Hypoxia-induced expression of HIF-1a and GPR30, in relation to survival, proliferation and apoptosis in MCF-7 and SkBr3 breast cancer cells.

Hypoxi-inducerat uttryck av HIF-1a och GPR30, i relation till överlevnad, proliferation och apoptos av MCF-7 och SkBr3 bröstcancerceller

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Abbreviations

BC	Breast cancer
BCC	Breast cancer cells
CBC	Contralateral breast cancer
EGFP	Enhanced green fluorescent protein
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
FACS	Fluorescence-activated cell sorting / flow cytometry
GPR30	G protein-coupled estrogen receptor 30
GPER	G protein-coupled estrogen receptor 1 or GPR30
GT	GPR30-transfected
HER2	Human epidermal growth factor 2
HG	Hypoxia group
HIF-1/2	Hypoxia-inducible factor 1 / 2
HRE	Hypoxia response element
MCF-7	ER-positive breast cancer cell line
MT	Mock-transfected
NG	Normoxia group
Skbr3	HER2 positive, but ER-negative, breast cancer cell line

Abstract

Introduction: Hypoxia that accompanies tumor growth induces the expression of hypoxia-inducible factor a (HIF-1a), in turn mediating the expression of other genes, including G protein-coupled receptor 30 (GPR30), to promote adaption to the hypoxic microenvironment and enable cell proliferation and survival. Nonetheless, this correlation has not been causally explained.

Objective: To further investigate how breast cancer cells with expression of GPR30 adapt to hypoxia, by studying survival, proliferation, and ability to induce apoptosis.

Method: Human breast cancer cells, MCF-7 (ER-positive) and SkBr3 (ER-negative), were seeded and transfected with either GPR30 or an empty pcDNA plasmid. Transfection efficiency was assessed by transfecting the cells with enhanced green fluorescent protein (EGFP). Cells were incubated in either a hypoxic or normoxic condition, and molecular biological methods as Western blot and FACS were applied to investigate the cell´s survival, proliferation, and apoptosis induction. Mann-Whitneys U test was performed to statistically analyze intra- and inter group variations.

Results: Recombinant expression of GPR30 was reduced in hypoxic cells, and β-actin staining of those cells, as well as their PARP-cleavage, suggested cell death. Significant increased cleaving of PARP in hypoxic GPR30+ SkBr3 cells (15.5% vs 62.95%, $p = 0.0286$) and a significant difference between hypoxic GPR30+ SkBr3 and MCF-7 cells ($p = 0.0286$). GPR30+ cells also had a lower cleavage of PARP. SkBr3 cellularity was affected negatively, whilst MCF-7 cellularity was generally increased, plus more MCF-7 cells in the S-phase, in hypoxia. GPR30+ cells had a lower survival rate in hypoxia, but an increased proliferation in normoxia.

Conclusion: Our results propose a trend showing an interaction between hypoxia and GPR30, a correlation between GPR30 and a promoted proliferation in normoxia, as well as a significant difference between SkBr3 and MCF-7 in those aspects. However, our results are not enough evidence of these correlations and requires additional research.

Populärvetenskaplig sammanfattning

Bröstcancer är av stor relevans i dagens samhälle på grund av dess höga frekvens och mortalitetsgrad. Behandling är härjämte individualiserad utifrån tumörtyp och uttryck av hormonreceptorer. Med upptäckten av en ny receptor, GPR30, som svarar på östrogen, har forskning drivits kring hur denna samverkar med hypoxi, hur det bidrar till tumörcellernas överlevnad samt dess prognostiska och prediktiva värde. Tidigare studier har kunnat påvisa ett samspel med HIF-1a, som uppregleras vid hypoxi, och denna interaktion uppges kunna bidra till cancercellernas överlevnad och tillväxt. För att vidare undersöka denna interaktion och få en bättre förståelse för receptorns betydelse i tumörutvecklingen, utförde vi en *in vitro* studie där vi transfekterade MCF-7 (ER-positiva) och SkBr3 (ER-negativa) bröstcancerceller med antingen GPR30 eller pcDNA (kontroll) plasmid. Transfektionseffektiviteten kontrollerades med EGFP (enhanced green fluorescent protein), där ökat upptag av plasmider noterades i SkBr3 i jämförelse med MCF-7 celler.

MCF-7 och SkBr3 cellerna placerades i antingen en hypoxikammare eller i normala förhållanden i 18 timmar. Cellerna och deras proteinuttryck analyserades sedan med Western blot, där mängden PARP i förhållande till klyvd PARP (apoptosmarkör), β-actin (proteinmängdsreferens) och GPR30 (endogent och rekombinant uttryck) kvantifierades. FACS (flödescytometri) tillämpades för att undersöka cellernas cellcykel och proliferation. Därutöver tillämpades Mann-Whitneys U test för att statistiskt analysera resultaten och undersöka variationer mellan och inom grupperna.

Det endogena GPR30 uttrycket var högre i hypoxiska celler i jämförelse med kontrollcellerna, medan det rekombinanta uttrycket var lägre. Detta i samband med nästan icke-befintligt uttryck av β-actin hos de hypoxiska cellerna och en signifikant ökad PARP klyvning hos GPR30+ SkBr3 cellerna (intensiteten för klyvd PARP/total PARP: 15.5% vs 62.95%, $p = 0.0286$), tyder på att de hypoxiska cellerna har dött. Dessutom noterades en signifikant högre PARP klyvning hos GPR30+ SkBr3 celler i jämförelse med GPR30+ MCF-7 celler i hypoxi ($p = 0.0286$) och att hypoxiska MCF-7 celler hade en betydligt högre överlevnadsgrad och fler celler i S-fasen. Tillsammans indikerar dessa resultat på att MCF-7 överlever bättre i hypoxi än SkBr3 cellerna. Vi noterade dessutom en trend av att GPR30+ celler hade en lägre PARP klyvning, och att fler befann sig i S-fas vid normoxi, vilket antyder en roll hos receptorn i överlevnad och proliferation.

Utifrån de resultat vi fått, tyder vår studie på att det finns en association mellan hypoxi och GPR30 i aspekter av proliferation och överlevnad, samt att det finns en skillnad mellan hur MCF-7 och SkBr3 adapterar i hypoxiska förhållanden, vilket skulle kunna reflektera en generell skillnad mellan ER-positiva och ER-negativa celler. Eftersom HIF-1a inte kunde framkallas, så kan denna studie inte uttala sig eller påvisa kausaliteten mellan HIF-1a och GPR30. För att få en klinisk eller vetenskaplig relevans krävs ytterligare studier och fler försök med större data.

1. Introduction

1.1 Background

1.1.1 Breast neoplasm

Breast cancer (BC) is the most frequently diagnosed neoplasm in women, being a leading cause of cancer-related mortality (1). Breast tumors are classified in different groups based on their expression of hormone receptors (HR): estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) (2).

1.1.2 Hypoxia and HIF-1 regulation

The development of a tumor leads to an abnormal microvascular function and eventually hypoxic environment due to structural and functional transformations that leads to inadequate perfusion and diffusion of oxygen (3, 4). According to Vaupel et al. (4) the median pO_2 in BC is 10 mm Hg, although it could be 2,5 mm Hg or lower in hypoxic fractions, compared to 65 mm Hg in normal breast tissue (3).

Decreased O_2 leads to over-expression of the heterodimeric transcription factors (TF), HIF-1 and HIF-2, which in turn affects the expression of the plethora of genes related to adaptation and survival in the hypoxic microenvironment (5, 6). HIF-1 consists of two PAS domain proteins, HIF-1a and HIF-1β. Suboptimal oxygen levels leads to accumulation of HIF-1a through inhibiting hydroxylation of asparagine and proline residues, which leads to less binding to von Hippel-Lindau (VHL) protein and thereby stabilization of the protein (3, 5). This is mediated by oxygen-dependent degradation domain (ODDD) (7). HIF-1a then translocates to the nucleus where it forms a complex with HIF-1β and activates the heterodimeric HIF-1 (8). HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), remains unaffected by hypoxia and is stably expressed (8, 9).

On the other hand, the HIF-2 complex consists of HIF-2a, a similar HIF-1a PAS domain previously termed HIF-like factor (HLF) or HIF-related factor (HRF), and HIF-1β (8). The difference between HIF-1a and HIF-2a is defined by a variation in the N-terminal transactivation domain (N-TAD) (9). As reported by Krieg M et al. (8) both HIF-1 and HIF-2 regulates transcription of other genes by binding to hypoxia responsive elements (HRE) in the promoters of target genes. However, they are differently activated at distinct sites; HIF-1 is characteristically activated and expressed at high levels in breast, colorectum, head and neck,

kidneys, testis and ovarium, as well as in melanoma and lymphoma (10), while HIF-2 is mainly expressed in liver, heart, lungs (8), endothelium and carotid body (7).

By binding to HRE in specific enhancer and promoter regions, HIF-1a regulates the transcription of distinct genes required for adaption and survival in hypoxic conditions (7). Among the genes that HIF-1a transcriptionally activate are vascular endothelial growth factor A (VEGF), glucose transporter 1 (Glut1), glycolytic enzymes and nitric oxide synthetase (8), as well as insulin-like growth factor-2 (IGF2) and transforming growth factor-a (TGF-a) (7). These genes regulate cell metabolism, stimulate proliferation, angiogenesis, metastasis and promotes survival and proliferation.

Paradoxically, as reported by Ke Q and Costa M (7), HIF-1 levels also correlates with caspase-3, Apaf-1-mediated caspase-9, Fas and Fas-L, pro-apoptotic factors that promotes cell death and apoptosis induction. Those pro-apoptotic factors are as well proteases that cleaves, among others, poly-(ADP-ribose) polymerase (PARP), a DNA repair protein, and thereby mediates apoptosis (11, 12). Contrarily, another *in vivo* study of chronic myeloid leukemic cells has found that PARP-1 mediates cell survival by forming a complex with HIF-1a and by co-activating its gene-expression (13).

HIF-1a and HIF-2a have many common target genes that they both regulate, nonetheless they may have distinct functions that probably is due to other factors present in the cell. A general difference is therefore difficult to pinpoint.

1.1.3 The role of hypoxia in BC development and in prognosis

According to Bao et al. (14) HIF-1a mediates epithelial-to-mesenchymal transition (EMT), a process that is critical to metastasis, by regulating specific TF, repressors, EMT-associated cytokines or signaling proteins. In breast cancer cells (BCC), those factors include ZEB1-MYB-E-cadherin, Jagged2, cyclooxygenase-2 (COX-2), urokinase receptor, long non-coding RNA (lncRNA), Ca2+ signaling (3) as well as E-cadherin, Snail, Slug and Twist (15).

