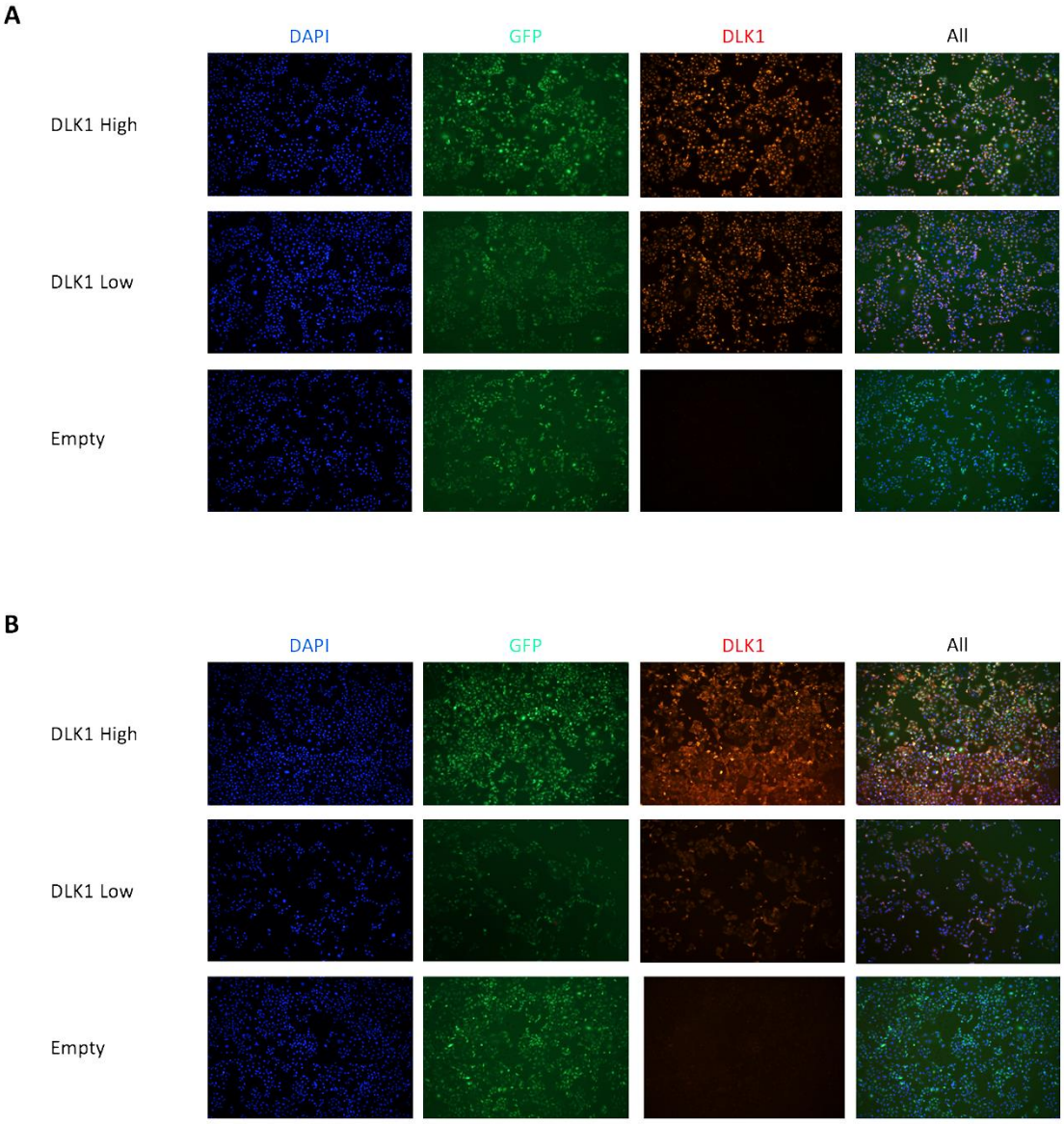

Figure 4: Verification of different expression levels of DLK1. *A) The results for permeabilized cells. A slight difference in expression of DLK1^{Low} and DLK1^{High} can be detected. B) The results for non-permeabilized cells. A significant difference in expression of DLK1^{Low} and DLK1^{High} can be detected.*

4.2.2 qPCR

To confirm the results from the immunocytochemistry a qPCR was performed. The results from the qPCR confirms that DLK1^{High} expresses high levels of DLK1, DLK1^{Low} expresses half the expression of DLK1^{High} and Empty does not express DLK1 (Figure 5).

The results from both immunocytochemistry and qPCR confirms that the DLK1 levels can be based on eGFP expression levels.

