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Immediate-Early Genes and Delayed Primary Response Genes Regulated by NmU in SKBR3 HER-2 Positive Breast Cancer Cell Line

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Immediate-Early Genes and Delayed Primary Response Genes Regulated by NmU in SKBR3 HER-2 Positive Breast Cancer Cell Line

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Abstract

Background

Breast cancer is a heterogeneous disease that consists of varying genetic, cellular and molecular subtypes with unique characteristics. Due to the multiple subtypes and molecular markers of breast cancer, successful clinical treatment is hampered by the lack of reliable biomarkers. HER2-positive breast cancer is an aggressive subtype associated with poor patient prognosis. Although survival rates have dramatically increased due to the development of Trastuzumab in 1997, many patients develop a resistance to this therapeutic treatment and relapse over time. Previous studies have associated the acquirement of resistance to HER2-treatment with Neuromedin U, but the mechanisms by which it works remain elusive.

Aim

The aim of this study was to investigate the effects of NmU on the regulation of immediate early and delayed primary response genes in HER2-positive SKBR3 breast cancer cells using RT-qPCR gene expression analysis. This information was then used to uncover related pathways that may be involved in the progression of this aggressive cancer due to NmU.

Results

Treatment of SKBR3 cells with endogenous NmU resulted in a significant change in the regulation of several cancer-associated genes. Jun expression was significantly downregulated after 30 minutes of NmU treatment, which increased significantly after 1 hour. EGR1 and NR4A1 expression levels were also significantly downregulated. EGR1 and NR4A1 act as tumour suppressors in certain human cancers, suggesting that NmU may drive cancer progression by inhibiting important tumour suppressors. Increasing regulation of SOD2 and DKK1 was observed due to NmU, suggesting that NmU plays a role in Wnt and MAPK signalling.

Conclusion

This project has identified a number of critical genes that may induced by NmU. Through further research, this could lead to the potential development of alternative therapies for HER2-positive breast cancer by targeting these genes.

Keywords: Breast Cancer, HER2-positive Breast Cancer, Neuromedin U, Immediate Early Genes, Gene Expression Analysis

1. Introduction

Breast cancer is a heterogeneous disease that consists of varying genetic, cellular, and molecular subtypes all with unique characteristics. Over 1 million new breast cancer patients are diagnosed yearly worldwide and accounts for the most cancer-related deaths in women (Jemal, *et al*., 2011). Due to the multiple subtypes and molecular markers of breast cancer, successful clinical treatment is hampered by the lack of reliable biomarkers. Human Epidermal Growth Factor Receptor 2 (HER2)-positive breast cancer is an aggressive subtype associated with poor patient prognosis. Although survival rates have dramatically increased due to the development of Trastuzumab in 1997, many patients develop a resistance to this therapeutic treatment and relapse over time. Rani *et al*. (2014), have recently associated the acquirement of resistance to HER2-treatment with a secreted neuropeptide called Neuromedin U (NmU), but the mechanisms by which this occurs have not been uncovered.

NmU is an endogenous neuropeptide belonging to a family known as the Neuromedins. These neuropeptides are involved in neuronal signalling and have implicated themselves in a range of physiological processes such as energy homeostasis and maintaining the biological clock (Budhiraja & Chugh, 2008). NmU was originally isolated from the spinal cord of a pig in 1985 and has since been found to be highly conserved in the gut and brain of a number of different species throughout evolution. NmU was first found to play a role in smooth muscle contraction of the uterus, hence its name, and has since been ascribed many peripheral and central activities It is ubiquitously distributed throughout the body with its highest levels found in the pituitary gland and gastrointestinal tract. Current research has suggested that NmU also plays a role in immune inflammatory diseases, stress, and cancer, however, the exact pathological roles of NmU have not yet been fully investigated and still remain ambiguous (Budhiraja & Chugh, 2008).

NmU is believed to act as an endogenous antagonist to its two identified receptors – NMUR1 and NMUR2. These are G protein coupled receptors (GPCRs), which transform extracellular stimuli into intracellular signals via their seven transmembrane domains (Kroeze, *et al.*, 2003). Activation of these GPCRs by NmU activates two heterotrimeric membrane-resident G proteins; Gqalpha-11 ($G_q\alpha_{11}$) and Gialpha ($G_i\alpha$). These G proteins consist of three subunits: α, β, and γ, which are anchored to the membrane via a fatty acid chain. When activated, these proteins are switched on by the conversion of guanosine diphosphate (GDP), found in the α subunit, to guanosine triphosphate (GTP). This allows the βγ subunit to dissociate from the α and move laterally across the membrane in search of a target (Fig. 1; Li, *et al*., 2002).

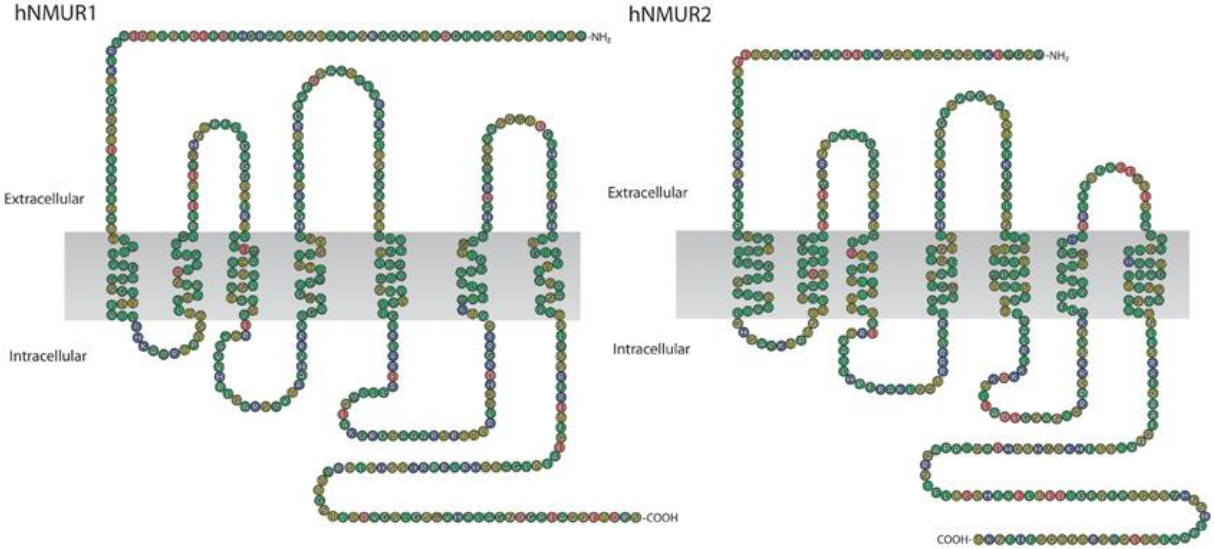

Figure 1: Schematic representation of NMUR1 and NMUR2 GPCRs (Malendowicz, Ziolkowska, & Rucinski, 2012; used with permission).

