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„Optimization of multi-reporter luciferase assays for simultaneous measurement of signaling pathway activities“

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Table of Content

1 Abstract

Cancer often evolves nor from single, but rather from several mutations or dysregulated pathways. A complex biological system of dysfunctional pathways, malfunction of the immune system or mutations on different genes occur. Sometimes multiple causes go hand in hand. Thus, it is all the more important that scientist have useful tools to study multiple elements simultaneously which contribute to malignancy. Signaling pathways like Sonic Hedgehog, Notch and Wnt are all suspected to play a key role cancer development and progression. Moreover, it is assumed that those pathways are dysfunctional in cancer stem cells and thereby involved in therapy-resistance of many cancer types. Hence, gaining knowledge about the specific activity of each pathway in different cancer cell types, but also their interplay with each other is all the more important. Multi-reporter plasmids can be very useful tools to detect their activity and interplay in cancer cells.

This thesis focuses on a multi-luciferase reporter plasmid (3P-Luc, based on Firefly, Nanoluc and Gaussia luciferase) where each luciferase reporter is under control of components for a specific signaling pathway and thus activity of three specific pathways can be analyzes simultaneously, namely Sonic Hedgehog, Notch and Wnt. 3P-Luc plasmid based polyplexes were used for in vitro transfection in a variety of cell lines and the read-out of different luciferases from the same well was optimized within one assay, namely Multi-Luc/BCA assay. Experiments were performed with different cell lines – HEK293T, MDA-MB-231 WT, MDA-MB-231 LM2-4, CT26 and LS174T – using three different concentrations, while measuring at two different time points. Results show that the simultaneous read-out of cells using Multi-Luc/BCA assay works precisely and reliable in all the cell lines tested. Pathway activity differences across cell lines were compared by normalizing the Multi-Luc/BCA read-out with either Firefly luciferase transfection efficiency (by co-transfection with LucSH plasmid) or percentage of EGFP positive cells after 3P-Luc transfection (separately measured by flow cytometry). Thus, the optimized Multi-Luc/BCA assay is quite useful for estimating multiple pathway activities simultaneously and the pathway-interplay of different cell lines can be investigated using this method.

2 Zusammenfassung

Krebs entsteht nicht durch einzelne, sondern viel mehr durch eine Vielzahl an Mutationen oder fehlregulierten Signalwegen. Es resultiert ein komplexes biologisches System fehlregulierter Signalwege, eine Fehlfunktion des Immunsystems oder Mutationen verschiedener Gene daraus. Dadurch ist es umso wichtiger, dass Forschern nützliche Methoden zur Verfügung stehen, um zahlreiche Komponenten, welche mit Malignität in Verbindung stehen, gleichzeitig messen zu können. Signalwege wie Sonic Hedgehog, Notch und Wnt spielen vermutliche eine große Rolle bei der Krebsentwicklung und -progression. Des Weiteren wird angenommen, dass die Signalwege in Krebsstammzellen fehlreguliert und dadurch an der Therapieresistenz vieler verschiedener Krebsarten beteiligt sind. Daher ist es umso wichtiger, Wissen über die Aktivität der einzelnen Signalwege in unterschiedlichen Zelllinien, sowie ihr Zusammenspiel zu erlangen. Multiple Reporterplasmide können sehr hilfreiche Werkzeuge sein, um die Aktivität und das Zusammenwirken zu messen.

Diese Arbeit konzentriert sich auf ein multiples Luciferase-Reporterplasmid (3P-Luc, basierend auf Firefly, NanoLuc und Gaussia Luciferase), wobei jeder Luciferase-Reporter von Komponenten eines spezifischen Signalweges kontrolliert wird, wodurch die Aktivität drei spezifischer Signalwege gleichzeitig gemessen werden kann, nämlich Sonic Hedgehog, Notch und Wnt. 3P-Luc Plasmid basierte Polyplexe wurden in mehreren Zelllinien für in-vitro Transfektionen verwendeten und die unterschiedlichen Messungen aus einem Well in einem Assay optimiert, genannt Multi-Luc/BSA Assay. Experimente wurden mit unterschiedlichen Zelllinien – HEK293T, MDA-MB-231 WT, MDA-MB-231 LM2-4, CT26 und LS174T – durchgeführt, wobei drei unterschiedliche Konzentrationen zu zwei verschiedenen Zeitpunkten gemessen wurden. Die Ergebnisse zeigen, dass die simultane Messung mittels Multi-Luc/BCA Assay präzise und zuverlässig in allen getesteten Zelllinien funktioniert. Unterschiede der Signalwegaktivitäten der einzelnen Zelllinien konnten durch die Normalisierung des Assays anhand der Transfektionseffizienz der Firefly Luciferase (durch Co-Transfektion mit LucSH-Plasmid) oder mittels Prozentsatz der EGFP positiven Zellen nach Transfektion mit 3P-Luc (unabhängig mit dem Durchflusszytometer gemessen) verglichen werden. Somit ist der optimierte Multi-Luc/BCA Assay hilfreich zur simultanen Bestimmung mehrerer Signalwegaktivitäten und das Zusammenspiel der einzelnen Signalwege unterschiedlicher Zelllinien kann zusätzlich untersucht werden.

3 Aim of the Thesis

This thesis focused on the goal of using a multi-luciferase plasmid (3P-Luc) for simultaneous detection of multiple pathway activities in cells, which led to the need of optimizing a multi-luciferase readout along with protein estimation by BCA assay (Multi-Luc/BCA assay). Towards this, the aims of this thesis were as follows:

1. Multi-Luc/BCA assay Optimization to detect pathway activity:
    a. Replace the normalization method from CellTiter Fluor to protein normalization via BCA (Bicinchoninic Acid) assay. In previous experiments, CellTiter Fluor was used for normalization of the cell count. The first aim was to see if the normalization based on BCA would also work for this experiments, as it is more economic. Because cells needed to be lysed for further analyses anyway, the need for cell lysis for BCA assay was no disadvantage.
    b. Investigate multiplexing by reading all assays from same well, namely BCA, Firefly luciferase, Gaussia luciferase and NanoLuc after 3P transfection. Because of the multiplexing and the aim to read BCA for protein normalization, but also Firefly and Gaussia Luciferase as well as NanoLuc from one well, it was necessary to adjust the volume of Passive Lysis Buffer (PLB). The standard volume was 30µl, but that way the capacities for each measurement would have been too small. Hence, it was customized to 40µl, which also had to be considered during calculations.
    c. Investigate different amounts of 3P-Luc plasmid and also different transfection durations to detect significant signals for pathway activity of Hedgehog, Notch and Wnt pathway. For measurements of the pathway activity of Hedgehog, Notch and Wnt only 50ng of 3P-Luc were used in previous studies, leading to only a minor detectable rise in signal compared to untreated cells. Therefore, another aim was to transfect cells with higher plasmid concentrations to see if this would result in changes in pathway activity. For this thesis, concentrations of 50, 100 and 200ng per well were tested. Since the transfection with 3P-plasmid is a transient one, it was also of interest to check the pathway activity at different time points. For this thesis, 24 hours and 48 hours post transfection were chosen.

2. Screen Hedgehog, Notch and Wnt pathway activity in HEK293T, MDA-MB-231 WT, MDA-MB-231 LM2-4, CT26 and LS172T cells and compare them using the optimized multi-Luc/BCA assay. In previous studies the plasmid was only tested on HEK293T cells, since it is known that the activity of the three pathways in question is increased in embryonic cells. Therefore, another goal was to detect if signals would be even higher in cancer cell lines than in HEK293T cells. Hence, the question arose if different cancer cell lines are comparable regarding their pathway activities or if various signaling pathways show differing results in divergent cell lines.

4 Introduction

4.1 Cancer Signaling Pathways

The development of cancer involves continuous genetic and epigenetic mutations, which may be inherited or arise spontaneously. For instance, as a result of DNA damage, due to environmental carcinogens or simply from replication errors, that allow cells to escape homeostatic controls which would suppress inappropriate proliferation and also kill those inappropriately proliferated cells. In solid tumors, these mutations typically support progression from just a benign group of proliferating cells to a mass of cells with aberrant morphology, cytological appearance and cellular organization. After the expansion of a tumor, the tumor core loses access to oxygen and nutrients, which often leads to angiogenesis and thereby a new way of supplying the cells with oxygen and nutrients. As a result, tumor cells can enter tissue beyond their normal boundaries, enter the circulation and form metastases at other sites – the defining feature of malignancy. This short description of events is clearly a simplification of the complex process of cancer development, but provides a useful framework to highlight the critical role of dysregulated signaling in processes connected to the initiation and progression of cancer.

Tumor cells also develop a lot of well-defined features, which not only include increased cell proliferation, but also resistance to apoptosis or other forms of cell death, metabolic changes, genetic instability, induction of angiogenesis and increased migratory capacity. These features are mainly subject to dysregulations of cellular signal transduction pathways. (Salazar & Yamamoto, 2018)

This thesis mainly focuses on the Sonic Hedgehog, Notch and Wnt pathways and their activity in different cell types.

4.1.1 Sonic Hedgehog Pathway

The Hedgehog (Hh) pathway plays an essential role during normal embryogenesis and is involved in the regulation of embryonic mammary gland induction, development of ductal architecture and differentiation in lactation, while dysregulation is entangled in the development and proliferation of breast cancer. There are three ligands known to be part of the Hh protein family – Sonic Hedgehog (SHH), Indian Hedgehog (IHH) and

Desert Hedgehog (DHH); SHH is the essential one for this thesis. Moreover, this complex pathway can be divided into canonical and non-canonical components. The canonical pathway – which is the one in question for this thesis – starts with the release of its ligand, SHH in this case as shown in Figure 1. SHH binds, and as a result, inhibits a transmembrane receptor – Patched (PTCH1) –, leading to the activation of the pathway. Unbound, PTCH1 inhibits the transmembrane transducer Smoothened (SMO). After ligand binding to PTCH1 and in consequence a relief of repression of SMO by PTCH1, SMO translocates to the primary cilium. This induces an intracellular signal cascade that enhances dissociation of suppressor of fused (SUFU) from glioma-associated oncogene (GLI) and thereby results in activation of transcription factors. Then, activated GLIs translocate from the cytoplasm to the nucleus and support transcription of Hh target genes. There are three GLI proteins – GLI1, GLI2 and GLI3. GLI1 acts as a transcriptional activator, GLI3 as a transcriptional repressor and GLI2 as both, activator and repressor. A lot of other target genes have already been described which are also involved in cell cycle regulation, proliferation, apoptosis, angiogenesis, epithelial-mesenchymal transition and stem cell regulation or self-renewal. (Bhateja, Cherian, Majumd, & Ramaswamy, 2019)

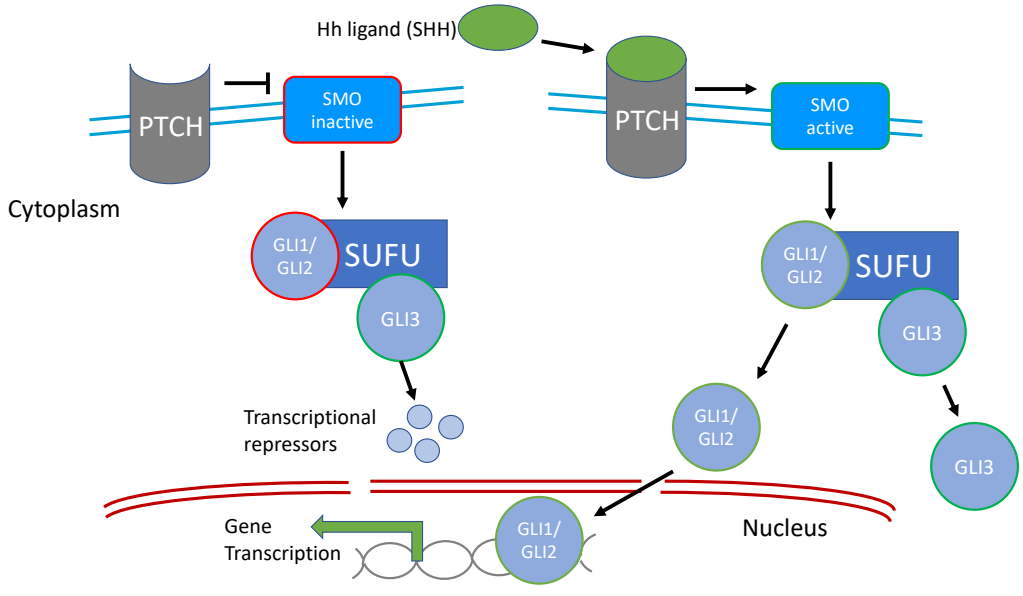


Figure 1: Inactivated and activated HH pathway through SHH (derived from (Bhateja, Cherian, Majumd, & Ramaswamy, 2019))

Left: No SHH, GLI stays outside the nucleus by binding to SUFU and therefore is inactivated.

Right: SHH binds to PTCH, SMO is no longer suppressed, GLI dissociates from SUFU, translocates to the nucleus and activates gene transcription

After birth, this pathway is turned off and only remains active in specific progenitor/stem cells, where it is needed for tissue repair and regeneration. Its aberrant reactivation during the future life can lead to cancer. A persistent pathway activation has been reported in several cancer types, also including solid tumors and hematological malignancies, where it was related to tumor development, progression and recurrence after chemotherapy through the regulation of residual cancer initiation cells. The deregulation of this pathway can have multiple reasons, such as

- ligand-independent activation due to inactivating mutations in PTCH1 or SUFU, activating mutations in SMO, or gene amplifications of GLI;
- autocrine/juxtacrine ligand-dependent activation, which leads to a higher Hh ligand expression of the tumor cells, but also a higher response to the same Hh stimulation in a cell-autonomous manner;
- paracrine ligand-dependent activation, where tumor cells secret Hh ligands and those ligands turn on Hh signaling in the surrounding stroma, which, again, stimulates growth and survival of the tumor and vice versa. (Pietrobono, Gagliardi, & Stecca, 2019)

Studies showed that the non-canonical Hh pathway is activated in hormone receptor positive breast cancer – especially in tamoxifen resistant tumors. There also seems to be a connection between an increase in the expression of SHH, SMO and GLI1, which correlates with the level of tamoxifen resistance in breast cancer cell lines. This also means that a knockdown of Hedgehog pathway components inhibits growth of tamoxifen resistant cells. Moreover, an increased expression of sonic hedgehog correlates with poorer overall survival in triple negative breast cancer (TNBC). This breast cancer type is characterized by the lack of estrogen, progesterone and human epidermal growth factor receptor 2 (HER2/neu) and the worst prognosis of survival among breast cancer subtypes. Further on, basal expression levels of GLI1 and GLI2 are higher in TNBC than in hormone receptor positive (HR+) breast cancer, leading to the suggestion that this pathway may be especially relevant to this subtype. In conclusion, Hh pathway activation leads to a poor prognosis in both cancer subtypes and also to several aggressive features like epithelial-mesenchymal transition (EMT), development and maintenance of cancer stem cells, angiogenesis and invasiveness. (Bhateja, Cherian, Majumd, & Ramaswamy, 2019)

4.1.2 Notch Pathway

The Notch pathway plays a pivotal role when it comes to the regulation of cellular identity, proliferation, differentiation and apoptosis by means of cell-cell interactions. Moreover, it is also essential for a wider array of developmental processes, such as regulation of neurogenesis, myogenesis, angiogenesis, hematopoiesis, epithelial to mesenchymal transition and tissue homeostasis (BOC Science, 2020).

For the proper activation of the Notch pathway, two cells need to be juxtaposed – one signal receiving and one signal sending cell as shown in Figure 2. The Notch receptor is expressed in the signal receiving cell and undergoes a lot of posttranslational modifications in the endoplasmic reticulum (ER) and the Golgi apparatus, such as O-glycosylation (ER) and N-glycosylation (Golgi), but also modulations by the first (S1) proteolytic cleavage (Golgi). Then, the receptors are transferred to the plasma membrane, where they act as cytoplasmatic receptors with four homologous proteins – Notch1, Notch2, Notch3 and Notch4 – and can interact with ligands secreted by their neighboring signal-sending cells. Regarding signal-sending cells, there are two families known – the Delta-family, consisting of Delta-like 1, 3 and 4, and the Serrate-family, consisting of Jagged 1 and 2. As a result, multiple ligands can be expressed in the same tissue and bind the four receptors with various affinities.

After ligand binding, the signal sending cell takes the ligand up by endocytosis, generating a traction that releases a second cleavage site (S2) on the Notch receptor. Without the traction, three Lin-12/Notch Repeat (LNR) domains would limit the access of a disintegrin and metalloprotease (ADAM), hence preventing it from cleaving the S2 site. After the majority of the Notch extracellular domain has gotten shed off, a membrane-tethered portion of the Notch receptor, referred to as Notch extracellular truncation (NEXT), stays in the membrane, where a γ-secretase performs its S3 cleavage to release the Notch intracellular domain (NICD) from the membrane. It's unclear whether γ-secretase processes NEXT at the cell membrane, within endocytic vesicles or both. After processing, NICD translocates to the nucleus and forms a transcriptional complex with various factors, such as Recombination signal Binding Protein for immunoglobulin kapa J region (RBPJ) or Mastermind-like 1-3 (MAML1-3). This active complex recruits transcriptional co-activators to initiate transcription of downstream target genes.

Signal termination of the Notch pathway is connected to ubiquitin-proteasomal degradation of the Notch intracellular domain (Salazar & Yamamoto, 2018).

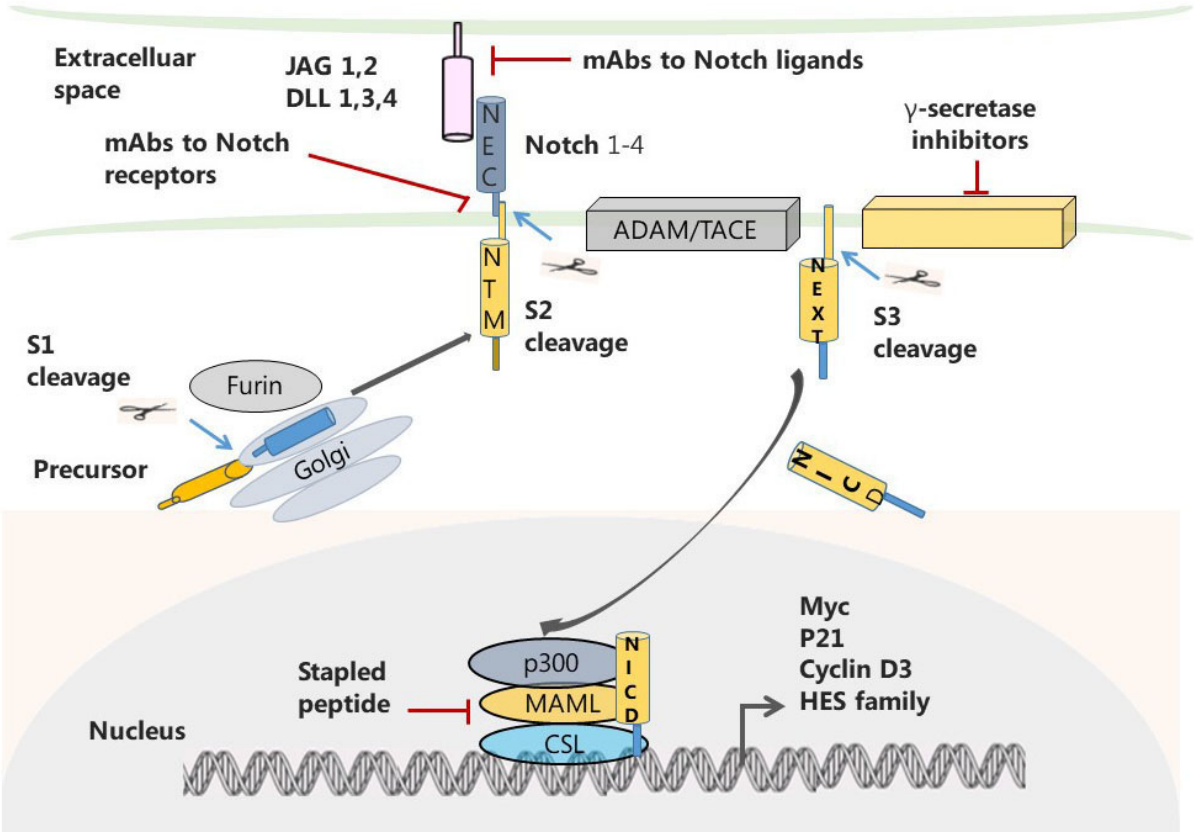


Figure 2: Schematic representation of the Notch signaling pathway ((BOC Science, 2020) – Notch Signaling Pathway).

Medical researches show a strong connection between Notch and numerous human diseases. Mendelian disorders, in other words genetic disorders caused at a single genetic locus, as an example, are known to be caused by inherited or de novo mutations in human genes which code core components of this pathway, such as receptor, ligand or transcription factors and downstream target genes. There is also growing evidence that a deregulation of the Notch pathway can lead to diseases ranging from neuropsychiatric to metabolic disorders. Somatic mutations in genes related to this pathway and/or deregulation of its signaling activity have been associated with oncogenesis and tumor progression in different cancer types. (Salazar & Yamamoto, 2018)

Moreover, Notch is necessary for the tumorigenicity of Cancer stem cells (CSC) by controlling and regulating the development of stem cells in terms of growth, differentiation and development of these CSC due to the abnormal activity of the pathway. These CSC are the reason for relapse of cancer, since they are chemo and radio resistant and can develop metastases several years after the treatment. Such

cancer stem cells have been identified in leukemia, prostate, ovarian, lung, melanoma, pancreatic, colon and brain cancer, only to name a few (Venkatesh, et al., 2018).

4.1.3 Wnt Pathway

The Wnt signaling pathway plays a major role when it comes to aspects of cell fate determination, cell migration and cell polarity. The large family of secreted glycoproteins consists of nineteen proteins in humans, forming a complex of signal regulation, function and biological output. The pathway can further be divided into a canonical or Wnt/ß-catenin dependent and non-canonical or ß-catenin-independent one.

The ß-catenin dependent pathway is the crucial one for this thesis, hence the key for pathway activation is the translocation of ß-catenin into the nucleus as shown in Figure 3. Without the Wnt ligand, the adherends junction-associated protein ß-catenin gets degraded by a destruction complex consisting of Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α). After phosphorylation within this complex by GSK3 and CK1α and ubiquitination, ß-catenin is proteolytically destructed by the proteasomal machinery.

The Wnt receptor complex is assembled by Frizzled (Fz) and low-density-lipoprotein-related protein 5/6 (LRP5/6) and gets activated by Wnt binding. As a result, the complex necessary for ß-catenin destruction, consisting of Axin, APC and GSK3 gets separated. Binding of the Wnt ligand to its receptor also leads to the membrane translocation of an important negative regulator of Axin, which responds by Axin-binding to the cytoplasmatic tail of LRP5/6 catalyzed by GSK3 or CK1γ-mediated phosphorylation of LRP5/6. This shows that GSK3 and CK1γ play different roles – when it comes to LRP5/6, their influence is positive, while their influence towards ß-catenin is negative. Moreover, Axin-binding also activates the phosphoprotein Dishevelled (Dsh), though the precise mechanism is still unclear. Activated Dsh inhibits the activity of GSK3 and enables a series of events that lead to the stabilization and prevention of degradation of ß-catenin. As a result, ß-catenin translocates into the nucleus, where it acts as a transcriptional co-activator. LEF/TCF DNA-binding transcription factors are among its binding partners and bind to the promoter of target genes. These target genes include those important for organizer formation through

embryogenesis (Siamois and Twin), but also genes engaged in oncogenesis during cancer formation such as Myc and CyclinD1. (Komiya & Habas, 2008)

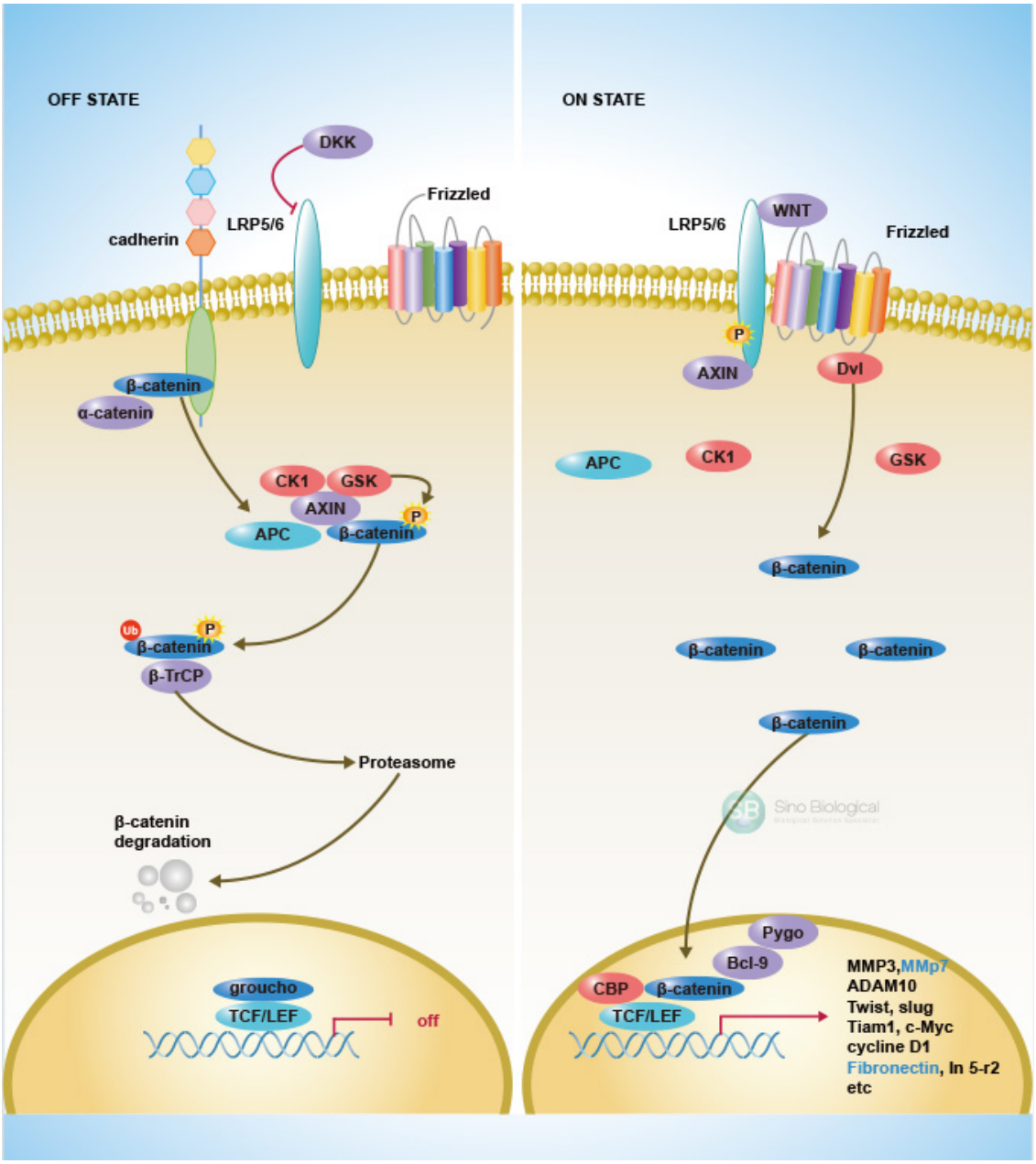


Figure 3: Inactivated and activated Wnt pathway through Wnt

Left: No Wnt, destruction complex is formed, ß-catenin gets degraded and therefore there is no gene transcription.

Right: Wnt binds Fz and LPR5/6, no degradation complex is formed, therefore ß-catenin translocates to the nucleus and activates gene transcription

The Wnt pathway regulates anterior head formation and neural patterning. Various Wnt inhibitors are expressed and secreted during gastrulation in order to modulate formation of the anterior of the embryo and promote anterior head formation. Moreover,

Wnt-signaling is important for formation of a number of organ systems such as heart, lungs, kidney, skin and bone, but also for stem cell renewal. There seems to be no organ system which is not at least peripherally touched by the canonical Wnt pathway during embryogenesis. Deregulation of this pathway has therefore disastrous consequences for the developing embryo on one hand, but also causes a lot of pleiotropic human pathologies on the other hand. These pathologies include cancer of the breast, colon and skin. (Komiya & Habas, 2008)

4.2 Luciferase Based Reporter Plasmids

Bioluminescence conduces many natural purposes, as defense, camouflage, feeding or mating, especially for marine animals living in the dark of the ocean (Thermo Fisher Scientific). Luciferase, in general, is a term for enzymes catalyzing visible light emission by living organisms, the so-called bioluminescence (Marques & Esteves da Silva, 2009). In this process, light is a secondary product resulting from the oxidation of specific substrates named *luciferins.* Such light emission can easily be identified and measured, hence making luciferase a perfect tool for developing reporter plasmids (Tzertzinis, Egaña, & Shea, 2010). Luciferase-based reporter plasmids are widespread in drug development, pharmaceutical and biomedical research and biochemistry to study gene expression and cellular incidents linked to gene expression at the transcriptional level. The method is often used because it is suitable, fairly inexpensive and gives quick results. This kind of bioluminescent reporters are also a good tool because there is typically no endogenous activity in host cells able to interfere with the quantification. Moreover, the emission of light is conditional on the chemical reaction between substrate and luciferase, which means the background signal should be insignificant, in comparison to fluorescence.

In easy words, the reporter gene is merged with the gene of interest and cloned into an expression vector. The expression vector, in return, is transferred into cells of interest.

Gene expression in the cells shall be measurable directly through the activity of the reporter protein itself or its enzymatic activity. Since the gene of interest is merged to the reporter gene, the luciferase quantity can be directly correlated with the quantity of the expression of the gene of interest.

A good reporter is highly sensitive and can be measured quantitatively after expression with a simple luminometer consisting of multiple photomultipliers or a charge-coupled device (CCD) camera (Man, 2019). Not only gene quantities can be studied, but also a lot of other cellular components and events that are linked to the process of gene regulation. For this thesis the process of interest is the pathway activity. A lot of phyla are capable of bioluminescence, including coelenterates, echinoderms, mushrooms and insects, though only a few have been characterized in detail (Allard & Kopish, 2008).
In this thesis parts of following luciferin-based reporter plasmids are of interest:

- TOP-NLuc (pMuLE_ENTR_TOP-NLuc1.1_L5-L4) for Wnt pathway,
- Gli-FLuc (pMuLE_ENTR_12GLI-FLuc_R4-R3) for Sonic Hedgehog pathway and
- CBF-GLuc (pMuLE_ENTR_CBF-GLuc_L3-L2) for Notch pathway.

Parts of these three individual reporter plasmids are combined into a multi-gene plasmid, named 3P-Luc (pMuLE_EXPR_CMV-eGFP_TOP-NLuc1.1_GLI-FLuc_CBF-GLuc, Figure 4). As already mentioned, Hedgehog, Notch and Wnt play an important role during embryonic development and also in cancer stem cells. They act on their own, though there is also an intense crosstalk taking place between them. Therefore, it is attractive to be able to measure all three pathways by one vector at once and thereby show the crosstalk between the pathways (Maier, et al., 2019).
Moreover, by usage of 3P-Luc, an examination of activation or inhibition of those pathways is possible through the change in light signals – by activation, the light signal increases while there is a decrease of the light signal due to inhibition.

Figure 4: Map of 3P-Luc (Maier, et al., 2019)

Complete map of the 3P-Luc, with special attention to the Hedgehog pathway promotor GLI-Flu (orange), Notch pathway promotor CBF-GLuc (red) and Wnt pathway promotor TOP-NLuc (blue). In addition, the plasmid harbors a PGK promoter-driven Neomycin resistance for selection purpose and a CMV-driven EGFP expression cassette for future sorting, for example by FACS.