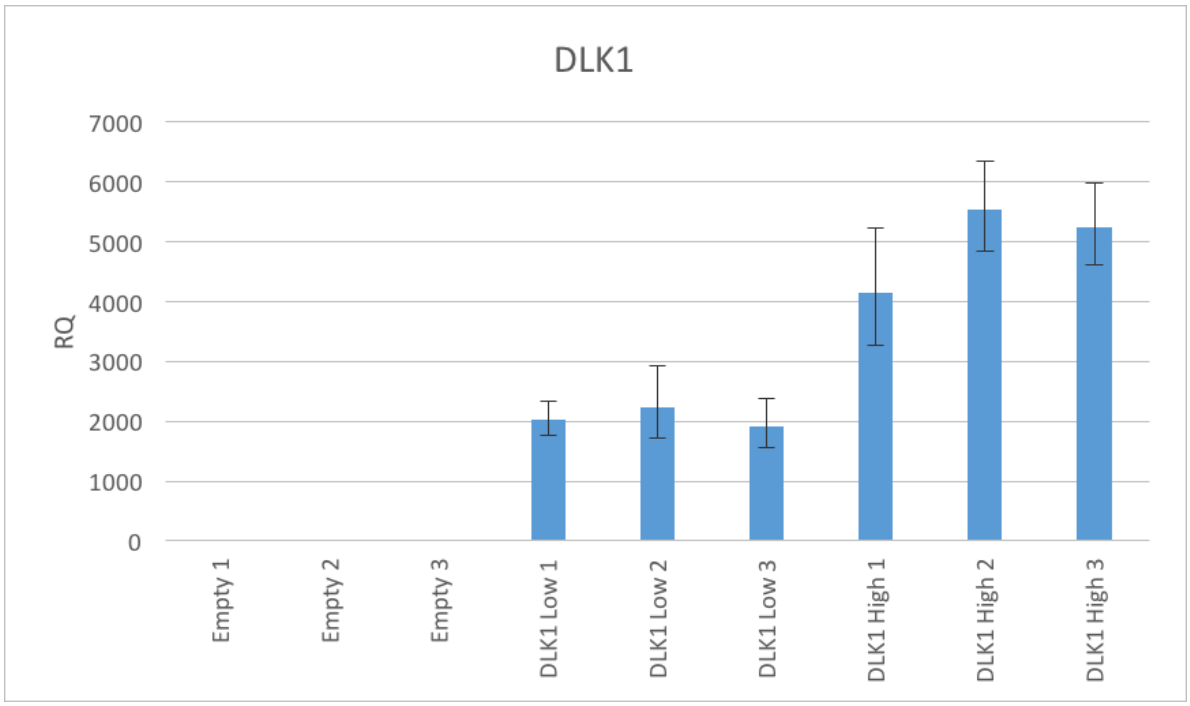

Figure 5: DLK1 expression levels verified using qPCR. *The qPCR results show that DLK1^{High} expression is twice the expression of DLK1^{Low}. The Empty cells do not show any DLK1 expression.*

4.3 Characterization of the cell lines.

4.3.1 Proliferation

To determine if the effect of different levels of DLK1 of the cell proliferation a proliferation analysis was carried out using the Incucyte®. Cell proliferation is quantified by capturing images and analyze the confluency percentage to give true cell growth rates. The results from the Incucyte® show that no phenotypic changes in proliferation between the $DLK1^{High}$, $DLK1^{Low}$ and Empty cell lines, which indicates that the cell lines proliferate practically at the same rate (Figure 6).