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NMUR1 and NMUR2 stimulation has been found to activate phospholipase C through the $G_q\alpha_{11}$ protein, allowing a flux of Ca^{2+} ions to pass through the cell membrane, subsequently activating a cascade of intracellular changes (Gajjar & Patel, 2017). These include the activation of Tyrosine-Protein Kinase Met (c-Met), which causes further signal transduction cascades such as the activation of pathways which have been heavily implicated in cancer progression. These are the phosphoinositide-3-kinase (Pi3K) and mitogen-activated protein kinase (MAPK) pathways, which have repeatedly been associated with increased cell migration, differentiation, and resistance to apoptosis (Ketterer, *et al*., 2009).

In recent years, it has been suggested that NmU may play an oncogenic role in cancer progression. Studies by Rani *et al.* (2014) have linked NmU with acquired resistance to HER2-targeted therapies in HER2-positive breast cancer. This study, which looked at the HER2-positive SKBR3 cell line, reported that NmU levels were significantly increased in patients who were resistant to HER-targeted treatments, and that NmU knockdown greatly improved the effectiveness of these treatments. Rani *et al.* (2014) suggested that NmU expression could be used as an independent prognostic factor for poor outcome. Subsequent research by Garczyk *et al.* (2017) found that NMUR2 expression levels were high in HER2-rich breast tumours. They also found that NmU over-expression increased motility of NMUR2-positive SKBR3 breast cancer cells. With this information, they suggested that NmU plays an oncogenic role, especially in the presence of the NMUR2 receptor. Little is known to date, however, on the pathways and molecules that NmU interacts with to induce this oncogenic effect. By uncovering which immediate early genes (IEGs) and delayed primary response genes (DPRGs) are regulated by NmU, we may be able to identify specific therapeutic targets to prevent the metastatic spread of this cancer.

The exact signal transduction of NMUR1 and NMUR2 is not yet known, however typically the binding of growth factors to cell surface receptors leads to the activation of signalling pathways involved in the mediation of cell proliferation, differentiation, and survival. These signalling cascades typically target transcription factors, which alter gene expression levels, resulting in changes in cell behaviour. There are two highly organised programmes of gene expression induced by growth factor stimulation: the regulation of primary response genes, which do not require *de novo* transcription factor synthesis; and the regulation of secondary response genes, which are regulated later by primary response genes and depend on the translation of primary gene mRNA to produce transcription factors for their activation (Tullai, *et al*., 2007). The initial response to growth factor stimulation is the rapid induction of these primary response genes, which are mediated by pre-existing transcription factors. These primary response genes are classified into IEGs and DPRGs. IEGs are regulated rapidly by growth factors and encode transcription factors, which in turn activate secondary response genes. While DPRGs are induced later than IEGs, they do not require further protein synthesis for their activation and are thus classified as delayed primary response genes and not secondary (Tullai, *et al*., 2007). There is little information about the effects of NmU on IEGs and DPRGs, and the pathways associated with them. This research project aims to uncover the role of NmU in cancer pathogenesis by examining the effect of NmU on the HER2-positive cell line – SKBR3 through the regulation of these genes. This could potentially lead to the identification of novel methods of treating HER2-positive breast cancer by targeting these pathways.

2. Materials and Methods

Materials

SKBR3 cell lines were obtained from Trinity College Dublin; Dulbecco's Modified Eagle's Medium – high glucose with 4500 mg/L glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate (Sigma); Fetal Bovine Serum Canada origin, sterile-filtered, γ-irradiated (Sigma); Penicillin-Streptomycin, stabilised with 10,000 units penicillin and 10 mg streptomycin/mL (Sigma); Trypsin BRP, European Pharmacopoeia (EP) reference standard (Sigma); Dulbecco's Phosphate Buffered Saline with MgCl2 and CaCl2, liquid (Sigma); Neuromedin U-25 (human) (NmU) (CAT no. ab141007 Abcam); Virkon disinfectant cleaner (Sigma); Ethanol (Fisher Scientific); Cell freezing medium-DMSO 1X (Sigma); Class II Biological Safety Cabinet; 37 °C incubator with 5% CO2; Corning® CELLBIND® Surface cell culture flasks (Sigma); Optika Vision Pro Software with camera lens; Cryogenic Tube (ALDRICH); Microscope (Olympus/CX23LEDRFS1); PureLink RNA Mini Kit (CAT no 12183018A, Invitrogen); Applied Biosystems High-Capacity cDNA Reverse Transcription kit (CAT no. 4368814); NanoDrop® ND-1000 spectrophotometer; SYBR™ Select Master Mix (Applied Biosystems, CAT 4472903); Applied Biosystems 7300 Real-Time PCR System; MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems).

Cell Culture and Treatments

SKBR3 cells were originally established in 1970 and were sourced from a Caucasian female 43 years of age who suffered from malignant breast adenocarcinoma. SKBR3 cells over-express the HER2 receptor. These cells were chosen as it has been earlier reported that NmU increases the stability of the HER2 receptor, thereby increasing the aggressiveness of the cancer (Rani, et al., 2014). SKBR3 cells were cultured in Dulbecco's minimal essential medium (DMEM), supplemented with 10% v/v fetal bovine serum (FBS), 1% v/v penicillin streptomycin (Pen-Strep), and 1% v/v L-glutamine. To induce expression of IEGs and DPRGs, $5x10^5$ cells were seeded in 25-cm^2 flasks, allowed to attach overnight, and subsequently treated with 5 µmol/L of NmU-25 for 30 minutes, 1 hour, and 24 hours respectively. Sterile water was used as a solvent to dissolve the NmU. Sterile water was also used as a solvent control for the untreated cells.

RNA Extraction and Quantification using Microvolume Spectrophotometry

Total RNA was extracted from the treated and untreated SKBR3 cells using the Invitrogen PureLink RNA Mini Kit as per the manufacturer's instructions. To lyse the cells for RNA extraction, 0.6 ml of lysis buffer was added to each sample and the lysate was passed through a 2.5 ml syringe needle 5 times for complete homogenisation. Extracted RNA was quantified using the NanoDrop® ND-1000 spectrophotometer. The A260/A260 ratio for all samples was 2.03-2.07 indicating the RNA was pure. Cell treatment and RNA extraction were performed once only.