4.2.1 NanoLuc Based Reporter

NanoLuc is originally derived from the deep-sea shrimp *Oplophorus gracilirostris* (Figure 5), which, when feeling endangered, secretes a luciferase that catalyzes the oxidation of Coelenterazine to emit blue light and was first cataloged in 1881 (Riss T. L.).

Figure 5: *Oplophorus gracilirostris* (Perez-Moreno, 2016)
Top: deep-sea shrimp *Oplophorus gracilirostris* in its natural state
Bottom: *Oplophorus gracilirostris* emitting its typical cloud of bioluminescent material

With 19.1 kDA it is a comparably small protein and remains in the cytoplasm after transcription. NanoLuc offers a few advantages over more established systems, such as enhanced stability as its half-life is 11.5 days at 37°C. In addition, it provides a more than 150-fold increase in luminescence compared to established systems and its codon is especially optimized for expression in mammalians. For the strong blue light signal, the enzyme only requires O2 and substrate, as shown in Figure 6, and is therefore ATP-independent. (England, Ehlerding, & Weibo, 2016).
Furthermore, the substrate for NanoLuc also shows higher stability and lower background activity. However, one restriction of this technology is the non-ideal emission for in vivo applications and its specific substrate (Urquiza-Garcia & Millar, 2019). One prominent substrate is the often used Furimazine, which provides maximal brightness at 460nm wavelength, a stability over 15 hours at 37°C and was also used for this thesis. For successful measurement of the Wnt pathway activity using a NanoLuc reporter, it is crucial to transfect cells with TOP-NLuc plasmids which also include Wnt pathway promotors.

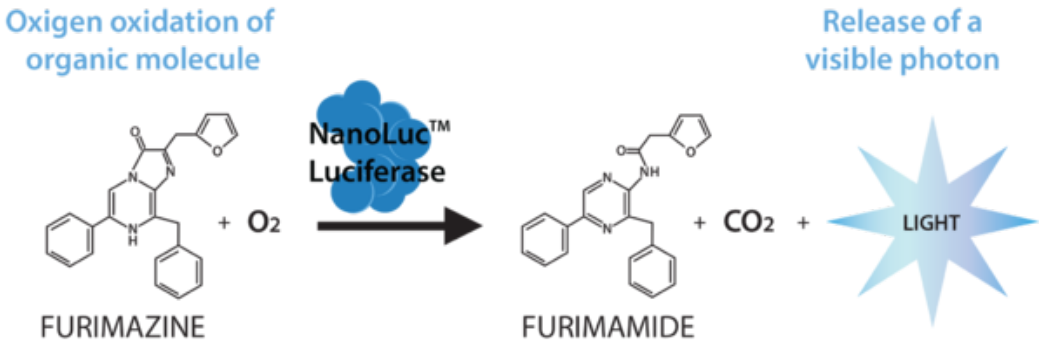


Figure 6: Furimazine gets catalyzed by NanoLuc and produces Furimamide and light (Chesnel, 2015).

4.2.2 Firefly Luc Based Reporter

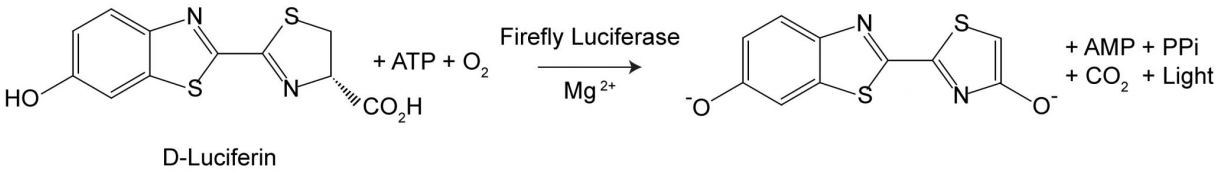
Firefly Luciferase is initially acquired from the firefly *Photinus pyralis* (Figure 7), which was first examined in 1917 and is nowadays the best known luciferin-luciferase-system with its substrate luciferin.

Figure 7: *Photinus pyralis* during flight, its bioluminescent parts are clearly visible (Die Chemie-Schule).

It is a very sensitive genetic reporter because there is no natural endogenous luciferase activity in mammalians. For this reason, Firefly Luciferase is a widely used bioluminescent reporter for studying gene regulation and function. With 61kDa it is much larger than NanoLuc, active as a monomer and does not require subsequent processing for its activity.

Firefly Luciferase uses ATP, magnesium and oxygen to catalyze D-Luciferin into oxyluciferin as shown in Figure 8, resulting in a light emission centered at 560nm

wavelength. The emitted green-yellow light is directly proportional to the number of luciferase enzyme molecules (Sigma Aldrich).



D-Luciferin

Figure 8: Catalyzation of D-Luciferin to Oxyluciferin by Firefly Luciferase, ATP and magnesium (Sigma Aldrich).

For measurement of the Sonic Hedgehog pathway activity using a Firefly Luc reporter, it is crucial to transfect cells with GLI-FLuc plasmids which also include Hedgehog pathway promoters. It should also be mentioned that Oxyluciferin stays in the cytoplasm and the cells therefore need to be broken for its measurement.

4.2.3 Gaussia Luc Based Reporter

Gaussia Luciferase was first used in a sensitive assay in 2002, hence being the most recently discovered protein which is naturally secreted by the marine copepod *Gaussia princeps* (Figure 9), resulting in blue luminescence.

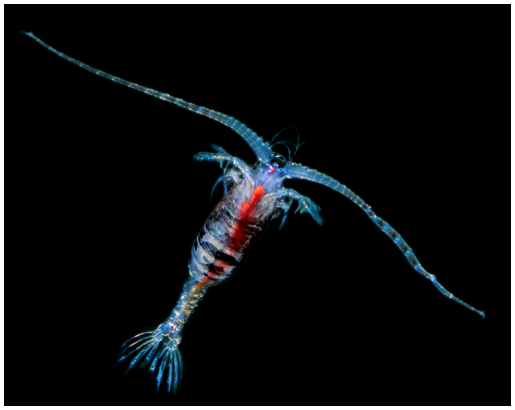


Figure 9: *Gaussia princeps* in its natural environment, blue luminescence clearly visible (Wrobel).

With 185 amino acids and 19kDa, it is the smallest luciferase, but its specific catalytic features make it the brightest, meaning the signals are a lot stronger than with other

luciferases. This reporter is naturally secreted in an active form from mammalian cells and therefore can be monitored in the corresponding medium of cultured cells, as it is the case in this thesis, but also in blood and urine of experimental animals. This has the advantage of avoiding cell lysis and as a result leaving the cells undamaged and available for other analyses or measurements at different time points. Moreover, the reporter protein is very stable in culture medium with a half-life of around 6 days, so samples can be stored at 4°C for several days without a significant change in its reporter activity, though its luciferase is only stable for two hours. The levels of GLuc in the medium – or blood or urine – are linear regarding cell number, growth and proliferation (Tannous, 2009).

Gaussia Luciferase catalyzes, ATP-independently, the oxidative decarboxylation of Coelenterazine resulting in the production of Coelenteramide and blue light at 480nm wavelength as shown in Figure 10. Those properties, in addition to the fact that recombinant GLuc produces the highest number of photons per mole of any luciferase, make it a highly sensitive reporter. (Tzertzinis, Egaña, & Shea, 2010)

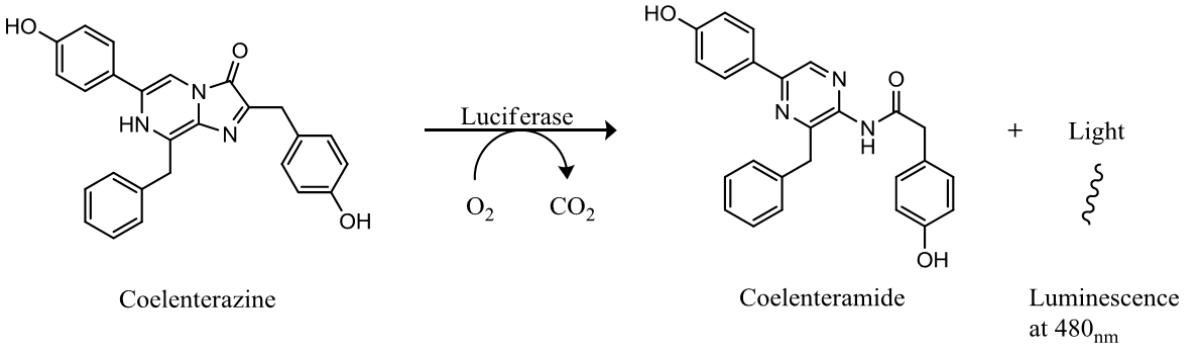


Figure 10: Coelenterazine catalyzed to Coelenteramide and light by Luciferase (Assay bio Tech).

For the measurement of the Notch pathway activity using a Gaussia Luciferase reporter, it is crucial to transfect cells with CBF-GLuc plasmids which also include Notch pathway promotors.

4.3 Transfection

Transfection is the process of getting foreign genetic material into cells. These genetic materials are mostly exogenous nucleic acids such as DNA, RNA or oligonucleotides, that are introduced into cells by several chemical, biological or physical methods, which can result in a change of features of these cells. Using physical methods like electroporation, sonoporation or microinjection might be tricky, since these processes are relatively toxic for the cells. Hence, using chemical compounds is a safer way of transfection to obtain good cell viability. (Polyplus, 2020)

Transfections can either be transient or stable: with transient transfections the foreign genetic material is only expressed for a limited time period and gets emitted when cells replicate once again, whereas with stable transfections the foreign genetic material integrates into the cellular genome and replicates when the host replicates itself.

Transfection is performed for therapeutic use, like human gene therapy, on one hand, or genomic studies, like enhancement or inhibition of gene expression in transfected cells on the other hand as well as for bioproduction, like virus and protein production.

Gene enhancement is performed to express a specific protein in cultured cells or animal models using a plasmid vector or mRNA. This way, correct protein folding and post-translational modifications of the recombinant protein can be guaranteed. Transfections with proteins already linked to detectable markers allow the study of promotor and enhancer sequences, protein-protein interactions or the activity of special proteins and thereby pathway activity.

Gene inhibition is performed to inhibit the expression of a specific protein in cultured cells through RNA interference. This RNA interference occurs because of endogenously expressed non-coding RNA in the way of microRNA (miRNA), which again is generated from a double-stranded RNA precursor. The mature microRNA becomes part of the RNA-induced silencing complex (RISC) which inhibits the translation of complementary target mRNA.

Another way of gene inhibition is via vector-based systems. This way, miRNA or short hairpin RNA (shRNA) precursors get digested by an endogenous machinery to form miRNA or shRNA.

A further possibility is to chemically manufacture short or small interfering RINAs (siRNA), which can also be accepted into the RNA-induced silencing complex and as a result induce gene silencing by targeting complementary mRNA for degradation. Changes to siRNA can help to forestall unwanted effects, but also to make sure that

the active strand of the dsRNA is charged into the RISC (Thermo Fisher Science, 2020).

The main steps of the whole process of transfection are the complexion of nucleic acid and interaction with the cellular membrane, followed by cell entry, release into the cytosol and finally the intracellular transport into the nucleus for the necessary expression as shown in Figure 11.
Regarding complexion of nucleic acid, it is important to mention that nucleic acids are negatively charged because of their polyphosphate backbone and therefore are unable to interact with the also negatively charged cell membrane. Cationic transfection reagents, such as polymers like polyethyleneimine (PEI) can consequently form a complex with these nucleic acids. This phenomenon is sometimes called “protein sponge effect”. As a result, those polymers protect the nucleic acid from nuclease degradation, but also allow them to interact with the cell membrane. This interaction causes cellular uptake through endocytosis, enabling transfection complexes to enter the target cells in endocytosis vesicles. After release of the nucleic acid into the cytoplasm by rupture or fusion, for example, many nucleic acids such as siRNA, miRNA etc., function directly in the cytoplasm, whereas nucleic acids made for gene transfer, like plasmid DNA, enter the nucleus and as a result are expressed transiently or permanently after genome integration (Polyplus, 2020).

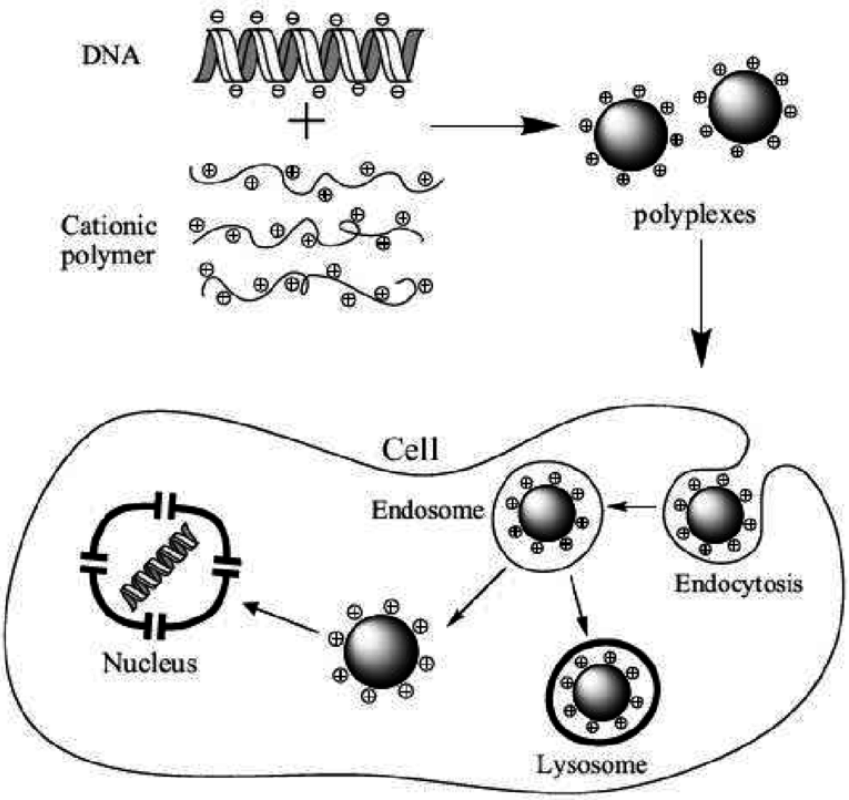


Figure 11: Schematic illustration of cationic polymer-mediated gene delivery (Gerges, 2009). Negatively charged DNA forms a complex with cationic polymers resulting in polyplexes, followed by endocytosis of the polyplexes. DNA is released from the Endosome and transferred from the cytoplasm into the nucleus by nuclear trafficking.

PEI is available in different molecular weights and configurations, including branched or linear structures. Those differences lead to different levels of cytotoxicity. It is suspected that the high transfection efficiency of PEIs leads back to the above mentioned “proton sponge effect” based on their high surface charge. With the increase of molecular weight not only the transfection capability but also the cytotoxicity increases, with linear PEI showing higher efficiency and lower cytotoxicity than the branched version (Yu, et al., 2016).

4.3.1 Transfection Controls

Transfection controls are an important tool for reliable results, since they show the constant features of cells and therefore demonstrate if the measurements are specifically associated with the trial or accidental factors. Moreover, they help to identify problems within the experiment and can be useful for further optimizations.

There are different types of plasmid controls. Usually, for plasmid-based transfection experiments, negative, positive and replicate controls are used.

Transfection controls display transfection efficiency and give information about factors influencing the transfection, as the used vector or process. There are two possibilities for transfection controls – empty vector or internal controls. Empty vector controls are plasmids without the variable in question and as a result show if the transfection reagents or processes are in any way cytotoxic for the target cells. Internal controls measure transfection efficiency by a reporter protein which is either co-transfected with the test plasmid or transfected into a separate well of cells (Cortez, 2015). For this thesis, both methods were of relevance. As an empty vector control pUC19, which was first created by Joachim Messing and is shown in Figure 12, was chosen. It is one of the most important plasmids, since cells with foreign DNA inhibit a specific enzyme, which is necessary for a signaling cascade resulting in a change of colonie-color from white to blue. As a result, transfected cells can easily be distinguished based on color differences, if needed, which is described in **Fehler! Verweisquelle konnte nicht gefunden werden.**. (Carl Roth, 2020) Internal controls were the already mentioned reporter proteins.

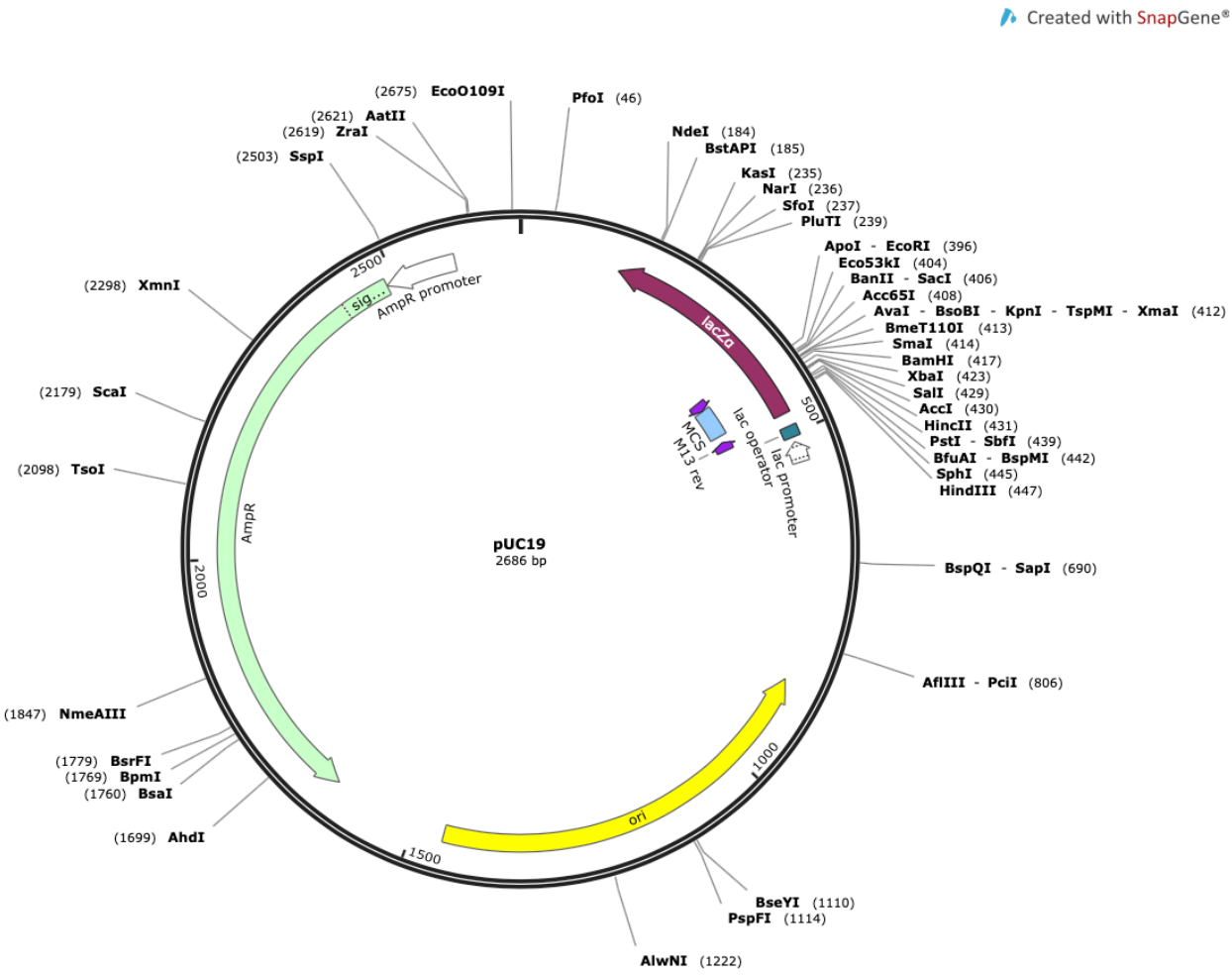


Figure 12: Map of pUC19 (New England Biolabs, 2020)

The origin of replication (ori) is visible in yellow, the ampicillin resistance gene (AmpR) in light green, the ß-galactosidase gene (lacZ) in purple. The multiple cloning site (MCS) is split into codons 6-7 of the lacZ gene. Foreign DNA can be introduced by inserting it into the MCS region (Wikipedia, 2020).

Negative controls include untreated cells, empty vector controls and non-targeting controls. Those controls should show no effect. In every experiment using plasmids, untreated controls should be used since they show the baseline of the host cells. Empty vectors in this context show if the vector itself has any effect on gene expression. Non-targeting controls should be used for experiments including gene targeting or genome editing technologies such as CRISPR, but since these technologies weren't used in this thesis, they are not described in further detail.

Positive controls are supposed to show the expected effect and include the already mentioned internal control vector. Other possible positive controls are dependent on the experiment and need to be chosen accordingly.

Replicates can be separated into technical and biological ones and serve the reproducibility of experiments. Technical replicates are not independent, since they are derived from one source. One example is the transfection of multiple wells within one plate with plasmids from the same aliquot. This kind of control doesn't demonstrate the reproducibility of the effect in question since the used cells, plasmid and medium were derived from the same source and also were incubated on the same plate at the same time. Nevertheless, it is very important, because it shows the consistency of the hands working on the experiment. Biological replicates are independent and more time-consuming, but also of higher importance. At best, the replicates should be performed using fresh medium each time, independent aliquots of cells and plasmids and on different days, but in practice there are limitations to this ideal situation. Experiments should at least be performed multiple times and on different times, using different aliquots of plasmid and cells. For this thesis both methods were relevant.

4.4 Multi-Reporter Plasmids

For studying complex coherences in cellular signaling pathways, efficient and unique tools are necessary to study changes in gene activation or repression (Hughes, Choi, Dobbs, Narahari, & Webb, 2012). As already mentioned above, reporter genes were used for this purpose. Most assays rely on single transfection to identify the phenomena in question. Due to the complexity of biological systems additional screening assays must be performed to gain more information, though this is linked to tedious assay development, more time and higher costs. In addition, autonomously performed screens may lead to increased overall errors. Multi-reporter plasmids might be able to address these limitations.

In case of multi-reporter plasmids, cells are transfected with only one plasmid containing multiple reporter proteins allowing multiple readouts from one sample. As a result, the gain of a more detailed cellular signature, and therefore the difference between healthy and disease-associated events is possible. Even subtle changes in

signaling pathway activity (Sarrion-Perdigones, et al., 2019), as well as the simultaneous analysis of their interplay can be surveyed using multiple luciferase reporters, as it was the case for this thesis. For multi-reporter plasmids, it is also important to compare their performance to the corresponding single-reporter plasmids and to show that there is no cross-reactivity between substrates. Previous studies showed that there are indeed cross-reactions. Cell lysate of both TOP-NLuc and CBF-GLuc transfected cells gave a positive signal with coelenterazine as a substrate, while when analyzing the supernatant only CBF-GLuc transfected cells gave a positive signal. For this reason it was decided to measure FLuc and NLuc and therefore the pathway activities of Hedgehog and Wnt from cell lysate, but GLuc and thus Notch pathway activity from supernatant. These experiments were already performed previously by Maier and colleagues (Maier, et al., 2019).

Literature also shows that a plasmid with the genetic information of six reporter proteins implemented on one vector already exists, whereby each unit gets copied equally often in each transfected cell. Classical cotransfection, in contrast, provides confidence that each plasmid gets equally uptaken by every cell, resulting in substantial fluctuations. A comparison between both methods shows that solotransfection and therefore the use of multi-reporter plasmids leads to more consistent results. Another advantage is the simplification of the transfection process, since only one transfection is needed. In the experiment performed by Sarrion-Perdigones and colleagues, all measurements were done on one well successively with the help of a quencher. The experiments for this thesis, in contrast, were also solotransfected with a plasmid containing the genetic information of three different reporter plasmids, but the readouts were performed differently. While one luciferase was read from the supernatant, the cell lysate was split into different wells for the measurements of the remaining readings, as illustrated in Figure 15. Another distinction between the experiments is the transfection reagent: while Sarrion-Perdigones et al. (2019) used Lipofectamine 3000, LPEI was used for this thesis. A similarity is though that the size of the used plasmids may be a concern regarding cell lines which are hard to transfect, since both plasmids are relatively large with 13.4 kb (Sarrison-Perdigones et al., (2019)) and 15.5 kb (Maier, et al., (2019)). (Sarrion-Perdigones, et al., 2019).

4.5 Multiplexing

In the complex field of cell analysis, multiplexing is a precious tool since it describes the process of gaining different sets of data from the same sample or well. This means that multiple experimental elements can be simultaneously evaluated, leading to an increased throughput of measurements and therefore minimizing time and cost while investigating individual components. Multiplexing also often goes hand in hand with the usage of multi-reporter plasmids. Researchers are combining more and more assays to study intricate biological questions for different purposes like pathway signaling events as it was the case for this thesis. Technological advances, for example in flow cytometry or the use of plate readers, make multiparameter measurements of one sample at the single cell level feasible. Additional technologies will increase the parameters which can be analyzed even further. (Behind The Bench Stuff, 2019) Due to the technical progress in developing diode pumped lasers, there are already easy to use flow cytometers available equipped with three or more lasers. In most cases the excitation wavelength ranges from near UV (405 nm) to dark red (638 nm), enabling coincident measurements of a number of distinct fluorescent proteins.
Combined luciferase reporters can differ concerning their substrate requirement, cell trafficking (secreted or cytoplasmatic) or enzyme kinetics (Maier, et al., 2019). Advantages of luciferase over fluorescent proteins are higher sensitivity, wider dynamic range and the lack of auto-luminescence in mammalian cells. A lot of multiplex luciferase experiments use a combination of FLuc and Renilla luciferase or NLuc. In these cases, FLuc is used to detect the cellular signaling event in question, while the other luciferase is used as an internal control. This way both luciferases can be sequentially quantified in the same sample, while preventing pipetting or other handling errors which could arise if readings were performed from different samples. In recent studies four criteria were used to determine luciferases for which a detection in a single multiplex experiment would be possible, those were the following:

1. Favor of using a single substrate over any other and capability of luminescence quenching.
2. If the same substrate is preferential, a minimal overlap in emission spectrum is necessary that can be differentiated using emission filters and/or mathematical calculations.
3. Steady light emission through the experiment.

4. A wide dynamic range of light emission for detecting slightest changes in pathway activity. (Sarrion-Perdigones, et al., 2019)

4.6 Transfection Normalization Methods

Another important part of the workflow is the normalization of biological raw data. Without normalization an accurate interpretation of results cannot be guaranteed. Normalization to a common shared parameter is needed for a valid comparison, whether for comparing different cell types, genetic modifications or compound treatments. It can relate to various features, e.g. cell number, genomic DNA or total cellular protein (Kam, Rogers, Jastromb, & Dranka, 2018).

4.6.1 Bicinchoninic Acid (BCA) Assay

The BCA assay consists of two components and is a high-precision protein assay for the detection of protein concentrations. This assay is affected less than other dye-binding methods when it comes to protein compositional differences, hence ensuring greater concentration precision.
Because of the calorimetric aspect first ratings can be made visually, exact measurements can then be performed using a standard spectrophotometer or, as it was the case for this thesis, a plate reader at 562 nm wavelength. It reveals less protein-to-protein variations than the Bradford assay, for example, while being unaffected by normal concentrations of most ionic and non-ionic detergents. The incubation time is 30 minutes, which is four times quicker than standard Lowry methods. The assay ranges from 20 to 2000 µg/mL, while it can identify a minimum of 5 µg/mL using improved protocols.

The method works by merging the protein driven reduction of Cu^{2+} to Cu^{1+} in an alkaline medium with the extremely sensitive and selective colorimetric verification of Cu^{1+} by bicinchoninic acid.
First, copper and peptides with three or more amino acid residues form a chelate in an alkaline setting encompassing sodium potassium tartrate, resulting in a light blue complex and a reduced cuprous cation. This first reaction is known as the Biuret reaction. In the following step, bicinchoninic acid reacts with the reduced cation so that two molecules of BCA form a chelate with only one cuprous ion, yielding an intense purple color. This complex is water-soluble, exhibits a linear absorbance with rising

protein amount at 562nm wavelength and is about 100 times more sensitive than the complex resulting from the first step.
The reduction is biased by the amino acid residues cysteine, cystine, tyrosine and tryptophan, but only at room temperature. At 60 degrees Celsius the peptide backbone is responsible for the reduction and therefore for the color formation, leading to a minimization of variability caused by different protein compositions (Thermo Fisher Scientific , 2020).

4.6.2 CellTiter-Fluor

Cell viability assays are frequently used when it comes to screening compounds which could have an effect on cell proliferation or show cytotoxic effects which would lead to cell death. Irrespective of the detailed cell-based assay being used, it is of great importance to know the cell number of the viable cells at the end of each trial.

As one marker of cell viability, CellTiter-Fluor (CTF) indicates the presence of conserved and constitutive protease activity of those cells. It uses a fluorogenic cell-permeable protease substance to selectively track down this protease activity which is limited to viable cells. The mentioned substrate is glycylphenylalanyl-aminofluorocoumarin (GF-AFC). It has the ability to intrude into living cells after which cytoplasmic aminopeptidase activity removes its amino acids glycine and phenylalanine and thereby unleashes aminofluorocoumarin (AFC). AFC in return, produces a fluorescent signal which is proportional to the viable cell count. Only living cells have this aminopeptidase activity, since the protease activity disappears rapidly as soon as cells die.

The GF-AFC substrate is fairly non-toxic to cells in culture, but a long-range exposure of cells leads to slight changes in viability measured by ATP as a marker. Due to the non-toxic features it is suitable for multiplexing using also other assay technologies, following a consecutive assay protocol (Riss, et al., 2013).

5 Materials and Methods

5.1 Equipment

List of equipment:

- Biological safety cabinet (Thermo Fisher Scientific)
- Centrifuge (Thermo Fisher Scientific)
- Centrifuge tube 15ml (CT-15) (Starlab)
- Centrifuge tube 50ml (CT-50) (Starlab)
- Counting chamber (Neubauer-improved, Paul Marienfeld GmbH & Co.KG)
- EppendorfR ResearchR plus pipettes (Sigma-Aldrich)
- Eppendorf tubes (Nerbe plus GmbH)
- Freezer (-20°C, -80°C) (Thermo Scientific)
- Incubator 37°C, 5% CO_2 (Thermo Fisher Scientific)
- Inverted microscope (Motic)
- Multichannel pipette (VWR International)
- Pipette boy (VWR International)
- Pipette tips (Nerbe plus GmbH)
- Plate reader (Infinite M200 Pro, Tecan)
- Plate shaker (Eppendorf)
- Reagent reservoirs (VWR International)
- Refrigerator (4°C) (Allectric)
- RPMI 1640
- Serological pipettes 5ml, 10ml, 25ml (Sarstedt)
- TC flask T75, standard, vent. cap. (Sarstedt)
- Transparent 96-well flat-bottom microplate, sterile (Greiner)
- Vacuum aspiration system (Integra Biosciences)
- Vortex mixer (Velp Scientifica)

- Water bath (Memmert GmbH)
- White 96-well flat-bottom microplate, transparent bottom, sterile (Greiner)

List of reagents:

- BCA Protein Assay Kit check
- Bovine serum albumin (Sigma-Aldrich)
- Cell lines:
    - HEK293T
    - MDA-MB-231 WT
    - MDA-Mb-231 LM 2-4
    - CT26
    - LS174T
- Coelenterazine
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)
- Dulbecco's modified eagle's medium (DMEM) high glucose (Sigma-Aldrich)
- Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldrich)
- Fetal bovine serum (FBS) (Sigma-Aldrich)
- HEPES buffered glucose (HBG, 5 % glucose w/v, 20 mM HEPES pH 7.4), MMCT (University of Vienna)
- HEPES buffered saline (HBS, 145 mM NaCl, 20 mM HEPES pH 7.4), MMCT (University of Vienna)
- L-Glutamine 200mM (Sigma-Aldrich)
- Linear polyethylenimine (LPEI), 10kDa, MMCT (University of Vienna)
- Luciferase assay reagent buffer + luciferin (LBL)
- MilliQ-water (Sartorius)
- Nano-GloR Luciferase Assay Buffer + Substrate (Promega)
- NanoLuc purified protein (0.4mg/ml in DPBS pH 7) (Promega)
- Passive Lysis Buffer 5x (Promega)
- Penicillin-Streptomycin (Sigma-Aldrich)

- pUC19, #50005 (Addgene plasmid)
- Trypsin-EDTA solution (Sigma-Aldrich)
- Versene (Gibco by Life Technologies)

The following plasmid was generated by Julia Maier in the course of her doctoral thesis and used for the experiments in this thesis: (Maier, et al. 2019)

- pMuLE_EXPR_CMV-eGFP_TOP-NLuc1.1_GLI-FLuc_CBF-GLuc, 3 pathway-luciferases, 3P-Luc plasmid

(These lists are derived from Elmenofi 2019 and Milenkovic 2019 and adapted.)