Another characteristic of a hypoxic phenotype is tumor cell invasiveness, mediated by upregulated expression of matrix metalloproteinase-2 (MMP-2) and MMP-9, proteinases that catalyze degradation of components of the extracellular matrix (ECM), mainly type IV collagen, by HIF-1a (3, 15). Contrarily, HIF-1a can also increase expression of specific hydroxylases, pro-collagen prolyl (P4HA1 and P4HA2) and lysyl (PLOD1 and PLOD2), that

mediate cell migration during invasion (3). Hypoxia thus provides necessary prerequisites for invasion, which correlates with poor prognosis in BC.

Extravasation of BCC into blood circulation or lymphatics has also been associated with HIF-1a dependent ANGPTL4 expression, a protein that permeabilize capillaries by disrupting cell junctions, and promote carcinogenesis in the lungs (3) and VEGF expression that also permeabilize the capillaries and promotes tissue (15). Furthermore, a study of the adherence of epithelial, human BCC line, MDA-MB-231, to endothelial cells (EC) reported that hypoxia, and primarily HIF-1a, increases the interaction between BCC and EC by up-regulating L1 cell adhesion molecule (L1CAM), a protein that binds integrins, neuropilin 1 or CD24 in EC (3, 16). This adherence, also called margination, is a crucial step in metastasis and extravasation of BCC. By mediating transcription of lysyl oxidase (LOX), enzymes that catalyze remodeling of ECM, hypoxia provides conditions to form a "metastatic niche" where the tumor seeds, promoting survival and colonization (3).

1.1.4 Prognostic and predictive value of HIF-1a

Clinical follow-up study of BC patients demonstrated a statistically significant correlation of HIF-1a with aggressive clinicopathological characteristics and phenotypes such as tumor size, metastasis, lymph node involvement and clinical prognosis (17), as mentioned earlier by molecular *in vivo* and *in vitro* studies (5, 14, 18). However, as reported by Cai et al. (17) the predictive value of HIF-1a in breast cancer is still unclear, and present evidence are not sufficient to predict the prognosis of BC patients, which is in concordance with other studies (19) regarding hypoxia used in tumor prognosis. Another clinical study of the prognostic value of HIF-1a in patients with colorectal cancer concluded that HIF-1a did not correlate with clinical parameters, but significantly correlated with worser prognose (20). Hence, it has a high prognostic value in patients with colorectal cancer.

1.1.5 Activation of GPR30 mediates tumor development and affects prognosis

GPR30 or GPER, is a G-protein coupled receptor that mediates an estrogen signaling which is both genomic and non-genomic (21, 22). The receptor is reported to be located both on the plasma membrane (23) but also in the endoplasmic reticulum (ER) and other intracellular membranes (24), and its function depends on the environment where it is expressed. Studies have shown that the receptor could promote proliferation, however, it has also been reported that GPR30 mediates apoptosis (25). The genomic pathway refers to the estrogen-dependent activation of GPR30 and regulation of gene transcription, as the receptor indirectly induces

expression of, among other genes, connective tissue growth factor (CTGF) in ER-negative (SkBr3) cells (26) and increases expression of c-Fos via the EGFR-MAPK-signaling (27). Nonetheless, induced expression of CTGF has been reported to promote cell proliferation and migration (26).

Thus, the role of GPR30 in cell proliferation may depend on the cell type as it has been demonstrated that it decreases cell proliferation in MCF7 cells (ER-positive BCC) but increase cell growth in SkBr3 cells (ER-negative) through increased intracellular Ca2+, sustainably or transitionally, respectively (25).

The non-genomic pathway on the other hand refers to a rapid onset of effect modulators. There have also been findings that suggest that GPR30 activated by estrogen is needed for the activation of extracellular signal-regulated kinase, Erk-1 and Erk-2, which is important for cell survival and proliferation (28, 29). Wang D et al. (21) refers to Vivanco et al. (30) study and suggest that estrogen-dependent activation of intracellular GPR30 receptor activates Src, which through a downstream series of intracellular events, consequently transactivates epidermal growth factor receptor (EGFR). EGFR activates in turn other modulators such as phospholipase C (PLC), mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI3K) (21). PI3K leads through second messengers to the AKT signaling, which is associated with cancer cell growth but also cell survival and proliferation. This pathway has also been proposed to stimulate HIF-1a expression (31), via translation dependent on mTor, in human prostate cancer cells (3). Another research (32) showed similar results as the activation of GPR30 by estrogen resulted in an increased production of EGF, promoting cell proliferation in ER-positive BCC.

Tutzauer J et al. (2) found that GPR30 located to the plasma membrane ($GPR30_{PM}$) associates with markers for worse prognosis such as triple negative subtype (ER-negative, HER-2 negative and PR-negative) and high Ki67. This corresponds to the findings in Sjöström M et al. (33) retrospective cohort study where PM localized GPR30, but not total GPR30, strongly associated with poor prognosis in BC. The previous study (2) also concluded that PM localized GPR30 was associated with an increased risk of breast cancer death in patients with an independent, primary, contralateral breast (CBC) cancer and lymph node metastasis. These results regarding GPR30s association with worse prognosis, agrees with the finding in another research that suggests a positive association between GPR30 and tumor size, distant metastasis and HER-2/neu (34).

1.1.6 HIF-1a upregulates GPR30

GPR30 is one of the plethora of genes that HIF-1a transcriptionally activate by binding to HRE in its promotor region (35). Data from a previous study where GPR30 5´ promotor region was bioinformatically analyzed reported three different HRE sites (35). The transactivation of GPER consecutively stimulated neovascularization, proliferation and migration through expression of VEGF and activation of EGFR/ERK signaling pathway in BCC (35-37), but studies have also shown that CTGF is over-expressed as a consequence of the correlation between HIF-1a and GPR30 (37). Furthermore, Franscesco E et al. (37) claims that the interaction or cooperation between HIF-1a and GPER is required to hypoxia-dependently transcript VEGF, meaning that GPR30 or HIF-1a by itself will not activate the promoter of VEGF.

By way of explanation, several studies emphasize a correlation between HIF-1a and GPER (35-38), nonetheless, this correlation has not been causally explained or clarified.

1.1.6 Treatment resistance associated with hypoxia and GPR30

ER-positive BC are generally treated with tamoxifen, a selective ER modulator (SERM), that binds competitively to the binding site on the ER instead of estradiol and blocks its gene transcription (39). It has been suggested that hypoxia has a potential role in tamoxifen-resistance (6), however, findings of whether GPER also mediates resistance are controversial (24).

Research on CBC material from patients treated and not-treated with tamoxifen were collected and examined in a study, giving the results that among patients earlier treated with tamoxifen and who later developed CBC (a primary second BC independent of the first one), there was a higher frequency of HIF-1a (18) and HIF-2a (39). Alam M et. al (39) claims that this antiestrogen-resistance is mediated by a crosstalk between HIF-2a and EGFR/HER1, one of the plethora of genes over-expressed in hypoxic conditions. Furthermore, in an *in vivo* study, Jögi A et al. (18) proved a strong, positive correlation between HIF-1a and EGFR, too. Statistical analysis also showed that BCC treated with tamoxifen in hypoxic conditions (1% O_2) were antiestrogen resistant and survived significantly longer in comparison to control cells treated in normoxic conditions (21% O_2) that instead survived shorter and were sensitive to the treatment given (39).

According to an *in vitro* model studied by Ignatov A et. al (40), GPER mediates tamoxifen resistance by transactivating EGFR, and the results indicates a significant correlation between

the two biomarkers as well as a positive correlation between GPER and HER-2. Despite this finding, Tutzauer J et al.(2) means that there isn't enough evidence that could prove this correlation or causality between GPER and tamoxifen resistance (2).

1.2 Aim

Thus, the aim of this *in vitro* study is to further understand how breast cancer cells with expression of GPR30 adapts to hypoxia. Given the scope of the course, the timeframe and practical circumstances, this study will be limited to only examine survival, proliferation, and apoptosis induction in human breast cancer cells with expression of GPR30 in hypoxic conditions.

1.3 Research questions

- How does hypoxia affect MCF7/SkBr3 cells with expression of GPR30?
- How does the expression of GPR30 affects HIF-1a?
- What role does hypoxia have on the survival, proliferation, and apoptosis induction of breast cancer cells?
- What indication could the interaction between HIF-1a and GPR30 have for future prognostic and therapeutic purposes?

2. Materials and methods

2.1 Research design

For the purpose of this preclinical study and its research questions, an experimental *in vitro* study was carried out.

2.2 Seeding and counting MCF-7 and Skbr3

Human breast cancer cells, MCF-7 that expresses ER receptors, and SkBr3 that expresses HER2, but not ER, receptors, were provided by American Type Culture Collection (ATCC). MCF-7 and SkBr3 cells obtained from ATCC were grown in DMEM (provided by Gibco, cat. number 11885084) supplemented with 10% FBS, 5% Penicillin-Streptomycin, pyruvate inter alia, respectively McCoy (modified with L-glutanine and sodium bicarbonate with supplement of FBS and antibiotics as DMEM, by Sigma), in a humidified incubator with 5% CO_2.

MCF-7 and SkBr3 were each exclusively seeded in 4 x 6-well plates of 32 mm to achieve an optimal cell density with approximately 70-80% confluence (41). Cell suspensions were prepared by removing the medium from a flask culture with appropriate confluency, further

adding appropriate volumes of phosphate-buffered saline (PBS, provided by Gibco, #2205998) to rinse of interfering growth factors and cell debris, and 0.25% trypsin (provided by Gibco), a proteolytic enzyme that helps detach the cells from the surface. The flasks were incubated for 2-5 minutes at 37°C for trypsin to catalyze the degradation of proteins and detachment of cells to occur. When the cells seemed detached under microscope, specific medium, McCoy for SkBr3 cells and DMEM for MCF-7, were homogeneously added to neutralize trypsin. PBS, trypsin, and mediums were all pre-heated to 37°C before used.

Concentrations and density of cells were calculated for subsequent seeding, by pipetting 10 µl diluted (1:10) cell suspension into the space between the coverslip and a hematocytometer. The suspension filled the chambers by capillarity (42), and precise calculations were made using a light microscope by counting the mean amount of cells in one square, dividing it by the square´s predetermined volume of 10^{-4} cm^3 and further adjusting for the dilution by multiplying with a factor of 10.