Reverse Transcription PCR

cDNA was synthesised from each RNA sample using the Applied Biosystems High-Capacity cDNA Reverse Transcription kit, according to the manufacturer's instructions. A Master Mix was prepared with 12 µl of 10X RT Buffer, 4.8 µl of 25X dNTP Mix (100 mM), 12 µl of 10X RT Random Primers, and 6 µl of MultiScribe Reverse Transcriptase. 164.4 ng/µl of RNA from each treatment time was converted to cDNA. The reverse transcription reaction was carried out in a thermal cycler using the conditions laid out in Table 1.

Table 1: Thermal cycler conditions for cDNA reverse transcription.

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25 °C	37 °C	85 °C	4 °C
Time	10 minutes	120 minutes	5 minutes	∞

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RT-qPCR

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was carried out using SYBR green detection to determine gene expression in SKBR3 cells due to NmU. The analysis was performed using 2 µl of DNA, 1 µl of the relevant primer mix, and 7 µl of SYBR green and ultra-pure water. The real time reaction was carried out on a 96-well plate according to the conditions collated in Table 2, with the annealing temperature changing according to the Tm of the primers used (Table 3). Beta-actin was used as an endogenous control to allow for normalisation of the gene expression levels.

Table 2: RT-qPCR reaction conditions.

Stage	Temperature (°C)	Time
Holding Stage	95.0	10 min
Cycling Stage (40 Cycles)	95.0	15 sec
	60.0*	1 min
Melt Curve Stage	95.0	15 sec
	60.0	1
	95.0	15 sec

*Annealing temperature changed according to melting temperature (Tm) of primers.

Table 3: List of primers used with corresponding Tm.

Primer used	Average Tm of forward and reverse (°C)
Beta-actin	59.6
FOS	58.15
JUN	61.4
EGR1	60.6
IER2	59.35
NR4A1	63.6
SOD2	59.8
DKK1	57.45

Statistical analysis

Three biological replicates and three technical replicates were used for statistical analysis, and the relative quantification (RQ)/fold-change was calculated in *Excel* using the average values for each gene. The delta-delta cycle threshold (CT) method was used to calculate the RQ, and the results were compared to untreated controls to determine the size of the change in expression between the two conditions. Statistical analysis on RT-qPCR data was performed in Excel. Student t-tests were used to generate p-values, with $p < 0.05$ considered as statistically significant.

3. Results

RNA Quantification using Microvolume Spectrophotometry

The NanoDrop® ND-1000 was used to measure the concentration of 1 µl of RNA extracted from each of the SKBR3 samples after treatment with NmU-25 peptide. The ND-1000 software was used to automatically calculate the quantity of RNA in each sample in ng/µl (Table 4). Corresponding absorbance curves for each treatment times are available on request. Each RNA sample quantified had a A260/280 ratio of 2.03-2.07, indicating that there were no contaminants present and therefore deeming the RNA as pure (NanoDrop Technologies Inc., 2007).

Table 4: RNA concentrations of the control and NmU-treated SKBR3 cells as determined using the NanoDrop® ND-1000. The ratio of RNA sample absorbance at 260 nm and 280 nm was used to determine the purity of the RNA. All samples with a 260/280 ratio of 2.0 were deemed as pure.

Sample ID	ng/µl	A260/280	A260/230
Control	768.64	2.04	1.18
30 mins	164.40	2.07	0.78
1 hr	430.63	2.04	1.91
24 hrs	850.82	2.03	1.30

Gene Expression Analysis using RT-qPCR

Based on literature by Forget *et al*. (2007), Jiao et al. (2010), and Wu et al. (2017) , five IEGs and two DPRGs were selected, which have previously been linked with cancer and these were analysed using RT-qPCR. IEGs and DPRGs can be activated and transcribed rapidly after stimulation by external and internal stimuli. Many of these genes are considered proto-oncogenes and have been linked to the MAPK pathway, which is commonly involved in cancer mutagenesis (Bahrami & Drablos, 2016).

A sample RT-qPCR curve for one of the reactions carried out can be seen below in Figure 2. From this curve the CT values were extrapolated and used to calculate the RQ values of each gene. The fold change in gene expression levels due to NmU can be seen below in Table 5 for IEGs and Table 6 for DPRGs. Beta-actin was used as an endogenous control so that the gene expression levels of the target gene could be normalised. However, beta-actin showed some variability in its expression between NmU-treatment times, as shown in Table 5.

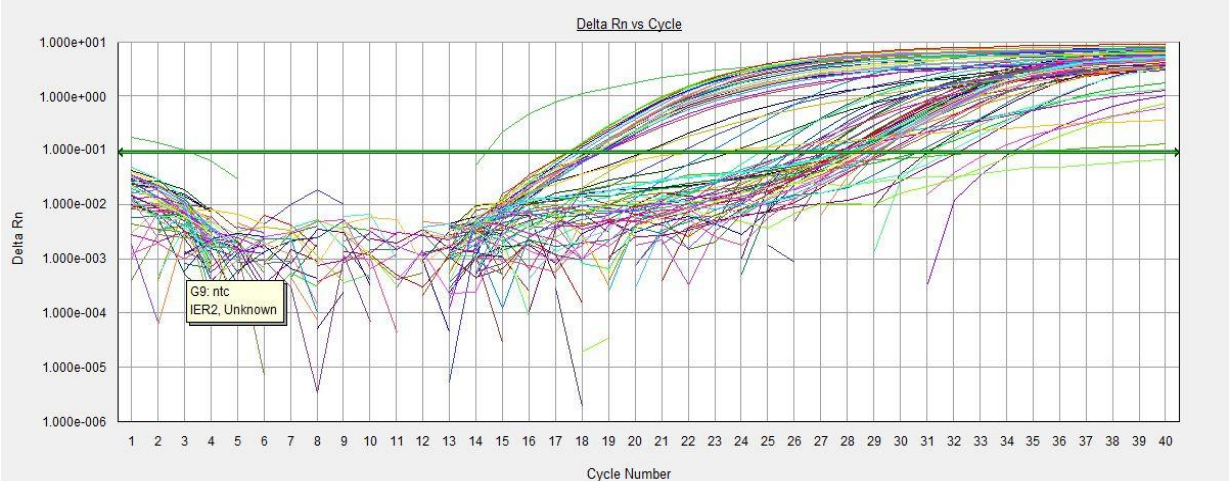

Figure 2: Sample image of the RT-qPCR analysis carried out of gene expression in SKBR3 cells due to NmU treatment at 30 minutes, 1 hour, 24 hours, and untreated controls. All curves over the baseline indicate a positive reaction.