5.2 General Workflow for a Multi-Reporter Assay Readout

To study the activity of signaling pathways using only one multi-reporter plasmid it is necessary to seed cells into a white 96-well-plate, transfect the cells with the plasmid, add the substrate and read the luminescence via different luciferase assays, as shown in Figure 13.

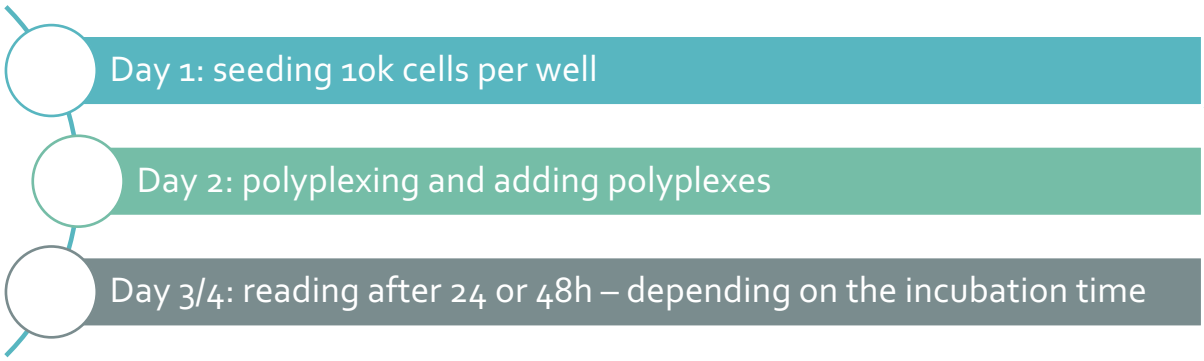


Figure 13: Workflow for the use of reporter plasmids

In preliminary trials, only HEK193T cells were used to test if the different optimized parameters worked as expected. It was of importance to know if the multiplexing worked, i.e. if BCA and the luciferase assays yielded the expected results when read from one well, adjusting the PLB1x-amount to 40µl. The reading of different luciferases was not done in one well but in different plates by dividing the cell lysate from one well into different plates. Moreover, it was essential to examine if different amounts of plasmid showed different results. Once this was clarified, other cell lines were also tested, while HEK293T served as a control in every run, since polyplexes from the same aliquot were always added to the cell line in question and HEK293T. Detailed information about the methods is given in the following subsections.

5.2.1 Cell Lines for Studying Signaling Pathways

HEK293T

In 1973, kidney cells were derived from an aborted human embryo by transformation using sheared Adenovirus-5-DNA. Several months after the isolation of a single transformed clone and many attempts, cell growth was detected – this cell line was named HEK293. It is known that a 4-kbp adenoviral genome integrated into its chromosome 19, which makes the cell line pseudotriploid in cyclogenetic analysis and encodes for E1A/E1B proteins. Those proteins interfere with cell cycle control pathways and therefore antagonize apoptosis.

There is a large number of different types of HEK293, out of which HEK293T was used for this thesis. The T in its name results from the expression of a temperature-sensitive allele of the SV40 T antigen, which enables the amplification of vectors incorporating the SV40 ori, which, as a result, makes this cell line highly transfectable for transient transfections.

SV40 T also forms a complex with p53, inhibits it and thus further increases genome integrity (Lin, et al., 2014).

Cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) with 10% fetal bovine serum (FBS), 1% L-Glutamine and 1% Penicillin/Streptomycin at 37 degrees Celsius and 5% CO_2.

MDA-MB-231 WT

The epithelial human breast cancer cell was assembled from pleural effusion of a 51-year-old female Caucasian with metastatic mammary adenocarcinoma. The cell line is highly aggressive, invasive and a triple negative breast cancer (TNBC), meaning there is neither an estrogen receptor (ER) nor a progesterone receptor (PR) expression and also no amplification of the human epidermal growth factor receptor 2 (HER2). The high invasiveness is due to a proteolytic degradation of extracellular matrix (Phe Cellculturecollections).

Cells were cultured in DMEM with 10% FBS, 2% L-Glutamine and 1% Penicillin/Streptomycin at 37 degrees Celsius and 5% CO_2.

MDA-MB-231 LM2-4

This human breast cancer cell line is a highly metastatic variant of the previously mentioned one and was generated in a process lasting nine months. The first step was to inject 2x106 MDA-MB-231 cells into the mammary fat pad (MFP) of adult 6-8 week old female CB17 SCID mice. This orthotopic transplantation was necessary, because human tumor xenograft transplants show a low number of rise in distant metastases when done ectopically or subcutaneously. The primary tumor was resected fully by skin incision 4 weeks later, when the tumor volumes were 300-500mm^3; afterwards the surrounding connective tissue was cleaned from tumor tissue. This is usually necessary to prolong survival times and thereby allow previously seeded metastatic cells to grow into macroscopic metastases. Those metastases can then be transplanted in order to develop a progressively aggressive metastatic variant (Fidler, 1991). Groups of mice were monthly sacrificed and checked for metastases in lungs, where full-blown diffuse metastatic spread was found after four months. Both lungs from one mouse were modulated to tissue culture and grown for three passages; the result was a cell line called MDA-MB-231 LM1. 2x106 cells of this new cell line were again injected into MFP of adult 6-8 week old female CB17 SCID mice. Around three weeks later the primary tumor had again reached the needed size of 300-500mm^3 and had to be resected as described above. Groups of mice were again sacrificed and checked for the presence of metastases in lungs, but this time mice with numerous macroscopic lung nodules spilling into the pleural cavity where already found after two months. Afterwards, several individual lung nodules were isolated and modified to tissue culture to derive established lines, one of which was MDA-MB-231 LM2-4. The cells were cultured in RPMI 1640-medium supplemented with 5% fetal bovine serum (Munoz, et al., 2006).

CT26

In 1975 BALB/c mice were exposed to N-nitroso-N-methylurethane (NMU) resulting in a grade 4 carcinoma, which is easily implanted and metastasizes readily – as a consequence the CT26 cells originate from a chemically induced tumor. This cell type in BALB/c mice is frequently used in in vivo test systems because it is a syngeneic one. Moreover, patterns of mutations in onco-relevant genes, gene expression signatures and regulated pathways of this cell line are in accordance with their colon

epithelia-origin. Mutations and expression profiles are alike to metastasis-prone human colorectal carcinoma (CRC) (Castle, et al., 2014).
Cells were cultured in RPMI-1640 with 10% FBS at 37 degrees Celsius and 5% CO_2.

LS174T
The epithelial colorectal adenocarcinoma, which served as a source for this cell line, was derived from a 58-year-old female suffering from Duke's type B adenocarcinoma in the colon. Another cell line, LS180, was deduced as the original one from the same tumor, while LS174T is the trypsinized variant and stains positively for cytokeratins (Cell Lines Service, 2020). Descendants of this cell line show microvilli and intracytoplasmic vacuoles. Moreover, they are tumorigenic in nude mice (Cell Bank Australia, 2020). LS174T cells are goblet-like and continuously produce high levels of mucin 2 (MUC2), which, when altered, is related to tumor development (Bu X. , Li, Tian, & Huang, 2013).
Cells were cultured in EMEM (Eagle's Minimum Essential Medium) with 1% L-Glutamine, 10% FBS at 37 degrees Celsius and 5% CO_2.

5.2.2 Cell Seeding

The cells were kept in the incubator in T75 flasks, and taken out forcell seeding. Color and turbidity of the medium as well as the adherence of the cells – which had to be between 85 to 90 percent – were visually checked. The flasks were transferred to the biological safety cabinet, where the medium was removed and the cells were gently washed with phosphate buffered saline (PBS). Between one to two ml of Versene or Trypsin – depending on the cell line – were added as detaching reagent and after a short incubation time of a few minutes at 37 degrees Celsius in the incubator the cells were transferred into CT15-tubes with complete medium and centrifuged. The supernatant was carefully removed and the cell pellet was resuspended in 1 ml of complete medium.

After this process the cells needed to be counted using a counting chamber and the calculation was made to know the necessary amount of cell suspension for a cell

number of 10,000 cells per well. The according amount of complete medium was pipetted into each well of triplicates of a white 96-well-plate using a multichannel pipette. In the next step the corresponding amount of cell suspension was added; all together the volume in each well needed to add up to 200µl.

After each well was visually checked under the microscope, the plate was put back into the incubator for 24 hours of incubation.

5.2.3 Polyplexing and Transfection

On the following day (after 24 hours of incubation), the seeded 96-well-plate was taken out of the incubator, medium was aspired using a Vacuboy – except from the wells needed as untreated controls – and basal Dulbecco's modified eagle's medium (DMEM) or RPMI 1640 was added into the other wells using a single pipette. Afterwards, the polyplexes had to be constructed by flash-pipetting equal amounts of linear polyethyleneimine (LPEI) to pDNA; both substances had been diluted in HEPES buffered glucose (HBG) before. To determine the needed amount of LPEI the following equation was used (Taschauer, et al., 2016):

$$m(LPEI) = \frac{m(pDNA) \times \ molecular\ weight\ of\ one\ LPEI\ subunit\ \times \frac{N}{P}}{average\ molecular\ weight\ of\ a\ nucleotid}$$

$$= \frac{m(pDNA)\ \times\ 43\ \times\ 9}{330}$$

Depending on the assignment of the wells to different treatment groups, different amounts of pDNA were used – 50ng, 100ng or 200ng, respectively as shown in Figure 14. After addition of the polyplexes, the total volume in each well had to be 100µl. Each plate was incubated for four hours at 37 degrees Celsius, afterwards another 100µl of complete DMEM were added, so that the total volume of each well amounted 200µl again. The 96-well-plate was put back into the incubator for another 20 or 44 hours, depending on the treatment.

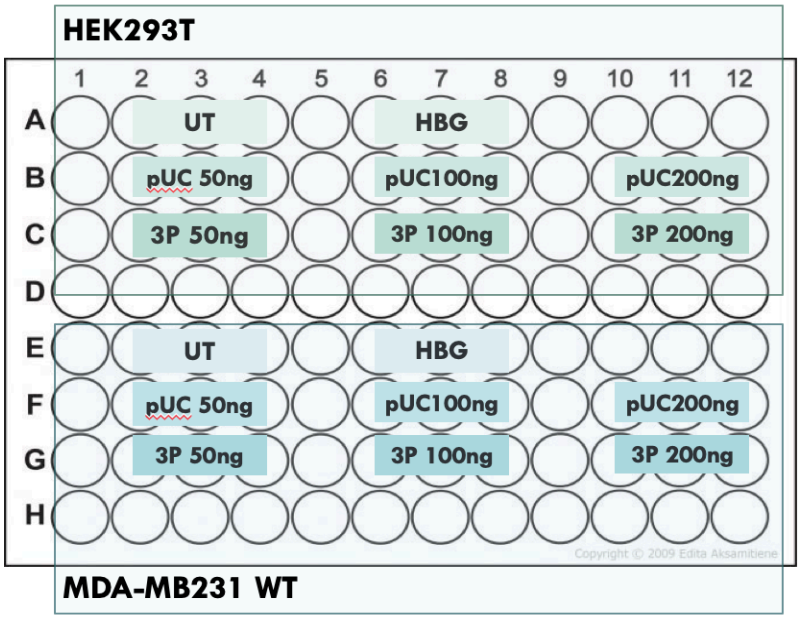


Figure 14: Schematic overview of a 96-well-plate showing different treatments

In preliminary trials only HEK293T cells were used. After all optimized were designed similar to this one, always having HEK293T cells to control if the polyplexes were working and on the bottom half of the plate the cell line in question.

5.2.4 Assay Reading

5.2.4.1 Luciferase

After a total incubation time of 24 or 48 hours, respectively, the white 96-well-plate (plate A) was taken out of the incubator and processed as shown in an overview in Figure 15 and in more detail in Figure 16.Figure **14** The supernatant was transferred into a transparent 96-well-plate (plate B), of which 20µl of each well were again pipetted into white 96-well-plate (plate C) for Gaussia Luc assay using a multichannel pipet and stored at four degrees.

The wells of the treated white plate (plate A) were washed with 200µl of PBS and 40µl of Passive Lysis Buffer (PLB) 1x premade by diluting PLB5x with MilliQ water, was added. Afterwards plate A was put on a shaker for 30 minutes with 500 rpm at room temperature.

After cell lysis, for BCA 20µl of each well were transferred into a transparent 96-well-plate (plate D), while for Firefly Luc assay 10µl were pipette into another white 96-well-plate (plate E) using a single pipette. The remaining 10µl were kept in plate A for NanoLuc assay.

Reading of all four plates (plate A, C, D and E) was done using a *Tecan Infinite M200 Pro* reader.

To measure the Relative Light Unit (RLU) by Gaussia Luc assay, 50µl of a mixture of 8.47µl Coelenterazine (stored at -80 degrees Celsius under light protection) and 4991.53µl of PBS + 5mM sodium chloride were injected directly into each well containing supernatant by the plate reader.

To measure the RLU by Firefly Luc assay, 100µl of hLAB (housmade Luciferin Assay Buffer) (stored at -80 degrees Celsius and kept under light protection) was injected directly into each well containing cell lysate by the plate reader.

To measure the RLU by NanoLuc assay, 40µl of a mixture of Nano-Glo Luciferase-Assay-Buffer + Furimazine substrate (Promega) in a 50:1 ratio and kept under light protection were also directly injected into each well of cell lysate by the plate reader.

For the experiments in this thesis, normalization of the RLU values was done by protein quantification with BCA.

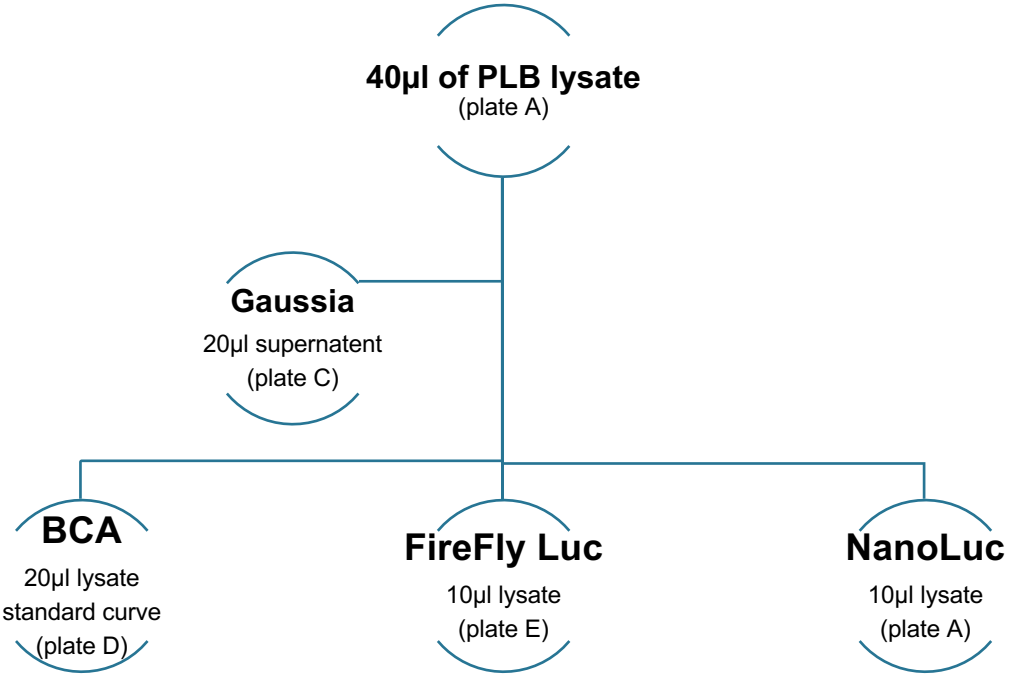


Figure 15: Schematic reading-overview for Multi-Luc/BCA assay

While BCA, Firefly Luc and NanoLuc are read from the cell lysate, Gaussia Luc is read from the supernatant. Corresponding volumes and plate names can be seen in the figure.

5.2.4.2 *Detailed Workflow*

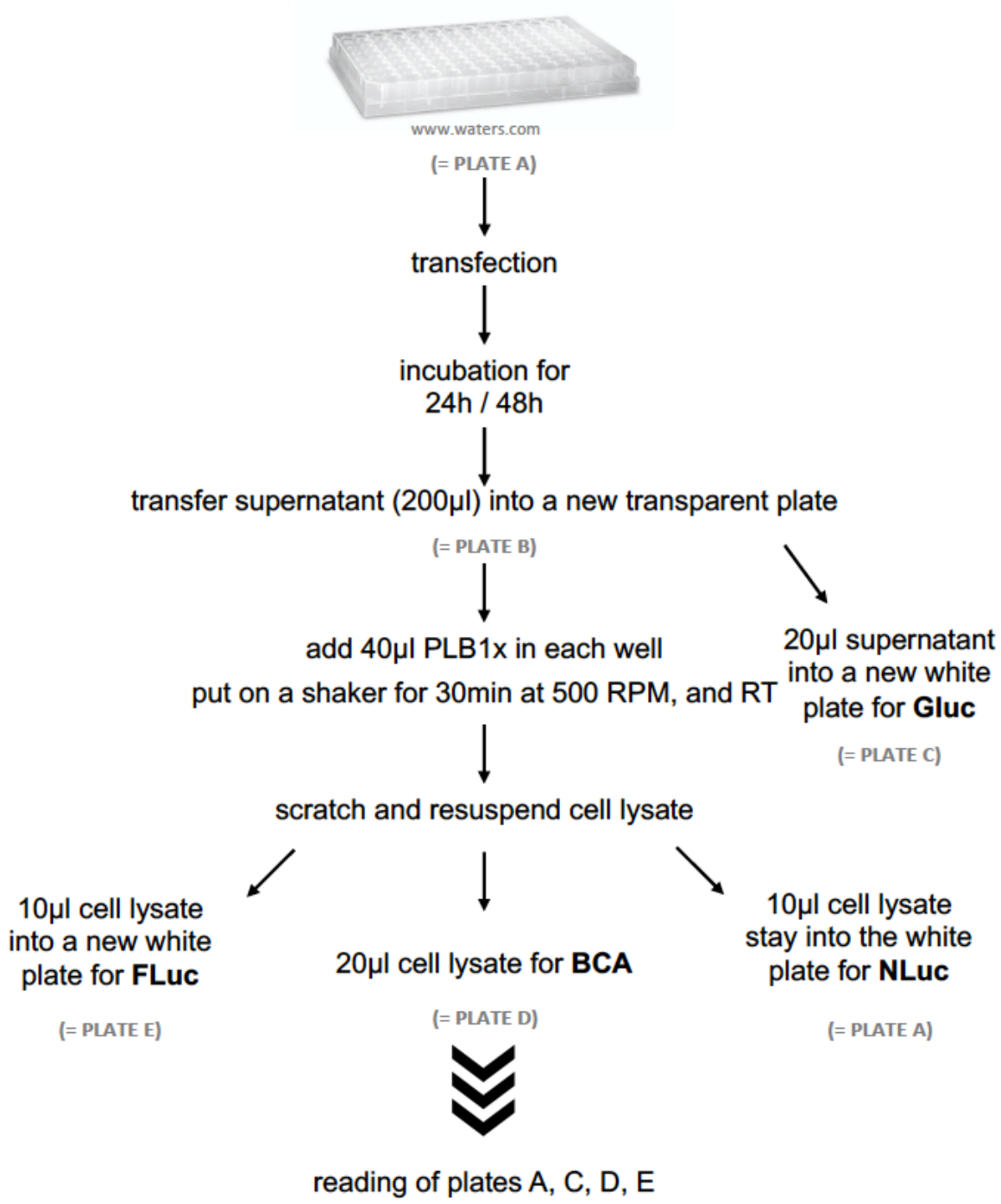


Figure 16: Detailed reading-workflow for Multi-Luc/BCA assay

5.2.4.3 BCA

In previous experiments, CellTiter-Fluor was used for cell normalization. Due to reasons like cost- and time-efficiency, a change from CellTiter-Fluor to bicinchoninic acid assay should be achieved. As shown in Figure 17, after addition of CTF into each well an incubation time of 2.5 hours is needed. Afterwards, the cell count is detectable in the supernatant. For further analysis of pathway activity cells have to be lysed, since some of the proteins in question are secreted into the cytoplasm as already described above. For BCA the 2.5 hours of incubation can be omitted, since cells lysate is anyway needed for further steps.

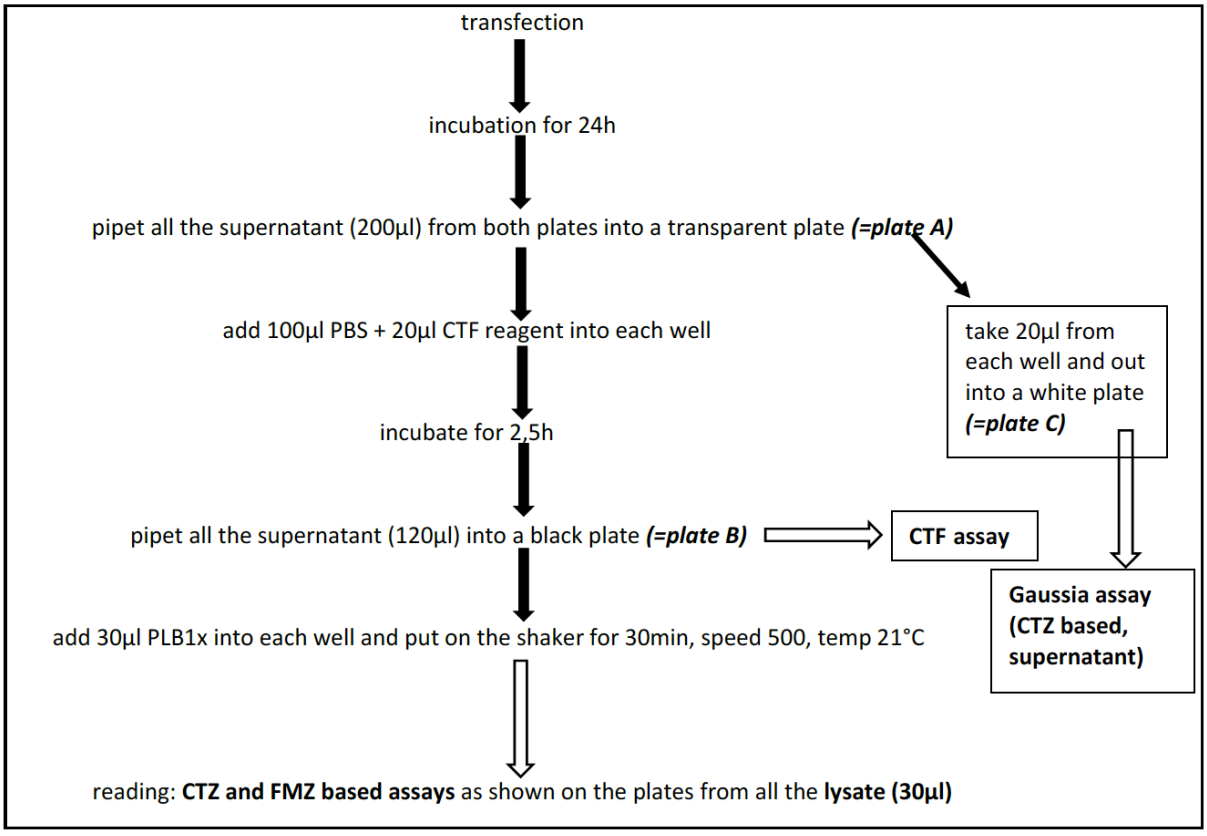


Figure 17: Preparation steps for reading based on cell count normalization using CTF (Elmenofi, 2019)

For normalization based on BCA, Bovine Serum Albumin (BSA) dilutions were prepared by mixing MilliQ-water, BSA 2mg/ml and PLB 2x in the concentrations given in Figure 18. PLB 2x was made by mixing PLB 5x with MilliQ-water.

Sample ID	MilliQ-water [µl]	BSA 2mg/ml from Kit [µl]	PLB 2x [µl]	BSA (µg)/well
2G	50,0	0	50,00	0
3F	45,0	5,00	50,00	2
4E	40,0	10,00	50,00	4
5D	30,0	20,00	50,00	8
6C	20,0	30,00	50,00	12
7B	10,0	40,00	50,00	16
8A	0	50,00	50,00	20

Figure 18: Modified BCA protocol (Elmenofi, 2019).

20µl of each dilution, starting with the lowest concentration (i.e. sample 2G) were transferred into a transparent 96-well-plate (plate D) in triplicates in order to produce a standard curve. 200µl of working reagent were added to each well of the plate D, shaken for 30 seconds at RPM 300 and room temperature and afterwards put in the incubator at 36 degrees Celsius for 30 minutes. During this time period, a change in color of the samples took place.

The color change traces back to the reduction of bivalent copper ions to monovalent ones. At room temperature mainly cysteine, cystine, tyrosine and tryptophan side chains would be responsible for the reduction, while at higher temperature all peptide bonds lead to the reaction, since the measurement is no longer dependent on the amino acid composition of samples.

At the end of the incubation time, the plate was wrapped in aluminum foil until plate reading. Reading tool place after a cooling-phase of ten minutes during which the plate reached room temperature. A *Tecan Infinite M200 Pro*-reader was used at 562nm wavelength, a bandwidth of 9nm and a flash-number of 25.

Preliminary trials were conducted with BSA only and without cell lysate to check if the measurements would result in a standard curve with a coefficient of determination of at least 0,9900.
For the actual measurements, 20µl of cell lysate of each well were also added to the measurement plate (plate D). For a precise determination of the protein amount the background levels of PLB1x used for lysing cells needed to be known. Therefore, it was essential to additionally measure triplicates of wells only consisting of 20µl of PLB1x.

To extrapolate the correct protein amounts in the total cell lysate from the different measurement volumes (20µL for BCA and Gaussia Luc and 10µL for Firefly and NanoLuc assay), the functional equation from the standard curve was used. RLU-measurements were normalized to RLU per 1µg of protein by adjusting them to the respective measurement volume relative to the total cell lysate amount and dividing by the corresponding protein amount in the lysate.

5.2.5 Normalization of 3P-Luc-Transfection for Transfection Efficiency Differences Across Cell Lines

All experiments were performed in triplicates.

It was suspected that not every cell line was equally transfectable. To check this hypothesis, special methods were chosen to check actual transfectability.

The workflow illustrated in Figure 13 was also used to determine transfectability of all cell lines under investigation.

5.2.5.1 Normalization using LucSH2

Two 96-well-plates with 20,000 cells per well, as described in *5.2.2 Cell Seeding,* of each used cell line were seeded using the pattern shown in Figure 19.

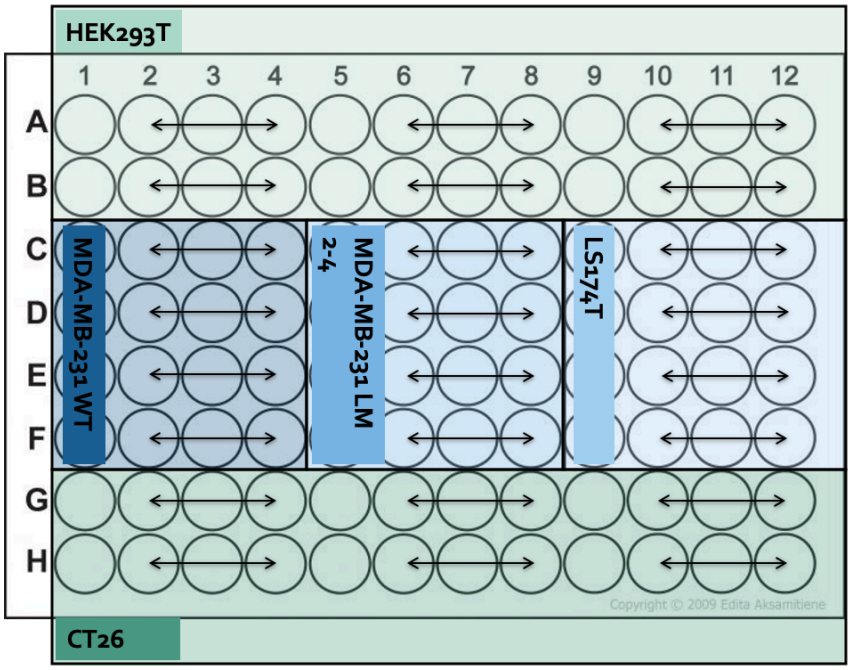


Figure 19: Seeding pattern, transfectability LucSH 2

Cells of each cell line which was checked for pathway activity were seeded, always in triplicates. The used cell lines were HEK293T, MDA-MB-231 WT, MDA-MB-231 LM2-4, LS174T and CT26.

Afterwards the plates were put into the incubator at 36 degrees Celsius for 24 hours. On the following day, two polyplexes were developed consisting of LPEI and 200ng of LucSH2 in a N/P ratio of 9; both polyplexes were equal. Medium was aspirated from all wells – except the untreated controls – and corresponding basal medium was added. Polyplexes were added as shown in Figure 20, but in this experiment same

concentrations (200ng) were used for each well. After 4 hours of incubation at 36 degrees Celsius, complete medium was added.

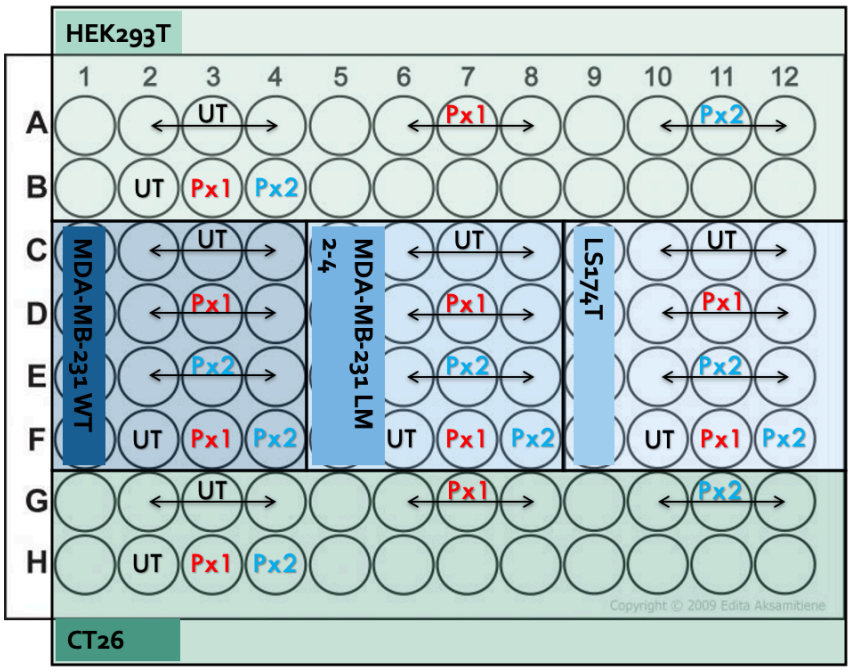


Figure 20: Transfection scheme, transfectability LucSH 2
UT marks the untreated cells, Px1 stands for polyplex 1, Px2 for polyplex 2.

