Regulation of signal transduction pathways by hypoxia in breast cancer subtypes

Liu, Q.

Chapter 1

General introduction and outline of this thesis

Breast cancer

As of 2020, female breast cancer is the most commonly diagnosed cancer and the second most common cause of cancer related deaths [1,2]. Breast cancer can be generally categorized depending on the histological profile of the carcinoma or cellular molecular markers [3]. Two histological subtypes can be identified, in situ carcinoma and invasive carcinoma. Invasive breast carcinoma has a significantly lower survival rate, and is associated with poor prognosis by resisting clinical therapy [4,5]. Invasive breast cancers can be histologically categorized into several subtypes including the most common subtype, the invasive ductal carcinoma (IDC), encompassing 70-80% of all invasive carcinomas [1]. Categorization based on molecular markers is exclusively applied to invasive carcinomas and is known to be of high predictive and prognostic significance regarding IDCs. Classification depends on the presence of the progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor Receptor 2 (HER2/neu) divided into 4 subtypes: 1) luminal A-like (PR and/or ER positive, and HER2/neu negative) accounting for 60-70% of breast cancers; 2) luminal B-like (PR and/or ER positive, HER2/neu positive) accounting for 10-20% of breast cancers; 3) HER2-positive (PR and/or ER negative, and HER2/neu positive), luminal B-like and HER2-positive accounting for 13-15% of breast cancers; 4) basal-like/triple negative (PR and/or ER negative, and HER2/neu negative) accounting for 10-15% of breast cancers [1,3] (Fig 1). In spite of making up a small percentage of breast cancers, basal-like/triple negative breast cancer (TNBC) has the highest metastatic ability and has a poor response to most therapies [3,6]. Therefore, investigating mechanisms regulating growth and progression of triple negative breast cancer is timely.

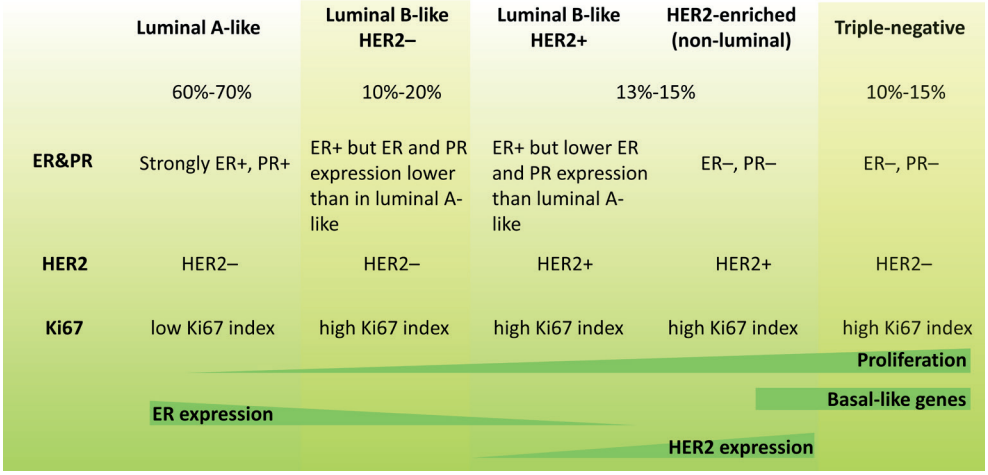

Figure 1. Classification of breast cancer based on molecular subtypes. According to the histology and immunohistochemistry expression of progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor Receptor 2 (HER2/neu) breast cancer is divided into 4 subtypes: 1) luminal A-like (PR and/or ER positive, and HER2/neu negative); 2) luminal B-like (PR and/or ER positive, HER2/neu positive); 3) HER2-positive (PR and/or ER negative, and HER2/neu positive), luminal B-like and HER2-positive; 4) basal-like/triple negative (PR and/or ER negative, and HER2/neu negative). (adapted from Harbeck N, *et al. 2019)*

Hypoxia

The tumor microenvironment (TME) plays a notable role in cancer progression. It includes pH and oxygen level, extracellular matrix (ECM), connective tissue, infiltrating immune cells, and the vasculature of the tumor. Recent studies indicate that hypoxia has a vital role in cancer metabolism and metastasis [7]. The proliferative capacity of cancer cells often causes tumors to outgrow the reach of their surrounding vascular system resulting in decreased oxygen availability, called hypoxia [8]. According to the exposure period, hypoxia can be divided into acute and chronic hypoxia. Acute hypoxia is from several minutes to 72 hours, and chronic hypoxia is characterised as hypoxia exposure up to several days or weeks [8]. A distinction is made between acute and chronic hypoxia because cancer cells exhibit different cellular responses and gene regulation depending on the duration and frequency of hypoxia exposure [8,9]. Acute hypoxia is associated with reversable changes, whereas chronic hypoxia is associated with long-term cellular changes potentially developing into mutagenesis and genetic instability [8].