Table 5: Statistical analysis of IEG expression was calculated using the delta-delta Ct method. The fold changes of each gene due to NmU are shown. A negative value infers gene downregulation due to NmU and a positive value signifies an upregulation of the gene. The fold changes of beta-actin treated and untreated and also included in this table, this housekeeping gene (HKG) was used for normalisation of the data.

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Gene	NmU Treatment Time	Average Fold Change	Standard Error of Mean (SEM)
Beta-Actin	Control	15.0844	0.69
Beta-Actin (Treated)	30 min	15.846	0.76
	1 h	15.58	0.49
	24h	15.3217	0.26
FOS	30 min	6.43	1.80
	1 h	5.57	0.87
	24 h	2.52	0.30
JUN	30 min	-97.25	0.79
	1 h	6.93	1.53
	24 h	2.49	0.32
EGR1	30 min	-73.48	6.82
	1 h	-7.48	1.33
	24 h	-93.88	2.18
NR4A1	30 min	1.46	0.11
	1 h	-1.33	0.19
	24 h	-2.12	0.26

Table 6: Statistical analysis of DPRG expression calculated using the delta Ct method. A negative value infers gene downregulation due to NmU and a positive value signifies an upregulation of the gene. The results for beta-actin collated above were used for data normalisation.

Gene	NmU Treatment Time	Average Fold Change	Standard Error of Mean (SEM)
SOD2	30 min	-1.35	0.17
	1 h	2.02	0.19
	24 h	1.72	0.14
DKK1	30 min	-2.16	1.11
	1 h	73.76	2.84
	24 h	15.57	0.54

Previous research by Rani *et al.* (2014) found that NmU-25 had no cytotoxic effects on SKBR3 cells when 1 x 10^6 cells seeded in a 25-cm^2 flask were treated with 1 µmol/L of NmU. As such, a similar method was followed in this research project, to ensure the cells were not adversely affected by the peptide. While cell morphology and proliferation rate were not a focus of this experiment, all cells treated with NmU-25 appeared to have reduced colony formation when examined under a microscope. However, this would need to be further examined using colony formation and migration assays. To determine the effect of NmU on the regulation of the genes listed in Tables 5 and 6 in HER2-positive breast cancer and whether they are linked to resistance in HER-targeted therapies, relevant primers for each gene were used in this study.

Analysis of Fos & Jun expression following NmU treatment.

NmU treated SKBR3 cells were analysed using RT-qPCR to determine the change in expression levels of the Fos oncogene (Fig. 3). However, these results obtained were not statistically significant, suggesting that Fos may not be directly impacted by NmU expression *in vitro*.

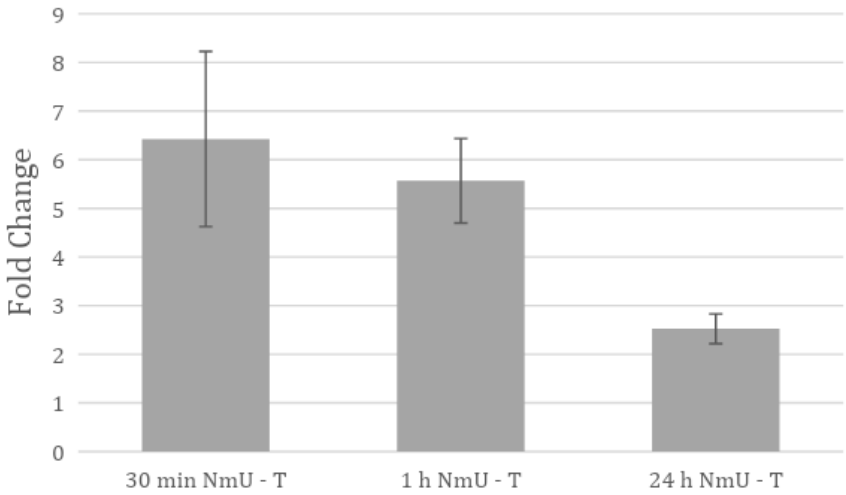

Figure 3: Decreasing expression of Fos was observed due to NmU treatment at 30 minutes (6.43-fold ± 1.80), 1 hour (5.57-fold ± 0.87, p=0.66), and 24 hours (2.52-fold ± 0.30, p=0.08). Fold change was calculated in Excel using the delta-delta Ct method. Results are presented as mean ± SEM; n=3, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

Jun, which is known to form a transcriptional complex with Fos, was also examined in this project. After 30 minutes of treatment with NmU, a large downregulation of Jun by -97.25-fold was observed, followed by a highly significant upregulation to 6.93-fold after 1 hour, which again decreased significantly to 2.49-fold after 24-hours (Fig. 4). This variation in gene expression may be due to the cyclic manner in which some genes are transcribed within the nucleus. It may also be due to an activating protein (AP-1) complex that is formed by the dimerisation of Fos and Jun, suggesting that Jun may rely on the formation of this complex for its upregulation.

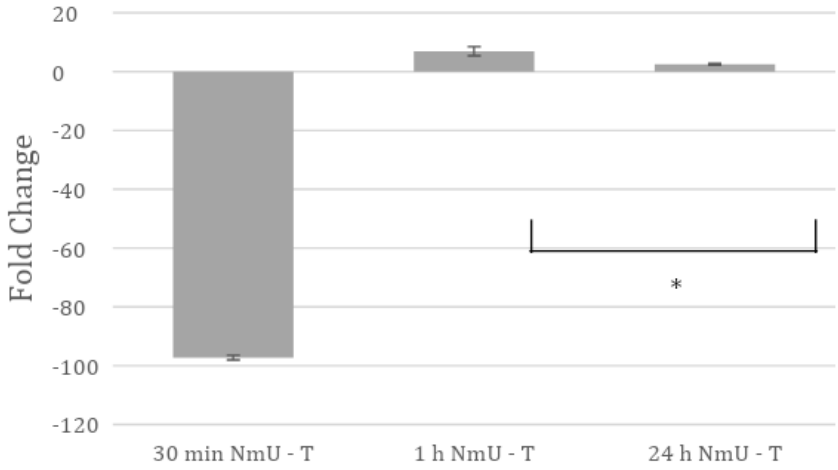

Figure 4: Regulation of Jun expression following NmU treatment at 30 minutes (-97.25-fold ± 0.79) , 1 hour (6.93-fold ± 1.53, p=0.000017), and 24 hours (2.49-fold ± 0.32, p=0.05). Fold change was calculated in Excel using the delta-delta Ct method. Results are presented as mean ± SEM; n=3, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

Analysis of EGR1 expression following NmU treatment.
Treatment of the SKBR3 cells with NmU resulted in a significant downregulation of EGR1 after 30 minutes and 24 hours (Fig. 5).