After another 20 hours of incubation at 36 degrees Celsius and 24 hours after the addition of the polyplexes, one plate was taken out of the incubator and reading was performed as described in *5.2.4 Assay* Reading, but with some modifications. For this experiment only BCA and Firefly Luc assay were needed, meaning that instead of 40µl only 30µl of PLB1x were required – 20µl for protein normalization, 10µl for Firefly Luciferase. RLU-values and the protein amount of each well were normalized accordingly.

Results normalized by 3P-Luc-transfection were derived by dividing already normalized light units of cells treated with 3P-Luc and pUC19 by the net RLU per 1µg of protein of the corresponding cell line.

5.2.5.2 Normalization Using FACS

As already mentioned before, the 3P-Luc plasmid also harbors a CMV-driven EGFP expression cassette which makes cell sorting by Fluorescence-activated Cell Sorting (FACS) possible.

Two 24-well-plates with 50,000 cells per well, as described in *5.2.2 Cell Seeding,* of each used cell line were seeded using the pattern shown in Figure 21.

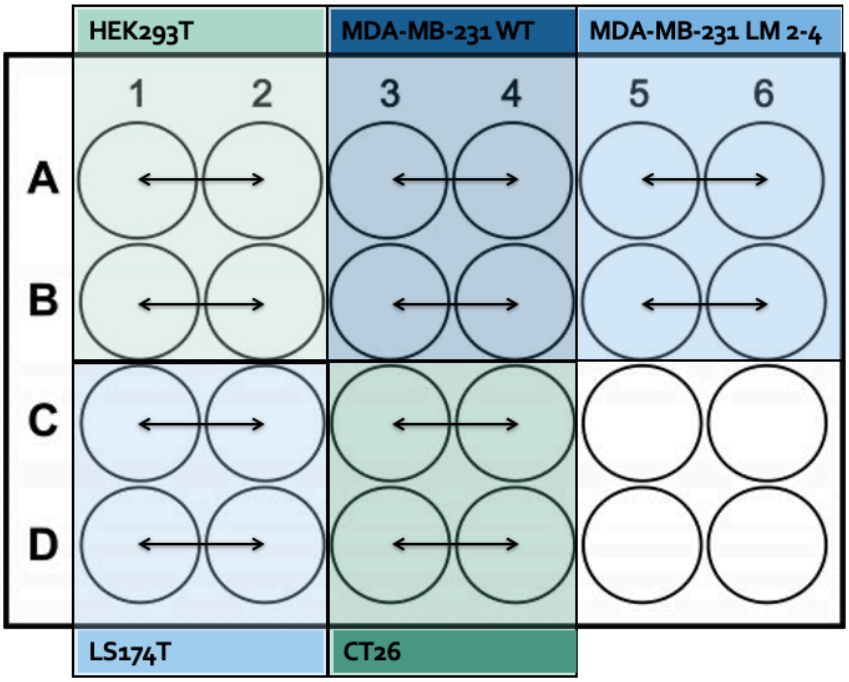


Figure 21: Seeding pattern, transfectability FACS

Cells of each cell line which was checked for pathway activity were seeded as shown. The used cell lines were HEK293T, MDA-MB-231 WT, MDA-MB-231 LM2-4, LS174T and CT26.

Afterwards the plates were put into the incubator at 36 degrees Celsius for 24 hours. On the following day, a polyplex was developed consisting of LPEI and 200ng of 3P-plasmid in a N/P ratio of 9. Medium was aspirated from all wells – except the untreated controls – and corresponding basal medium was added. Polyplexes were added as shown in Figure 22, but in this experiment same concentrations (200ng) were used for each well. After 4 hours of incubation at 36 degrees Celsius, complete medium was added.

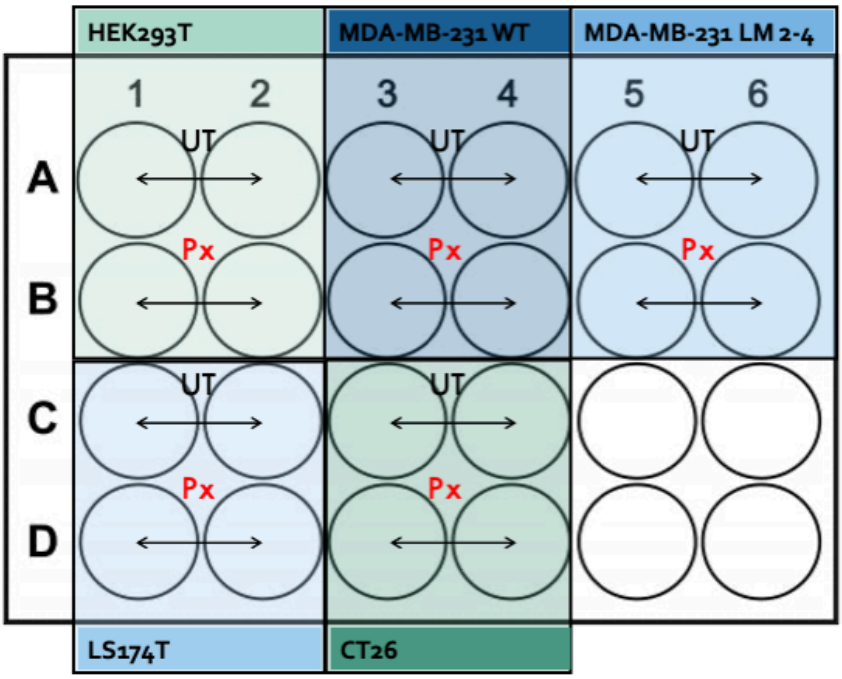


Figure 22: Transfection scheme, transfectability LucSH 2

UT marks the untreated cells, Px stands for polyplex consisting of LPEI and 200ng of 3P-Luc plasmid.

After another 20 hours of incubation at 36 degrees Celsius and 24 hours after the addition of the polyplexes, medium was aspired from all wells. After cells of each well were washed using PBS, cell lysis was performed. EGFP-signal of untreated as well as treated cells was measured using FACS.

5.2.5.3 Statistical Tests Used

As already mentioned above, extrapolations were made to in order to obtain the correct protein amounts in the total cell lysate from the different measurement volumes. Values of different cell lines were also normalized regarding their different transfectabilities and statistical significance was checked.

A legend for the symbols describing the different levels of significance used in further graphs can be found in Figure 23. Significance was calculated using the Mann-Whitney U Test Calculator and the T-Test Calculator for 2 independent means by Social Science Statistics (Statistics, 2020).

Mann-Whitney U Test Calculation			T-Test Calculator for 2 Independent Means		
			Two-tailed	0.1	+
Two-tailed	0.05	*	Two-tailed	0.05	++
Two-tailed	0.01	**	Two-tailed	0.01	+++

Figure 23: Legend for different levels of significance

6 Results

6.1 Multi-Luc/BCA Assay Optimization

6.1.1 BCA Normalization with 40µl Cell Lysate

As already mentioned above, one of the goals was to replace cell number normalization method via CTF assay with protein based normalization via BCA assay. Since cell lysing is needed for luciferase assays, using part of the lysate for BCA assay does not pose any disadvantage. Luciferase assays give signals for the whole cell lysate (including non-removed dead cells, which have nevertheless been removed as much as possible during multiple washing steps using PBS before lysing) and protein amount of the same lysate can be estimated via BCA assay. Towards this, 40µl of passive lysis buffer was attempted so that BCA assay and other luciferase assays can be read from the same lysate as shown in Figure 16. To be able to determine the protein amount under these lysis conditions of 40µl of passive lysis buffer, a reproducible standard curve had to be developed. Several sequential standard curves like the representative one shown in Figure 24 had to be produced with a coefficient of determination of 0,9900 at least until it was used on cells.

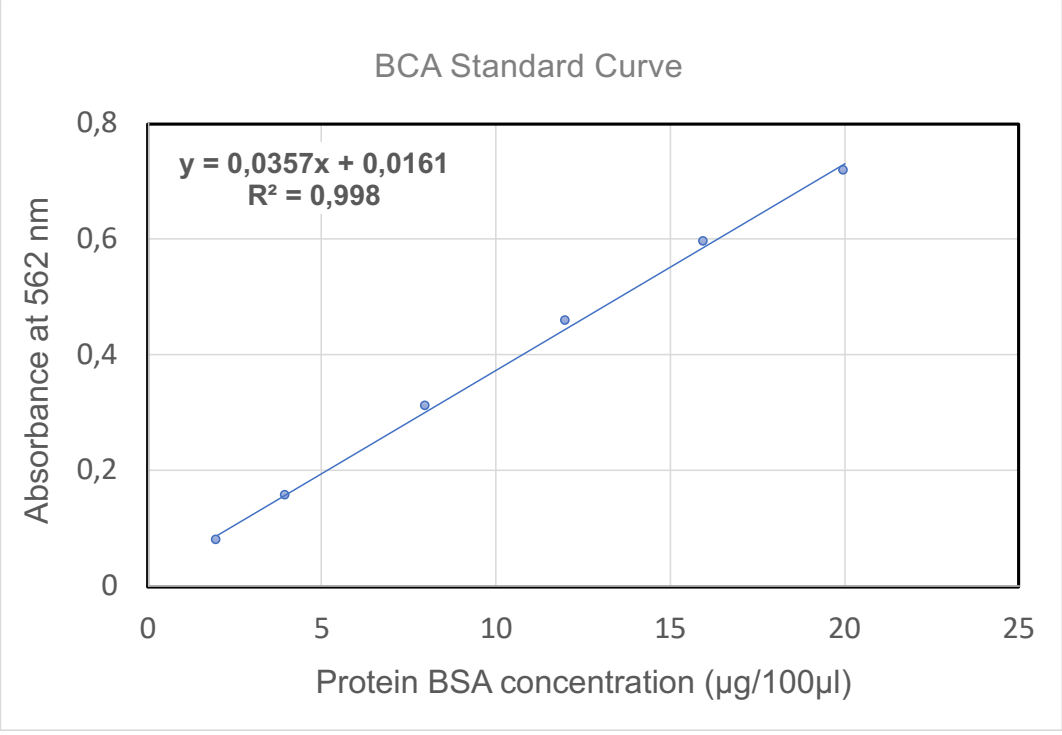


Figure 24: Representative standard curve used for protein amount determination using a Tecan Infinite M200 Pro-reader at 562 nm wavelength.

Stable transfected cells were seeded in different densities (10.000, 20.000, 40.000 and 80.000 cells per well), lysed after 24 hours and subjected to BCA and Firefly Luciferase assay, respectively, as shown in Figure 25 and Figure 26. Cells were normalized based on a corresponding standard curve. The main goal of this experiment was to see if wells with a higher number of seeded cells resulted in a higher cell density confirming that cell seeding yielded precise and reproducible results. Simultaneously, accuracy of the BCA assay could be checked using this method. To train the simultaneous handling of different assays, the experiment was repeated three times.

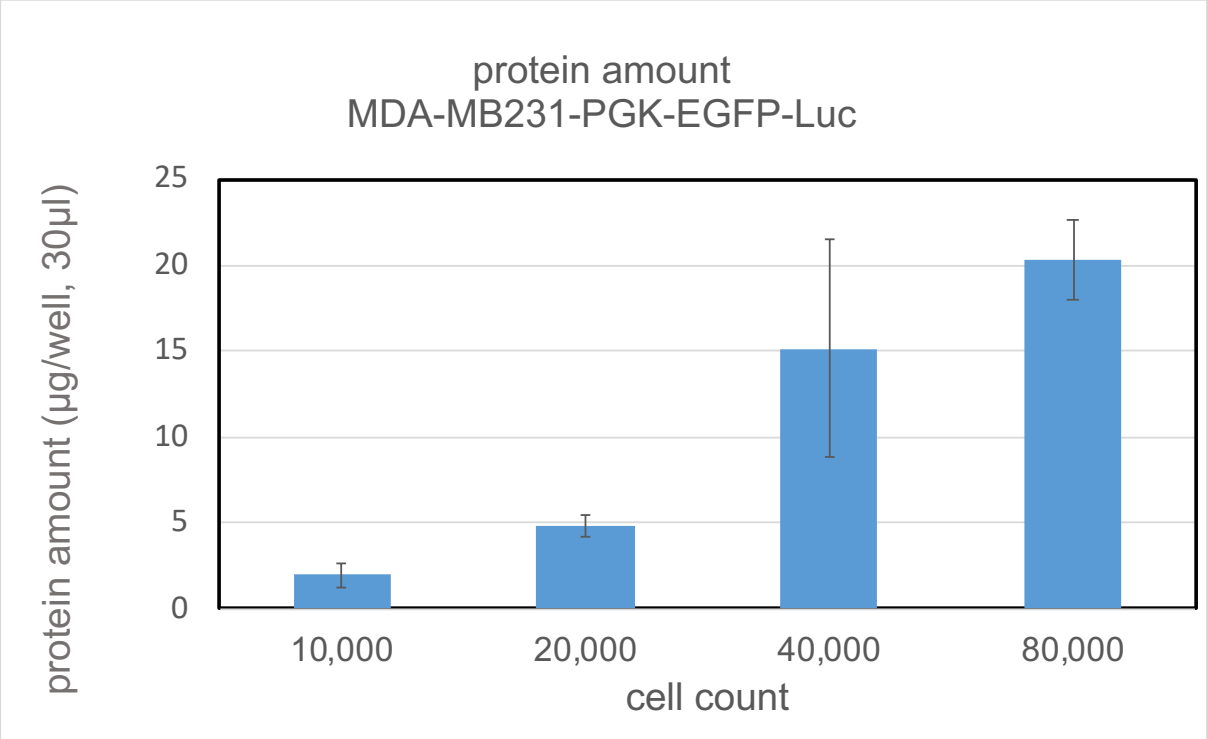


Figure 25: Representative graph of checking the reproducibility of cell seeding using BCA assay. Cells were seeded in shown densities, BCA assay was performed. The main goal was to see if cell seeding yields precise and reproduceable results. Simultaneously, accuracy of the BCA assay was checked.

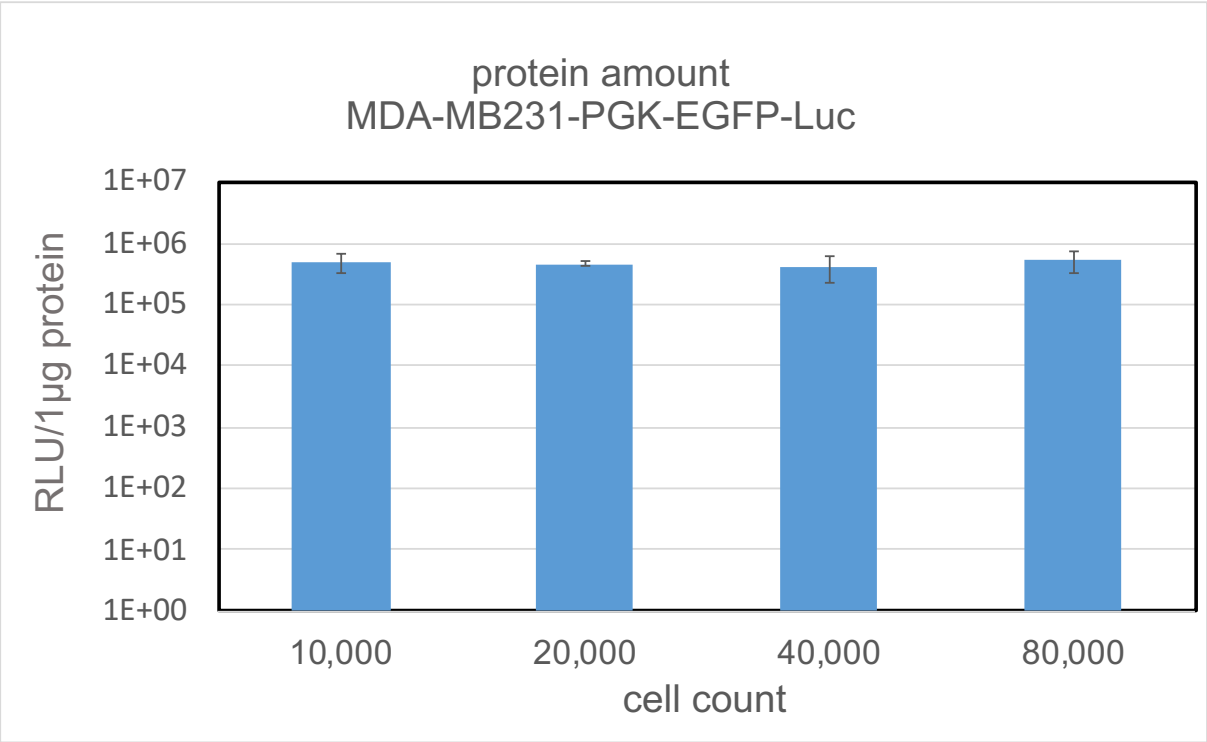


Figure 26: Representative graph of checking the reproducibility of cell seeding using Firefly assay. Cells were seeded in shown densities, Firefly assay was performed, cells were normalized using BCA assay. The main goal was to see if cell seeding yields precise and reproduceable results. Simultaneously, accuracy of the BCA assay was checked.

6.1.2 Multiplexing of Multiple Luciferases with BCA Read-Out: Multi-Luc/BCA Assay

This subsection describes the combination of BCA for protein normalization and Firefly luciferase assays with Gaussia luciferase and NanoLuc assays to achieve the esteemed multiplexing of three different luciferase assays. In the previous section, handling of reproducible BCA and Firefly luciferase assays was explained, which then had to be combined with the other two assays still ensuring reproducibility of the whole process as shown in Figure 45 in Appendix.

After a reproducible protocol was established for each assay, all four assays were combined and tested on HEK293T cells, since previous work was also conducted with this cell line which is known to be easily transfectable.

Multi-Luc/BCA Assay for Detecting Pathway Activity

One of the main aims of this thesis was to investigate the activity of the Hedgehog, Wnt and Notch pathway simultaneously in different cell lines. 3P-Luc plasmid transfections were performed in different cell lines separately and multi-Luc/BCA assay was used to estimate pathway activity. Towards this, transfections were performed using different amounts of the plasmid and measuring at different time points to get to know the minimum amount of plasmid needed for significant signal detection. This project builds on research carried out by the former PhD student Julia Maier, who generated the 3P-Luc plasmid used for the experiments at hand (Maier, et al., 2019).

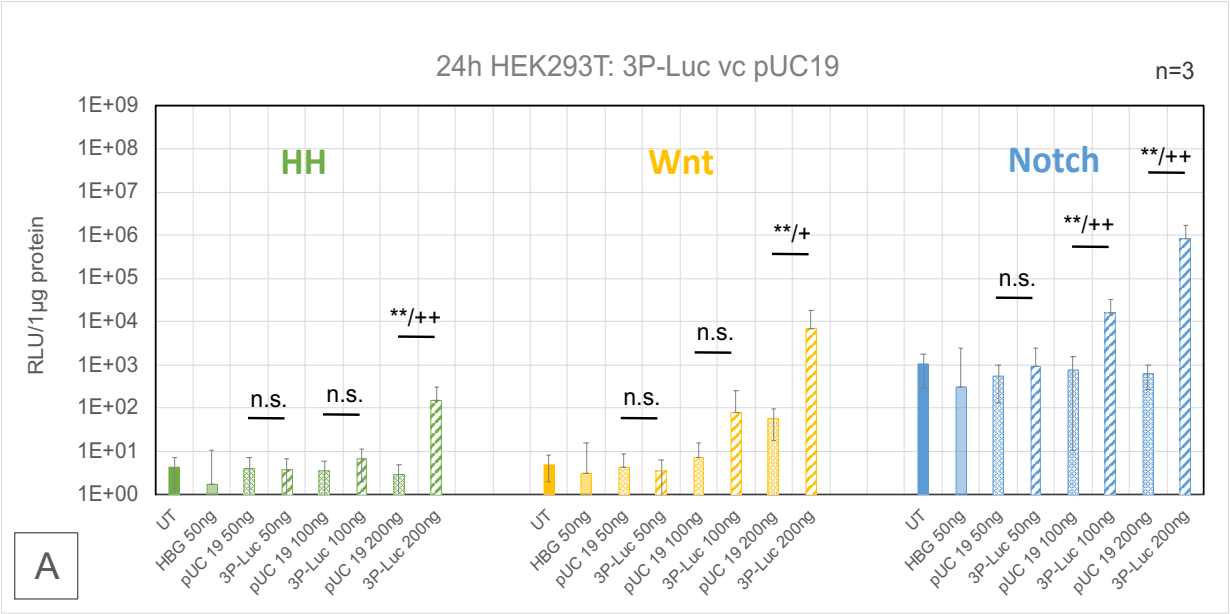
Figure 27 shows the activity of Sonic Hedgehog, Wnt and Notch pathways for HEK293T cells after transfection with 3P-Luc plasmid and measuring different luciferase read-outs with multi-Luc/BCA assay. Values of untreated cells (UT, untreated control), cells treated with only HBG (corresponding to 50ng pDNA polyplexes, buffer control) and plasmid-based polyplex treated cells can be seen. The polyplex treated cells are either treated with 3P-Luc reporter plasmid or with pUC19 plasmid (which served as a transfection control). For each plasmid, three different doses per well were tested.
Figure 28 also shows that simultaneous measurement of all three pathways is possible using the 3P-Luc multi-reporter plasmid as each pathway gave strong signals when measured with corresponding substrate. With an increase in 3P-Luc plasmid amount there is also a noticeable rise in luciferase signal, which is linked to the activity of the respective pathway. Moreover, different luciferase readouts show different background activities at both timepoints. To be more specific, Firefly readouts, which indicate Hedgehog activity show the least background activity, though it is comparable to NLuc readouts, which state Wnt pathway activity. Notch readouts, which show Gaussia activity, in contrast, show higher background activities.

After 24 hours of transfection, pathway activity can be significantly detected for 200ng 3P-Luc as the signals for each pathway are statistically different from the pUC19 transfection controls at similar concentration of 200ng. For Notch pathway, detection of activity was even possible at 100ng 3P-Luc as observed in Figure 28A.

After 48 hours of transfection, the normalized RLU-values are approximately one logarithmic unit higher than after 24 hours. In addition, the pathway activity detection

results were statistically significant already at 100ng for Wnt and Notch pathways when compared to transfection control at 100ng. For Notch even 50ng 3P-Luc gave significant signal detection. A direct comparison between both treatment times is valid since plasmids from the same aliquots were used for both experiments.

All experiments were conducted using an embryonic but immortalized cell line which is known to show overexpression of all three pathways of interest providing an environment analogous to the one in dysregulated cancer stem cells. The results show that the investigated plasmid enables measuring all three pathways of interest simultaneously in such cells using multiplexing. (Takebe, et al., 2015).



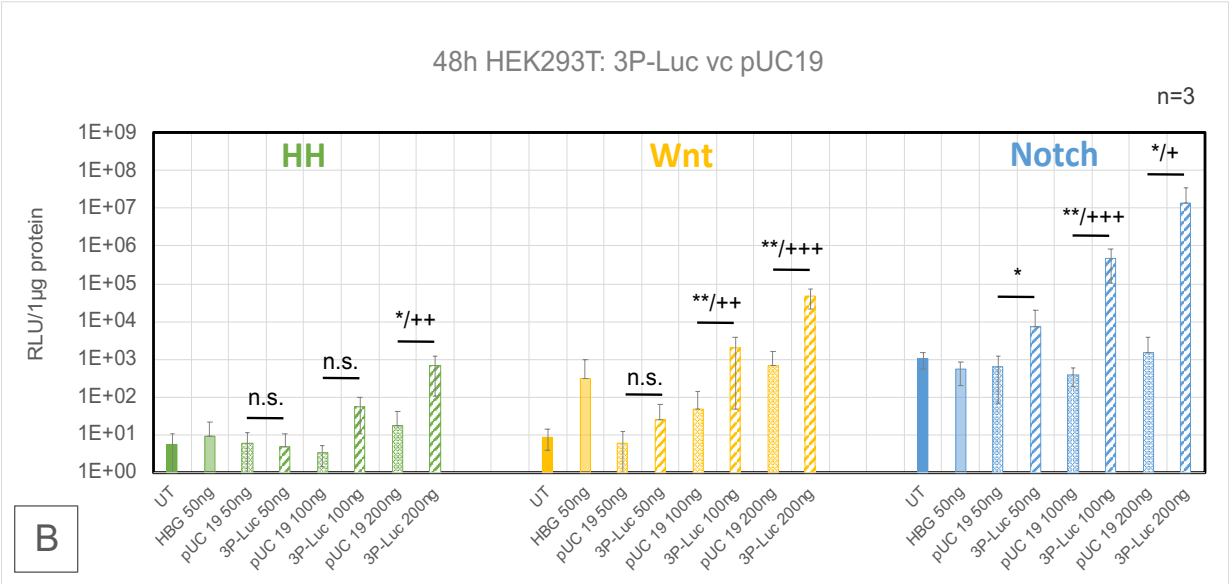


Figure 27: HEK293T

Detection of pathway activity by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways in HEK293T cells after (A) 24 and (B) 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein for cells which were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). (*P< .05, **P< .01, u-test; +P< .10, ++P< .05, +++P< .01, t-test; n.s.= not significant at both.) Experiments were performed in triplicates, n=3.

After it could be confirmed that the 3P-Luc plasmid yielded the expected results in HEK293T cells, in different amounts as well as for different durations, the plasmid was also tested on other cell lines. Plasmid of the same aliquot as used on the cells in question was also tested on HEK293T cells as positive controls in every run. Both, control and sample cell lines were seeded on the same plate as can be seen in Figure 14.

Figure 28 shows the activity of Sonic Hedgehog, Wnt and Notch pathways for MDA-MB-231 WT cells after transfection with 3P-Luc plasmid and measuring different luciferase read-outs with multi-Luc/BCA assay. Values of untreated cells (UT, untreated control), cells treated with only HBG (corresponding to 50ng pDNA polyplexes, buffer control) and plasmid-based polyplex treated cells can be seen. The polyplex treated cells are, again, either treated with 3P-Luc reporter plasmid or with pUC19 plasmid. For each plasmid, three different doses per well were tested.
Moreover, it shows that simultaneous measurement of all three pathways is possible using the 3P-Luc multi-reporter plasmid as each pathway gave strong signals when measured with corresponding substrate. With an increase in 3P-Luc amount there is a visible rise in luciferase signal at both timepoints, which is linked to the activity of the respective pathway, especially for Wnt. Different luciferase readouts show different background activities at both timepoints. In this case,too, Firefly readouts, which indicate Hedgehog activity show the least background activity, though it is comparable to NLuc readouts, which state Wnt pathway activity. Notch readouts, which show Gaussia activity, in contrast, show higher background activities.

At both timepoints, the results for Wnt and Gaussia pathway are statistically different from the transfection controls with pUC19 at a dose per well of 200ng. There is also a difference in activity visible for Hedgehog pathway between 200ng of 3P-Luc plasmid and pUC, though not statistically significant. A direct comparison between both incubation times is valid since plasmids from the same aliquots were used for both experiments.

Findings of several other studies confirm these results, since it has been already shown that the Wnt pathway affects the proliferation of breast cancer cell lines like MDA-MB-231 WT. The pathway is deregulated by autocrine mechanisms. A lot of breast tumors also have hypermethylated secreted Frizzled-related protein 1 (sFRP1)

promotor regions, which again lead to low expressions of the WNT antagonist and therefore higher WNT pathway activity. (Matsuda, Schlange, Oakeley, Boulay, & Hyned, 2009)

Dysregulated Notch pathway activity is indicated to regulate cell cycles, maintain stem cells and affect cell transformation and breast cancer. It has been already shown that an inhibition of the pathway in MDA-MB-231 cells leads to a reduction in their invasiveness (Bolós, et al., 2013). We were able to confirm these results, since a statistically significant rise of signal was detectable when comparing 200ng of 3P-Luc plasmid to 200ng of pUC19.

In contrast to the results of this thesis, other studies have found that the Sonic Hedgehog pathway also plays a role when it comes to cell viability, proliferation and migration capacities of MDA-MB-231 cells (Song, et al., 2016). One reason for the divergent results could be traced back to the preparation steps for the plate reading method used for the experiments of this thesis. For cell lysis it was necessary to scratch the cells. But, due to the physical proximity of the wells containing pUC19 and 3P-Luc, as shown in Figure 14, it cannot be excluded that no overspill happened during scratching. This would mean that droplets from the well containing 3P-Luc plasmid splattered into the wells containing pUC, which would explain the surprisingly high signals of pUC. The low value of UT cells compared to the high values of 3P-Luc-treated cells would also provide the overspill-thesis, since values of transfection controls (pUC19) should be comparable to those of untreated cells. Therefore, it would be necessary to replace the scratching part or separate the wells further for subsequent experiments.

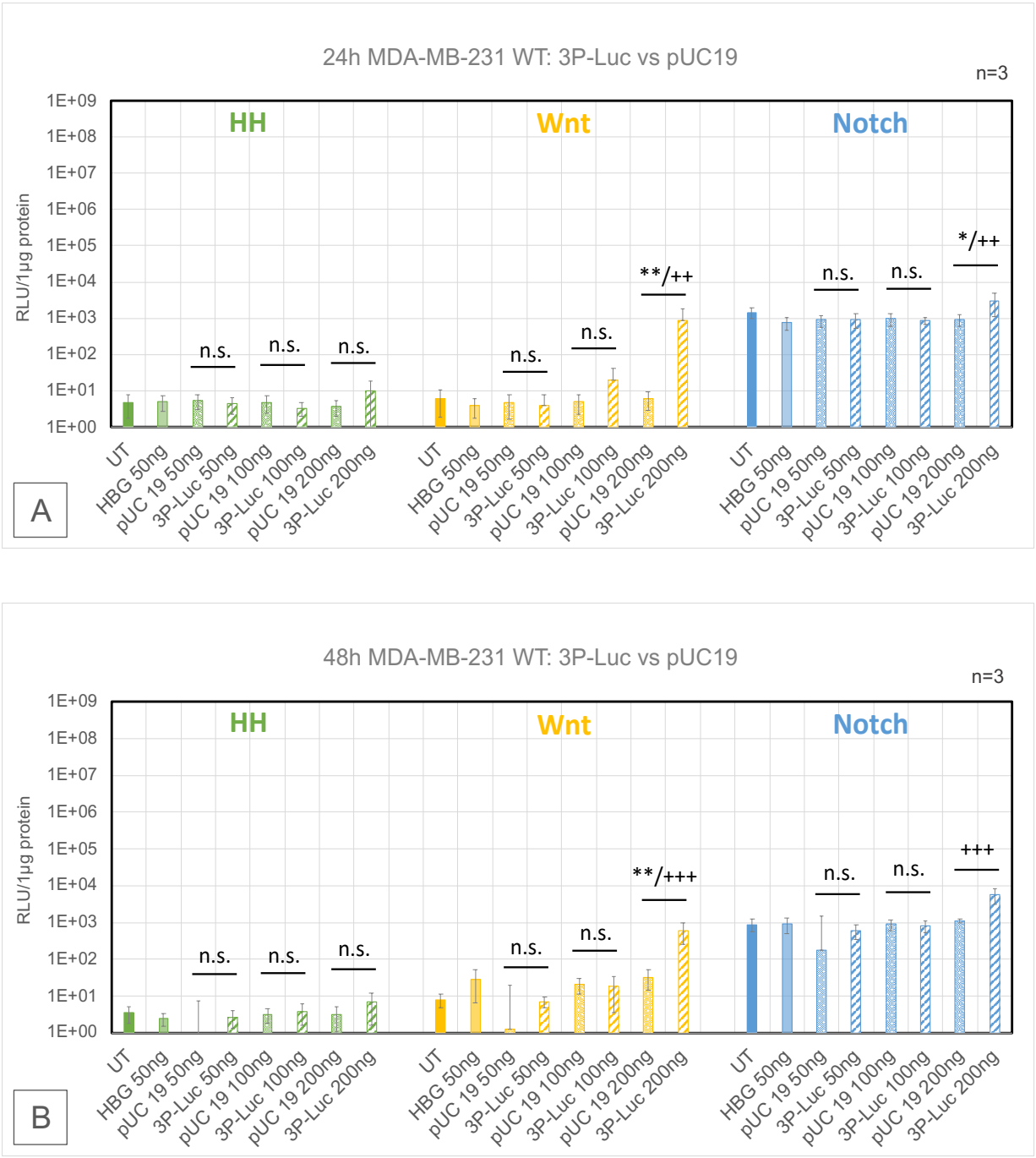


Figure 28: MDA-MB-231 WT

Detection of pathway activity by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways in MDA-MB-231 WT cells after (A) 24 and (B) 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein for cells which were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). (*P< .05, **P< .01, u-test; ++P< .05, +++P< .01, t-test; n.s.= not significant at both.) Experiments were performed in triplicates, n=3.