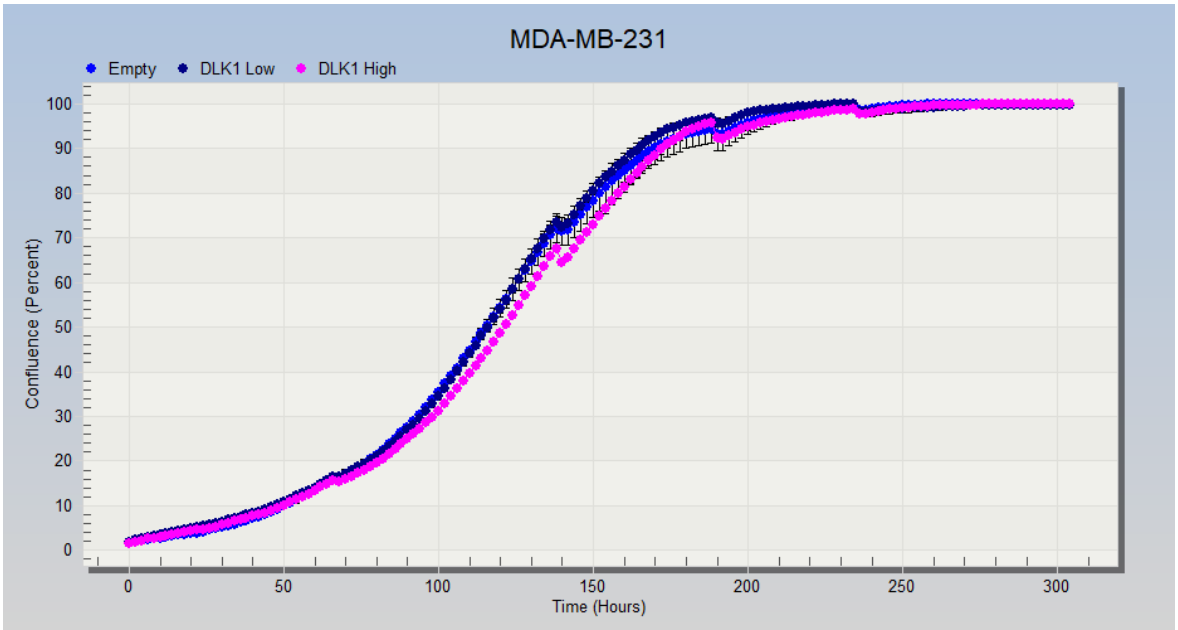

Figure 6: Proliferation of the $DLK1^{High}$, $DLK1^{Low}$ and Empty cell lines. *The results show no difference in proliferation between the cell lines.*

4.3.2 Migration and invasion assays

Invasion and migration potential of the cell lines were measured using a transwell assays with or without matrigel, respectively. These assays were used to detect if different levels of DLK1 had an effect on these properties.

First a migration transwell assay was performed. During this assay, MDA-MB-231 cells are placed on the upper layer of a transwell filter that contains 8 µm pores. Following incubation, the cells had migrated on to the other side of the filter, through the pores, the non-migrating cells were removed and the migrating cells stained and counted. After counting the migrating cells, more of DLK1^{High} cells had migrated through the 8 µm pores in the filter compared to DLK1^{Low} and Empty cells. This indicates that DLK1^{High} migrates faster than DLK1^{Low} and Empty, and that DLK1^{Low} migrates faster than Empty (Figure 7).

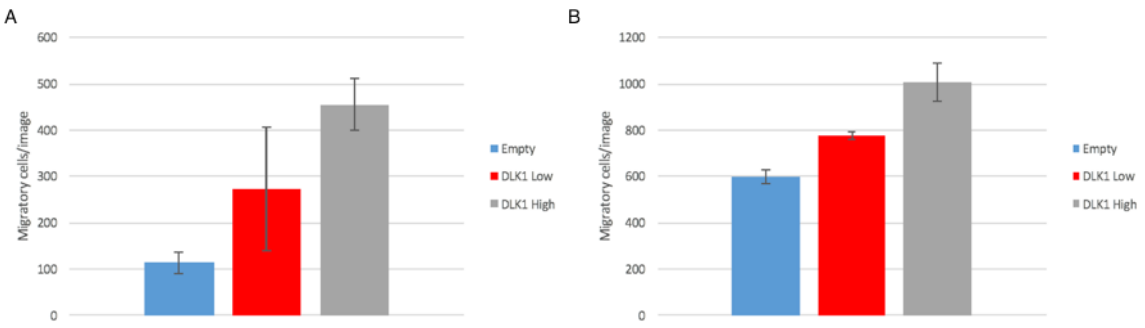

Figure 7: Migration transwell assay indicates that DLK1^{High} migrates faster than DLK1^{Low} and Empty. *The figure shows two migration transwell assays. The results in Figure 7A and Figure 7B show that DLK1^{High} migrates faster than DLK1^{Low}, and that DLK1^{Low} migrates faster than Empty. The results in Figure 7A are more significant compared to Figure 7B, however, the error bars are higher in Figure 7A compared to 7B.*

To confirm these results, a migration scratch assay was performed using the wound maker. The wound maker scratches the confluent cell culture, the Incucyte® images the migration of the cells and counts the migrating cells in the wound. The results from the Incucyte® demonstrates that DLK1^{High} migrates faster than DLK1^{Low} and Empty. DLK1^{High} cells show faster migration and are more confluent than DLK1^{Low} and Empty after 30 hours. However, different from the transwell assay no difference could be detected between DLK1^{Low} and Empty, which indicates that they migrate almost at the same rate (Figure 8).