Depending on when the transfection was planned, a cell density of 150 000- (3 days later), 300 000- (2 days later) or 400 000 cells (the following day) were desired per well. To calculate the volume of medium needed to be added to dilute the cell suspension to obtain desired concentration, a simple equation was useful ($c_1v_1 = c_2v_2$). 1 ml medium and appropriate amount of newly diluted cell suspension was then pipetted in each well, and homogeneously mixed. For each cell type, in 2 out of the 4 6-well plates, only 4 wells were seeded. Thus, 6 wells were seeded in 2 plates, and 4 wells in the other 2 plates, giving the total of 20 seeded wells for each cell type. The plates were later placed in a humidified 37°C incubator with 5% CO_2 for 24, 48 or 72 hours, depending on when the transfection was planned. The seeding procedure was repeated 3 times, yielding 3 experiment with 20 seeded wells for each cell type in each experiment.

2.3 Transfection

Transfection is a molecular biological method aimed to express a specific gene through transferring DNA plasmids, extrachromosomal circular DNA molecules, to the nucleus where it could implement in the genome and subsequently be expressed. Seeded BCC cultures were transfected with 2 µg/well GPR30 plasmids or mock transfected with empty pcDNA 3.1 containing plasmids. The solution used for the transfection contained plasmids (either containing the GPR30 gene or not); a transfection lipopolyplex reagent called LT-1 (2 µl/µg plasmid, provided by Mirus, prod. No. Mir 2305) that mediates the transport of the plasmid

across the cell membrane and ensures an efficient delivery of the plasmid DNA in mammalian cells (43, 44); as well as DMEM (provided by Gibco^{TM}, cat. number A1443001) free of serum and antibiotics.

To assess transfection efficacy, enhanced green fluorescent protein (EGFP) or mock was transfected in Skbr3 and MCF-7 cells, and visualized under microscope. To get a more accurate quantification of the effectivity, nuclear staining with propidium iodide (PI) >94.0% (provided by Sigma Aldrich, #P4170) was conducted on the transfected cells to calculate the transfection ratio.

2.3.1 Overexpressing EGFP and controlling effectivity

Seeded SkBr3 and MCF7 plates were transfected with EGFP to control transfection effectivity. Cells were either transfected with 2 µg/well of an empty control plasmid called pcDNA 3.1 or 2 µg/well of plasmids with EGFP. Accordingly, DMEM was mixed with the plasmids as well as 2 µl/µg LT-1, to obtain a solution of 260 ul per well. This transfection solution was thereon incubated for 15 minutes in room temperature (RT). This step is necessary for the formation of a transfection complex, consisting of LT-1 and plasmid, which facilitates the reaction between the cell´s DNA and the transfected plasmid. The solution was pipetted into each well respectively, and then placed to incubate under normal growth conditions as describes above for 48h.

The nuclear staining started off with rinsing of culture medium and adding 4% paraformaldehyde (PFA) (by Sigma Aldrich, #16005), a fixator, to incubate in a dark place for 15 minutes. Using PBS, PFA was rinsed of, and 2% Triton™ X-100 (Sigma-Aldrich, catalog No. T8787) was added in a 1:10 dilution with PBS and incubated for 10 min. Triton™ X-100 permeabilize the cell membranes by inducing pores. A solution of RNAse A diluted to 50 µg/µl was added to treat the cells, before adding PI (100 µg/ µl), a mutagen nuclear stain. Due to light sensitivity, all solutions with PFA, Triton™ X-100 and RNAse A, were placed to incubate in a dark place at RT. A fluorescence microscope was used to visualize the cell staining.

2.3.2 Overexpressing GPR30

Three attempts were done to seed SkBr3 and MCF-7 cells, and all seeded plates were transfected with 2 µg/well of plasmid containing either N-terminally FLAG-tagged human wild-type (WT) GPR30 or an empty pcDNA 3.1 plasmid. Flag-tag is a specific sequence that could be recognized by specific anti-FLAG antibodies and is thus used in this study to

distinguish the plasmids GPR30 from the natural GPR30 that some cells express. DMEM was mixed with the plasmids and LT-1 following the same protocol as EGFP.

2.4 Hypoxia induction

Cell cultures transfected with GPR30 respectively mock transfected with pcDNA two days earlier were placed in a hypoxia chamber to mimic a physiological hypoxic condition. To obtain an optimal humidification level cloths soaked with water were placed in the chamber (45). For each cell type, and in each of the 3 experiments conducted in this study, one seeded 4-well- and one seeded 6-well plate were placed and exposed to hypoxic condition for 18h, contrarily, the equivalent other two plates were placed in normoxia as controls.

Transfection medium in each well was rinsed off with PBS, and another medium consisting of DMEM (without pyruvate) including 25 mmol HEPES, a chemical agent that buffers for changes in CO_2, and 10% FBS, was added. Oxygen level in the chamber was modulated to 0.1-0.8%. After 18h incubation in hypoxia, levels of HIF-1a, GPR30 and PARP (an apoptosis marker), were measured and compared to the levels in normoxia.

2.5 Protein extraction and western blot analysis

Western blot, a semi-quantitative method used to detect specific proteins through an electric field (46), was conducted to identify and quantify the expression of GPR30, PARP and HIF-1a in cultures incubated in both hypoxic and normoxic conditions for 18h. The results of GPR30, PARP and HIF-1a were normalized to β-actin (ACTB), a housekeeping gene, that is expressed to the same extent in all cells, and therefore used as a reference protein to exclude non-specific staining (1).

Cell cultures were placed on ice to stop biological processes and prevent degradation of HIF-1a which has a half-life of approximately 5 minutes (47). Proteins were extracted from MCF-7 and SkBr3 cells using a lysis buffer consisting of radioimmunoprecipitation assay (RIPA) buffer (80 µl/well, provided by ThermoFisher Scientific, catalog No. 89900), a detergent that lyses cells, and Complete™ Protease Inhibitor Cocktail (Roche). Extracted proteins were thermally denatured in NuPAGE™ LDS Sample Buffer (ThermoFisher, #NP0007) diluted to 10:4 with reducing agent dithiothreitol (DTT, 60 mM, provided by Sigma Aldrich), and placed to incubate in 70°C for 10 minutes.

SDS-PAGE was performed according to a predetermined protocol. A precast NuPAGE™ 4-12% Bis-Tris SDS gel (Invitrogen, ThermoFisher, catalog No. NP0322PK2) was placed in the

electrophorator, as well as the MES SDS Running buffer (Novex, #2035728) prepared. The comb was removed, and each lane was loaded with 30 µl of each sample (see *appendix 1*). Nonetheless, the first well was filled with the ladder Precision Plus Protein™ Dual Color Standard (Bio-Rad). An electrical field of 200V was subsequently used to separate the proteins based on size and molecular weight, through gel electrophoresis.

By 7 minutes electrotransfer, the proteins were then transferred to a membrane to be visualized as bands. Blotting was thenceforth performed by first blocking the membrane with 5% low fat milk (LFM) diluted with Tris-buffered saline tween (TBST) for 3 hours in room-temperature (RT), to prevent non-specific binding of antibodies to the membrane. Primary antibodies, goat anti-GRP30 (1:200, R&D #AF15534), rabbit anti-PARP (1:1000, Cell Signaling, #9542), anti-HIF-1a (1:1000, BD Biosciences, #610959) or rabbit anti-β-actin (1:10 000, provided by Cell Signaling, #8457), diluted in a 5% LFM solution as the one previously described, were then added and incubated with the membrane overnight at 4°C. Secondary antibodies, anti-rabbit (ECL™ Anti-Rabbit IgG, Horseradish Peroxidase linked whole Antibody (from donkey), by Amersham™, #NA934, dilution 2.6:10 000), anti-mouse (ECL™ anti-mouse IgG, Horseradish Peroxidase linked F(ab)s fragment (from sheep), dilution 2.6:10 000) or anti-goat (rabbit anti-Goat IgG, Invitrogen, #UA276835, dilution 2.0:10 000) were respectively added to the membrane and incubated at RT for 1h. Unbound primary and secondary antibodies were washed off 3x10 minutes using TBST.

A chromo-genic solution was added, and proteins were analyzed in ChemiDOC™ MP Imaging System and quantified in Image Rad 6.0.1. The membranes were stripped with Restore™ Western Blot Stripping Buffer (Thermo scientific #21059) and re-blotted with rabbit anti-β-actin, mouse anti-HIF-1a, rabbit anti-PARP or anti-HIF-1a.

2.6 Cell cycle analysis by flow cytometry

Flow cytometry (FACS) was applied to qualitatively analyze and quantitively count cells based on molecular markers of the cells and the light they scatter (47). The procedure began with ethanol fixation and permeabilization of MCF-7 and SkBr3, by firstly rinsing of the medium from the hypoxia treated cells roughly with PBS to later add 0.25% trypsin to detach the cells from the surface. Medium (DMEM without pyruvate and 10% FBS) was added and the solution in each well-type were transformed to polystyrene tubes before being placed in a centrifuge at 1200-1400 rpm for 5 minutes. The medium was rinsed of with PBS by centrifuging for 5

minutes and then decanting the solution from the pelleted cells. Appropriate amount of 70% ethanol was added in each tube and the cells were placed to incubate in 4°C

for 30 minutes. The ethanol was thereafter washed off with PBS 2 times following the same protocol as rinsing of the medium.

To sustain a single cell solution for a successful staining of cell antigens, cells were intermittently vortexed methodically and medium was rinsed of with PBS by pipetting up and down several times.

For the cell cycle analysis, 300 µl cell suspension was transformed to specific FACS tubes, prepared with 1.5 µg/ml RNAse, and stained with 31 µg/ml PI that easily permeates the cells. Samples were then analyzed by the flow cytometer, and the cell cycle was analyzed by quantifying and interpreting the amount of DNA in each stage.

2.7 Statistical analysis

Data from western blot and FACS were statistically analyzed in Prism 8.0.1. Standard error of the mean (SEM) was selected by default to measure the uncertainty in the collected data, as it considers several independent observations in comparison to standard deviation, as conducted in this study. As the obtained data were non-Gaussian distributed, Mann-Whitneys U test, a non-parametrical test was used to analyze intra- (MCF-7 vs. SkBr3 in normoxia respectively hypoxia) and inter- (normoxia vs. hypoxia) group variations. A p-value of $\leq 0{,}05$ was defined as statistical significance. Furthermore, Grubb's test could be used to define outliers and exclude values that did not pass this test; however, it was not implicated because of the few data obtained.

3. Ethical considerations

This research is a preclinical study, where interventions were not tested on humans nor on animals, and thus did not need to be ethically tested. However, the cells handled in the following study are extracted from patients with BC, and obtained from an independent resource, ATCC, who claims to adhere to all ethical standards when obtaining materials from humans (48). Whether these cells were extracted with the patient´s consent is nonetheless unclear which is an ethical aspect that should be considered. However, who the cells belong to, is not of knowledge in this study which contrarily is favorable as the patient's integrity is preserved.