Figure 29 shows the activity of Sonic Hedgehog, Wnt and Notch pathways for MDA-MB-231 LM2-4 cells after transfection with 3P-Luc plasmid and measuring different luciferase read-outs with multi-Luc/BCA assay. Values of untreated cells, cells treated with HBG and plasmid-based polyplex treated cells can be seen. The polyplex treated cells are either treated with 3P-Luc reporter plasmid or with pUC19 plasmid. For each plasmid, three different amounts were tested.
In this case, too, the simultaneous measurement of all three pathways is possible using the 3P-Luc multi-reporter plasmid as each pathway gave strong signals when measured with corresponding substrate. With an increase in 3P-Luc plasmid amount there is also a noticeable rise in luciferase signal, though less than in the previously discussed cell lines, which is linked to the activity of the respective pathway. Different background activities can also be noticed at both timepoints. To be more precise, Firefly readouts, which indicate Hedgehog activity show the least background activity, though it is comparable to NLuc readouts, which state Wnt pathway activity after 24 hours of incubation. After 48 hours of incubation, Wnt background activity is about one logarithmic unit above Hedgehog activity. Notch readouts, which show Gaussia activity shows the highest background activity at both timepoints.

The signals for Hedgehog, Wnt or Gaussia pathway are not statistically different from the transfection controls with pUC19 at different amounts after 24 or 48 hours of incubation.

Interestingly, there seems to be a partial decrease in Hedgehod and Notch pathway activity between both timepoints of incubation hinting at a reduction of Hedgehog and Notch activity between the first and the second time point of measurement. Wnt pathway activity steadily increased during the experiment as expected. At all amounts and in every pathway, high RLU values were detected above the background signals of untreated cells.

No studies were found during literature research regarding MDA-MB-231 LM2-4 cells and Hedgehog, Notch and Wnt pathway which would either underline or refute these results.

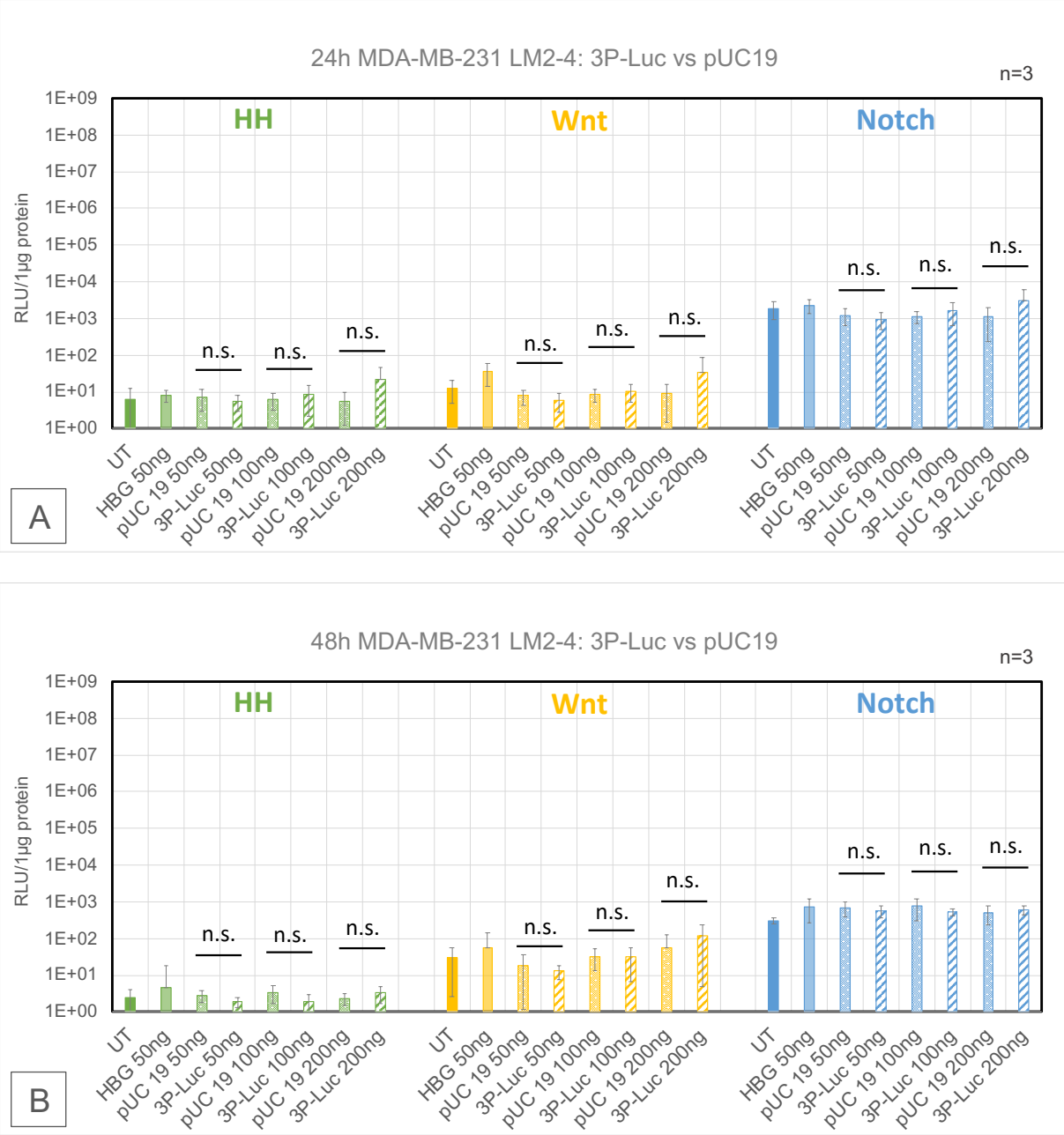


Figure 29: MDA-MB-231 LM2-4

Detection of pathway activity by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways in MDA-MB-231 LM2-4 cells after (A) 24 and (B) 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein for cells which were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). (*P< .05, **P< .01, u-test; +P< .10, ++P< .05, +++P< .01, t-test; n.s.= not significant at both.) Experiments were performed in triplicates, n=3.

Colorectal carcinoma cells were investigated as an additional cell type. The activity of Sonic Hedgehog, Wnt and Notch pathways after transfection with 3P-Luc plasmid and measurement of different luciferase read-outs with multi-Luc/BCA assays are visualized in Figure 30. Signals of untreated cells, cells treated with HBG and plasmid-based polyplex treated cells can be seen. The polyplex treated cells are either treated with 3P-Luc reporter plasmid or with pUC19 plasmid. For each plasmid, three different doses per well were tested.
The simultaneous measurement of all three pathways is valid using the 3P-Luc multi-reporter plasmid as each pathway gave strong signals when measured with the corresponding substrate. A rise of the 3P-Luc plasmid signal and therefore in pathway activity can be detected for each pathway at both timepoints. The pathways show different background activities at both timepoints. Regarding this cell line, NLuc readouts, which state Wnt background seems to show the lowest signal, though comparable to Firefly readouts, which indicate Hedgehog. GLuc and therefore Gaussia pathway indicates the highest background activity at both timepoints.

After 24 hours of transfection, Wnt and Notch pathway activity can be significantly detected for 200ng 3P-Luc as the signals for both pathways are statistically different from the pUC19 transfection controls at similar amounts of 200ng as observed in Figure 31A.

After 48 hours of transfection, the normalized RLU-values are not higher than after 24 hours. In addition, Notch pathway activity was statistically significantly for 200ng 3P-Luc when compared to transfection control at 100ng.

A direct comparison between both treatment times is valid since plasmids from the same aliquots were used for both experiments.

The reason for this lack of statistical significance in contrast to the clearly visible difference in signal activity between 3P-Luc plasmid and its correlating pUC19-plasmid could be lead back to the potential overspill while scratching and should be considered for further experiments.

Findings of several other studies emphasize these results, since Sun and colleagues, and also many others, have already shown that the Wnt pathway is upregulated in colorectal cancer. Depending on the pathways activity this could be advantageous or

harmful for the patients, since it has also been shown to be intertwined with carcinogenesis (Sun, et al., 2017). Studies regarding Hedgehog pathway and colorectal cancer are discordant in their findings. The majority concludes that the pathway is over-activated and therefore related to malignancy, while few others state it is not. Since the pathway is responsible for pattering, growth and differentiation of the gastrointestinal tract, an over-activation or mutations could lead to malignancy. Nevertheless, it is also accountable for the constant renewal of the intestinal epithelial cells in adults, which could also explain higher activity. Both options seem plausible. Some authors claim that the Hedgehog pathway activity may differ in different colorectal cancer types, which seems to be a valid conclusion (Wu, Zhu, Ruan, & Tao, 2017). Another study reports that LPS-activated macrophages from conditioned medium convey the phenotype of cancer stem cells in CT26 through activation of the Sonic-Hedgehog pathway and that those macrophages also increase chemoresistance (Fan, et al., 2018). Results of this thesis show that there is an increase in pathway activity with rising dose per well of reporter plasmid of about one logarithmic unit above the signal of untreated cells at 200ng of 3P-Luc plasmid, although not statistically significant.

Studies investigating Notch pathway also suggest that it is dysregulated in colorectal cancer and the pathways activity is obtained during tumorigenesis. When comparing colon cancer cells to colon cancer stem cells it is striking, that the stem cells show a 10- to 30-fold higher Notch activity (Vinson, George, Fender, Bertrand, & Sigounas, 2015). We were able to confirm these results as a continuous rise in signal with growing 3P-Luc amount could be shown for which RLU-values after 48 hours of incubation are significantly different from the controls.

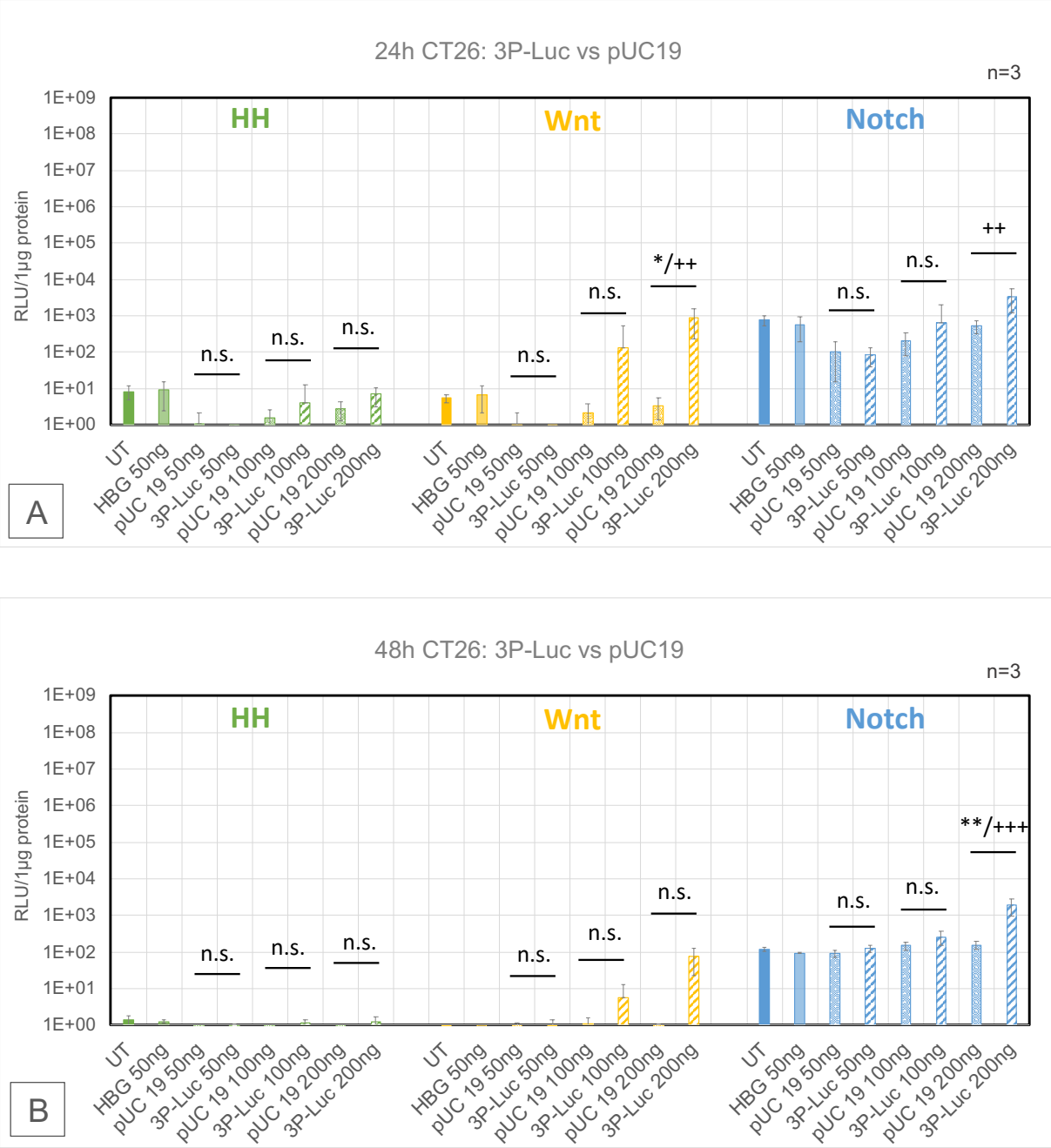


Figure 30: CT26

Detection of pathway activity by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways in CT26 cells after (A) 24 and (B) 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein for cells which were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). (*P< .05, **P< .01, u-test; ++P< .05, +++P< .01, t-test; n.s.= not significant at both.) Experiments were performed in triplicates, n=3.

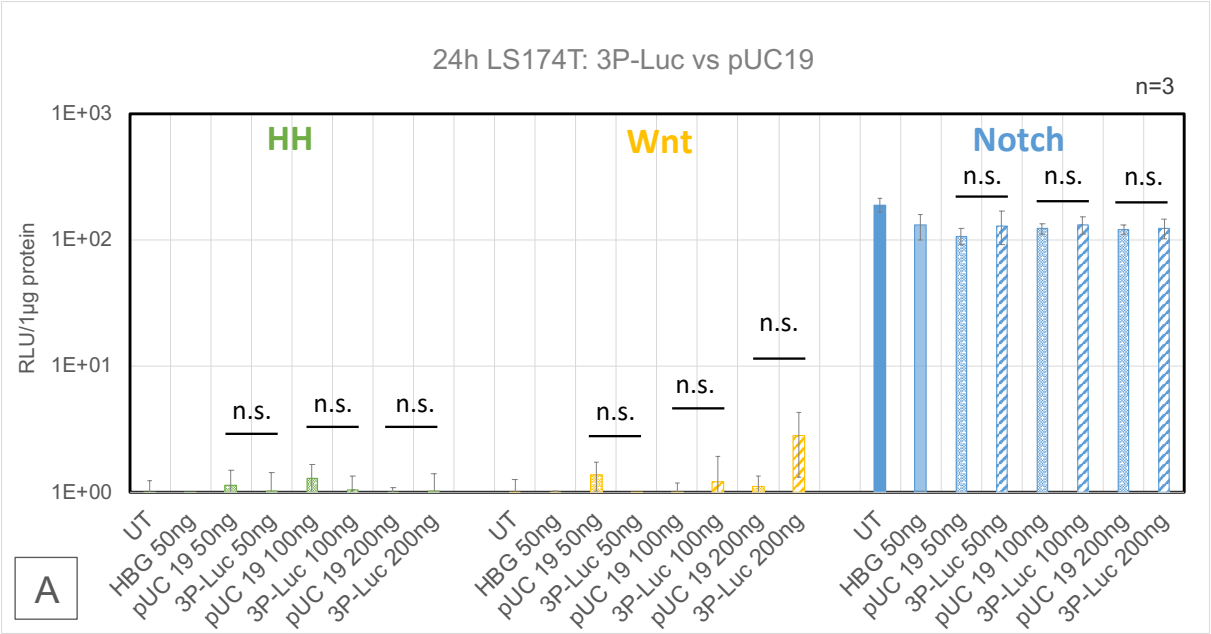
Another investigated colorectal cancer cell line was LS174T. Signals of the activity of Sonic Hedgehog, Wnt and Notch pathways after transfection with 3P-Luc plasmid and measurement of different luciferase read-outs with multi-Luc/BCA assays can be seen in Figure 31. Signals of untreated cells, cells treated with HBG and plasmid-based polyplex treated cells can be seen. The polyplex treated cells are either treated with 3P-Luc reporter plasmid or with pUC19 plasmid. For each plasmid, three different doses per well were tested.
The simultaneous measurement of all three pathways is valid using the 3P-Luc multi-reporter plasmid as pathways gave strong signals when measured with the corresponding substrate. It is striking that the background signals for Firefly readouts, which indicate Hedgehog pathway and NLuc readouts, which state Wnt pathway are only slightly above the zero point, while GLuc, which indicates Notch pathway shows a higher background activity. While analyzing this cell line it was conspicuous that there was only a rise in signal with an increase of plasmid amount for Wnt pathway. One reason for the missing significance could, again, be a potential overspill.

The results for Hedgehog, Wnt or Gaussia pathway are not statistically different from the transfection controls with pUC19 at different doses per well after 24 or 48 hours of incubation.
There is a notable difference in signal activity regarding Wnt pathway when comparing 200ng of 3P-Luc plasmid to 200ng of pUC19 after 24 hours of incubation. After 48 hours of incubation, this difference is even noticeable at 100ng, though not statistically significant.

Several other studies underline these results, stating an oncogenic mutation in ß-catenin, which results in a constitutive presence of the ß-catenin/TCF4 nuclear complexes (Mahmoudi, et al., 2009), which, in turn, leads to the constant transcription of target genes, mostly oncoproteins (Shang, Hua, & Hu, 2017).
The Notch pathway is crucial for the progression of the secretory cell lineages of the intestine. Loss of function of this pathway in the intestinal epithelium results in a massive transformation into goblet cells. A study by Bu and colleagues showed that due to a down-regulation of Notch pathway using gamma-secretase inhibitor the proliferation and invasion of LS174T cells was inhibited (Bu X. , Li, Tian, & Huang, 2011). Results gained during this thesis showed no rise in signal with an increase in reporter plasmid amount, although there was a noticeable difference in signal activity

between treated and untreated cells. For further experiments it would be interesting to also treat and measure healthy colorectal cells and compare the difference in pathway activity to carcinogenic cells.

As already mentioned above, literature research revealed contrasting results regarding Hedgehog activity in colorectal cancer cells. There is still a lack of studies dealing with the Hedgehog pathway in LS174T cells in particular. The results at hand showed no difference between cells treated with the reporter plasmid and the transfection control, neither between doeses per well nor between time points. The only significant difference could be found between treated and untreated cells.



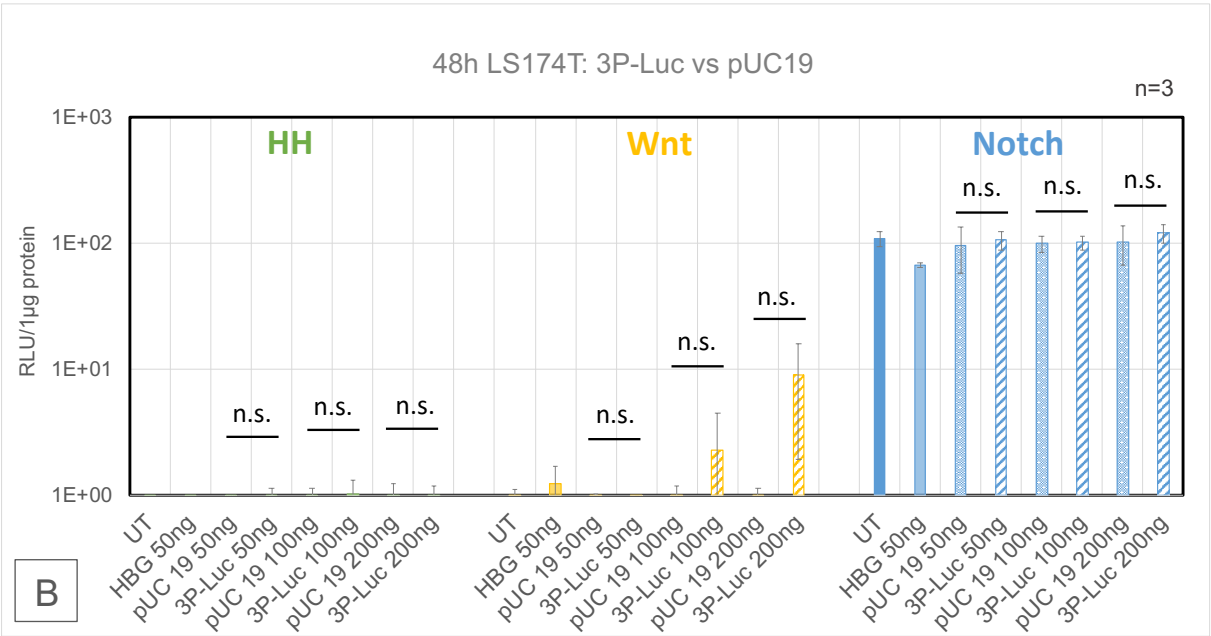


Figure 31: LS174T

Detection of pathway activity by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways in LS174T cells after (A) 24 and (B) 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein for cells which were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). (*P< .05, **P< .01, u-test; +P< .10, ++P< .05, +++P< .01, t-test; n.s.= not significant at both.) Experiments were performed in triplicates, n=3.

In summary, results show that BCA works for protein normalization and is therefore a good alternative to CTF. Moreover, it is proven that multiplexing using the 3P-Luc plasmid functions and that different readouts of differing cell lines need various minimum amount of plasmid for significant signal detection as shown in Figure 32. Previously mentioned experiments show that HEK293T cells give statistically significant signals for all pathways using 200ng of 3P-Luc plasmid. For MDA-231 WT cells 200ng of 3P-Luc plasmid are enough for detecting Wnt and Notch activity, though it is too less to detect the Hedgehog activity. MDA-MB-231 LM2-4 cells give no statistically significant signal at varying amounts and time points. In CT26 cells, Wnt activity can be detected using 200ng of 3P-Luc plasmid after 24 hours of incubation, while Notch is statistically different at both timepoints. LS174T cells give no significant signals at no time. The numbers in red in Figure 32 are compared to HEK293T cells at a later point.

		3P-Luc amount needed for significant signal detection		
		HH	**Wnt**	**Notch**
HEK293T	24h	200ng	200ng	100ng 200ng
	48h	200ng	100ng 200ng	50ng 100ng 200ng
MDA231 WT	24h	/	200ng	200ng
	48h	/	200ng	200ng
MDA231 LM2-4	24h	/	/	/
	48h	/	/	/
CT26	24h	/	200ng	200ng
	48h	/	/	200ng
LS174T	24h	/	/	/
	48h	/	/	/

Figure 32: Multi-Luc/BCA assay based estimation of 3P-Luc amounts needed for significant pathway activity detection of HH, Wnt and Notch pathways in different cell lines.

Comparison of Pathway Activity in Different Cell Lines by Multi-Luc/BCA assay

As already mentioned previously, after it could be proven that the 3P-Luc plasmid yielded the expected results in HEK293T cells, in different doses per well as well for different durations, the plasmid was also tested on other cell lines. The next step was to compare the statistically different signals of each cell line to HEK293T results, but also across different tumor cell lines.

When comparing MDA-MB231 WT cells to HEK293T cells, it is striking that signals are by far less intense than expected, while the background signals are comparable, which is shown in Figure 33 and Figure 34. For both cell lines, different luciferase readouts show different background activities at both timepoints. In detail, Firefly readouts, which indicate Hedgehog activity show the least background activity, though it is comparable to NLuc readouts, which state Wnt pathway activity. Notch readouts, which show Gaussia activity, in contrast, show higher background activities.
In contrast, plasmid treated MDA-MB-231 WT cells show less activity regarding each measured pathway at different amounts and timepoints compared to HEK293T cells.

Literature research revealed that the transfection efficiency between cell lines can differ significantly, since cells are not likely to react equally to a given transfection (Thermo Fisher Scientific, 2020). To compensate for this inequality, the transfection efficiency of each used cell was determined and taken into account during data analysis.

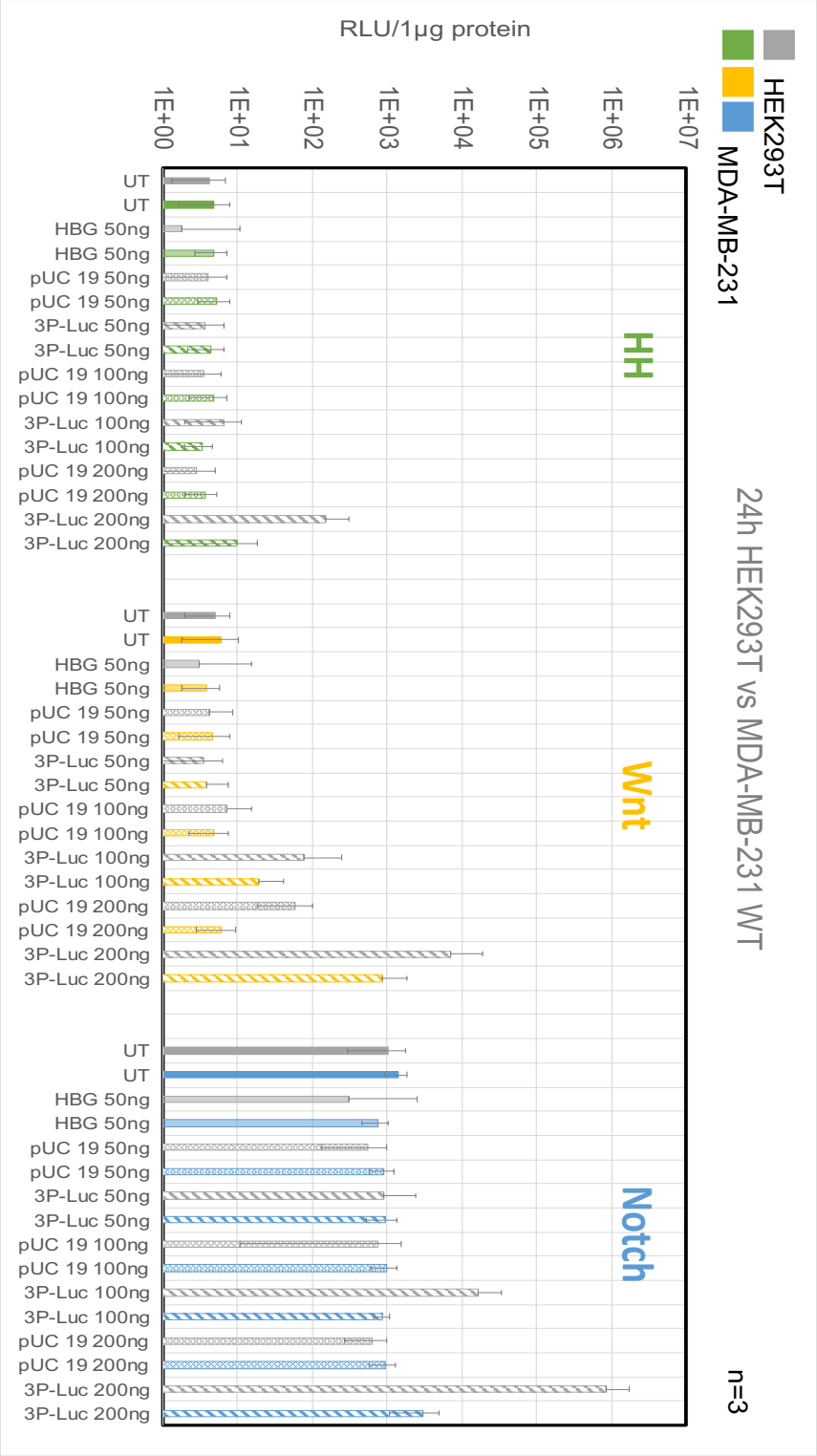


Figure 33: HEK293T versus MDA-MB-231 WT 24h

Comparison of pathway activity for HEK293T cells (grey) versus MDA-MB-231 WT cells (color) by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways after 24 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein for cells which were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). Experiments were performed in triplicates, n=3.

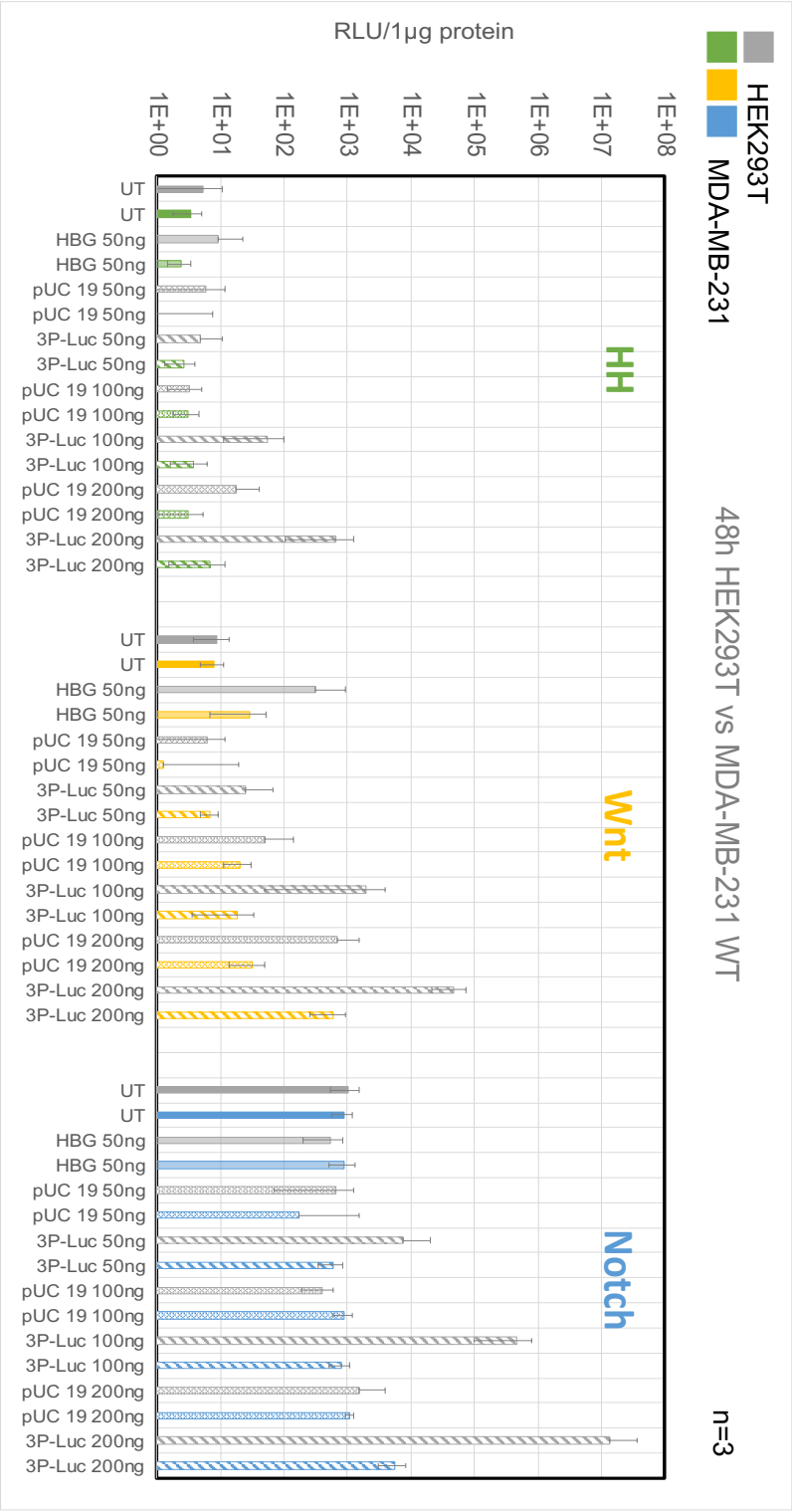


Figure 34: HEK293T versus MDA-MB-231 WT 48h

Comparison of pathway activity for HEK293T cells (grey) versus MDA-MB-231 WT cells (color) by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways after 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein for cells which were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). Experiments were performed in triplicates, n=3.