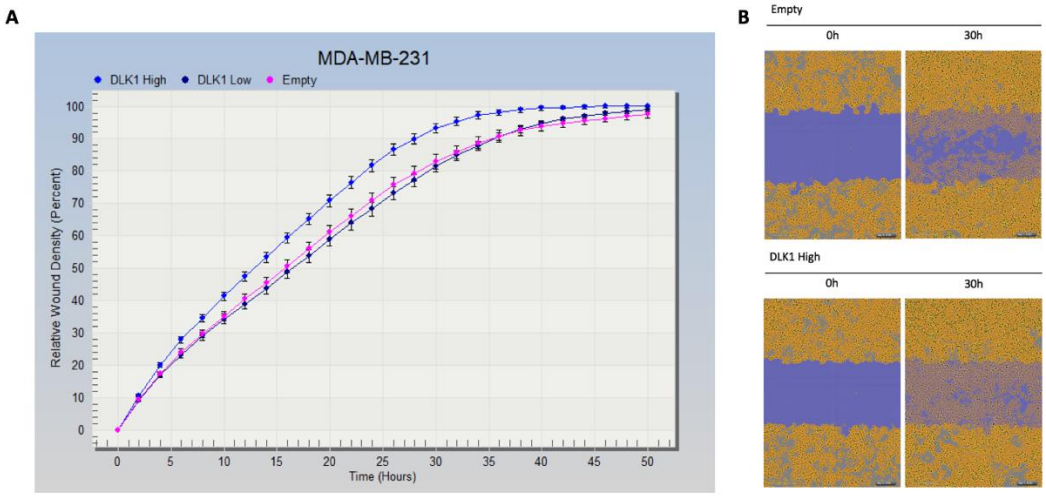

Figure 8: Migration scratch assay demonstrates that DLK1^{High} migrates the fastest.

The results from the migration assay show that DLK1^{High} migrates faster than DLK1^{Low} and Empty. Figure 8A shows that DLK1^{Low} and Empty migrate at the same rate. Figure 8B shows the difference in confluency between Empty and DLK1^{High}. DLK1^{High} is more confluent than Empty after 30 hours.

An invasion transwell assay similar to migration assay was performed to indicate if the different expression levels of DLK1 had an effect on the invasion properties. MDA-MB-231 cells were placed on matrigel, which covered the transwell filters. Following incubation, the cells had invaded through the matrigel and the 8 µm pores in the filter. The non-invasive cells were removed and the invasive cells stained and counted. After counting the invasive cells, more of DLK1^{Low} cells had invaded through the matrigel and the 8 µm pore filter, compared to DLK1^{High} and Empty. Therefore, these results indicate that DLK1^{Low} are more invasive then DLK1^{High} and Empty (Figure 9).

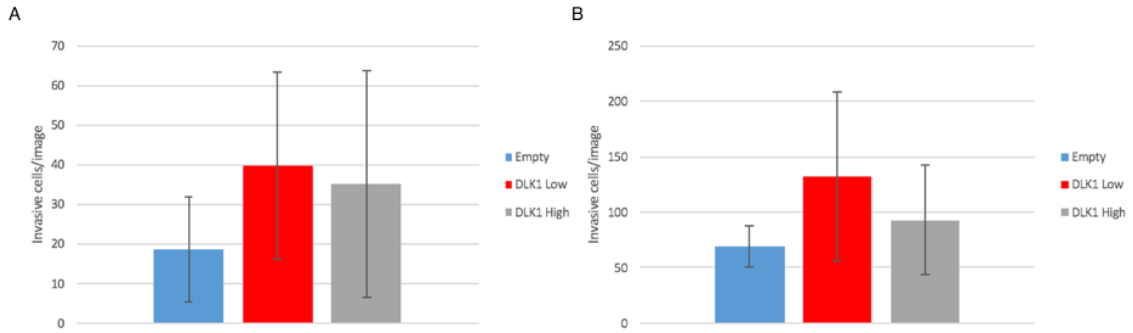

Figure 9: Invasion transwell assay indicates that DLK1^{Low} are more invasive than DLK1^{High} and Empty.

The figure shows two invasion transwell assays. In both cases the DLK1^{Low} show more invasive properties compared to DLK1^{High} and Empty. However, the error bars are high in both Figure 9A and 9B.

4.4 Gene expression

A gene expression analysis was performed, using qPCR, to see if different levels of DLK1 had an effect on selected genes, such as Notch signaling genes, Sox9 gene and EMT markers.

The selective genes for Notch signaling pathway were the Notch receptors; NOTCH1, 2 and 3, and the Notch downstream targets; HES1, HEY1 and HEY2. The results from the qPCR show that there is no difference in expression of these genes between the three cell lines. Which indicates that different levels of DLK1 does not affect the expression of these Notch signaling genes (Figure 10).