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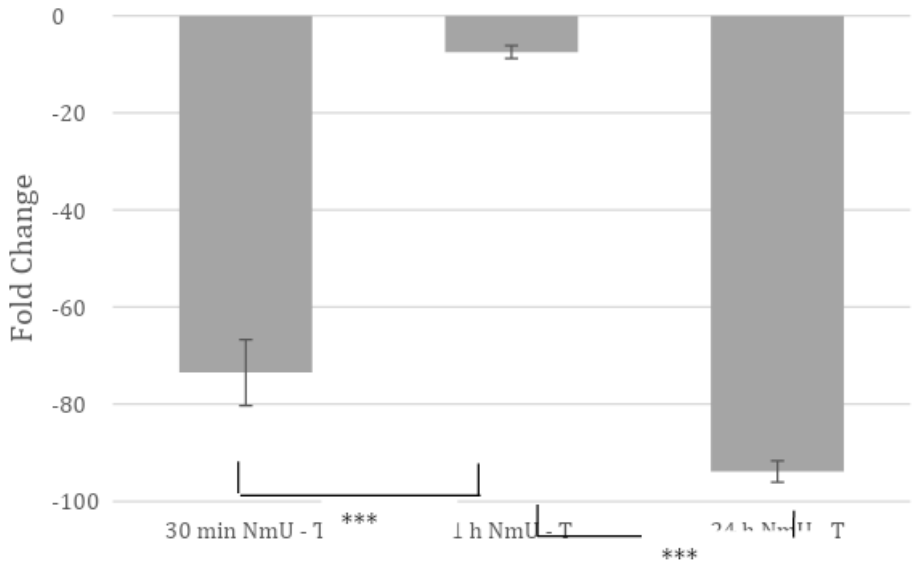

Figure 5: Downregulation of EGR1 due to NmU treatment at 30 minutes (-73.48-fold ± 6.82) , 1 hour (-7.48-fold ± 1.33, p=0.0007), and 24 hours (-93.88-fold ± 2.18, p=0.000005). Fold change was calculated in Excel using the delta-delta Ct method. Results are presented as mean ± SEM; n=3, * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.

Analysis of NR4A1 expression following NmU treatment.
Treatment of SKBR3 cells with NmU resulted in an initial upregulation of NR4A1 after 30 minutes of treatment, which decreased significantly after 1 hour (Fig. 6).

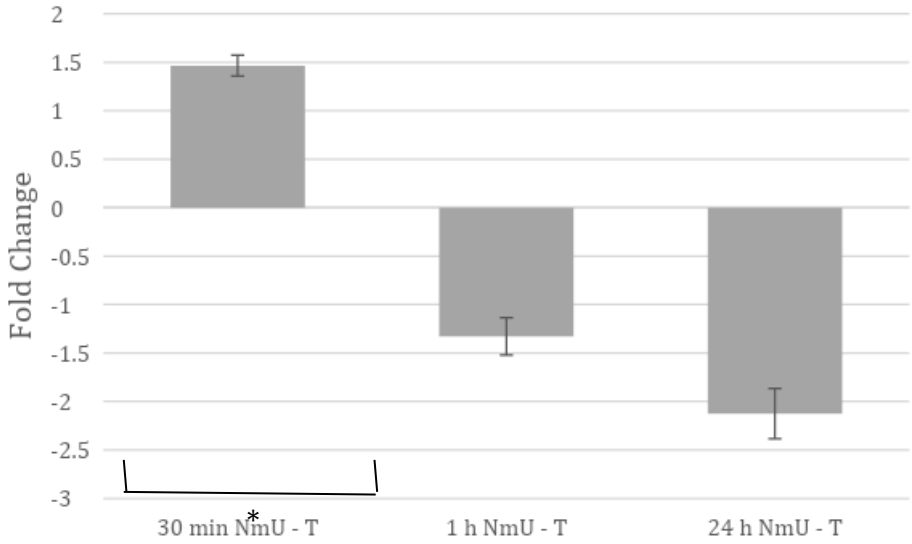

Figure 6: Regulation of NR4A1 due to NmU treatment at 30 minutes (1.46-fold ± 0.11) , 1 hour (-1.33-fold ± 0.19, p=0.03), and 24 hours (-2.12-fold ± 0.26, p=0.13). Fold change was calculated in Excel using the delta-delta Ct method. Results are presented as mean ± SEM; n=3, * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.

Analysis of SOD2 expression following NmU treatment.
Treatment of SKBR3 cells with NmU resulted in an initial downregulation of SOD2 gene expression after 30 minutes, which significantly increased after 1 hour (Fig. 7). This shows that in HER2-positive breast cancer with high levels of NmU, SOD2 is likely to play the role of a tumour promoter.

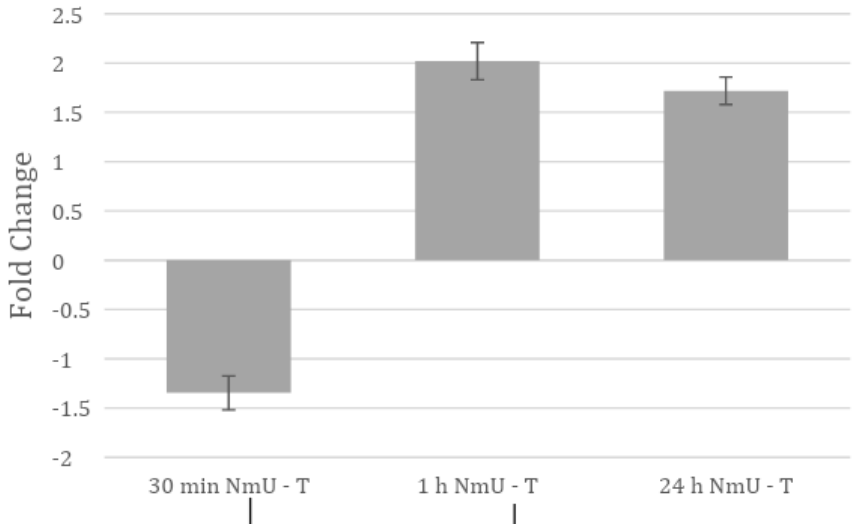

Figure 7: Regulation of SOD2 due to NmU treatment at 30 minutes (-1.35-fold ± 0.17) , 1 hour (2.02-fold ± 0.19, p=0.001), and 24 hours (1.72-fold ± 0.14, p=0.33). Fold change was calculated in Excel using the delta-delta Ct method. Results are presented as mean ± SEM; n=3, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