Investigation of transfection efficiency differences across cell lines: normalization of 3P-Luc readout for transfection differences

Transfection efficiency differences across cell lines were estimated either by LucSH transfections or by flow cytometry based detection of EGFP from 3P-Luc transfection in all the tested cell lines.

Figure 35 shows the differences in transfection efficiency measured in net RLUs per 1µg protein between untreated cells and those treated with 200ng of LucSH2 for every used cell line. Therefore, not only the comparison between untreated and treated cells is possible, but also between each individual cell line. The aim was the normalization of values regarding 3P-Luc-transfection. Here it can be observed that all cell lines show different degrees of transfection with HEK293T showing the highest transfection in comparison to other cell lines.

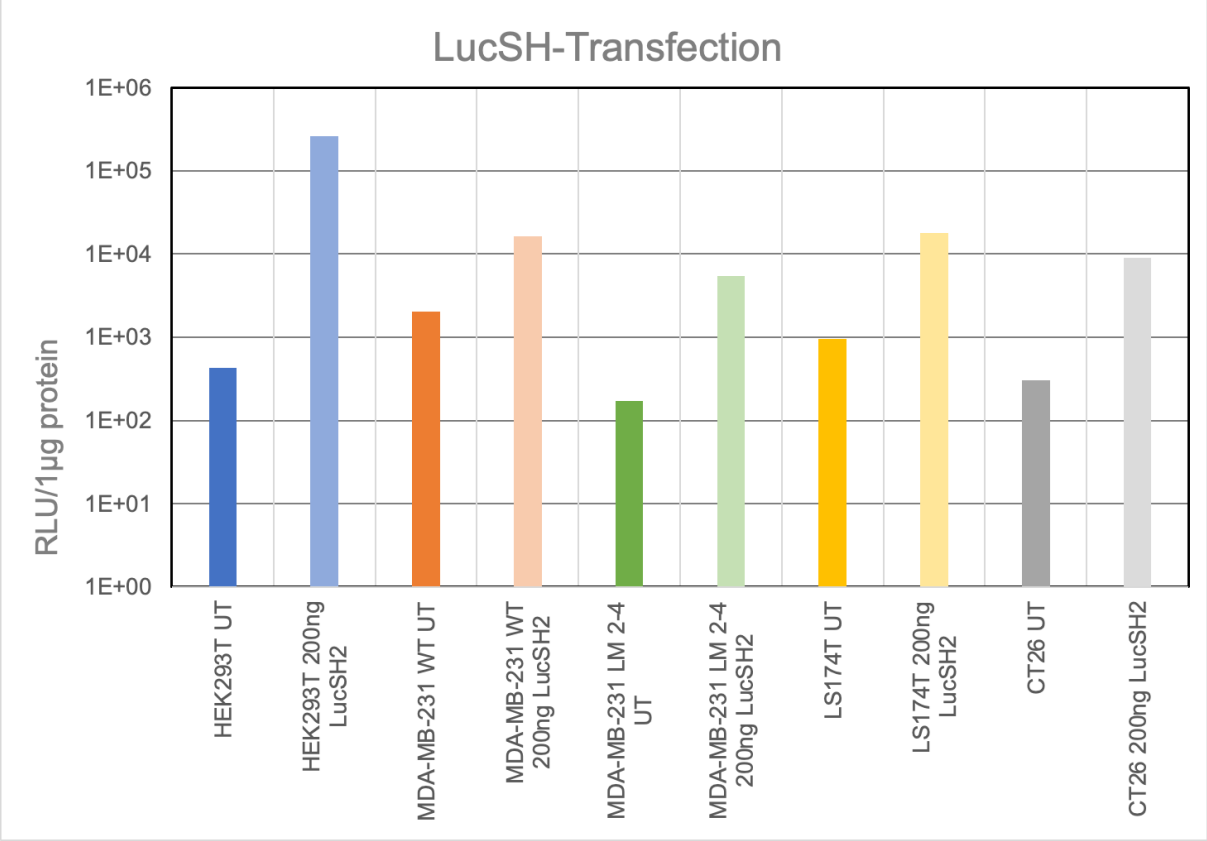


Figure 35: Transfection efficiency of different cells lines investigated by LucSH transfections and firefly assay.

All cell lines – HEK293T, MDA-MB-231 WT, MDA-MB-231 LM2-4, LS174T and CT26 – were transfected at the same time on the same plate to show the differences in transfection efficiency . Data shows the values of untreated cells and of cells treated with 200ng of LucSH2.

Another way of normalizing 3P-Luc-transfection was performed by measuring EGFP positive cells (using FACS) after transfection with 3P-Luc plasmid which also has EGFP reporter gene under constitutive expression, as already mentioned before. Figure 36 shows the percentage of EGFP positive cells.
LS174T cells were also treated, but cells grew in clusters, whereby measurement was impossible.

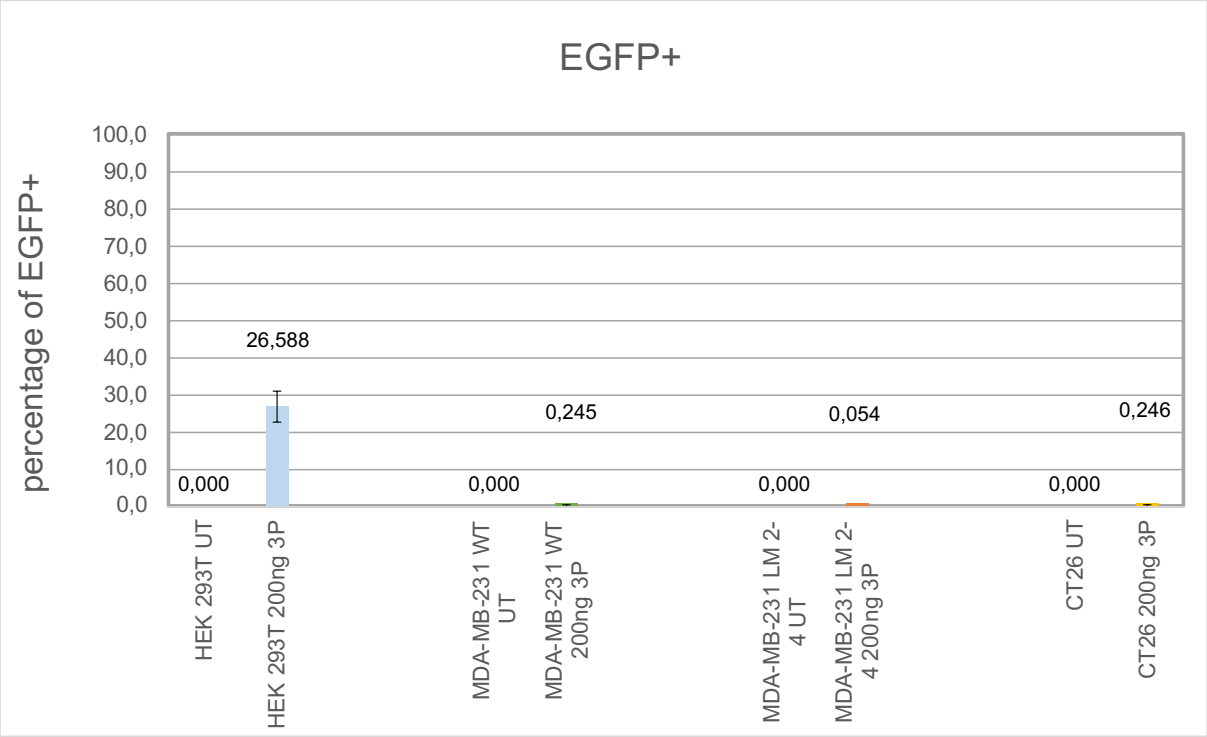


Figure 36: Transfection efficiency of different cells lines investigated by EGFP positive cells (flow cytometry) after transfection with 3P-Luc.
All used cell lines – HEK293T, MDA-MB-231 WT, MDA-MB-231 LM2-4 and CT26 – were transfected at the same time on the same plate to show the differences in transfection efficiency. Data shows the EGFP+ cells from untreated cells and of cells treated with 200ng of 3P-plasmid.

Figure 37 and Figure 38 show that the pathway activity of HEK293T cells is comparable to MDA-MB-231 WT cells after normalization regarding 3P-Luc-transfection using LucSH2. Strictly speaking, signals for Hedgehog and Wnt pathway are even higher at each amount regarding the 24-hour experiments, while there is only a low rise in signal regarding Notch pathway. Signals are indicated in RLU/1μg protein/LucSH, which means the Relative Light Units are normalized regarding protein amount and 3P-Luc-transfection for a better comparison between each cell line.

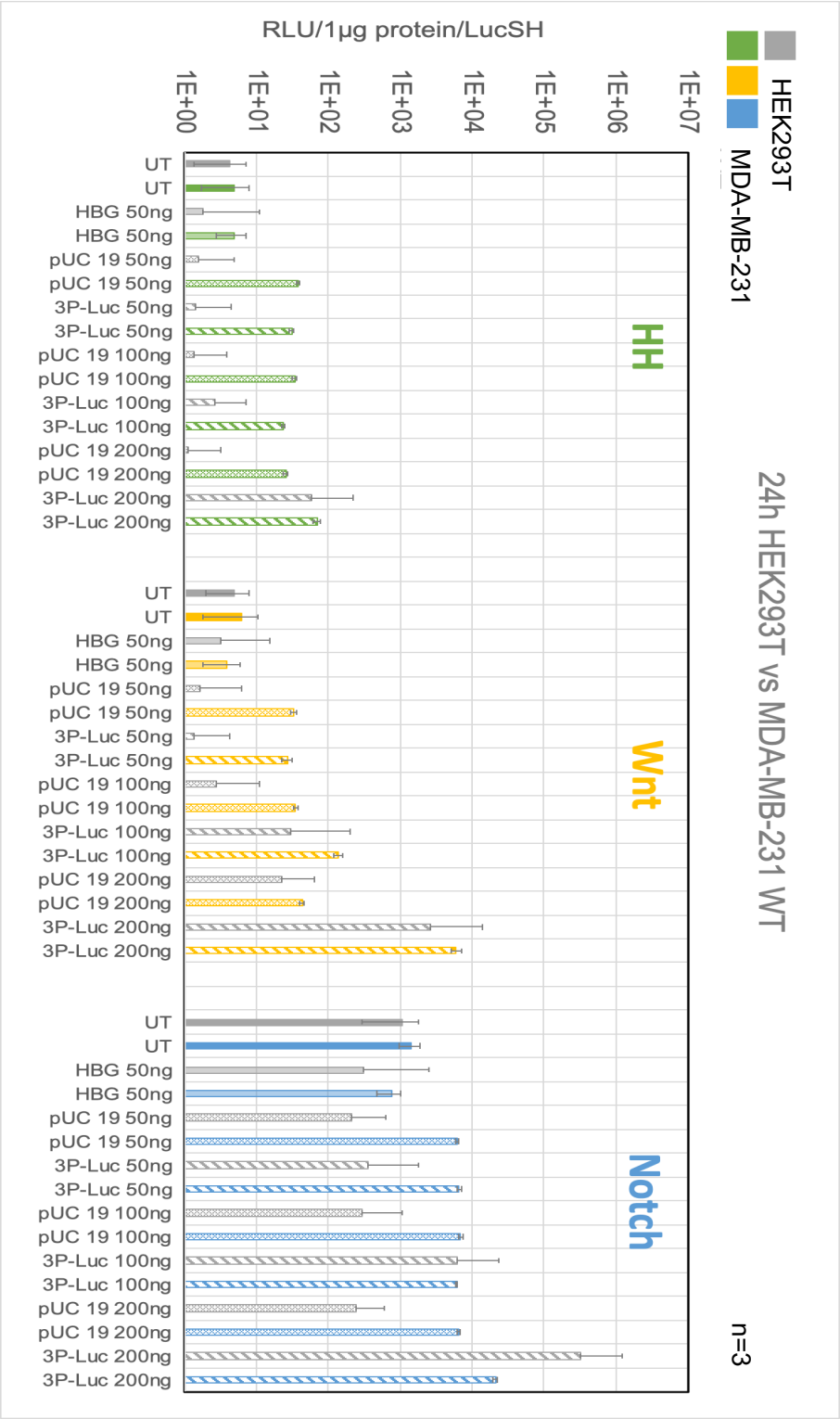


Figure 37: MDA-MB-231 WT vs. HEK293T – LucSH 24h

LucSH transfection based normalization of 3P-Luc readout for HEK293T cells (grey) versus MDA-MB-231 WT cells (color) by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways after 24 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein and further normalized for LucSH transfection. Cells were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). Experiments were performed in triplicates, n=3.

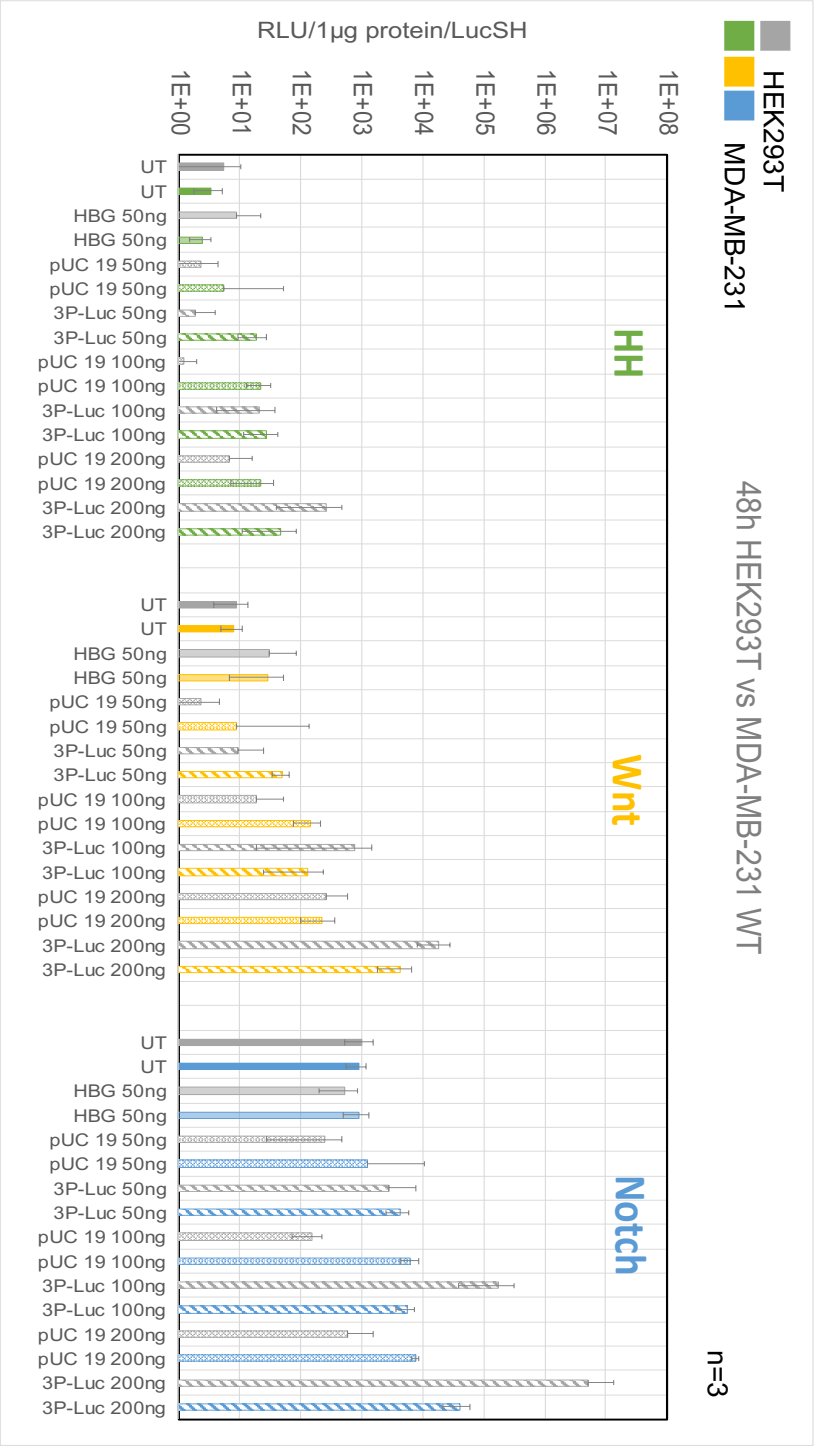
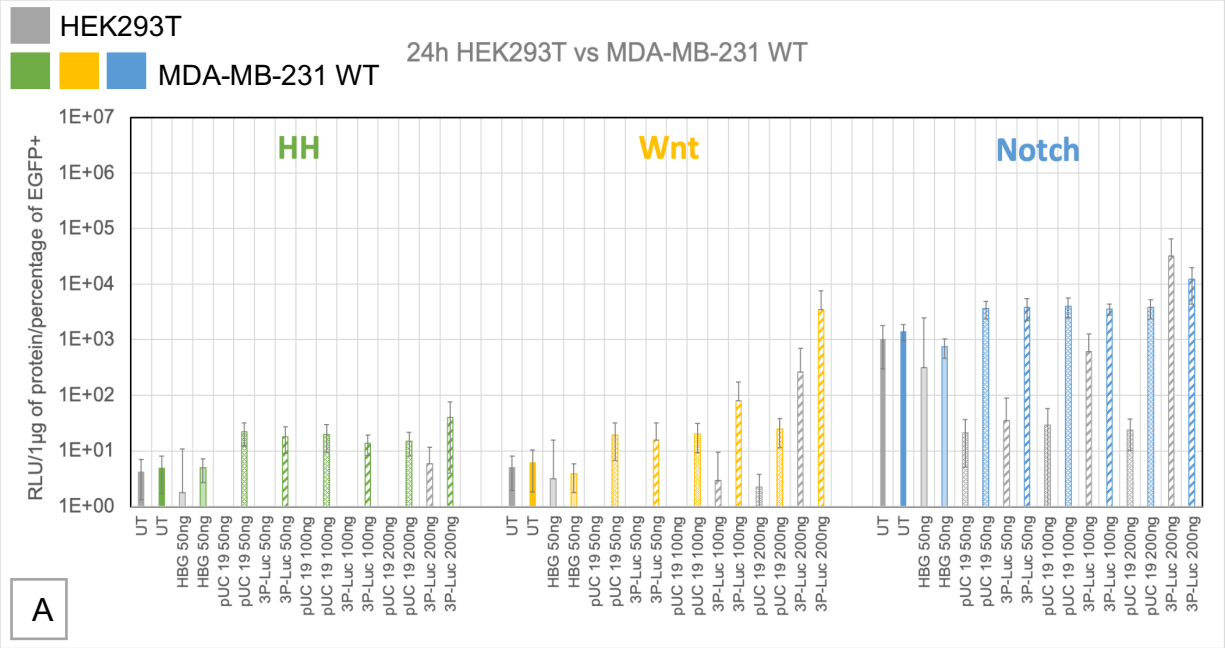


Figure 38: MDA-MB-231 WT vs. HEK293T – LucSH 48h

LucSH transfection based normalization of 3P-Luc readout for HEK293T cells (grey) versus MDA-MB-231 WT cells (color) by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways after 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein and further normalized for LucSH transfection. Cells were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). Experiments were performed in triplicates, n=3.

Normalization of 3P-Luc-transfection using FACS underlines these results, as shown in Figure 39. After 24 hours of incubation, Hedgehog and Wnt pathway show an even higher signal activity compared to HEK. In contrast to the normalization using LucSH, Hedgehog and Wnt pathway seem to be more active in MDA-MB-231 WT cells than in HEK293T cells after 48 hours of incubation.
Since FACS measurements have only been performed once in duplets, more research has to be done.



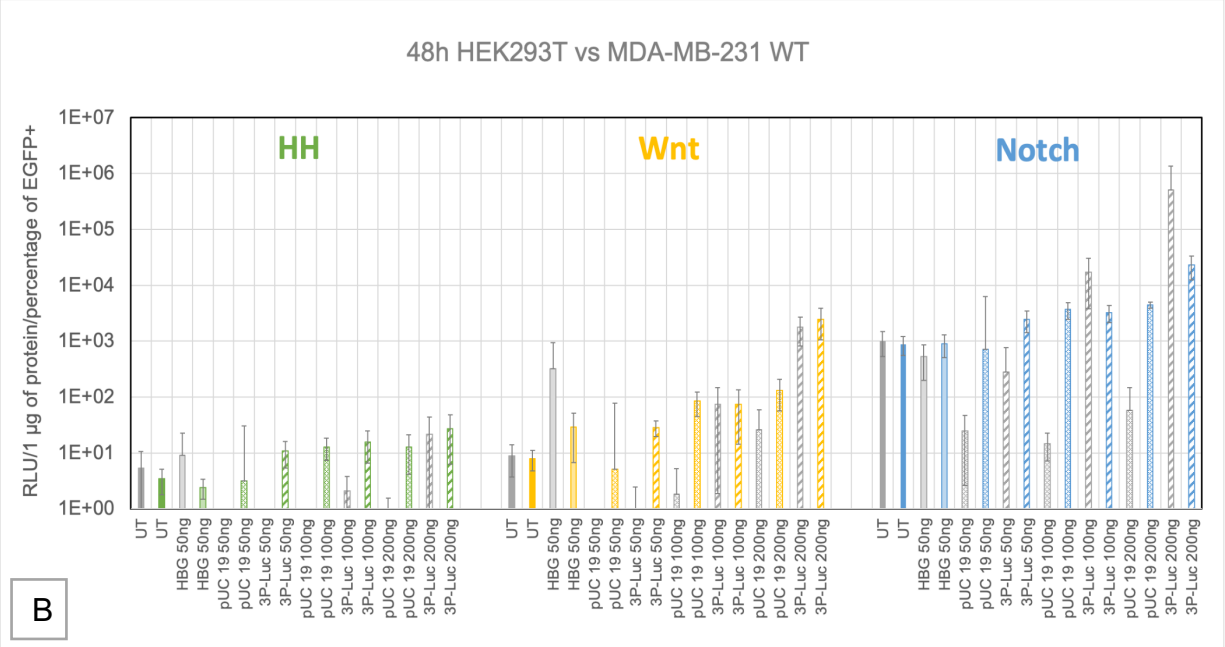
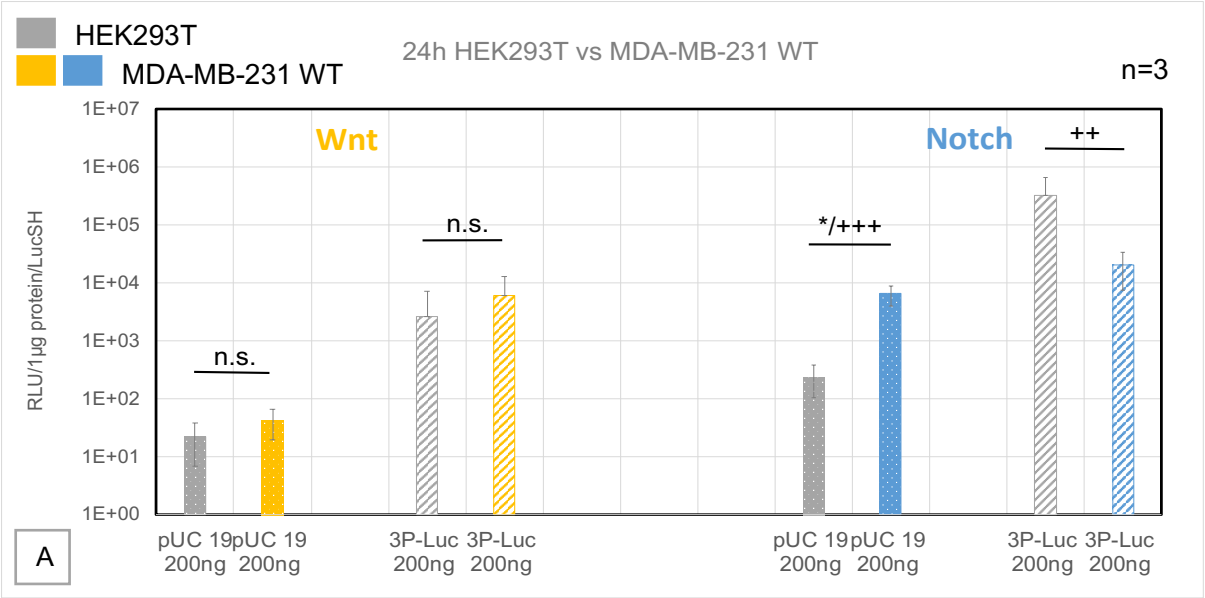


Figure 39: MDA-MB-231 WT vs. HEK203T – FACS

Percentage EGFP positive cells based normalization of 3P-Luc readout for HEK293T cells (grey) versus MDA-MB-231 WT cells (color) by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways after 24 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein and further normalized by percentage EGFP positive cells as quantified by flow cytometry. Cells were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). Experiments were performed in triplicates, n=3.

As a consequence of these findings, each cell line was normalized regarding the 3P-Luc-transfection. In the following figures, significant values from Figure 32 are compared to HEK293T, as already mentioned before.

Figure 40 shows the statistically significant values of MDA-MB-231 WT cells compared to HEK293T. After 24 hours of incubation MDA-MB-231 shows a higher Wnt signal than HEK293T, even if it is not significant. Notch showed a higher activity in HEK293T cells at both time points, but a significant difference could only be detected after 24 hours of incubation. This could be due to the potential overspill. After 48 hours of incubation, HEK293T cells yielded a significantly higher Wnt-signal than MDA-MB-231 cells. The high and variable results of pUC19 might also be traced back to the potential overspill.



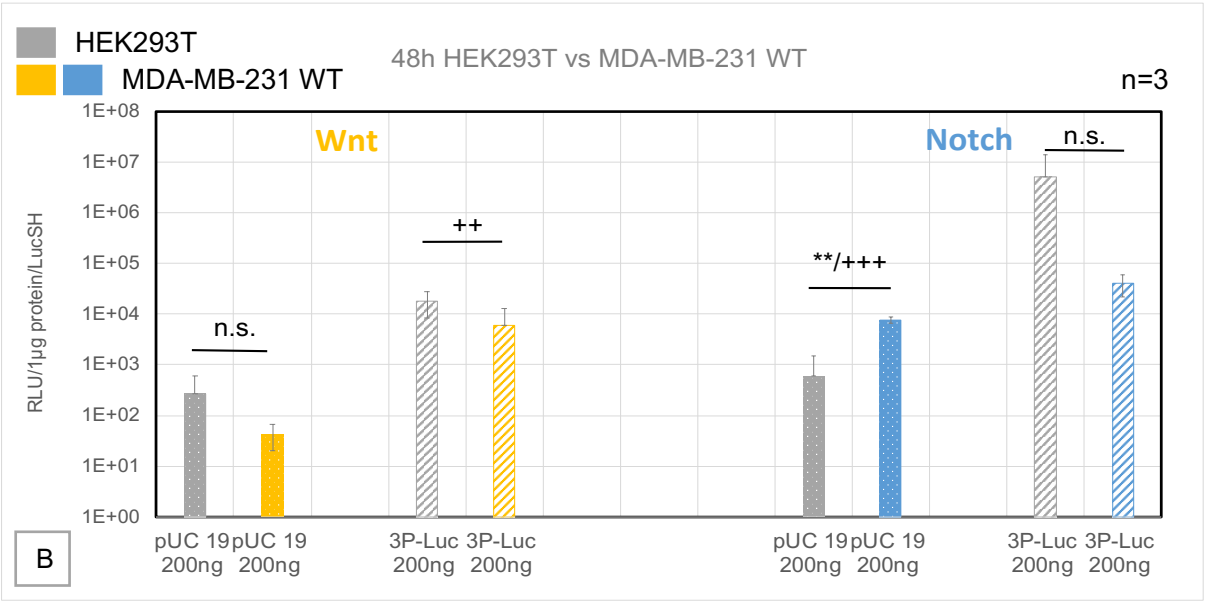


Figure 40: HEK vs. MDA, sign.

LucSH transfection based normalization of 3P-Luc readout for HEK293T cells (grey) versus MDA-MB-231 WT cells (color) by Multi-Luc/BCA assay for Wnt and Notch pathways after (A) 24 and (B) 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein and further normalized for LucSH transfection. Cells were treated with either 3P-Luc plasmid-based polyplexes (3P-Luc) or pUC19 plasmid-based polyplexes (pUC19). (*P< .05, **P< .01, u-test; ++P< .05, +++P< .01, t-test; n.s.= not significant at both.) Experiments were performed in triplicates, n=3.

MDA-MB-231 LM2-4 revealed no statistically different results when comparing 3P-Luc plasmid to pUC19. As a result, values were not compared to HEK293T cells. Due to the close relation between MDA-MB-231 LM2-4 and MDA-MB-231 WT cells it was decided to compare those cell lines to each other.

After normalization using the LucSH-method, MDA-MB-231 LM2-4 cells showed a higher activity when comparing each dose per well of Hedgehog and Notch pathway to MDA-MB-231 WT cells (Figure 41), except for Notch after 48 hours of incubation, which could give a hint to the high aggressiveness and invasiveness of the cell line. RLU-values for Wnt pathway, in contrast, are considerably smaller.

Cells normalized using the FACS-method confirm the findings as shown in Figure 42. What's more, the differences in pathway activity are even more noticeable and Notch also shows a higher signal in LM2-4 cells after 48 hours of incubation.

The lack of statistical significance due to the mismatch between treated and untreated cells could, again, be led back to the potential overspill during scratching. This should be taken into account for further experiments.