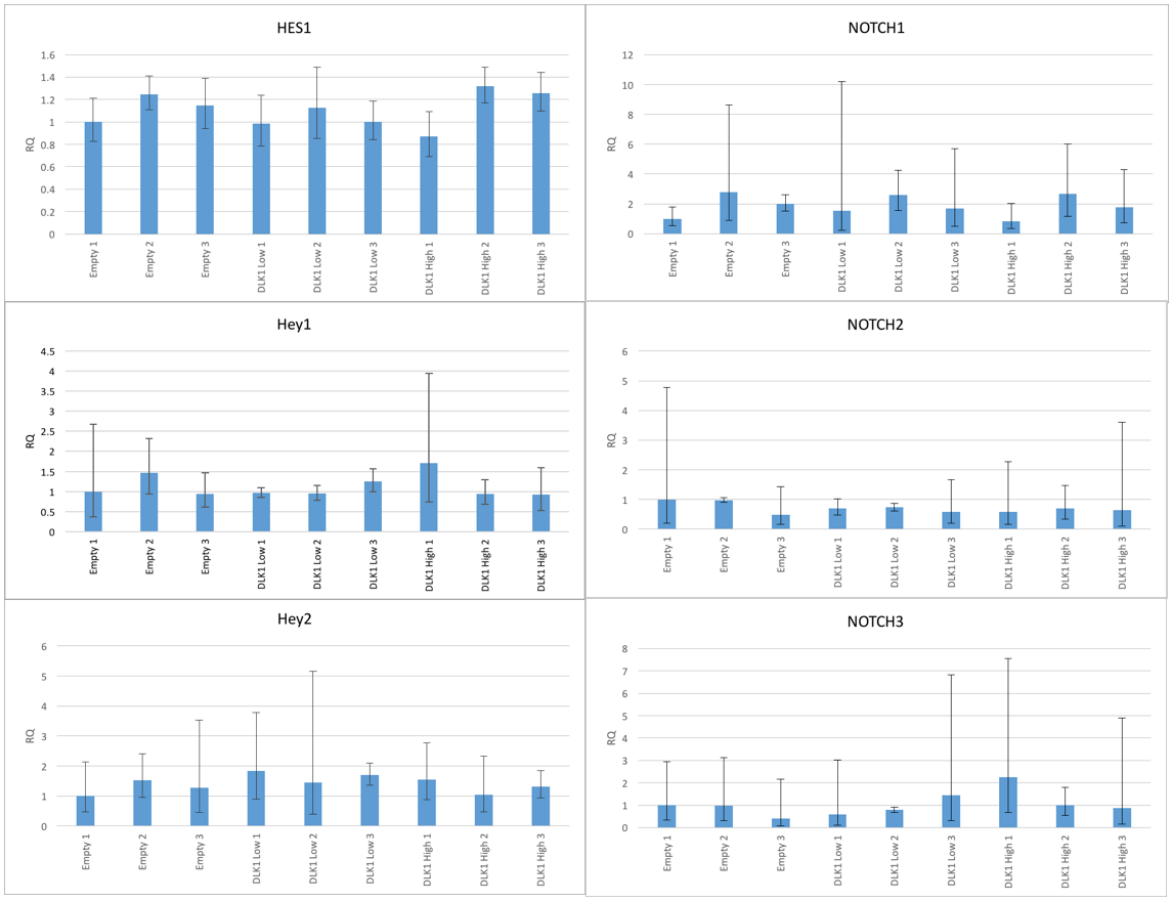

Figure 10: Different expression levels of DLK1 does not affect the expression of the Notch downstream targets HES1 and Hey 1, 2 nor the expression of the Notch receptors NOTCH1, 2 & 3. *The figure shows that there is no significant different in expression of the Notch genes between DLK1^{High}, DLK1^{Low} and Empty.*

After noticing that different levels of DLK1 did not affect selected Notch signaling genes, another signaling pathway was studied. An expression analysis on Sox9 gene was performed to determine if different levels of DLK1 had an effect on the expression of Sox9 gene. The results from the qPCR show that there is no significant change in the expression of Sox9 between DLK1^{High}, DLK1^{Low} and Empty cell lines (Figure 11).