On the other hand, by conducting this study we are contributing to the understanding of GPR30s interaction with hypoxia, as earlier studies proposed an interaction that could contribute to

tumor development. By gaining further understanding of this phenomenon, this knowledge could contribute to the development of more individualized and effective treatments, which could be beneficial for BC patients.

4. Results

4.1 Successful transfection in both cell types but inferior expression of transfected plasmids in MCF-7

To control the potential efficiency of the transfection, MCF-7 and SkBr3 were transfected with EGFP respectively mock-transfected with an empty pcDNA 3.1. To qualitatively evaluate the efficiency, the cells were visualized using a fluorescence- and phase-contrast microscopy. Both MCF-7 and SkBr3 transfected with EGFP emitted a green fluorescence (*appendix 2A-C*), which suggests a successful transfection. To further quantify the efficiency, cells were likewise nuclear stained with PI. Microscopical observations showed that SkBr3 had a higher expression of EGFP than MCF-7 *(appendix 2D-E*).

4.2 Normoxic cells transfected with GPR30 had a higher expression of the protein than hypoxic cells

Imaging of MCF-7 and SkBr3 lysates immunostained with goat-GPR30 antibodies showed an endogenous (natural) expression of the protein in both ER-positive (MCF-7) and ER-negative (SkBr3) cells (Figure *1*). This endogenous expression corresponded to the first band at molecular weight (MW) 50 kDa, as theoretically, plasmid-expressed proteins receptors are modified and cleaved post-translational, which results in proteins with different MW that separates and migrates differently along the membrane. A visual inspection of the imaging of MCF-7 and SkBr3 in our first experiment suggested a stronger immunoreactivity of endogenous GPR30 in cells exposed to hypoxia, however those results were not confirmed by our second or third experiment (Figure *1*).

GPR30 antibody-staining gave indications of a recombinant overexpression of the receptor in both MCF-7 and SkBr3 transfected with the N-terminally FLAG-tagged human WT GPR30 plasmid, exposed to normoxia (Figure *2A-B*). This, thus, indicates a successful expression of recombinant GPR30. Contrarily, cells incubated in a hypoxic condition for 18h showed a reduced expression of GPR30. Nonetheless, a quantitative analysis of GPR30 transfected cells normalized to its correspondent mock in experiment 1 (analysis of 37-50 kDa) showed a higher recombinant GPR30 expression in SkBr3 compared to MCF-7 cells (Figure *2C*).

As we failed to develop corresponding β-actin (a reference protein that gives information of the amount of living cells in each sample) blots for the hypoxic cells (Figure *3*, *appendix 3-4*), while re-blotting for all membranes and samples (Figure *3D)*, quantification of recombinant and endogenous GPR30 was not possible. Failed or reduced β-actin expression could thence indicate cell death.

4.3 Increased cleaving of PARP in hypoxic cells

Imaging of membranes probed with rabbit-anti-PARP indicated a higher expression of the DNA repair-protein in cells in normoxia, and qualitatively no expression in cells incubated in hypoxia, which suggests that PARP is cleaved in response to hypoxia (Figure *4*). There was a statistical significance ($p = 0.0286$) for cleaved PARP between GPR30+ SkBr3 in normoxia and GPR30+ SkBr3 in hypoxia (cleaved PARP in mock-transfected (MT) SkBr3 in normoxia: 0.251%; MT in hypoxia: 0.7385%; GPR30-transfected (GT) SkBr3 in normoxia: 0.155%; GT in hypoxia: 0.6295%). This corresponded to the images of β-actin expression in Figure *3*, which showed relatively extensive cell death. MCF-7 cells did not appear to have the same degree of cleaved PARP in the hypoxia samples (MT in normoxia: 40.1%; MT in hypoxia: 54.5%; GT in normoxia: 35.7%; GT in hypoxia: 39.7%) (Figure *5*).

A trend that was observed in our research was that cells transfected with GPR30 had a less quantitative cleavage of PARP, in comparison to mock-transfected cells (MCF-7: MT in normoxia: 40.1% vs GT in normoxia: 35.7%; MT in hypoxia: 54.5% vs GT in hypoxia: 39.7% respectively SkBr3: MT in normoxia: 25.1% vs GT in normoxia: 15.5%; MT in hypoxia: 73.85% vs. GT in hypoxia: 62.95%) (see Figure *5*). The effects came mainly from our second attempt, where the upper PARP bands (the non-cleaved PARP proteins) had a higher intensity compared to the lower ones, in the normoxic group (Figure *4*). In experiment 1, the effects seemed to stem mostly from the fact that cleaved PARP are higher in well 1 of mock-normoxia.

To further compare SkBr3 and MCF-7, each hypoxia sample was normalized to its corresponding normoxia sample (normalized to mean value = 1). This was done to isolate the effect that hypoxia exerts in each condition and to eliminate eventual inter-and intra variations. As the collected data were not normally distributed, Mann-Whitney U test was applied, and the results showed a statistically significant difference ($p = 0.0286$) between SkBr3 and MCF-7 cells transfected with GPR30 and exposed to hypoxia (see Figure *6*).

4.4 HIF-1a expression in MCF-7 and Skbr3

In one of four attempts to develop a HIF-1a blot, the imaging showed an expression of HIF-1a in all cells, in both groups, with a slightly increased expression in cells exposed to hypoxia (*appendix 6*). No visible bands were seen on the other membranes for the remaining blotting attempts (*appendix 7).* HIF-1a has a short half-time and is unstable in normoxia, which means that it degrades quickly. This may thus explain the failed attempts of its blot development.

4.5 GPR30 promotes proliferation of BCC in normoxia

To analyze the cell´s proliferation and survival, FACS was adopted. After gating for single cell events of the correct size, an average of 39.6% events among MCF-7 and 28.9% amid SkBr3 cells remained for analysis (MCF-7: hypoxia group (HG): 36.2%: normoxia group (NG): 43.0%; SkBr3: HG: 25.9%: NG: 30.5%).

All three experiments were represented in our cell cycle analysis. However, a trend that was recurring in the second and third experiment was that when exposed to hypoxia, SkBr3 cellularity (interpreted as cells of interest) was affected negatively (exp.2, mock transfected Skbr3: -40%; exp.3: mock transfected SkBr3: -95%; exp.2: GPR30 transfected SkBr3: -88%; exp.3 GPR30 transfected SkBr3: -98%). Contrarily, MCF-7 cellularity was generally increased after 18h incubation in hypoxia (exp.2, mock transfected MCF-7: +290%; exp.3: mock transfected MCF-7: +1,2%; exp.2: GPR30 transfected MCF-7: +1527%; exp.3 GPR30 transfected MCF-7: -19%). All values are approximate and rounded upwards, for the exact measurements see *appendix 8.*

Accordingly, the mean decrease in number of measurable cells in hypoxia among mock-transfected SkBr3 cells (based on statistics of two experiments) is approximately -78%, while the mean decrease among GPR30+ SkBr3 cells is -93%. For mock-transfected MCF-7 cells the mean increase was +32% compared to +17% in GPR30+ cells. Hence, this data suggests that GPR30 could be associated with lower survival in both SkBr3 and MCF-7.

FACS also revealed that after 18h incubation in hypoxic condition, more MCF-7 GPR30+ were in the S-phase (NG: 6.28%; HG: 10.69%) (Figure *7B*, *appendix 5A*). SkBr3 analysis of GPR30 transfected cells gave opposite results, where fewer cells were observed in the S-phase (NG: 26.65% vs HG: 17.16%) (*appendix 5B*). Nonetheless, SkBr3 analysis revealed that more GPR30+ cells were in the S-phase compared to mock-transfected, in normoxia (exp.1: MT: 2.66% vs GT: 18.75%; exp. 2: MT: 6.12% vs GT: 45.8%; exp. 3: MT: 13.36 vs GT: 15.39%) (Figure *7A).*

Those results were also confirmed by the analysis of MCF-7 cells (MT: 4.693% vs GT: 4.797%), which suggests that GPR30 promotes proliferation of BCC in normoxia.

5. Discussion

Breast carcinoma is a major cause of cancer-related death globally and affects a relatively large proportion of women annually. Several studies have suggested that GPR30 has a role in the prognosis, development, and treatment resistance of ER-positive BC. This *in vitro* study investigates the interaction between hypoxia and GPR30 that has previously been proposed to exist, as it could hold importance for future treatment of breast cancer. Both SkBr3 (ER-negative) and MCF-7 (ER-positive) cells were used to enable generalization of our research results.

Transfection of breast cancer cells (BCC) with EGFP allowed a qualitative assessment of the transfection efficiency, while the nuclear staining allowed a quantitative analysis to be performed. MCF-7 had a lower estimated transfection efficiency since SkBr3 had a quantitatively higher expression of GPER (Figure *2C*). The nuclear staining was however not useful in the quantitative analysis as the cell nuclei of the EGFP-transfected cells could not be separated, instead, we quantitatively assessed the GPR30 expression in a specific area between 37-50 kDa by normalizing to the β-actin signal in experiment 1. The results were considered to be representative to the whole band as the MCF-7-GPR30 bands were weaker than SkBr3, which is in line with what we saw in the fluorescence microscopy (*appendix 3B-C*).

More efficient transfection among SkBr3 cells may be due to a higher permeability, which makes it easier for the plasmid to be transported into the cell and to interact with the nucleus, but it could also be explained by the fact that the plasmid has a specific promoter that has different affinities to diverse transcriptional complexes. Another reasonable explanation for low transfection efficiency is a high confluency on the day of the transfection, as MCF-7 cells tend to adhere to each other and form a cell layer, giving over-confluent cultures. This is also clarified by ThermoFisher Scientific (49), meaning that high confluency leads to lower transfection efficiency by the consequent contact inhibition of the cells.

Western blot was performed to analyze proteins of interest qualitatively and quantitatively. By staining for GPR30, we found an increased endogenous expression in both hypoxic MCF-7 and SkBr3 cells in the first experiment (Figure *1*). β-actin staining (housekeeping protein) from the same attempt showed no significant expression in the hypoxic cells (Figure *3*), indicating that the cells are dead and that intracellular protein have leaked out, and that the strengthened

endogenous expression of GPR30 probably is membrane-bound GPR30 (24) extracted from membrane residue during cell lysis. The finding was not confirmed in the other attempts, which limits its impact and significance in our study.