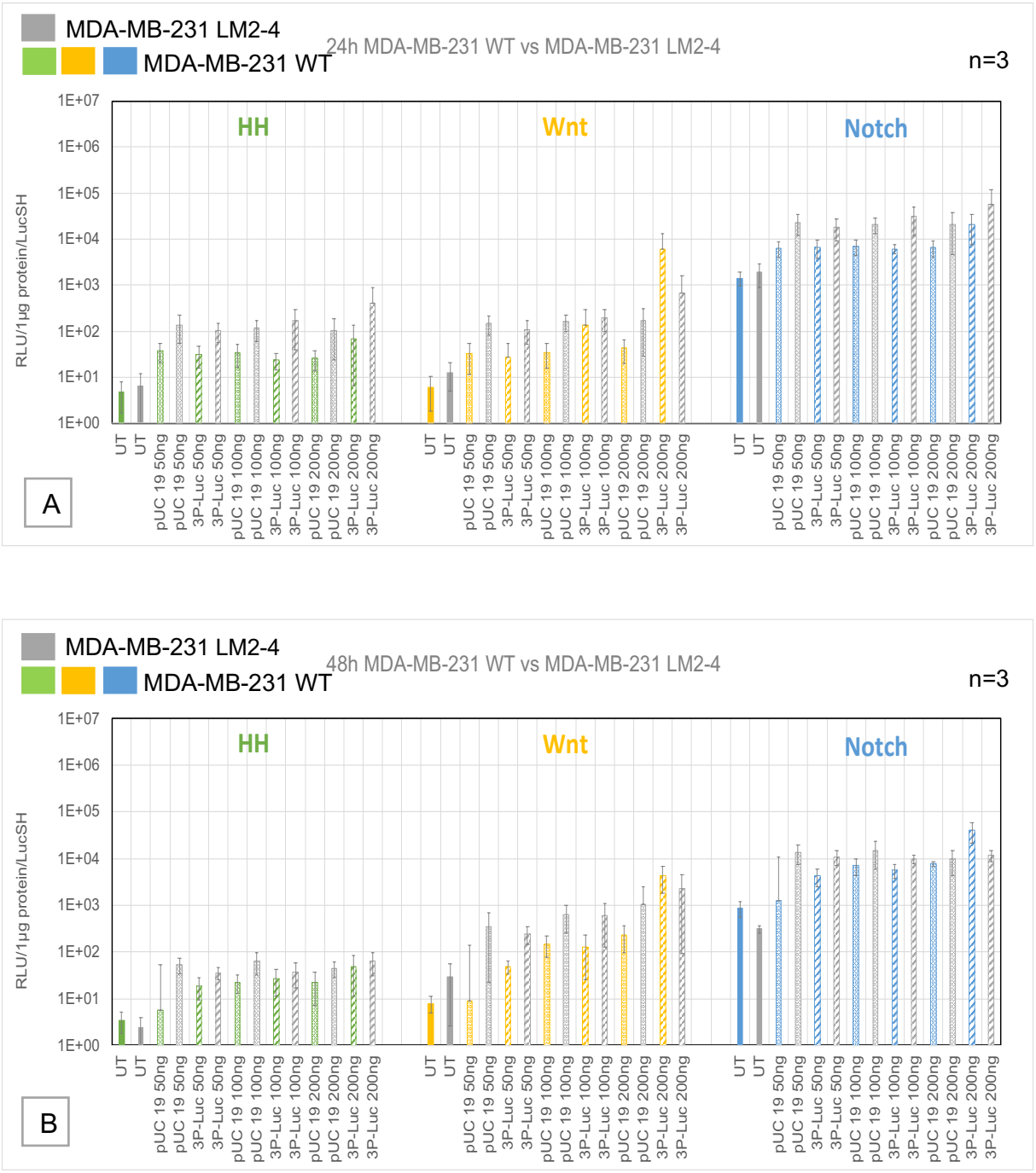


Figure 41: MDA-MB-231 WT vs. MDA-MB-231 LM2-4

LucSH transfection based normalization of 3P-Luc readout for MDA-MB-231 LM2-4 cells (grey) versus MDA-MB-231 WT cells (color) by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways after (A) 24 and (B) 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein and further normalized for LucSH transfection. Cells were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). Experiments were performed in triplicates, n=3.

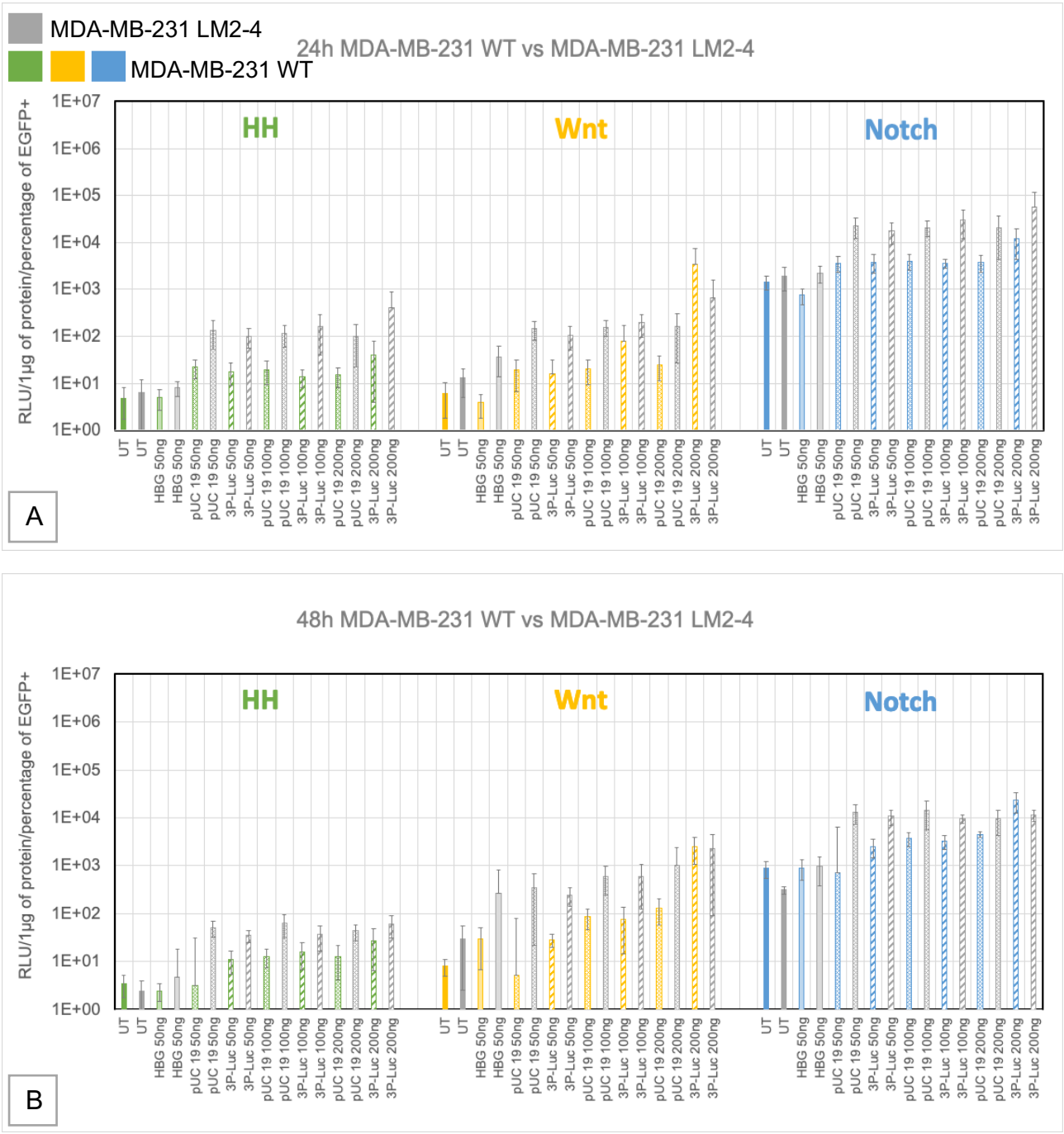
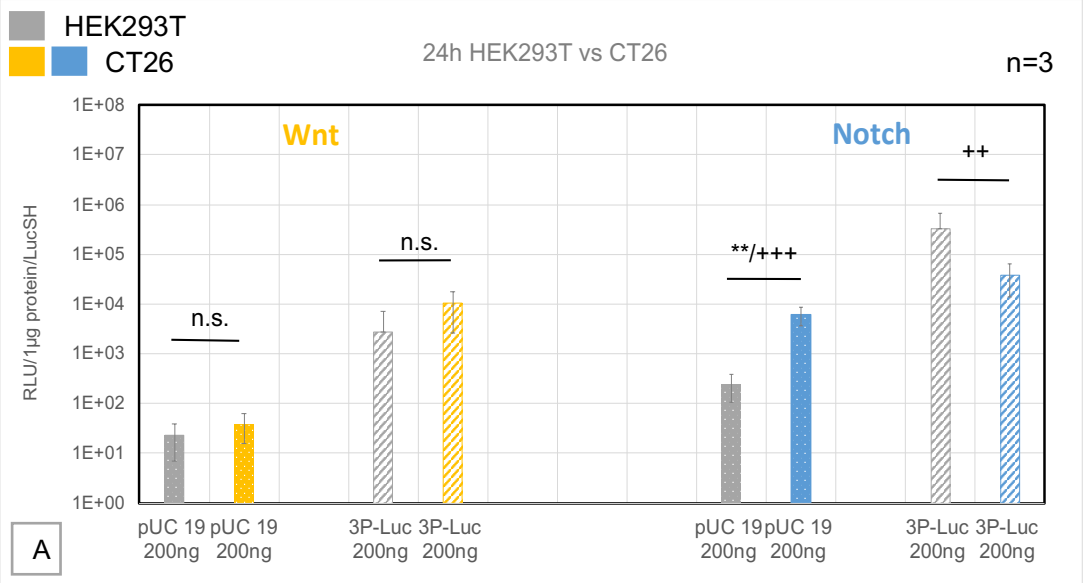


Figure 42: MDA-MB-231 WT vs. MDA-MB-231 LM2-4 – FACS

Percentage EGFP positive cells based normalization of 3P-Luc readout for MDA-MB-231 LM2-4 cells (grey) versus MDA-MB-231 WT cells (color) by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways after 24 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein and further normalized by percentage EGFP positive cells as quantified by flow cytometry. Cells were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). Experiments were performed in triplicates, n=3.

Figure 43 shows the statistically significant values of CT26 compared to HEK293T cells normalized using LucSH. After 24 hours of incubation, CT26 cells show a higher Wnt signal than HEK293T, though not statistically significant, while after 48 hours of incubation, HEK293T cells show a significantly higher Wnt signal. Notch shows a high activity after 48 hours of incubation, although not significant when compared to HEK293T cells. The high, and variable, results of pUC19 could be due to the potential overspill while scratching.



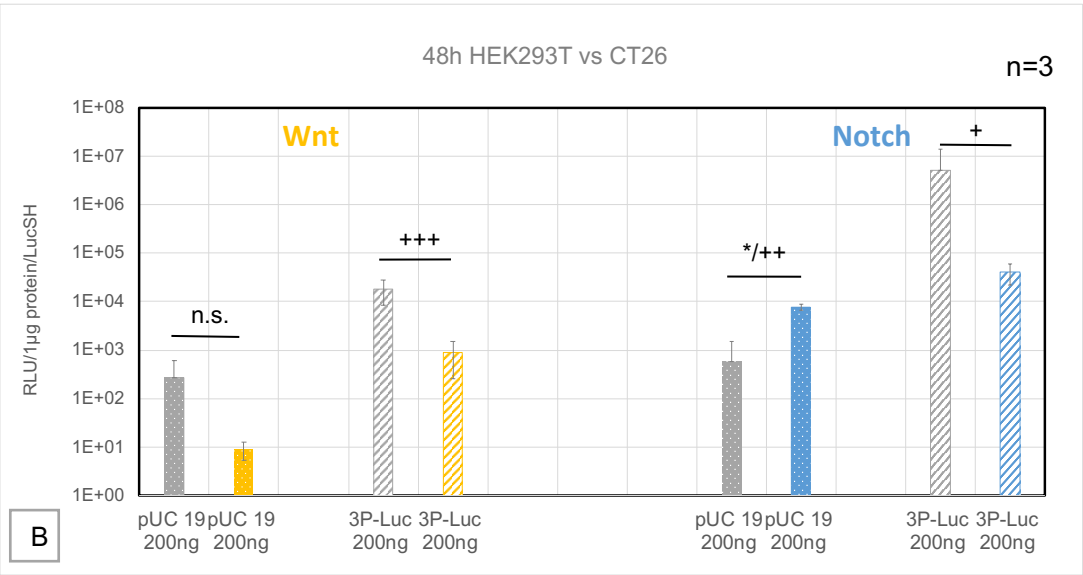


Figure 43: HEK vs. CT, sign.

LucSH transfection based normalization of 3P-Luc readout for HEK293T cells (grey) versus CT26 cells (color) by Multi-Luc/BCA assay for Wnt and Notch pathways after (A) 24 and (B) 48 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein and further normalized for LucSH transfection. Cells were treated with either 3P-Luc plasmid-based polyplexes (3P-Luc) or pUC19 plasmid-based polyplexes (pUC19). (*P< .05, **P< .01, u-test; +P< .10, ++P< .05, +++P< .01, t-test; n.s.= not significant at both.) Experiments were performed in triplicates, n=3.

Normalization using EGFP positive cells, and therefore FACS, could confirm these findings. Figure 44 shows that at both timepoints, 24 and 48 hours after incubation, the pathway activity is higher for CT26 cells when comparing them to HEK293T cells.

There is also a visible rise in signal regarding Notch pathway, though the signal does not exceed the HEK-signal.

Figure 44: HEK293T vs. CT26 – FACS

Percentage EGFP positive cells based normalization of 3P-Luc readout for HEK293T cells (grey) versus CT26 cells (color) by Multi-Luc/BCA assay for Wnt, Hh and Notch pathways after 24 hours of transfection with multi-luciferase based plasmid 3P-Luc. Luciferase expression normalized by protein is plotted as RLU/1µg protein and further normalized by percentage EGFP positive cells as quantified by flow cytometry. Cells were untreated (UT), buffer-treated (HBG), 3P-Luc plasmid-based polyplex treated (3P-Luc; different doses of plasmid per well), or pUC19 plasmid-based polyplex treated (pUC19; different doses of plasmid per well). Experiments were performed in triplicates, n=3.

7 Conclusion

Multiple recent studies focus on pathway activities in cancer stem cells. While not only each pathway is investigated on its own, entanglements are also of great interest. For this purpose, multiplexing and therefore multiple-reporter plasmids are an ideal tool for in-vitro-experiments.

For this thesis, three signaling pathways were of interest: Sonic Hedgehog, Notch and Wnt – all of which are suspected to play a role in carcinogenesis and malignancy. We could not only show that the 3P-Luc-multi-reporter plasmid generated by the former PhD student Julia Maier is suitable for simultaneous measurement of all three pathways at different minimum amounts and altering time points, but also that there are differences in pathway activities between various cancer cell lines.
While investigating pathway activities for differing cell lines, we could also show that protein normalization based on BCA assay is a good alternative for CTF.
Moreover, we could also prove that normalization of 3P-Luc-transfection can be performed using (at least) two different methods, whereby results of both point the same direction. To be able to make more accurate statements, more repeats of the FACS-experiment have to be performed.

One limitation for this thesis was the potential overspill while scratching. For further experiments a cell-lysing-method without the need of scratching would be desirable. Another solution to this problem could be the physically separation of wells, which would require a high number of well-plates.
Moreover, a comparison of malignant to their corresponding healthy cells could give information about the origin of degeneration. As a result, knock-down of those pathways could be a very good approach fur further treatment developments.

8 List of Figures

9 References

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10 Appendix

Figure 45 shows one of the training steps for the multiplexing process. Different substances (AuNP: nanoparticles with gold, Px NP: nanoparticle-polyplexes, AuPx: gold-polyplexes) were used for transfection. The goal of this experiment was to learn the handling of BCA, Gaussia Luciferase and NanoLuc.

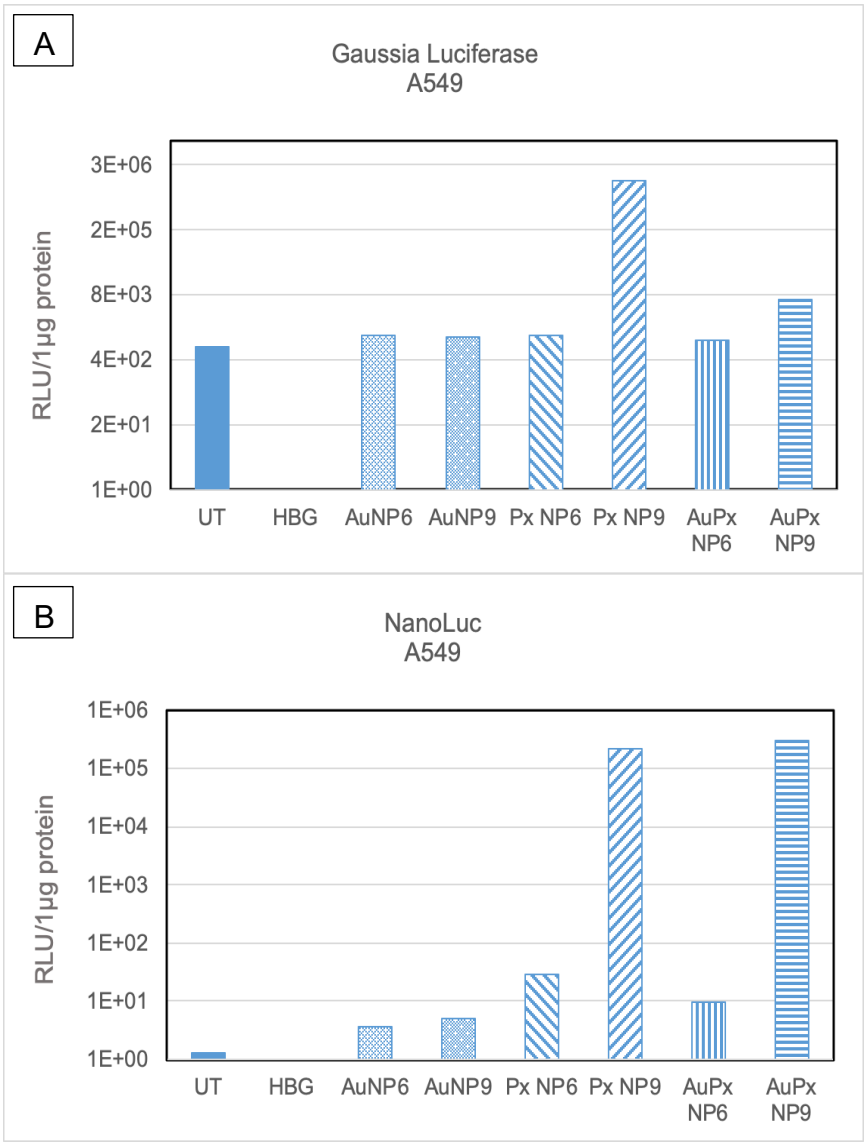

Figure 45: Combining GLuc and NLuc assays.

A: Results of Gaussia Luc assay, B: Results of NanoLuc assay. Both figures show the same experiment combining the two assay using multiplexing.

Values are RLU/1µg protein. UT: untreated cells, AuNP6/9: gold nano particles with a N/P ratio of 6 or 9. PX NP6/9: polyplexes with a N/P ratio of 6 or 9, AuPx NP6/P9: polyplexes containing gold nano particles in a N/P ratio of 6 or 9.

10.1 General Workflow for Studying Knock-Down Experiments Based on siRNA

To study the gene knockdown based on anti-luc siRNA-based formulations, cells were needed which already produced the gene of interest. For this study, MDA-MB-231Luc cells were chosen. The general workflow included seeding cells, transfecting them with polyplexes, adding the substances and reading the luminescence as shown in Figure 46.

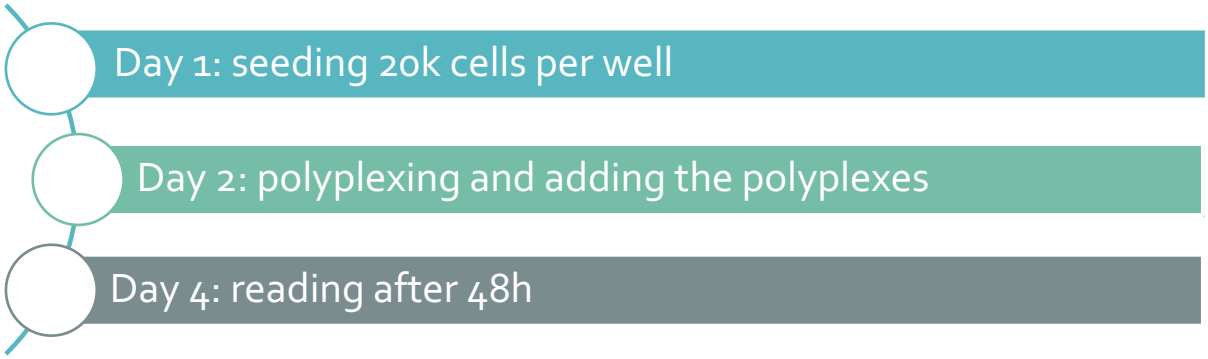

Figure 46: Workflow for knockdown based on siRNA

10.1.1 Cell Line for Studying Knock-Down

The cell line used for this experiment was the stable transfected MDA-MB-231Luc (short form of MDA-MB-231 pGK, EGFP, Luc), which was originally derived from MDA-MB-231 cells, described in chapter *0.*

PGK stands for Phosphoglycerate kinase, which is an important enzyme in the aerobic glycolysis pathway and therefore in the cell metabolism regulation which exists in all organisms. There are two isoforms, PGK1 and PGK2. While PGK2 is encoded by an autosomal gene and only expressed during spermatogenesis, PGK1 is encoded on the X-chromosome and thereby expressed ubiquitously in all cells. Subsequently, PGK1 is referred to as PGK. It catalyzes the reversible transmission of a phosphate group to ADP and further to ATP. Moreover, it is involved in multiple biological activities, as angiogenesis, autophagy and DNA repair, which makes its involvement in cancer development fairly complicated.

Aberrant expression levels are linked to disease occurrence. Deficits are related to parkinsonism, neurological impairment and myopathy. Overexpression can be found in synovial tissues and blood of rheumatoid arthritis, leading to the suggestion that it plays a part in pro-inflammation. High intracellular expression rates of the gene lead to tumor cell proliferation, whereas high extracellular expression rates lead to suppression of angiogenesis and as a result suppress cancer malignancy. In addition, it is also linked to chemoradiotherapy resistance and poor prognosis of cancer patients. Especially under hypoxic conditions it helps to generate ATP for tumor cells, which is associated with development and progress of various cancer types (He, et al., 2019).

EGFP and Luc function as reporters, either for fluorescence and luminescence, respectively.

Cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) with 10% fetal bovine serum (FBS), 2% L-Glutamine and 1% Penicillin/Streptomycin at 37 degrees Celsius and 5% CO_2.

10.1.2 Cell Seeding for Knock-Down

The cells were kept in the incubator in T75 flasks. For cell seeding the adherence, color, and turbidity of the medium were checked visually. Medium was removed, cells were washed with PBS and Versene was added. After four minutes of incubation time the cell suspension was transferred into a CT15-tube and centrifuged. The pellet was resuspended in complete medium, cells counted and 20,000 cells were seeded per well. A more detailed description of the steps can be found in *5.2.1 Cell Seeding.*

10.1.3 Polyplexing and Transfection for Knock-Down

After 24 hours, the seeded 96-well-plate was taken out of the incubator, medium was aspired and basal Dulbecco's modified eagle's medium was pipetted into the wells. Afterwards the polyplexes had to be made by flash pipetting equal amounts of branched polyethyleneimine (BPEI) to siRNA; both substances had been diluted in HEPES Buffered Saline (HBG) before.

Depending on the wells, different amounts of siRNA and negative control (NC) were used (Figure 47): 0.2, 0.4 and 0.6µg. After addition of the polyplexes, the total volume of each well had to reach 200µl. The 96-well-plates were put back into the incubator for 48 hours.

A more detailed description of the steps can be found in *5.2.3 Polyplexing and Transfection.*

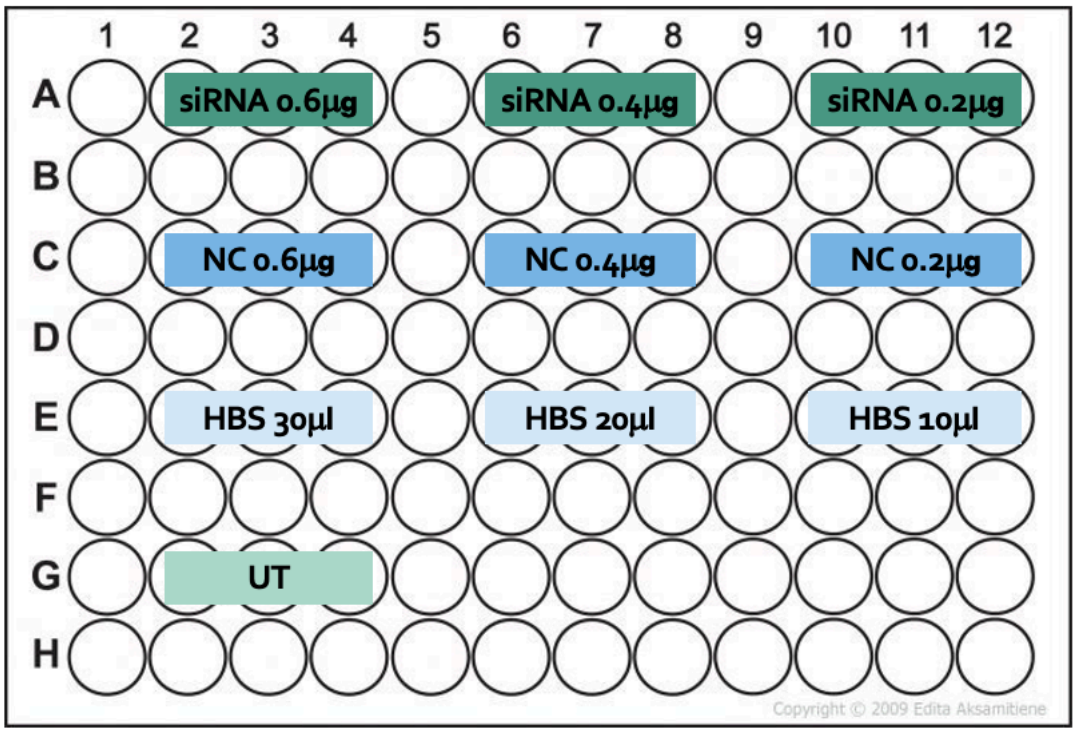

Figure 47: Schematic overview of the experimental design for knock-down treatment.

10.1.4 Reading of the Knock-Down Experiment

10.1.4.1 Luciferase

After 48 hours of incubation, the white 96-well-plate was taken out of the incubator. The supernatant was removed, the wells were washed with PBS and Passive Lysis Buffer (PLB) 1x, which was prepared by diluting PLB5x with MilliQ water was added. For this experiment, only 30µl of PLB were needed, since only BCA and Firefly luciferase assays were performed as shown in Figure 48.

Afterwards, the plate was put on the shaker for 30 minutes at 500 rpm and room temperature.

After cell lysis, 20µl of each well were pipetted into a transparent 96-well-plate for BCA, the remaining 10µl were kept in the original plate for Firefly Luc assay.

Both plates, one for BCA assay and one for luciferase assay, were read using a *Tecan Infinite M200 Pro* plate reader.

To measure the RLU by Firefly Luc assay, 100µl of Luciferin (stored at -80 degrees Celsius and kept under light protection) was injected directly into each well of the cell lysate by the plate reader.

For the experiments in this thesis, normalization of the RLU values was done by protein quantification with BCA.

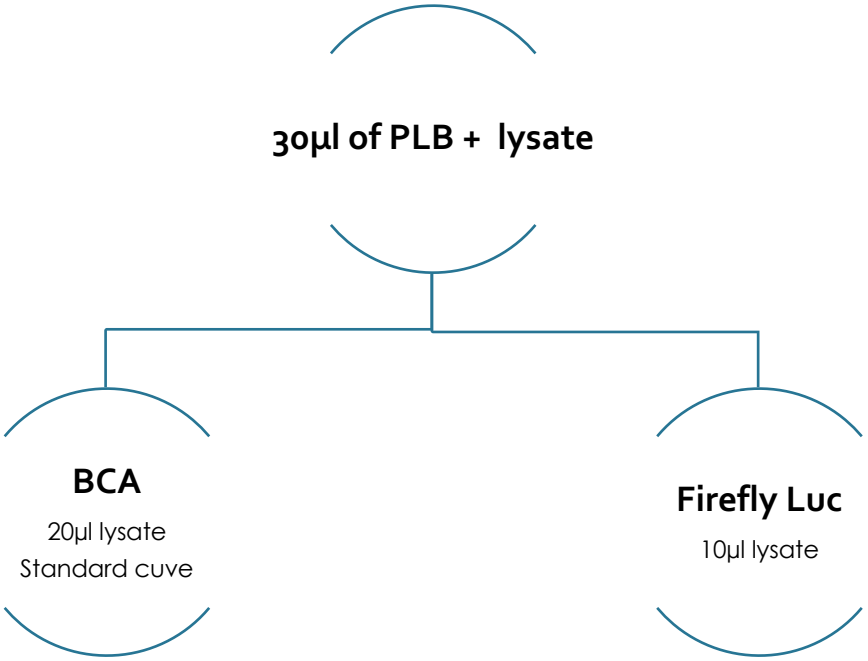

Figure 48: Schematic reading-overview for knock-down-experiments

10.1.4.2 BCA

For normalization based on BCA, Bovine Serum Albumin (BSA) dilutions were prepared by mixing MilliQ-water, BSA 2mg/ml and PLB2x in concentrations as already described in *Figure 18: Modified BCA protocol (Elmenofi, 2019).*. PLB 2x was made by mixing PLB 5x with MilliQ-water.

20µl of each dilution were added into a transparent 96-well-plate in triplicates in order to derive a standard curve. 20µl of cell lysate of each well were also added to the measurement plate. 200µl of working reagent were added into each well of the transparent plate, shaken for 30 seconds at RPM 300 and room temperature and afterwards put in the incubator for 30 minutes, in which a change of color happened.
At the end of the incubation time, the plate was wrapped in aluminum foil until plate reading. Reading tool place after a cooling-phase of ten minutes during which the plate reached room temperature. A *Tecan Infinite M200 Pro*-reader was used at 562nm wavelength, a bandwidth of 9nm and a flash-number of 25.
A more detailed description of the steps can be found in *5.2.4.3 BCA.*

To extrapolate the correct protein amounts in the total cell lysate from the different measurement volumes (20µL for BCA and 10µL for Firefly assay), the functional equation from the standard curve was used. RLU-measurements were normalized to RLU per 1µg of protein by adjusting them to the respective measurement volume relative to the total cell lysate amount and dividing by the corresponding protein amount in the lysate.

10.1.5 Results

Based on the measurements the percentage of Relative Light Units (RLU) were calculated, as described in chapter 10.1.4.2. Cells treated with anti-luc siRNA-based formulations were normalized to cells treated with noncoding-based siRNA-controls (Taschauer, et al., 2020). Wells with a concentration of 0.4µg of siRNA showed the lowest light signals and therefore the highest knock-down in relation to the negative control, followed by 0.2µg of siRNA. Surprisingly, wells with a concentration of 0.6µg of siRNA showed the least knockdown, which can be seen in Figure 49.