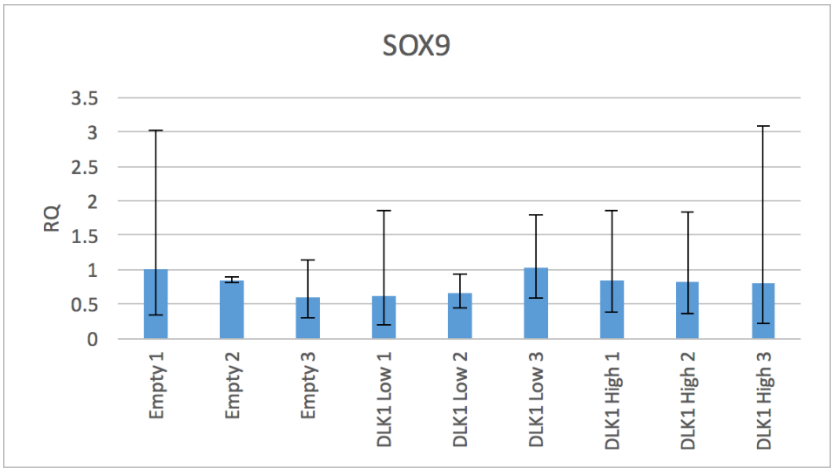

Figure 11: Different levels of DLK1 does not affect the expression of Sox9 gene. *The figure shows that there is no significant difference between DLK1^{High}, DLK1^{Low} and Empty.*

To determine if different levels of DLK1 had an effect on the expression of a cell-surface markers, an expression analysis was performed on the EMT markers CDH1 (also known as E-cadherin) and CDH2 (also known as N-cadherin). The results from the qPCR show that there is no significant difference in the expression of the markers between DLK1^{High}, DLK1^{Low} and Empty (Figure 12).

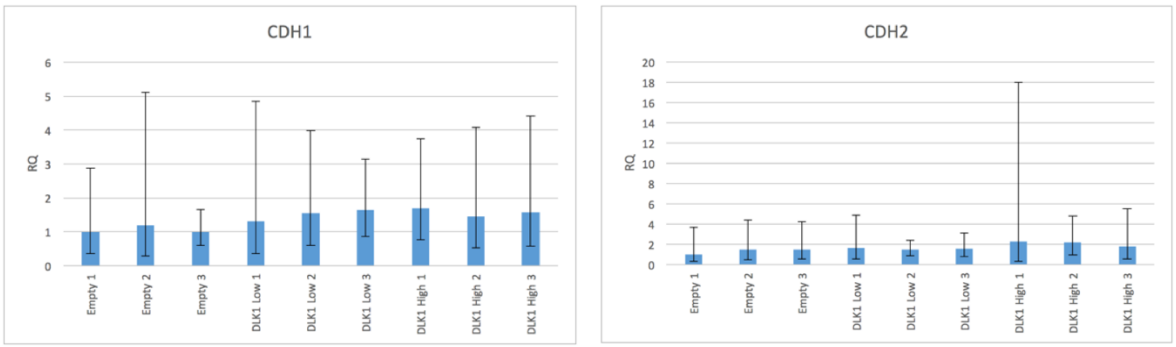

Figure 12: Different levels of DLK1 does not affect the expression of the EMT markers, CADH1 and CADH2. *The figure shows that there is no significant difference in the expression of the EMT markers between DLK1^{High}, DLK1^{Low} and Empty.*

5 Discussion

The results from this project have demonstrated that DLK1 expression levels in our DLK1 overexpressing MDA-MB-231 cells can be based on eGFP expression and provided clues about the characterization of DLK1 in this tumorigenic cell line. The experiments that verify that DLK1 expression levels can be based on eGFP, show clearly that $DLK1^{High}$ cell line have high expression of DLK1, $DLK1^{Low}$ cell line have lower expression of DLK1, although still high, and that Empty cell line have no expression of DLK1. That indicates that the three cell lines have different expression levels of DLK1. After that had been determined the phenotypic and functional effects of DLK1 could be assessed.

In this study the aim was to characterize phenotypic and functional effects of DLK1 overexpression in MDA-MB-231 cells, and elucidate what role differential expression levels of DLK1 play for their oncogenic potential. These properties were assessed by studying proliferation, migration, invasion and the expression of a selected genes. Studies have shown that high expression of DLK1 in tumorigenic MDA-MB-231 cells led to inhibition of Notch signaling and decreased proliferation and invasion and that low expression of DLK1 led to lower inhibition of Notch signaling and an opposite effect on proliferation and invasion (Nueda et al., 2017). From our results we can detect that low expression of DLK1 leads to increase in invasion and that high expression of DLK1 decreases it to similar levels as the Empty (see figure 9). However, different from the study of Nueda et al., our studies show that different levels of DLK1 does not seem to affect proliferation nor the Notch signaling.

DLK1 is a non-canonical NOTCH ligand and inhibits NOTCH signaling through a direct interaction with NOTCH1 receptor and therefore inhibits binding of other proteins such as Notch ligands e.g. DLL1. Normally when proteins bind to the NOTCH1 receptor, it activates and a part of the receptor will be cut of and transports into the nucleus, where it works as a transcription factors and activates particular Notch target genes for example the downstream targets HES1 and HEY1,2. When DLK1 binds NOTCH1 and therefor inhibits the expression of the downstream targets, it would therefore be expected that the overexpression of DLK1 would decrease the expression of the downstream targets HES1 and HEY 1, 2. Our results show that DLK1 overexpression does not affect these downstream targets which is the opposite of the results from Nueda et al.