In our second experiment, we found an equal endogenous expression at 50 kDa in both hypoxic and normoxic cells. Immunoreactivity to β-actin was also higher, though a weak expression and presumably quantitatively irrelevant, in the hypoxia lanes (see *appendix 3-4*). Taken together, the finding is in line with previous studies (35-38) that propose a role of hypoxia in upregulating the transcription of GPR30 by the binding of HIF-1a to its HRE promoter. However, as a large proportion of cells have died, we do not know if those who survived are representative of the population, or if the number of cells that survived in hypoxia are quantitatively relevant. Cancer cells are also genetically unstable, and cells that survived may very well be a subgroup of mutated cells with a promoted survival.

Lack of β-actin expression could mean that there are no proteins in the sample and that the protein extraction was not performed correctly. However, as the same technique and method was used for lysis and scraping of cells, and as we got a clear and strong expression of β-actin in our control group (the normoxic cells), the shortage seems not to be in the protein extraction. The shortage is likely a result of the cells lysing and that cytoplasmic proteins have leaked during the incubation in the hypoxic chamber. As we manually had to put wet cloths inside the chamber to buffer for the liquid and keep the humidity at optimal levels, we noted that in our first attempt, the previously wet cloth was completely dry. Consequently, because of the low humidity in the chamber, the medium containing the cells could potentially have evaporated, increasing the osmolarity outside of the cells and causing them to lysis and their receptor to relocate to the solution. When the medium was later removed as a part of the preparation, these receptors were lost as well. This is strengthened by the results of an *in vivo* hypoxic model that automatically regulates and controls O_2, CO_2, temperature and humidity levels, studied by Elvidge and colleagues, that revealed that MCF-7 cells survived 16 hours in hypoxia without any cell damage (50).

No recombinant GPR30 expression was noted in the BCC in hypoxia (Figure *2*), which could indicate cell apoptosis induced by hypoxia or a decreased expression of the protein to a non-detectable amount. The non-visible band of recombinant GPR30 could therefore confide on that there were not enough cells expressing it or that there are other mechanisms underlying its repression.

Furthermore, increased cleavage of PARP was noted in both GT and MT SkBr3 and MCF-7 cells placed in the hypoxia chamber. This finding is in accordance to Ke et al. (7) research that reported that levels of HIF-1a correlated with inter alia caspase 3 and 9, the proteases that cleaves PARP and hence mediated apoptosis (11, 51). Mann-Whitney U test performed to analyze the difference between the two conditions showed a significant increased cleavage of PARP in GT SkBr3 in hypoxia, in comparison to GT SkBr3 in normoxia. In fact, increased cleaving of PARP is a marker for ongoing apoptosis, which means that apoptosis induction was significantly increased among SkBr3 cells in the particular hypoxic condition (Figure *5A*). Increased cleaving of PARP was also noted in MCF-7 cells, however the results were not significant (Figure *5B*). Taken together with our β-actin (Figure *3A-D*), where β-actin was not expressed in a quantifiable amount in both cell types in hypoxia, the results hence indicates cell death in both MCF-7 and SkBr3. Those findings also propose that other proteins involved in signaling cellular damaged and programmed cell death may have been involved in the case of MCF-7, but also highlights the possibility that MCF-7 may lack specific enzymes, such as caspase-3, or that the cells require a higher hypoxic exposure to become apoptotic.

Additionally, as presented in the statistical analysis, there was a significantly larger cleavage of PARP in GPR30+ SkBr3 cells as to GPR30+ MCF-7 cells, in hypoxia. This suggests that MCF-7 may have defense mechanisms to hypoxia that promotes its adaption and survival, or that SkBr3 are less stable and consequently induces apoptosis at less favorable conditions. Less favorable conditions include increased osmotic pressure and evaporation of medium. Our FACS results also confirms this hypothesis, were the cellularity of SkBr3 decreased in hypoxia, whilst MCF-7 cellularity increased, together with the results that a larger portion of GPR30+ MCF-7 were in the S-phase after 18h in a hypoxic condition (Figure *7*). This analysis supports the theory that estrogen-dependent activation of GPR30 activates the PI3K/Akt signaling pathway that is associated with cell proliferation and survival, as reported by Vivanco et al. (30). Which could add on to the effects of the estrogen receptors expressed in the MCF-7 cells, to promote proliferation. However, other proliferative and survival signaling pathways may also have been involved.

One also has to consider that the gating of our cells may have not included all cells that survived, as cells that underwent mitosis at the specific moment or for other reasons did not fulfill the gating criteria were not included in the analysis. On the hand, considering that we have measured the cell´s viability through expression of β-actin and analysis of the cell cycle, this

points out that we have sufficient evidence to state that amount of measurable cells is approximately equal to survived cells.

When observing the expression of PARP and its relation to cleaved PARP in our analysis we found that both GPR30+ MCF7 and SkBr3 cells, hypoxic and control, had a lesser amount of cleaved PARP in relation to mock cells (see Figure *5A-B*). This trend could indicate a role of GPR30 in survival and adaption of the cells, in normoxia as reported by (21, 28, 29, 32), but is also in agreement to other studies proposing a cooperation between GPR30 and hypoxia (35-38). One could also speculate whether GPR30 exhibits an anti-apoptotic effect.

An unexpected finding in our study associates GPR30+ with poorer survival in hypoxia. This finding is based on FACS analysis of our first and second experiment were BCC transfected with GPR30 survived inferiorly in comparison to mock-transfected corresponding cells. Survival rate was thus lower for GT cells (*appendix 8)*. This finding contradicts to our earlier presented finding that PARP cleavage was lower among GT cells (Figure *5)*. However, these results are not significant, and it requires larger data to draw general conclusions. This finding needs to be strengthened or rejected with further research and experimental trials.

Our results of how hypoxia could both mediate survival and apoptosis in BCC strengthens the paradox that was earlier described, which is interesting from a cancer perspective. This paints a picture that hypoxia can be favorable or unfavorable depending on other genetic factors in the cell, and that a factor that is lethal to a cell becomes a strength for another one. This could also suggest a genetic shift at the point where the tumor becomes so large that the vascular supply is no longer adequate.

The finding that a larger portion of hypoxic GT MCF-7 cells were in the S-phase than normoxic cells also emphasizes an interaction between GPR30 and hypoxia, as previous studies has shown that HIF-1a directly (7, 8), as well as indirectly via inducing expression of GPR30 (30, 35-37), stimulates proliferation. These results could on the other hand also be interpreted as the MCF7 cells not being able to pass over to the next phase, G2. Although, considering that there were more GT hypoxic MCF7 cells in the G2-phase than the S-phase, this seems rather unlikely.

When investigating the results of the SkBr3 cells, the opposite was instead noted with more GPR30+ control SkBr3 cells in the S-phase compared to GPR30+ hypoxic SkBr3 cells (Figure *7*). This is in coincide with our other findings that SkBr3 cells have a lower survival rate based on lesser cellularity after hypoxia in comparison to normoxia (*appendix 8*), as well as an increased amount of cleaved PARP in hypoxic cells, and a significant increased apoptosis induction

compared to MCF-7. Taken together, this data points to a worse survival and adaption of GPR30+ SkBr3 cells to hypoxia.

In regard to the proportion of control cells in the S-phase, there was a larger proportion of GT SkBr3 and GT MCF-7 compared to its correspondent mock (Figure *7)*. This could be interpreted as a function of GPR30 promoting cell proliferation in normal, non-hypoxic, conditions, as several other studies has previously proposed. Quantitatively, more SkBr3 cells were in the S-phase as to MCF-7 (*appendix 8*), which correspond to Ariazi et al. (25) study that found that GPR30+ mediates proliferation of SkBr3 and reduces growth of MCF-7 cells.

5.1 Strengths and limitations

As two candidates performed all experiments, measurements and protocols were thoroughly followed and monitored by both parts. This also prevented eventuall systematic errors from occurring. The using of two breast cancer cell lines, differing by their expression of the estrogen-receptor, enabled generalization of the results to different types of breast cancers, as well as it isolated the effect of GPR30, without interaction of the estrogen-receptor.

Our study aimed to investigate the relationship between HIF-1a and GPR30, but as we were not able to develop any visible expression of HIF-1a on our Western blot membranes, it is challenging to draw any conclusions and prove any causality regarding their interaction. Instead, we are able to discuss the relationship between the conditions in the hypoxic chamber and the expression of GPR30 in the cells. As previously mentioned, it is a protein with low stability and therefore it demands fast and careful handling to prevent its degradation. Further trials are therefore needed to assess this, possibly with other, or more optimized, methods.

5.2 Future aspects

The clinical and scientific relevance of our results are nevertheless unclear and requires additional research and larger data to be fully understood. However, we have shown trends that either proves or contradicts previous studies. This may be a good framework for future, supplementary studies, to further evaluate, improve and individualize the treatment of breast carcinoma.

6. Conclusions

Taken together, our results propose a trend showing an interaction between hypoxia and GPR30 as well as a correlation between GPR30 and a promoted proliferation in normoxia. However, our findings are not enough to prove this correlation. Also, we noted a significant difference

between SkBr3 and MCF-7 cells in aspects of proliferation and survival in hypoxic conditions, which could be due to that MCF-7 is ER-positive, and that GPR30 exerts its effect through a different signaling pathway, that is to say, the difference between genomic and non-genomic pathway.

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8. Declaration of Original Work

Both students contributed equally to the conduction of the experimental parts of the project as well as the writing of the article. Working with two cell lines demanded parallel working hours, and hence, all laboratory work was performed simultaneously and cooperatively by the students. Even though the writing was divided, the students and the supervisor contributed with constructive feedback to all parts, which were taken into account. Statistical analysis and figures were performed and given by Julia Tutzauer, and these were discussed and analyzed thoroughly. Nonetheless, the finished article was revised and approved by both students.

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Figures and tables

	Experiment 1	Experiment 2	Experiment 3
Blotting	PARP, GPR30	GPR30	HIF-1a, GPR30
Re-blotting	HIF-1a, β-actin	PARP, β-actin	PARP, β-actin

Table 1 The various antibodies selected in each experiment, as well as which antibodies were used for re-blotting. This was applied to both cell types in all 3 experiments.