Analysis of DKK1 expression following NmU treatment.
Treatment of SKBR3 cells with NmU resulted in a highly significant upregulation of DKK1 expression after 1-hour of treatment (73.76-fold), which decreased to 15.57-fold after 24 hours (Fig. 8). This suggests that the DKK1 gene is also transcribed in a cyclic manner, as was seen with Jun.

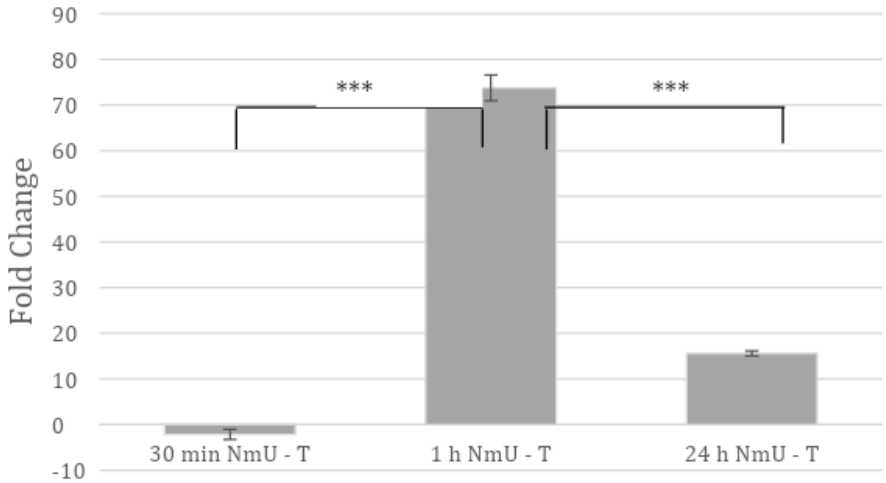

Figure 8: Regulation of DKK1 due to NmU treatment at 30 minutes (-2.16-fold ± 1.11) , 1 hour (73.76-fold ± 2.84, p=0.00002), and 24 hours (15.57-fold ± 0.54, p=0.0006). Fold change was calculated in Excel using the delta-delta Ct method. Results are presented as mean ± SEM; n=3, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

4. Discussion

NmU has previously been associated with HER2-positive breast cancer, but little is known about its effects on IEGs and DPRGs, and the role it plays in cancer progression. A number of IEGs and DPRGs have been associated with the progression and increased aggressiveness of cancers such as HER2-positive, however, the role that NmU plays in this has not yet been uncovered. The genes listed in Table 3.2 were selected as they have previously been linked to human cancers, but it has not been determined whether NmU plays a role in their activation. By determining the role of NmU in the regulation of these genes, it will be possible to make

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connections between specific genes and pathways, and potentially uncover new targets for HER2-positive breast cancer treatment.

Endogenous Control gene

Beta-actin was used as an endogenous control so that the gene expression levels of the target gene could be normalised. Beta-actin is a HKG, whose expression levels should remain constant throughout NmU treatment. However, in this study a small level of variability in expression between NmU-treatment times was observed. HKGs are often used for the normalisation of mRNA levels between different samples, however, their expression levels can vary among cells and tissues and may change under specific circumstances; this is likely in this case. Good reproducibility was observed throughout the experiment with other samples and primers used, confirming that the variability seen in beta-actin between treatment times is likely due to the gene itself, and not due to errors made in sample preparation. Aithal & Rajeswati (2015) have shown that beta-actin varies considerably during some gene expression profiling and is thus not the most suitable candidate for gene expression analysis.

Fos

This study analysed the effects of NmU on the Fos oncogene, which plays a distinct role in the regulation of cell growth, differentiation, and transformation. It is characterised as an IEG, as its expression is rapidly induced by external stimuli such as mitogens and hormones (He, *et al.*, 2001). Transcription of Fos is activated by serum and growth factors and is linked to the transcription factor Elk-1 – a target of the MAPK pathway. Numerous studies have implicated Fos in enhanced cell proliferation and differentiation and have found it to regulate many genes involved in tumorigenesis by causing a downregulation of tumour-suppressor genes (Bakin & Curran, 1999). Members of the Fos family have long since been represented in gene profiles associated with early relapse and reduced survival rates in many cancer types (Meinhold-Heerlein, *et al*., 2005). Furthermore, Fos has been seen to induce epithelial-mesenchymal transition (EMT), leading to the metastatic growth of mammary epithelial cells (Fialka, *et al*., 1996). There was no significant evidence however, to suggest that Fos was directly impacted by NmU (see Figure 3.3). This was surprising as Jun, which is known to form a heterodimeric AP-1 transcriptional complex with the nuclear protein encoded by Fos, was found to be significantly downregulated due to NmU. The AP-1 complex is a transcription factor formed in response to external stimuli by the dimerisation of c-Fos and c-Jun (Maggiolini, *et al*., 2004). This complex controls a number of cellular processes seen in cancer development, such as apoptosis, proliferation, and differentiation. It acts by binding to the promoter region of specific target genes and converting extracellular signals into changes in gene expression (Mahner, *et al*., 2008). According to Eferl & Wagner (2003), some Jun and Fos family proteins can actually suppress the formation of tumours and whether AP-1 is oncogenic or not depends on the cell type and differentiation state.