On the contrary, it has not been shown that DLK1 inhibits the expression of the NOTCH receptors, neither NOTCH1, 2 or 3. Our results show that different levels of DLK1 does not affect the expression of the NOTCH receptors.

When studying proliferation, the result show that the three cell lines all proliferate at the same rate in the same RMPI medium containing serum. Therefore, it is probable that neither overexpression of DLK1 nor the different levels of its expression stimulates cell growth. It would be interesting to see if the overexpressing cells continue to proliferate after full confluent of the cells have been achieved, and to see if the different expression of DLK1 would affect it.

As previously mentioned, our results indicate that $DLK1^{Low}$ are more invasive compared to $DLK1^{High}$ and Empty. In contrast, $DLK1^{High}$ migrated faster than both $DLK1^{Low}$ and Empty. Two methods were used to study migration, transwell assay and scratch assay. The migration assay was performed three times due to a lack of authenticity. The first two migration experiments showed two different results, however, the third one showed similar results as the first experiments. The possible reasons for the lack of authenticity and high error bars (see figure 7 and 8) could be pipetting, counting and calculation errors. For this reason, a migration scratch assay was performed in the Incucyte. This assay is more reliable than the transwell assays. The results from the scratch assay show that $DLK1^{High}$ migrates at the fastest rate, similarly to the transwell assay. However, the results from the scratch assay show that $DLK1^{Low}$ and Empty migrate at the same pace which the transwell assay did not. The transwell assay showed that $DLK1^{Low}$ migrates faster than Empty. Therefore, it would be convenient to perform the scratch assay again.

Similar to migration a transwell assays were performed to study invasion, where the cells needed to invade through a matrigel. When studying invasion, three experiments were needed to be performed. The first two did not show the same results, however, the third and the first one showed similar results, that $DLK1^{Low}$ cells are most invasive. The same applies here as in migration, the results showed lack of authenticity. The possible reasons for the lack of authenticity and high error bars (see figure 9) are the same as in migration, pipetting, counting and calculation errors. It is interesting that $DLK1^{Low}$ cells are more invasive than $DLK1^{High}$ even though $DLK1^{High}$ cells migrates faster.

It is well established that invasive properties of breast cancer are related to the expression of transcription factors that regulate the expression of cell adhesion proteins such as E- and N-cadherin (Hajra, Chen, & Fearon, 2002) and that Notch signaling activates this progress (Fender, Nutter, Fitzgerald, Bertrand, & Sigounas, 2015). For this reason, we wanted to see if different levels of DLK1 would affect the expression E- and N- cadherin. Our result show that different levels of DLK1 did not affect the expression of E- nor N-cadherin.

DLK1 is also known to interact with other signaling pathways such as ERK/MAPK pathways. Studies have shown that DLK1 activates ERK/MAPK pathway and upregulates Sox9 expression (Hudak & Sul, 2013). Therefore, it would be interesting to see if different levels of DLK1 would affect the expression of Sox9. However, our results show no difference in expression between the three cell lines. Which indicates that different levels of DLK1 does not affect the expression of Sox9.

It is possible that there is an actual difference in expression of the genes we studied, it would therefore be better to perform a western blot to verify the results from the qPCR. However, due to lack of time this experiment was not performed.

In this study we demonstrate that by using the pWPI bicistronic lentiviral vector to overexpress DLK1, different levels of DLK1 can be based on eGFP expression. Furthermore, our results indicate that $DLK1^{High}$ migrates the fastest of the three cell lines and the $DLK1^{Low}$ cells are most invasive. Furthermore, our results showed that different levels of DLK1 does not affect proliferation nor the expression of the genes we studied. In future studies, it would be immensely interesting to perform the same experiments on non-tumorigenic human breast (MCF10A) cells to asses better the oncogenic potential of Delta-like 1 homolog (DLK1).

6 Reference

Alberts, B., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K., & Walter, P. (2015). *Molecular Biology of the Cell*. New York: Garland Science.

Baladrón, V., Ruiz-Hidalgo, M. J., Nueda, M. L., Díaz-Guerra, M. J. M., García-Ramírez, J. J., Bonvini, E., … Laborda, J. (2005). Dlk acts as a negative regulator of Notch1 activation through interactions with specific EGF-like repeats. *Experimental Cell Research*, *303*(2), 343–359. https://doi.org/10.1016/j.yexcr.2004.10.001

Baldassarre, G., & Belletti, B. (2016). Molecular biology of breast tumors and prognosis. *F1000Research*, *5*(0), 711. https://doi.org/10.12688/f1000research.8158.1

Buchholz, T. A., & Wazer, D. E. (2002). Molecular biology and genetics of breast cancer development: A clinical perspective. *Seminars in Radiation Oncology*, *12*(4), 285–295.