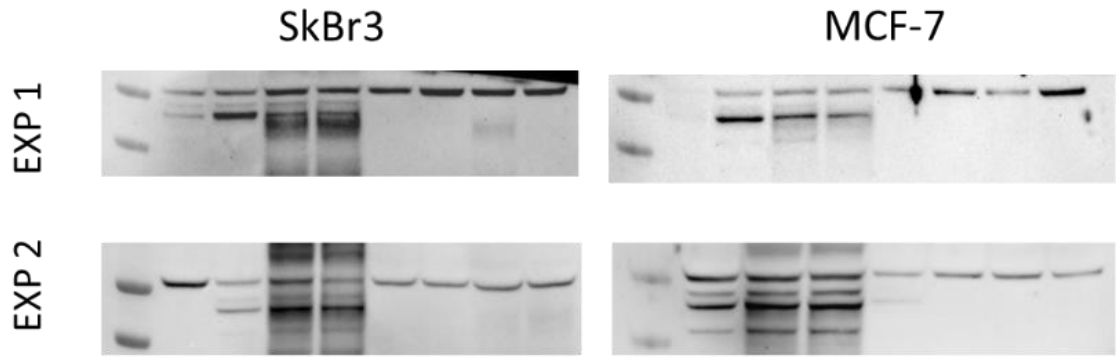


Figure 1 GPR30 staining from experiment 1 and 2 showing the endogenous expression of GPR30 at 50 kDa. In experiment 1 for both SkBr3 and MCF-7 cells, a slightly stronger GPR30 immunoreactivity can be seen in the hypoxia wells (the four wells to the right).

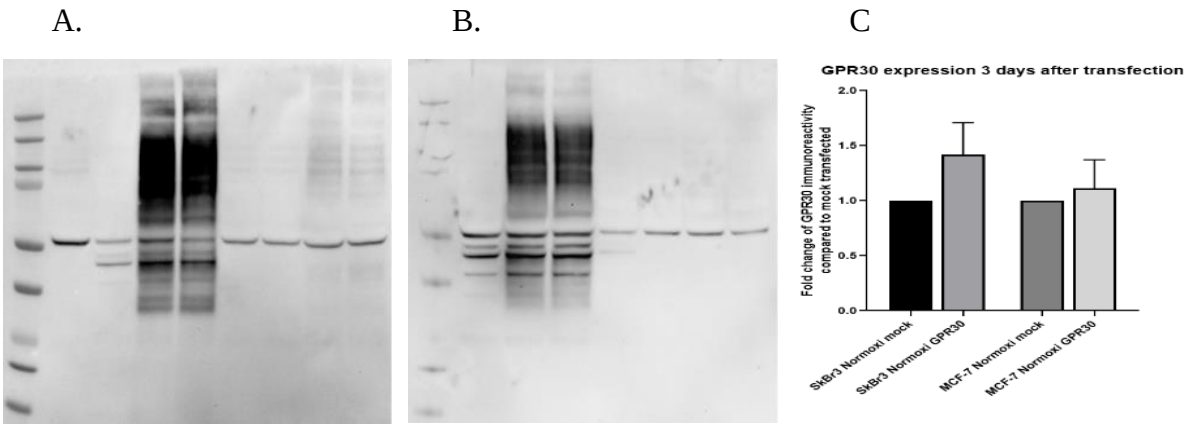


Figure 2 GPR30 staining from experiment 2 in A: SkBr3 cells, and B: MCF-7 cells. A recombinant expression of GPR30 in plasmid transfected cells is seen as darker visible bands with different molecular weight (MW). As noted, non-detectable expression of recombinant GPR30 is visible in hypoxic cells. C. A quantitative analysis of GPR30 expression 3 days after transfection, showing a higher recombinant expression in SkBr3 cells compared to MCF-7, and a reduced expression in mock transfected cells.

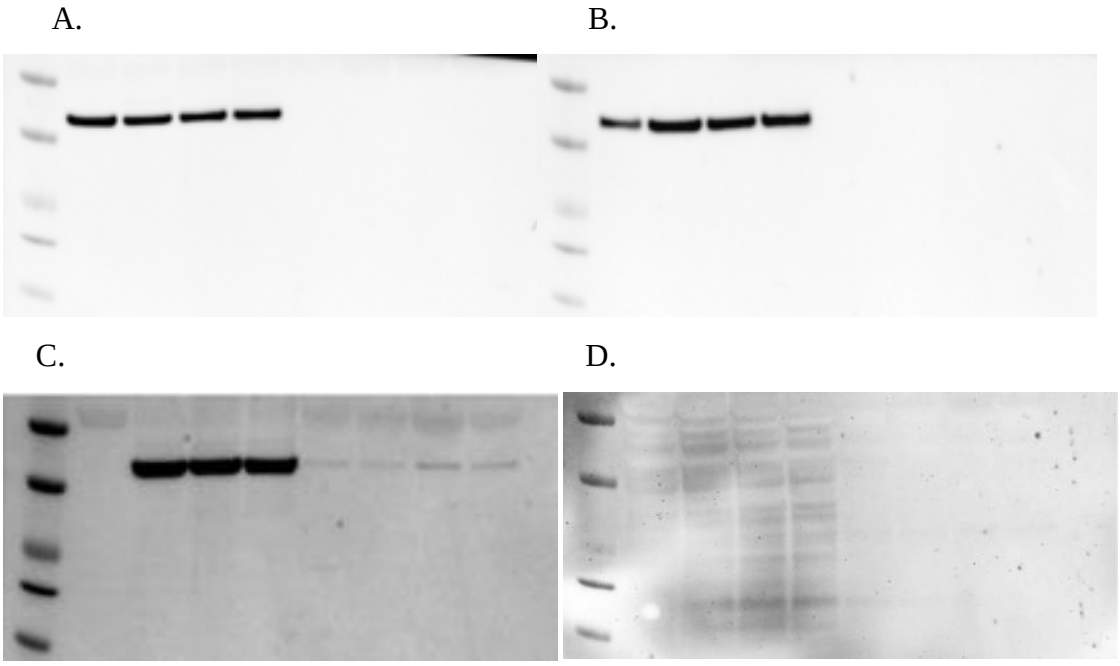


Figure 3 β-actin staining of A: SkBr3 cells in exp. 1, B: MCF-7 cells in exp. 1, C: SkBr3 cells in exp. 2. D: MCF-7 cells in exp. 2. The four wells to the left in A and B, as well as the three left wells in C and D, are the normoxia-group. Lack of expression of β-actin in the hypoxic wells indicates cell death.

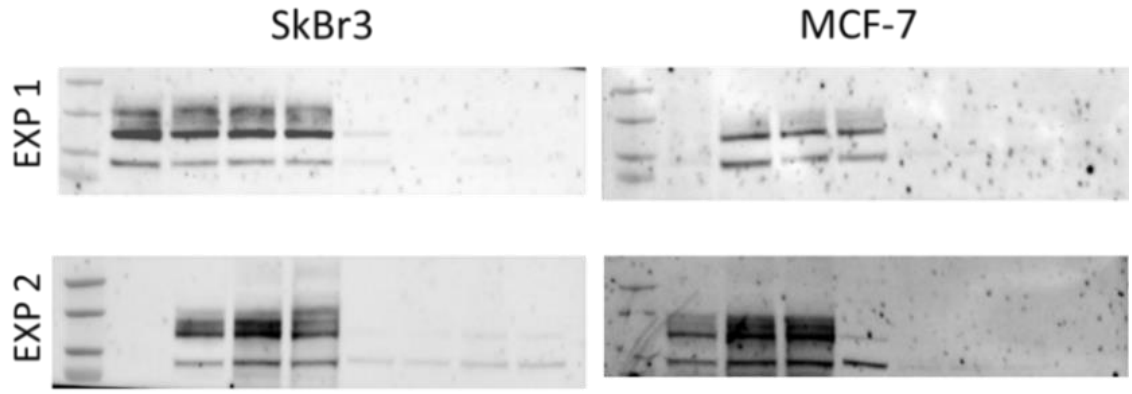


Figure 4 PARP staining of SkBr3 and MCF-7 cells from exp. 1 and 2. The upper band represent non-cleaved PARP. PARP cleaving (represented by the lower band) is observed as reduced or lacked expression of the protein and is mainly seen in the hypoxic wells (the four wells to the right).

A. B.

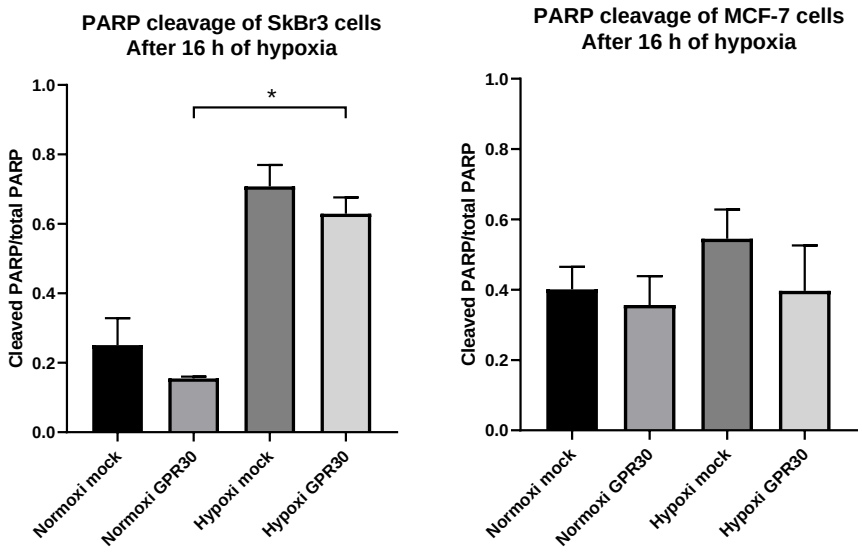


Figure 5 Bar chart presenting cleaved PARP relative to the total PARP per condition for A: SkBr3 cells and B: MCF-7 cells. Standard error of the mean (SEM) is presented by error bars and indicates the uncertainty of the data. A star above a bar indicates a statistically significant result, as seen between GPR30+ SkBr3 in normoxia and GPR30+ SkBr3 in hypoxia.

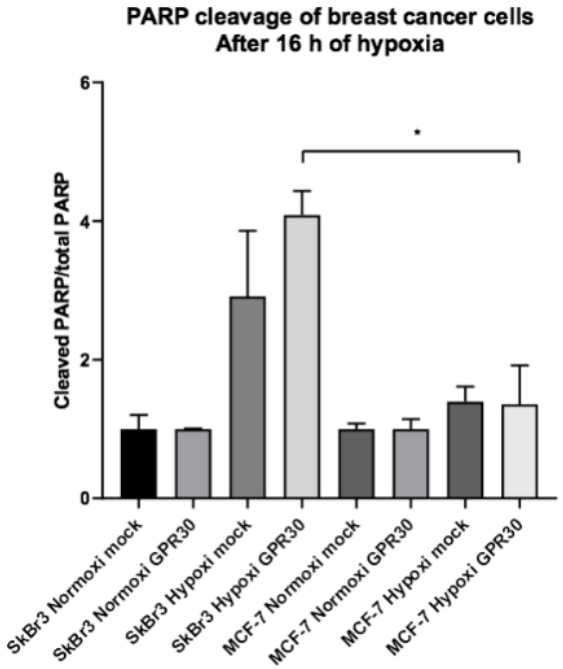


Figure 6 Bar chart presenting cleaved PARP for both MCF-7 and SkBr3 to enable a comparison between the two cell types. A significant difference was found between GPR30+ SkBr3 and GPR30+ MCF-7 in hypoxia.