Jun

Jun is a proto-oncogene that produces the transcription factor c-Jun, which forms part of the AP-1 complex discussed above. c-Jun is overexpressed in human breast cancer and has been linked to cancer progression through activation of the MAPK pathway. Studies by Jiao *et al.* (2010) have shown that c-Jun promotes cellular invasiveness and stem cell expansion in HER2-positive and other aggressive breast cancer subtypes. As Jun is also associated with cell proliferation, the significant downregulation of this gene observed here (see Figure 3.4) due to NmU may explain the reduced cell growth and proliferation observed by Garczyk *et al.* (2017) in cells with increased levels of NmU mRNA. The AP1 complex, formed by Fos and Jun, is also associated with increased cell motility. While this study has not found NmU to be linked

with Fos expression, it may still play a role in the promotion of AP-1 through the stimulation of Jun, suggesting that NmU may be involved in the increased motility associated with HER2-positive cells, allowing them to disseminate from the primary tumour site and invade other parts of the body. This postulation may explain why Garczyk and his colleagues found that NmU reduced cell-Matrigel adhesion of NMUR2-positive SKBR3 cells in an anchorage-independence assay (Garczyk, *et al*., 2017). As changes in tumour cell migration are often accompanied by alterations in cell adhesion, this warrants for further investigation into the role of the AP-1 unit in HER2-positive breast cancer and the genes and pathways associated with it. If there is a link associated with NmU and the AP-1 unit, it may be possible to design a method to reduce the risk of cancer metastasis by targeting this complex.

EGR-1

EGR1 is an important transcriptional regulator that acts as a master switch for a variety of extracellular stimuli involved in long-term cellular responses such as growth, proliferation, and apoptosis (Gregg & Fraizer, 2011). EGR1 is found in low abundance in many cancers such as lymphoma and breast cancer, indicating that it plays a role in tumour suppression. Studies have shown that it acts through targeting two main tumour suppressors – Phosphatase and tensin homolog (PTEN) and p53, and also the pro and anti-apoptotic factor c-Jun. This suggests that the absence of EGR1 allows cells to grow unchecked and proliferate out of control (Gregg & Fraizer, 2011). EGR1 was found to be significantly downregulated after 30 minutes and 24 hours of treatment with NmU (see Figure 3.5). This correlates with Gregg & Fraizer (2011), who found the EGR1 gene to be absent or in low prevalence in cancers such as breast cancer. This is also in concordance with previous research carried out to analyse the effects of the growth factors heregulin and EGF on HER2-positive breast cancer (Murphy, 2017). This project found that treatment of SKBR3 cells with EGF, the main growth factor involved in EGR1 regulation, reduced their ability to proliferate (Murphy, 2017). This suggests that NmU may enhance the invasive property of SKBR3 cells by downregulating EGR1 and allows cells to move through the cell cycle unchecked. This warrants for further investigation into the possible link between NmU and EGF.

NR4A1

Nuclear hormone receptor 4 (NR4A1) belongs to a family of nuclear hormone receptors (NHRs) that are involved in the regulation of physiological and pathological processes in the human body. NHRs are transcription factors that are induced by hormones in the body to regulate their target gene expression. NR4A1 however, is an orphan receptor as it does not have any identified hormones or ligands. It can be activated by various extracellular stimuli such as growth factors, cytokines, and neurotransmitters (Maxwell & Muscat, 2006). This IEG has pleiotropic physiological roles and has been implicated in the regulation of functions involving the central nervous system (CNS), inflammation, and metabolism (Maxwell & Muscat, 2006). In cancer, conflicting reports have been made about its role, suggesting that the complex function of this transcription factor may be specific to each subtype of this disease (Wu, *et al*., 2017). Several studies have reported NR4A1 acting as a tumour suppressor. For example, Lenz *et al.* (2008) found that the over-expression of NR4A1 in lymphoma cells induced apoptosis and inhibited further growth of tumour cells in mice. The significant downregulation of NR4A1 observed in this study (see Figure 3.6) is supported by Wu *et al.* (2017) who found that expression of this gene in TNBC cells inhibited cell proliferation, migration, and invasion (Wu, *et al*., 2017). The downregulation of NR4A1 observed after 1 hour of treatment with NmU correlates with studies by Wu *et al.* (2017), where NR4A1 expression was significantly downregulated in triple-negative breast cancer (TNBC). The low levels of NR4A1 protein in the TNBC tissue samples was associated with advanced tumour stage, lymph node metastasis, and disease recurrence

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(Wu, *et al*., 2017). This leads to the suggestion that NmU may increase the aggressiveness of HER2-positive breast cancer by down-regulating tumour suppressors such as NR4A1, thereby allowing the cells to proliferate and avoid cell cycle checkpoints.

Superoxide dismutase 2

Superoxide dismutase 2 (SOD2), a DPRG, is an antioxidant enzyme that plays an important role in the defence of reactive oxygen species (ROS) (Kang, 2015). ROS are known to cause DNA damage in cells, generating genetic lesions, which can ultimately lead to the initiation of tumorigenesis (Bulteau, *et al.*, 2005). SOD enzymes are one of the most important enzymes involved in the mediation of ROS induced damage (Yamakura & Kawasaki, 2010), with SOD2 being the only one found within the mitochondrial matrix. SOD2 has been found to play a dichotomous role in cancer, acting as both a tumour suppressor and promoter. Traditionally, SOD2 was considered a tumour suppressor due to its cytoprotective role of scavenging harmful superoxide anion free radicals ($O_2\bullet^-$) in the mitochondrial matrix (Kim, *et al.*, 2017). This observation was made due to the reduced expression of the gene found in a variety of tumour types (Zhong, *et al.* 1997), and forced expression of SOD2 in a number of murine tumours also resulted in a significant tumour growth reduction (Weydert, *et al*., 2003). More recently however, it has been shown that SOD2 expression is entirely dependent of the tumour type, with some cancers actually displaying an increase in SOD2 levels (Miar, *et al*., 2015). A reduction in SOD2 expression is primarily seen at the initiation of cancer and in non-metastatic cancer cell lines, however, during metastasis, SOD2 levels have been seen to increase (Hempel, *et al.*, 2011). It has been found that an increase in the antioxidant enzymes encoded by SOD2 are required to enable tumour cells to cope with the added stress of detaching from the ECM. (Liu, *et al*., 2015).The increase in SOD2 levels observed in this study (see Figure 3.7) correlates with Hempel *et al.* (2011), who found that SOD2 levels were increased in metastatic cancers. As HER2-positive breast cancer can become metastatic, it is likely that this is in part due to the elevated levels of SOD2 induced by NmU, which helps the cells to cope with the stress of detaching from the ECM.