Chillakuri, C. R., Sheppard, D., Lea, S. M., & Handford, P. A. (2012). Notch receptor-ligand binding and activation: Insights from molecular studies. *Seminars in Cell and Developmental Biology*, *23*(4), 421–428. https://doi.org/10.1016/j.semcdb.2012.01.009

Falix, F. A., Aronson, D. C., Lamers, W. H., & Gaemers, I. C. (2012). Possible roles of DLK1 in the Notch pathway during development and disease. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1822*(6), 988–995. https://doi.org/10.1016/j.bbadis.2012.02.003

Fender, A. W., Nutter, J. M., Fitzgerald, T. L., Bertrand, F. E., & Sigounas, G. (2015). Notch-1 Promotes Stemness and Epithelial to Mesenchymal Transition in Colorectal Cancer. *Journal of Cellular Biochemistry*, *116*(11), 2517–2527. https://doi.org/10.1002/jcb.25196

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., … Bray, F. (2015). Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer*, *136*(5), E359–E386. https://doi.org/10.1002/ijc.29210

Garcés, C., Ruiz-Hidalgo, M. J., Bonvini, E., Goldstein, J., & Laborda, J. (1999). Adipocyte differentiation is modulated by secreted delta-like (dlk) variants and requires the expression of membrane-associated dlk. *Differentiation*, *64*(2), 103–114.

https://doi.org/10.1007/s002580050265

Gubina, E., Ruiz-Hidalgo, M., Baladron, V., & Laborda, I. (2000). Assignment of dlk (Dlk1) to mouse chromosome band 12E-F1 by in situ hybridization. *Cytogenetics and Cell Genetics 88: 322-323*.

Gunnhildur Ásta Traustadóttir. (2015). *The Molecular Mechanism of Delta-like 1 Homolog (DLK1) in Development and Regeneration.* University of Southen Denmark.

Hajra, K. M., Chen, D. Y., & Fearon, E. R. (2002). The SLUG zincfinger protein represses E-cadherin in breast cancer. *Cancer Res. 62, 1613-1618*.

Hudak, C. S., & Sul, H. S. (2013). Pref-1, a gatekeeper of adipogenesis. *Frontiers in Endocrinology*, *4*(JUL), 1–6. https://doi.org/10.3389/fendo.2013.00079

Khoury, H., Suarez-Saiz, F., Wu, S., & Minden, M. D. (2010). An upstream insulator regulates DLK1 imprinting in AML. *Blood*, *115*(11), 2260–2263. https://doi.org/10.1182/blood-2009-03-212746

Laborda, J. (1993). dlk, a Putative Mammalian Homeotic Gene Differentially Expressed in Small Cell Lung Carcinoma and Neuroendocrine Tumor Cell Line. *The Journal of Biological Chemistry*, *268*(2), 3817–3820.

Nueda, M.-L., Naranjo, A.-I., Baladrón, V., & Laborda, J. (2017). Different expression levels of DLK1 inversely modulate the oncogenic potential of human MDA-MB-231 breast cancer cells through inhibition of NOTCH1 signaling. *The FASEB Journal*, *31*(8), 3484–3496. https://doi.org/10.1096/fj.201601341RRR

Smas, C. M., & Sul, H. S. (1993). Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. *Cell*, *73*(4), 725–734. https://doi.org/10.1016/0092-8674(93)90252-L

Traustadóttir, G. Á., Jensen, C. H., Thomassen, M., Beck, H. C., Mortensen, S. B., Laborda, J., … Andersen, D. C. (2016). Evidence of non-canonical NOTCH signaling: Delta-like 1 homolog (DLK1) directly interacts with the NOTCH1 receptor in mammals. *Cellular Signalling*, *28*(4), 246–254. https://doi.org/10.1016/j.cellsig.2016.01.003

Wang, Z., Li, Y., Ahmad, a, & Azmi, A. (2010). Targeting Notch signaling pathway to overcome drug resistance for cancer therapy. *...)-Reviews on Cancer*, *1806*(2), 258–267. https://doi.org/10.1016/j.bbcan.2010.06.001.Targeting

Zang, S., Ji, C. H., Qu, X. U. N., Dong, X., Ma, D., Ye, J., … Guo, D. (2007). A study on notch signaling in human breast cancer. *Neoplasma*, 304–310.