A. B.

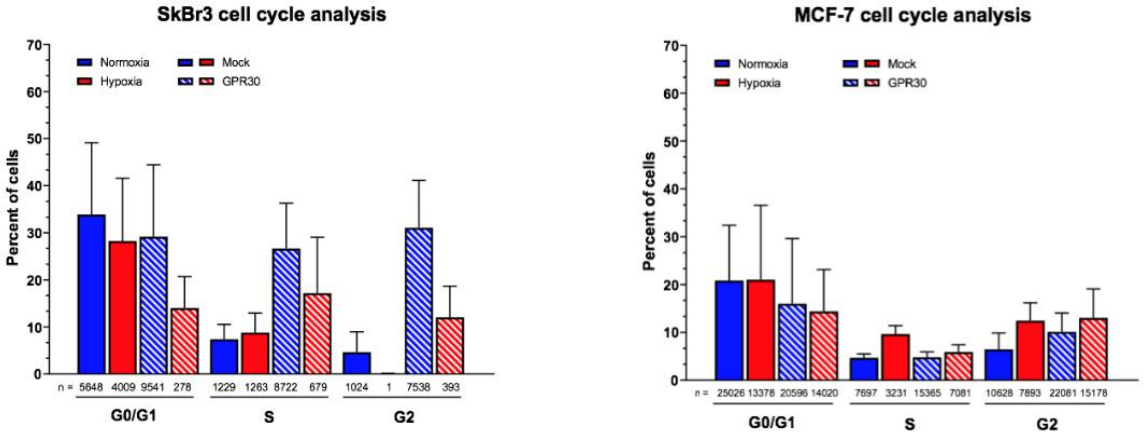
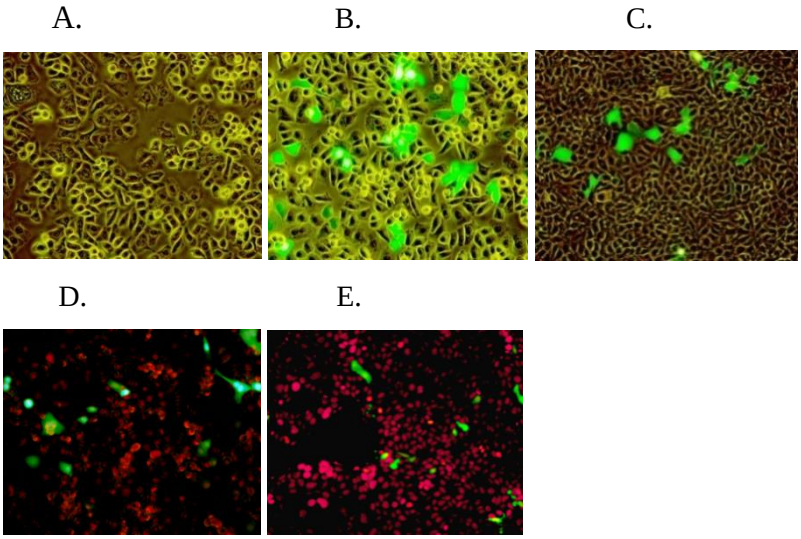


Figure 7 A bar chart of the percent of cells (y-axis) and the stages of the cell cycle (x-axis) in A: SkBr3 cells, B: MCF-7 cells. The monochromatic bars represent the mock-transfected cells, although GPR30+ cells are presented by the stripped bars. Blue bars are cells placed in normoxia, while red represents hypoxic cells. As seen in A; more GPR30+ cells are in the S phase compared to mock, and hypoxia effects the percent of cells negatively. Contrarily, as seen in B: hypoxia seems to have a positive effect on the percent of cells in the S phase.

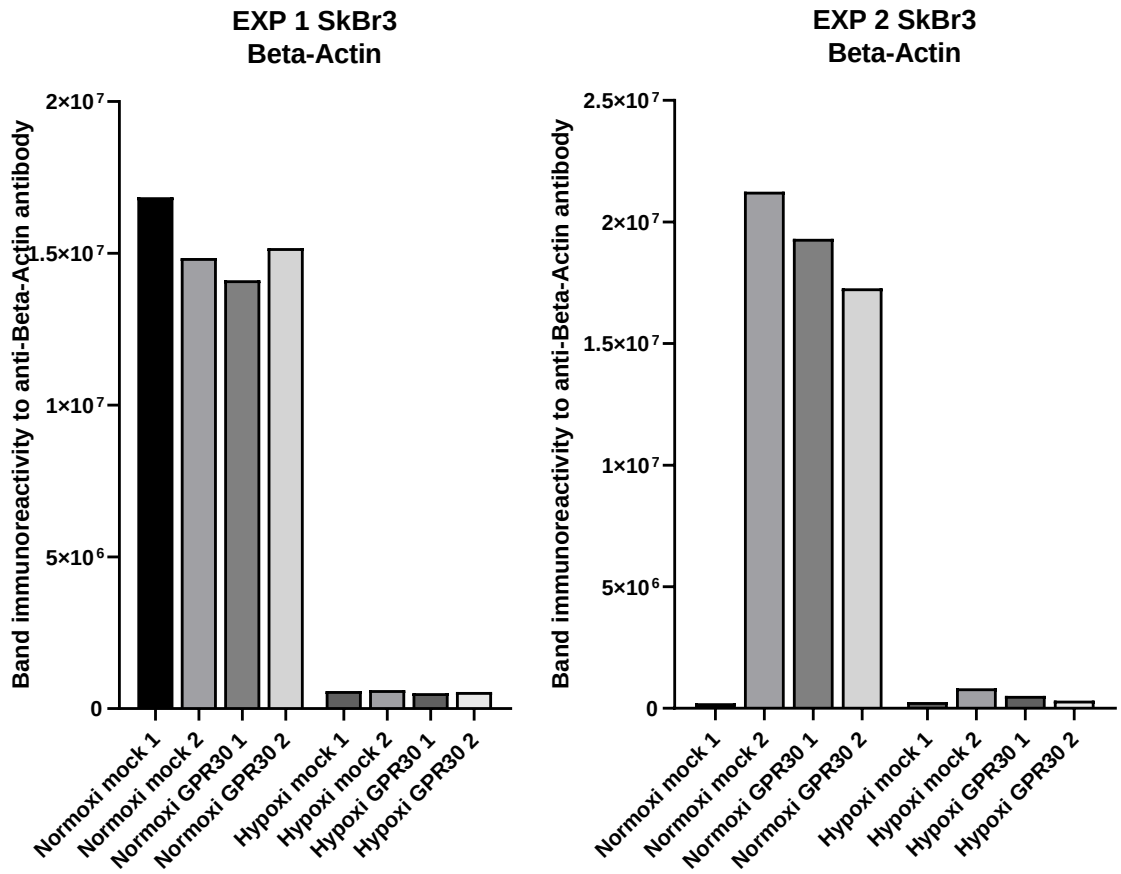
Appendices

Ladder	**Mock-Normoxia**	**Mock-Normoxia**	**GPR30-Normoxia**	**GPR30-Normoxia**	**Mock - Hypoxia**	**Mock-Hypoxia**	**GPR30 - Hypoxia**	**GPR30 - Hypoxia**

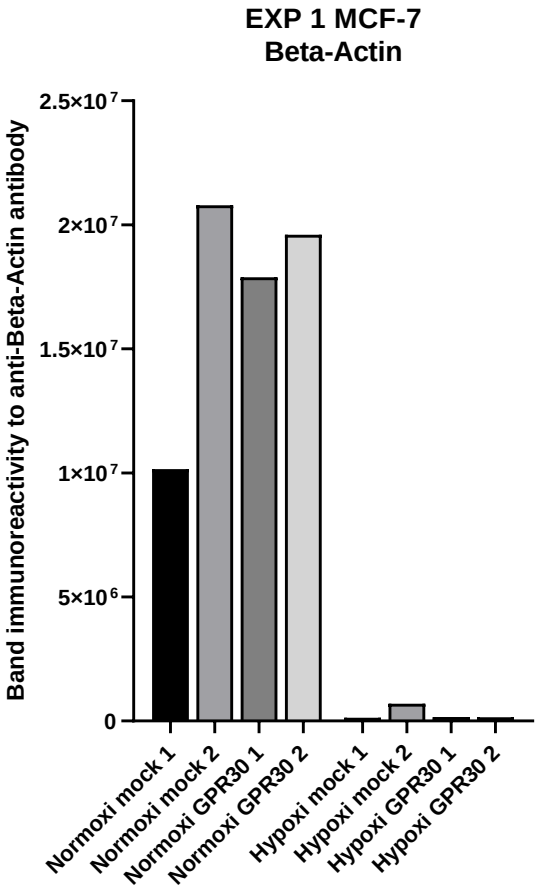
Appendix 1 All samples, for both SkBr3 and MCF-7, were pipetted in the well for the SDS Page following this model. The first well was filled with the ladder Precision Plus Protein™ Dual Color Standard (Bio-Rad).



Appendix 2 Cells observed using fluorescence- and phase-contrast microscopy. A: Skbr3 transfected with pcDNA (mock-transfected). B; Skbr3 transfected with EGFP, presented by the green, fluorescent staining. C: MCF-7 cells transfected with EGFP, D: Nuclear PI staining of EGFP transfected Skbr3 and E; MCF-7; under microscope. Transfection efficiency in SkBr3 was higher than in MCF-7 cells, suggesting that other factors could have interfered with the transfection of MCF-7 cells.