DKK1

DKK1 encodes a member of the dickkopf family of proteins – dickkopf-related protein 1. This soluble protein is secreted into the microenvironment of the bone and is important in embryonic development and bone formation. This protein is known to inhibit the beta-catenin dependent Wnt signalling pathway and has been found to be overexpressed in multiple human cancers, possibly promoting invasion in cancer cell lines (Forget, *et al*., 2007). In the Wnt signalling pathway, Wnt-1 protein binds to both the frizzled (fz) receptor and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6), which triggers cellular signalling from proliferation via beta-catenin. When DKK1 is overexpressed, it binds to LRP5/6, thus blocking it from interacting with Wnt-1, ultimately leading to beta-catenin degradation and alterations in proliferation (Forget, *et al*., 2007). Conflicting reports have been made about DKK1's role in cancer progression, with studies suggesting it prevents metastasis through downregulating the Wnt signalling pathway. Forget *et al.* (2007), however, have described DKK1 to be a potential diagnostic marker of aggressive breast cancer subtypes due to its high prevalence in human breast cancer with negative hormone expression. In their study, high DKK1 expression was found in MCF-7 HER2-positive cell lines along with DKK1 protein expression, leading to the suggestion that elevated DKK1 expression may contribute to the invasive phenotype associated with aggressive breast cancer cells (Forget, *et al*., 2007). The upregulation of DKK1 observed in this study (see Figure 3.8) correlates with the enhanced expression of this gene observed in MCF-7 cells by Forget *et al*. (2007). This suggests that NmU plays a role in the upregulation of DKK1 by signalling intracellular cascades such as the Wnt pathway. Garczyk *et al*. (2017) have

recently suggested that a crosstalk of NmU signalling with cancer-associated pathways such as Wnt and MAPK cascades may result in the altered expression of DKK1, thus contributing to cell growth inhibition and the promotion of cell migration.

The DPRGs investigated in this project followed a similar trend of an initial downregulation at 30 minutes, followed by a significant upregulation. As DPRGs are regulated later than IEGs, it is likely that it took one hour for this activation to occur inside the cells. This is in concordance with the literature available on DPRGs, which has shown that they require the upregulation of other primary response genes for they themselves to be activated.

Proposed pathways involved in NmU activity

To fully understand the mechanisms by which NmU increases resistance to treatment and progresses cancer, it is important to understand the proteins and genes that it interacts with. Gene expression analysis performed in this study allowed for the identification of critical genes and pathways associated with NmU. In light of this retrospective data discussed in this paper, a number of these potentially associated genes were evaluated using the String Database (see Fig. 3). This investigation identified several interactions between these critical cancer-genes that are regulated by NmU. Fos, Jun, EGR1, and NR4A1 were all found to be related through intracellular interactions with strong data support. This new-found information will pave the way for further research into these genes, the pathways they are involved with, vital protein-protein interactions, and the potential of their use as biomarkers to determine the onset of metastatic breast cancer. This also leads to the postulation that the targeting of NMUR2 could prevent NmU from regulating these oncogenic genes, thus preventing cells from becoming invasive and resistant to HER2-targeted therapies.

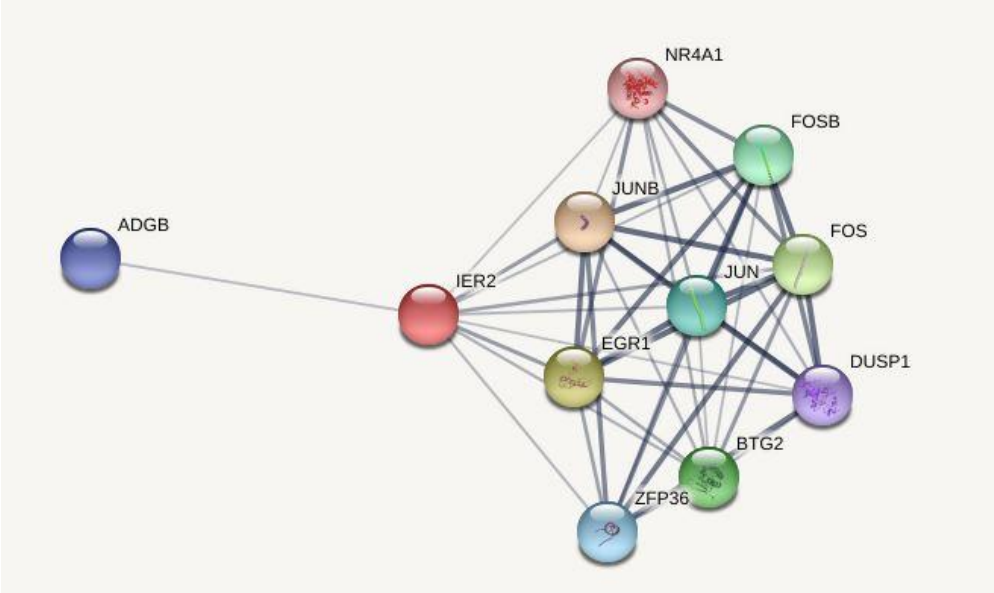

Figure 3: Gene Ontology (GO) network diagram, created using the String Database, showing known and predicted interactions between Fos, Jun, IER2, EGR1, and NR4A1. Line thickness indicates the strength of data support.

5. Conclusions

Through gene expression analysis, this research project has, for the first time ,identified a number of critical genes and pathways induced by NmU, which is believed to act through its receptor NMUR2, as discussed previously by Garczyk *et al.* (2017). This may be the missing link in determining the mechanism by which HER2-positive breast cancer becomes metastatic

and lays the groundwork for the development of alternative therapies by targeting these genes. This study also suggests that NmU may play a role in inducing breast cancer metastasis through the activation of genes which increase cell motility. This leads to the postulation that NMUR2 could potentially be used as a druggable target to block NmU from activating these genes. To verify these results found, the experiments detailed here will need to be repeated using a more suitable HKG, due to variability observed in beta-actin.

6. Future Work

In future studies, screening for suitable house-keeping genes should be performed to ensure the accuracy of the gene expression analysis. As the selection of HKGs with the most stable expression is crucial to accurately analyse gene regulation, several endogenous controls should be compared using RT-PCR to determine the most appropriate candidate.

The relationship between NmU and these genes should also be investigated in other HER2-positive cell lines with innate-resistance/susceptibility to various HER-drugs, this would help to consolidate the results observed in this study. To identify further pathways and genes associated with NmU, more cancer-associated genes should be investigated to determine the effect NmU has on their reaction. Once a large group of specifically regulated genes has been identified, Western Blotting and BioID could be used to screen for protein-protein interactions involved in HER2-positive breast cancer with high NMUR2 levels. As Rani *et al.* (2014) suggested a link between NmU and HER2-resistance, gene expression analysis could then be performed in HER-Drug resistant cell lines so that a co-relation between NmU and HER-drug resistance could also be detected.

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