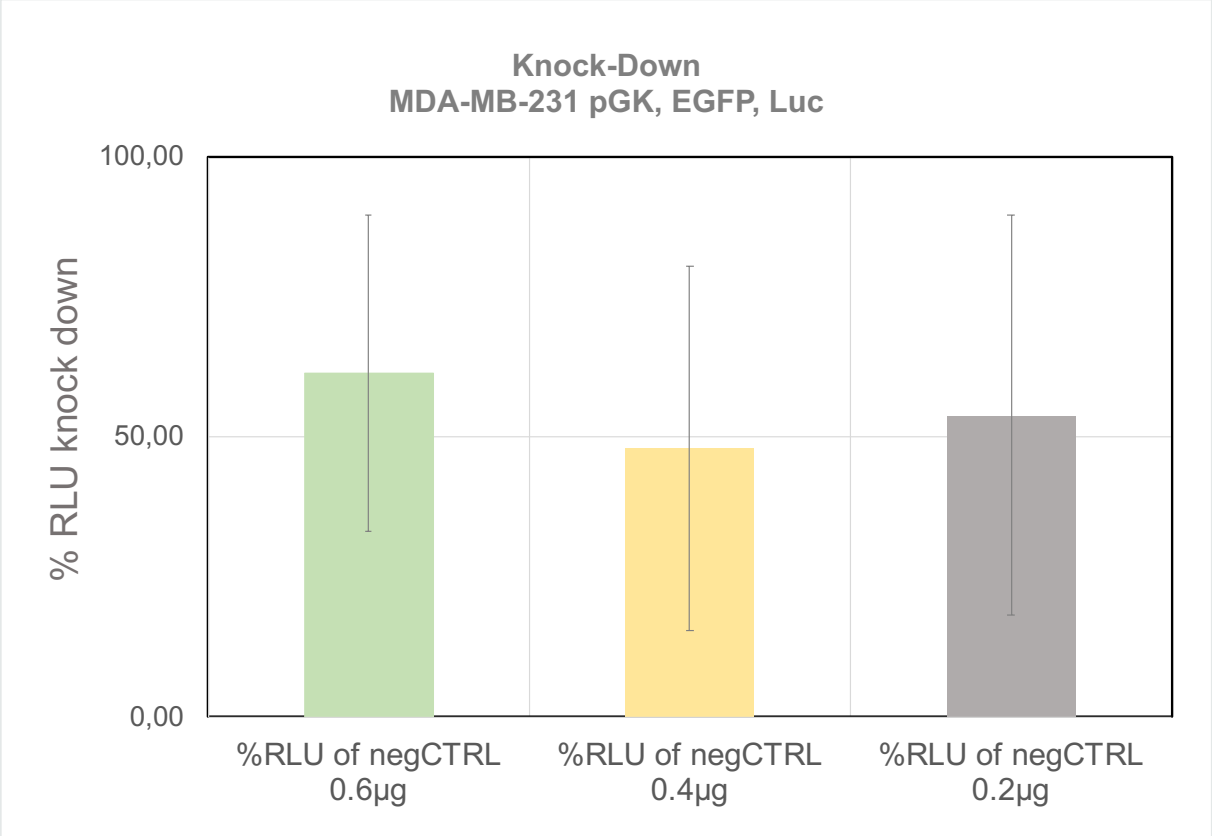

Figure 49: %RLU knock down

Values of RLU of knock-down are portraited in percent. Different amounts (0.6, 0.4 and 0.2µg) of siRNA were tested.

Afterwards the cell count was included and thus normalization was performed. Cells treated with highest concentration of siRNA, which was 0.6µg, showed the least signal with under 50% RLU knock-down in relation to the negative control, meaning those cells indicate the highest knock-down. Cells treated with 0.4µg of siRNA show a moderate increase in light units, indicating less knock-down. Signals for cells treated with 0.2µg of siRNA were higher, but knock-down is still clearly visible, implying that the experiment worked in each concentration, as shown in Figure 50.
Several studies performed by Taschauer and colleagues confirm these results. (Taschauer, et al., 2020)

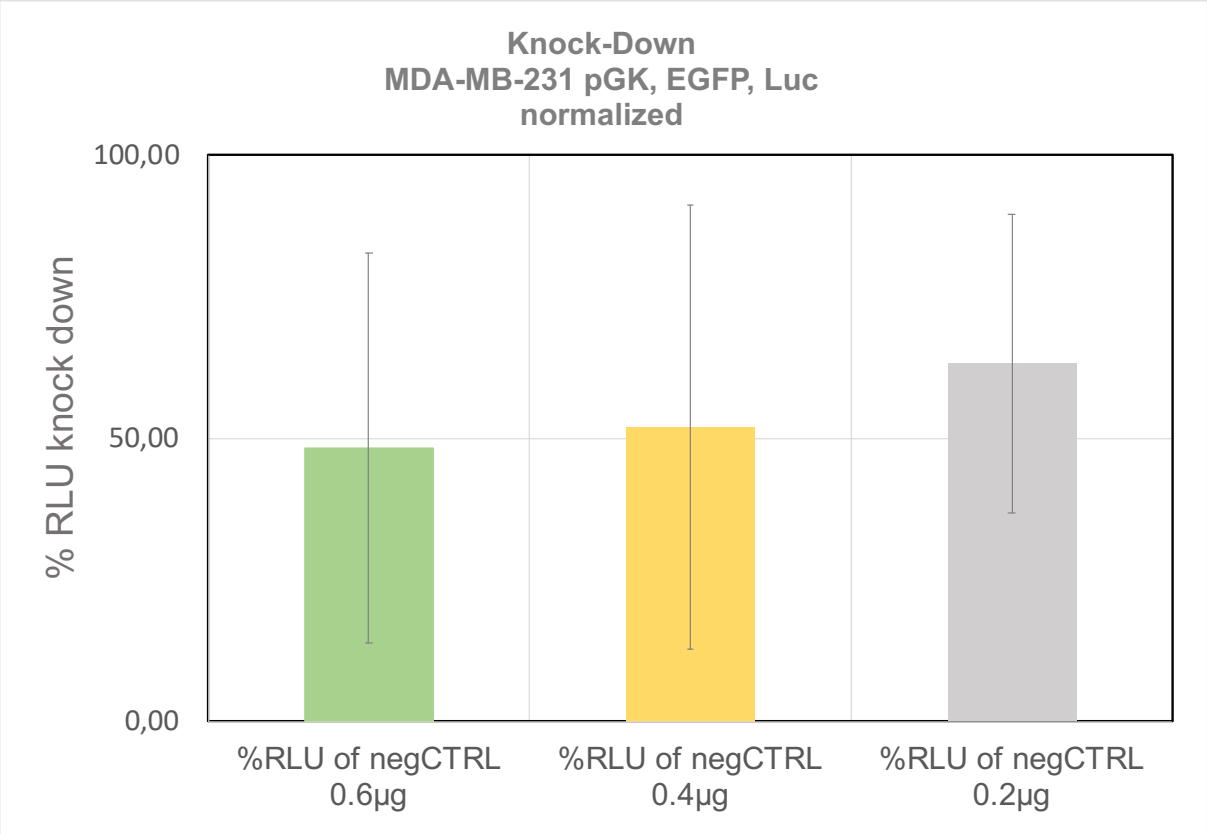

Figure 50: %RLU knock down normalized

Values of RLU of knock-down are portraited in percent. BCA assay was used for normalization regarding the cell count. Different amounts (0.6, 0.4 and 0.2µg) of siRNA were tested.

10.2 General Workflow for Studying Cell Uptake of Gold Nano Particle (Prof. Michael Reithofer)

To study the cell uptake of gold nano particles the workflow needs to consist seeding cells into transparent 24-well-plates, adding gold nano particles (AuNP), adding the substrate for reading and reading the luminescence (see Figure 51).

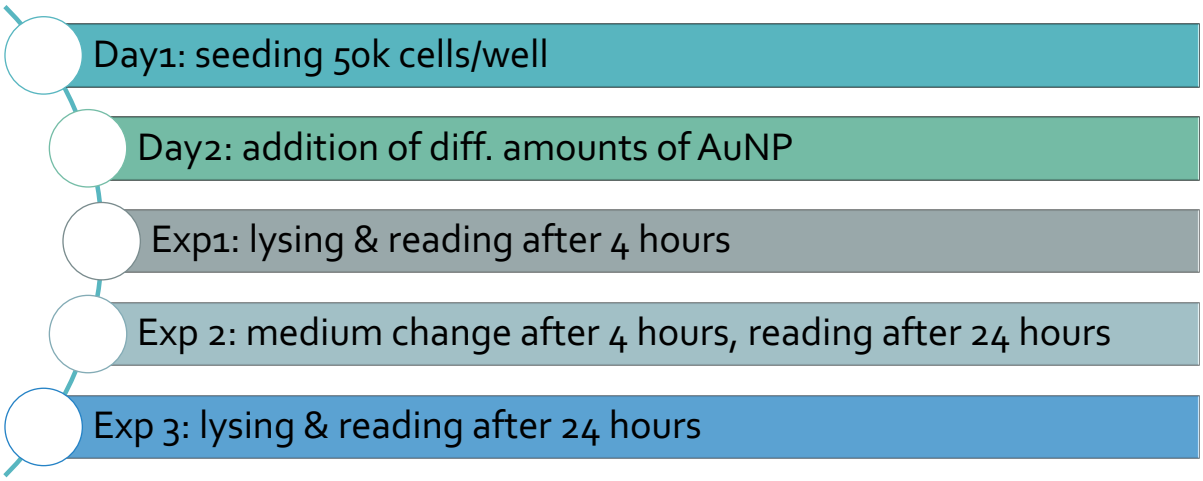

Figure 51: Workflow for studying the cell uptake of AuNP

10.2.1 Cell Line for Studying AuNP Cell Uptake

Two of the three cell lines used for studying AuNP-uptake were the above mentioned HEK293T and MDA-MB-231 WT. Additionally, the cell line A549 was used.

A549

The A549 cell line was developed in 1972 by the removal and culturing of cancerous lung tissue of a 58-year-old Caucasian male. In humans the alveolar basal epithelial cells are responsible for the diffusion of substances like water and electrolytes across the alveoli. The adenocarcinomic human alveolar basal epithelial cells are used as models for lung cancer studies and therefore also for the development of drug therapies against this disease (Wikipedia, 2020).

10.2.2 Cell Seeding for the AuNP-Uptake Experiment

The cells were kept in the incubator in T75 flasks. For cell seeding the adherence, color, and turbidity of the medium were checked visually. Medium was removed, cells were washed with PBS and Versene or Trypsin, depending on the cell line, was added. After four minutes of incubation time the cell suspension was transferred into a CT15-tube and centrifuged. The pellet was resuspended in complete medium, cells counted and 50,000 cells were seeded per well into a transparent 24-well-plate. After this the right amount of cell suspension was added; all together there needed to be a volume of 500µl in each well. Three plates needed to be seeded. A more detailed description of the steps can be found in *5.2.1 Cell Seeding.*

10.2.3 Transfection for the AuNP Experiment

The gold nanoparticles were kindly provided over by Prof. Michael Reithofer. The concentration was 200µg/ml, for each experiment a new aliquot with a ratio of 1:10 was prepared using PBS. The seeded 24-well-plate prepared one day before the experiment was taken out of the incubator, medium was aspired and 1000µl basal or complete medium was pipetted into the wells. Afterwards the diluted formulation was added.

Depending on the wells, different amounts of AuNP were used (see Figure 52 and Figure 53) – 0.2ng, 0.4ng, 0.8ng. Both plates were incubated for four hours, afterwards each plate was treated differently.

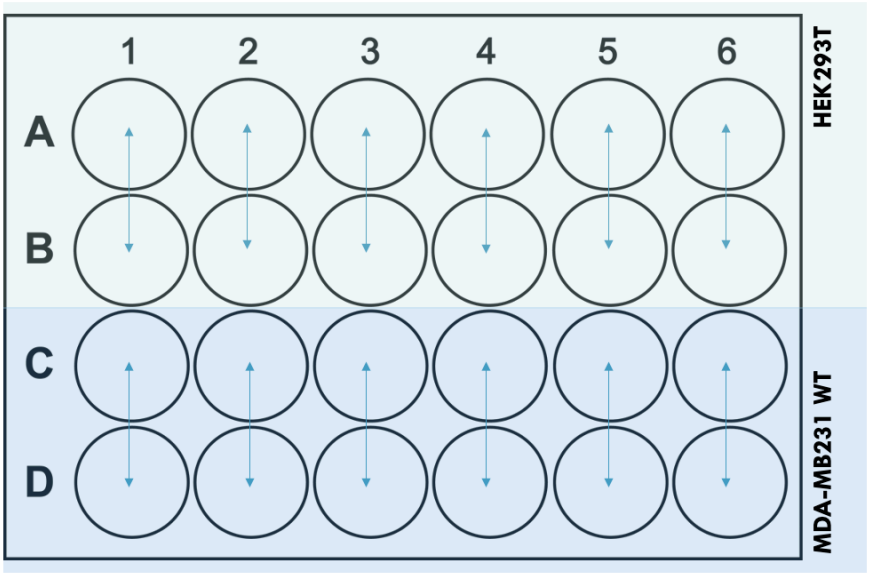

Figure 52 - Transfection map for the 4-hour-AuNP-experiments

The numbers indicate the column numbers:

1: BM + 0.4ng AuNP, 2: BM + 0.8ng, 3: BM

4: CM + 0.4ng, 5: CM + 0.8ng, 6: CM

AuNP: gold nano particle, CM: complete medium, BM: basal medium

Two plates were transfected following this scheme.

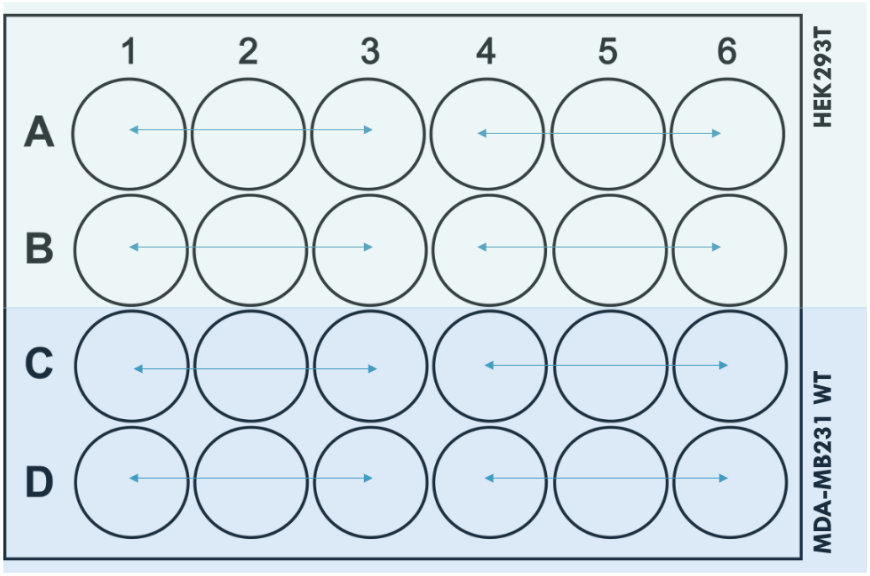

Figure 53 - Transfection map for the 24-hour-AuNP-experiments

For this experiment only complete medium was used. Letters indicate row names:

A&C 1-3: 0.2ng AuNP, A&C 4-6: 0.4ng AuNP

B&D 1-3: 0.8ng AuNP, B&D 4-6: UT

AuNP: gold nano particle.

One plate was transfected following this scheme.

From one of the plates that was prepared following the transfection scheme shown in Figure 52 medium was aspired after 4 hours and 1000µl of complete medium were added and this plate was put back into the incubator for another 20 hours of incubation at 36 degrees Celsius. Cells in the other plate were lysed after 4 hours. The plate which was prepared following the transfection scheme shown in Figure 53 was incubated for 24 hours without any medium change.

10.2.4 Measurements of AuNP

10.2.4.1 BCA Assay

For normalization based on BCA, Bovine Serum Albumin (BSA) dilutions were made by mixing MilliQ-water, BSA 2mg/ml and PLB 2x. The used concentrations can be seen in
Figure 18: Modified BCA protocol (Elmenofi, 2019). PLB 2x was made by mixing PLB 5x with MilliQ-water.

20µl of each dilution were added into a transparent 96-well-plate in triplicates in order to derive a standard curve. 20µl of cell lysate of each well were also added to the measurement plate. 200µl of working reagent were added into each well of the transparent plate, shaken for 30 seconds at RPM 300 and room temperature and afterwards put in the incubator for 30 minutes, in which a change of color happened.
At the end of the incubation time, the plate was wrapped in aluminum foil until plate reading. Reading tool place after a cooling-phase of ten minutes during which the plate reached room temperature. A *Tecan Infinite M200 Pro*-reader was used at 562nm wavelength, a bandwidth of 9nm and a flash-number of 25.
A more detailed description of the steps can be found in *5.2.4.3 BCA.*

To extrapolate the correct protein amounts in the total cell lysate from the different measurement volumes (20µL for BCA and 10µL for Firefly assay), the functional equation from the standard curve was used. RLU-measurements were normalized to RLU per 1µg of protein by adjusting them to the respective measurement volume relative to the total cell lysate amount and dividing by the corresponding protein amount in the lysate.

10.2.4.2 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

For quantifications of the AuNP cell uptake, ICP-MS measurements were performed by Mag. Simon Decker.

10.2.4.3 Detailed Workflow

The detailed workflow is shown in Figure 54.

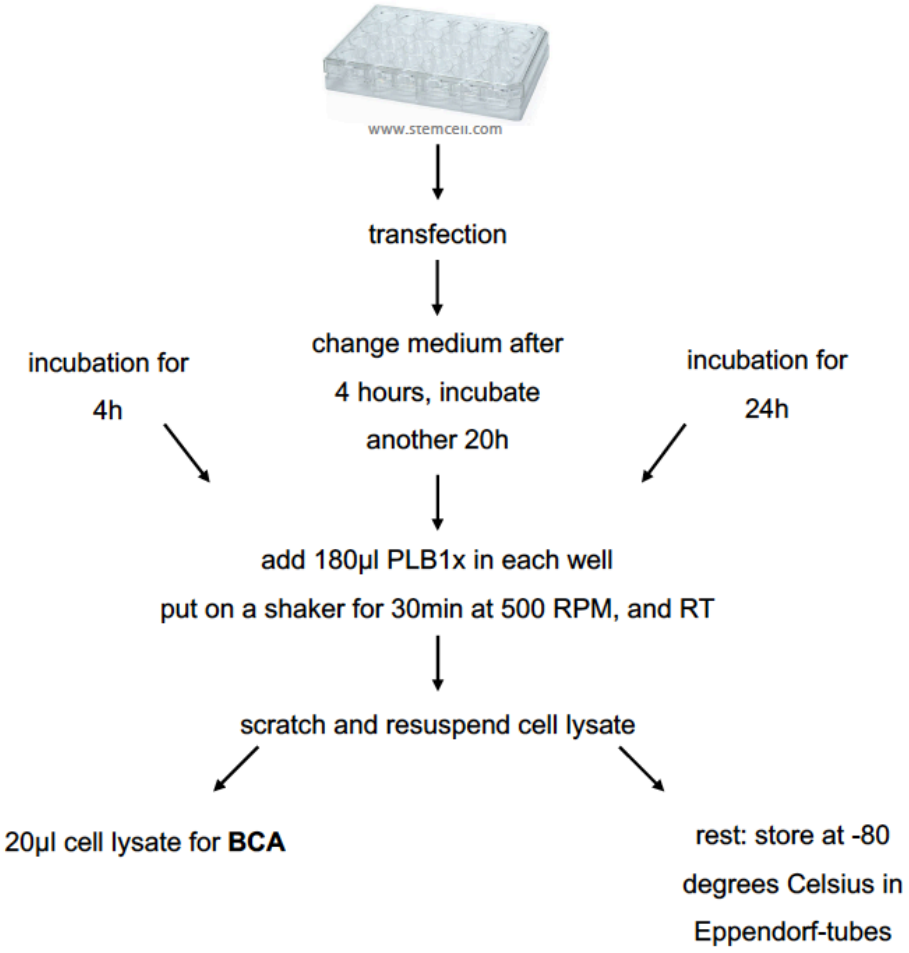

Figure 54: Detailed workflow for the experiments regarding cell uptake of gold nano particle

10.2.5 Results

For first trials HEK293T and MDA-MB-231 WT cells were used. The first round of experiments revealed a very low protein amount of HEK293T cells, indicating that these cells did not survive the treatment as shown in Figure 55.

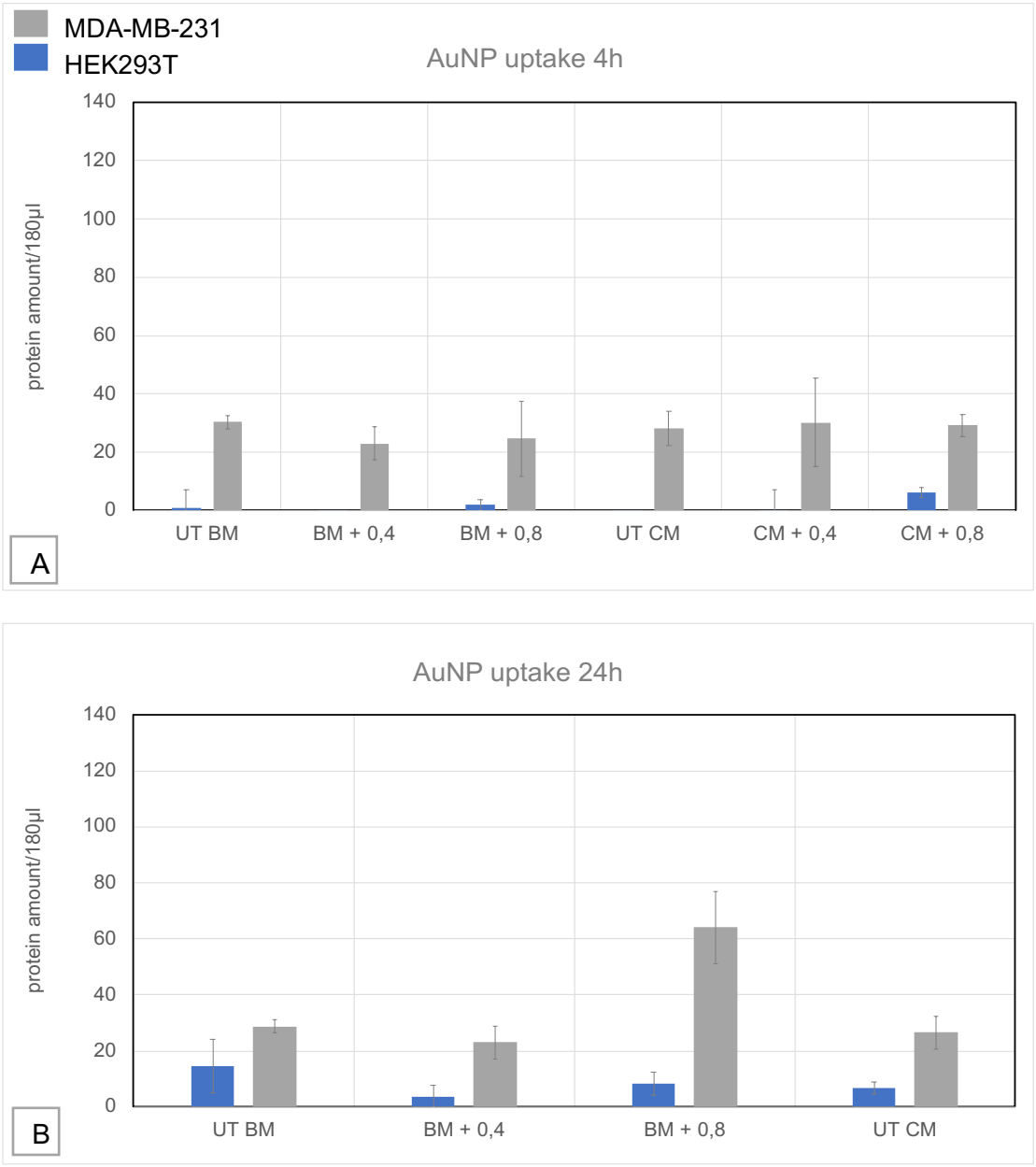

Figure 55: Example of the first round of experiments using AuNP

Results show the protein amount of the different treatments after 4 (A) and 24 (B) hours. Blue bars indicate HEK293T cells, grey bars MDA-MB-231 WT. CM: complete medium, BM: basal medium

As a consequence, it was decided to use A549 cells instead of HEK293T cells. Figure 56, Figure 57 and Figure 58 show that this cell line as well as MDA-MB-231 WT were suitable for the treatment.

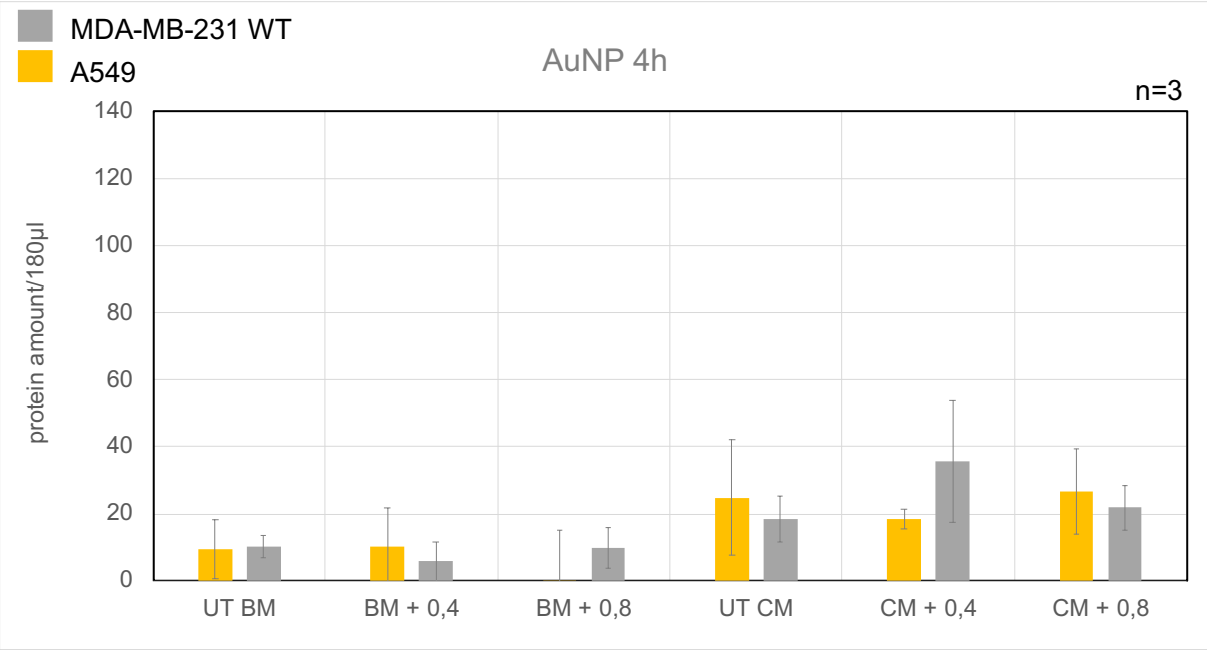

Figure 56: Protein amount of cells lysed four hours after the treatment.

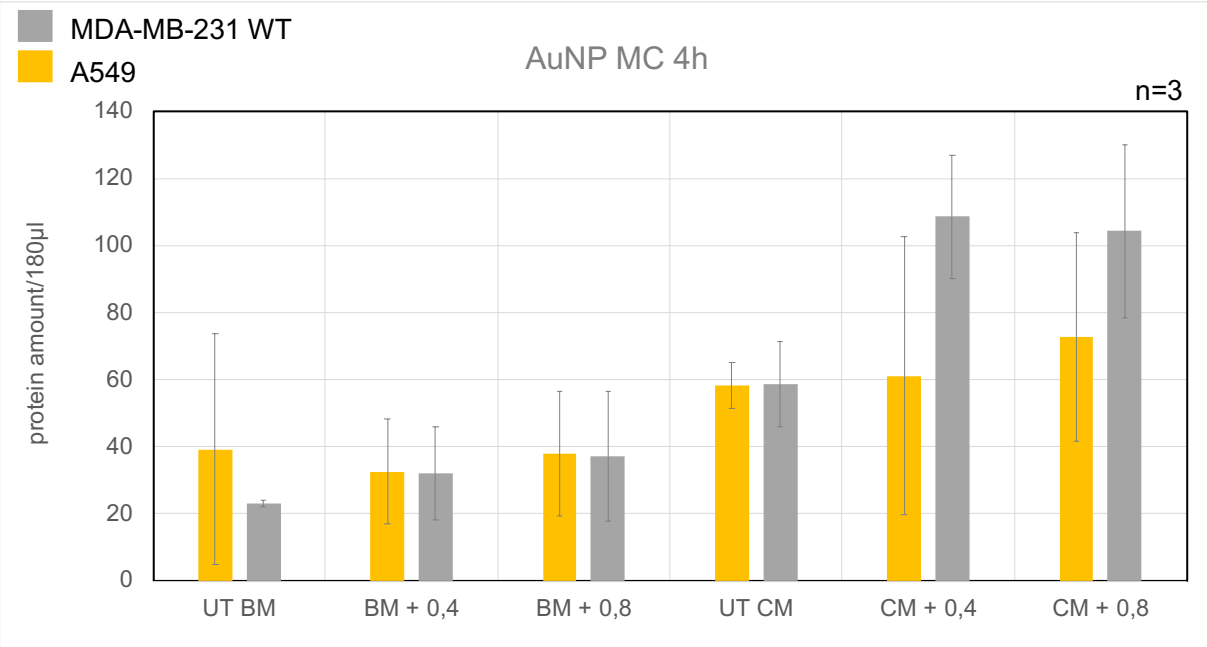

Figure 57: Protein amount of cells - four hours MC

After four hours the medium was changed, after another 20 hours of incubation the cells were lysed.

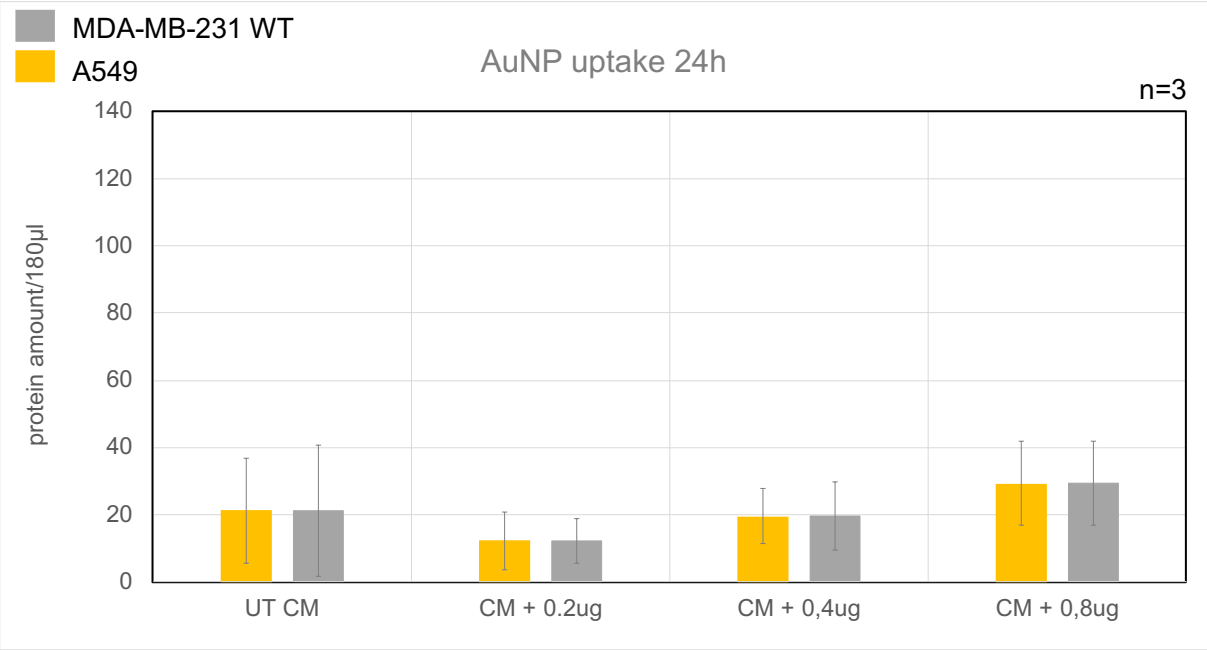

Figure 58: Protein amount of cells lysed 24 hours after the treatment.

All experiments were repeated three times.

During ICP-MS measurements it struck that the AuNP concentration in the cell lysate was under the detection level and therefore readings were stopped. For further plans it would be necessary to repeat the experiment with higher concentrations.