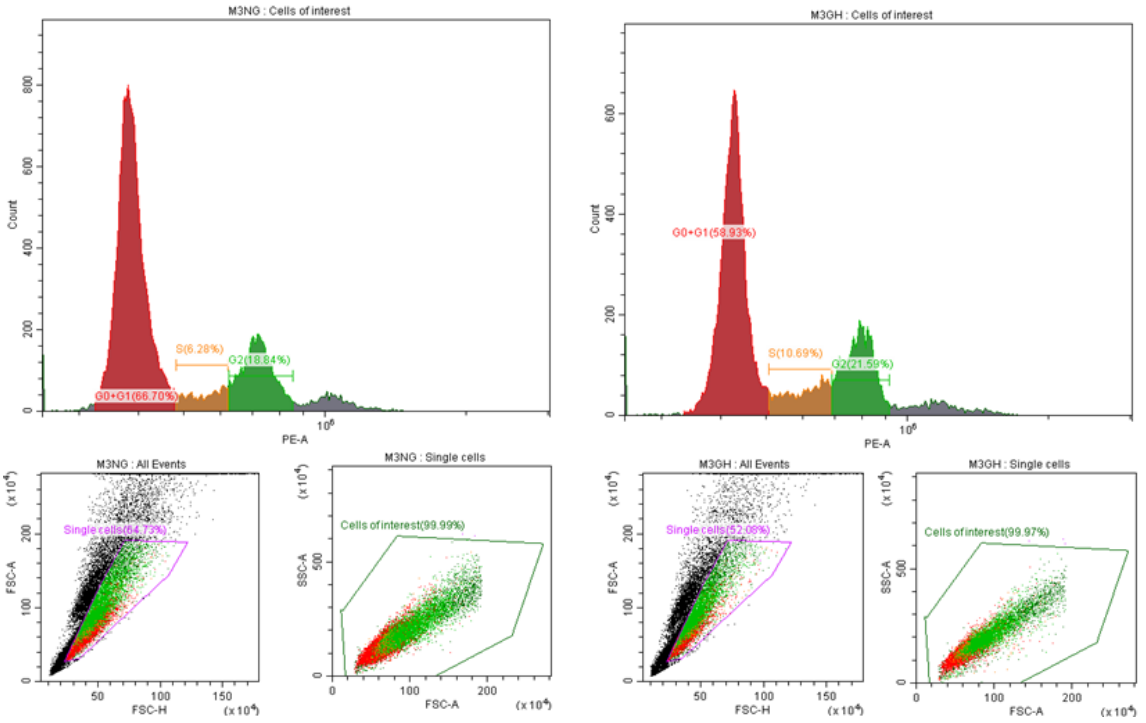


Appendix 3 Bar chart of the band immunoreactivity to anti-β-actin antibody for SkBr3 in both experiment 1 and 2. No or quantitatively non-relevant amount of β-actin was expressed in cells exposed to hypoxia for 18h. Failed or reduced β-actin expression indicates cell death.

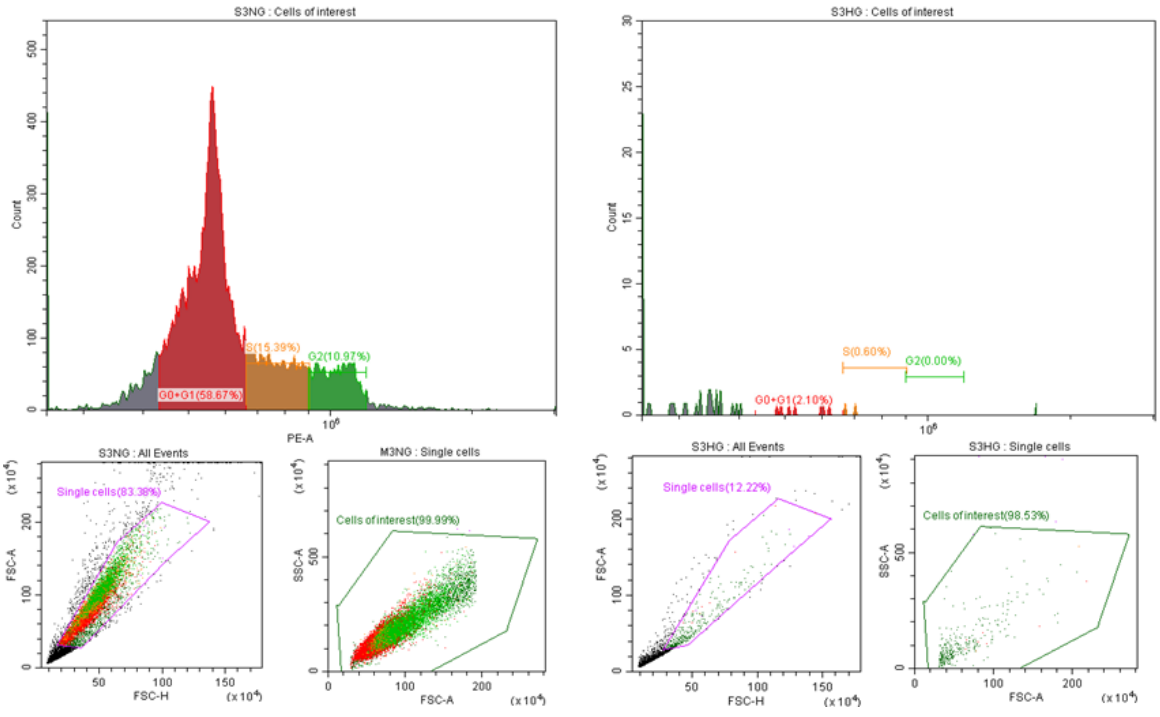


Appendix 4 A bar chart of the band immunoreactivity to anti-β-actin antibody of MCF-7 cells in experiment 1. No or quantitatively non-relevant amount of β-actin was expressed in cells exposed to hypoxia. Failed or reduced expression of β-actin indicates cell death.

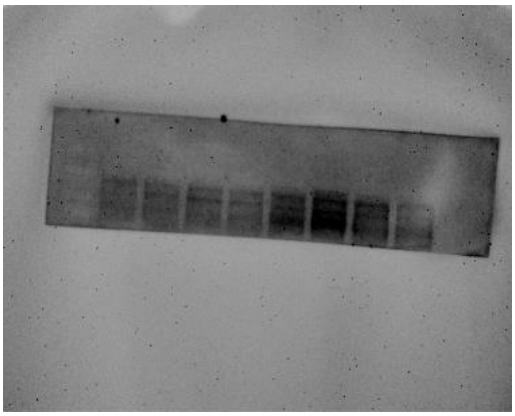
A.



B.

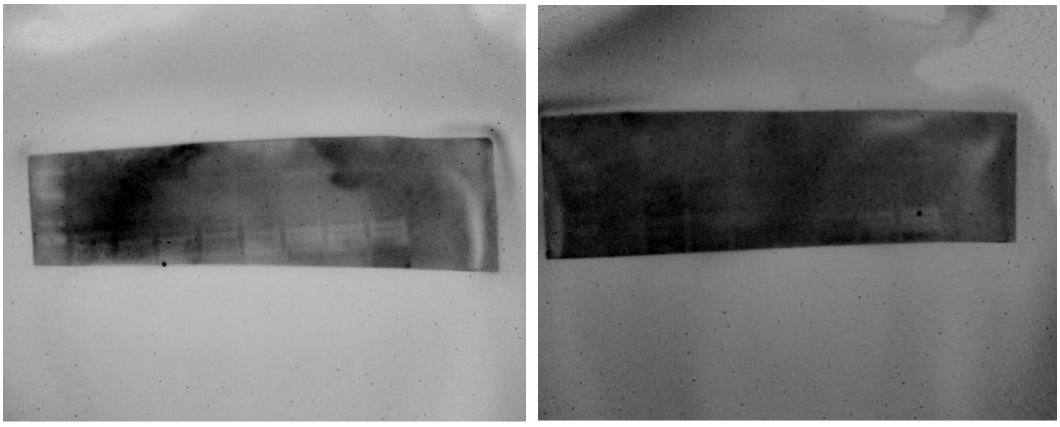


Appendix 5 FACS results of A: MCF-7 and B: SkBr3 cells in experiment 3. M3NG: GPR30 transfected (GT) MCF-7 cells in normoxia in comparison to M3GH; GT MCF-7 cells in hypoxia. S3NG: GT SkBr3 in normoxia. S3HG; GT SkBr3 in hypoxia. As illustrated in A: quantitatively more cells are in the S-phase after being incubated in hypoxia for 18 hours (10.69% vs 6.28%). B: quantitatively fewer hypoxic cells were in the S-phase. In the smaller figures, cells were gated to a specific size to include single cells and exclude double cells.



Appendix 6 Developed membrane of HIF-1a staining of MCF-7 cells in experiment 3. Imaging of the membrane showed an expression of HIF-1a in all lanes, in both cell the hypoxic and control group, with a slightly increased expression in cells exposed to hypoxia.

A. B.



Appendix 7 HIF-1a stained membranes of A: SkBr3 and B: MCF-7 in experiment 1. Imaging of the membrane resulted in no visible bands, that is, no expression of HIF-1a in any lane.

Experiment Nam	201217 Hanna Dianna			
Tube Name:	**All Events**	**Cells of inte**	**G0+G1 Events**	**S Events**
S1NM	3025	338	53	9
S1HM	26568	7722	3118	991
M1NM	94168	30104	11149	5748
M1HM	85325	27099	4383	5993
S1NG	3096	560	119	105
S1HG	1426	84	21	9
M1NG	27743	8247	1079	1946
M1HG	40878	20613	4782	3559
S2NM	11119	3446	751	211
S2HM	7368	2071	885	270
M2NM	18353	3621	1273	614
M2HM	55003	14202	3203	7128
S2NG	95388	14024	1060	6423
S2HG	7797	1663	250	668
M2NG	3922	464	35	130
M2HG	56372	4630	429	1924
S3NM	10663	7550	4844	1009
S3HM	2993	381	6	2
M3NM	28819	19765	12604	1335
M3HM	24970	20000	13010	2244
S3NG	17105	14253	8362	2194
S3HG	2775	334	7	2
M3NG	28407	18387	12264	1155
M3GH	28709	14949	8809	1598

Appendix 8 FACS results of both SkBr3 and MCF-7 in experiment 1-3. First two letters; S1 or M1; abbreviation for cell type and experiment. H or N; hypoxia or normoxia. Last letter; M or G; abbreviation for mock and GPR30. Cells of interest was interpreted as cellularity, and survival rate was calculated by counting the difference between the cellularity in normoxia vs hypoxia. SkBr3 cells had a lower survival rated based on lesser cellularity after incubation in hypoxia. Contrarily, MCF-7 hypoxic cells had a clear survival advantage, with an increased cellularity. BCC transfected with GPR30 survived inferiorly, that is, increased reduction in cellularity in Skbr3 and reduced increase in MCF-7 cells after hypoxia, based on experiment 2 and 3. In normoxia, more SkBr3 cells were in the S-phase as to MCF-7, indicating promoted proliferation in those cells in normoxia.