The magnitude of the hypoxic state of cancer cells is heavily dependent on the size and stage of the tumor. Hypoxia-inducible factors (HIFs) are transcription factors responsive to hypoxic stress comprising an oxygen sensitive alpha subunit and a constitutively expressed beta subunit that act as heterodimers [10–12]. There are 3 different members of the HIF family in humans: HIF-1, HIF-2, and HIF-3. The HIF-1α subunit is the most prominent of the HIFs mediating the response to hypoxia [13]. In normoxia, HIF-1α is hydroxylated at the oxygen dependent domains (ODD) by prolyl hydroxylase domain proteins (PHDs) [14]. Thereafter, HIF-1α is ubiquitinated by binding to the von Hippel-Lindau tumor suppressor protein (pVHL) and subsequently degraded [13]. Conversely, HIF-1α ubiquitylation is inhibited in hypoxic regions as the binding to pVHL is regulated by oxygen-dependent prolyl hydroxylation [13]. As a result, HIF-1α accumulates and forms a heterodimer with HIF-1β [13]. HIF-1 translocates to the nucleus and binds to hypoxia response elements (HREs) in the genome to activate transcription of target genes thereby regulating the hypoxic response associated with, but not limited to, proliferation, invasion and angiogenesis [8,10] (Fig 2). Hu *et al.* implied that HIF-2α also mediates gene regulation by hypoxia, thereby contradicting previous literature mentioning a function for HIF-2α restricted to endothelial cell-specific gene expression [15,16]. Even though HIF-2α is structurally very similar to HIF-1α as they share a 48% identical amino acid sequence, many studies utilizing various approaches show that they have unique roles and gene targets in both normal and cancer cells [17,18]. HIF-3α has a different amino acid sequence from both HIF-1α and HIF-2α. In fact, HIF-3α is often deemed a negative regulator of HIF-1α and HIF-2α target genes [19]. HIF-3α owes this status to its adverse effect on gene expression due to competitive binding between the HIFs to the HREs of target genes [19].

Besides HIFs, several hypoxia regulated genes are regarded as typical hypoxia biomarkers such as carbonic anhydrase (CA9), glucose transporter-1 (GLUT-1), lysyl oxidase (LOX) and erythropoietin (EPO) [20]. The proteins encoded by these genes are involved in tumor progression by regulating metabolism, tissue remodelling and erythropoiesis [20]. For example, CA9 is a target gene of HIF-1α, and the CA9 protein is absent in nearly all normal tissue, but is rapidly upregulated under hypoxic conditions leading to reduced pericellular pH and causing ECM disruption [13,21].

YAP/TAZ in Hippo pathway

Yes-associated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ), two transcriptional co-activators, are often hyperactivated in a variety of cancers [22]. In breast cancer, hyperactivation can promote multiple features of cancer progression such as epithelial to mesenchymal transition (EMT), invasion and migration, induction of cancer stem cell like properties and chemotherapy drug resistance [23]. The transcriptional activity of YAP and TAZ is regulated by a wide variety of cues, including ECM stiffness [24], GPCR signalling [25], cell polarity [26] and energy stress [27]. Most of these cues exert their effect on YAP and TAZ activity through modulating the Hippo pathway. However, more and more evidence suggest that the Hippo pathway is not solely responsible for YAP and TAZ inactivation, and distinct mechanisms independent of the Hippo pathway exist to activate YAP and TAZ in tumors [22].

YAP and TAZ are vital components in the Hippo pathway that is often deregulated in different types of cancers, including hepatocellular carcinoma, ovarian cancer, gastric cancer, colorectal cancer, non-small cell lung cancer and breast cancer, indicating its importance in cancer progression [28]. The Hippo pathway was first discovered in Drosophila, and is highly conserved in mammals [29]. The Hippo pathway consists of a large protein network and the core of the pathway consists of the following: two pairs of serine/threonine kinases which are called sterile 20-like kinase 1 (MST1) and 2 (MST2), and large tumor suppressor 1 (LATS1) and 2 (LATS2) [23]. When the Hippo pathway is on, MST1/2 are phosphorylated and bind to Salvador homologue 1 (SAV1) to form an enzymatic complex phosphorylating and activating LATS1/2 and the scaffold proteins MOB kinase activator 1A (MOB1A) and 1B (MOB1B), which in turn together phosphorylate YAP and TAZ (Fig 2). The process prevents nuclear entry of YAP and TAZ and their binding to multiple transcription modulators including TEA domain-containing sequence specific transcription factors (TEAD1-4) and others. Intracellular localization of YAP and TAZ plays a pivotal role in their activity as transcriptional regulators [30,31]. The Hippo pathway acts as a tumor suppressor pathway of which the main function is to inactivate the oncoproteins YAP and TAZ by shifting the balance in nuclear cytoplasmic shuttling from the nucleus to the cytoplasm [31].

The Hippo pathway can suppress YAP/TAZ transcriptional activity through two mechanisms: 1) when YAP and TAZ are phosphorylated by the Hippo pathway at a single serine residue 14-3-3 proteins will bind resulting in 14-3-3 mediated nuclear export and cytoplasmic retention of YAP and TAZ [32,33]; 2) YAP and TAZ are first phosphorylated by LATS1/2, which primes further phosphorylation by CK1ϵ, which attracts the F-box protein β-TrCPv resulting in YAP/TAZ ubiquitination by the SCFβ-TrCP E3 ligase and subsequent proteasomal degradation [34,35]. Repressing YAP/TAZ transcriptional activity results in two of the best-known physiological functions of Hippo pathway: controlling organ size and tissue homeostasis [36]. Besides the Hippo signalling pathway, recent research has identified several other proteins that can mediate YAP/TAZ phosphorylation, including c-Abl, AMPK, CK1 and JNK1/2 [31].

When the Hippo pathway is off, YAP and TAZ will reside in the nucleus and bind to transcription factors. Although YAP and TAZ predominantly bind to TEAD1-4, they can also bind to other transcription factors such as SMADs, RUNXs, HIF-1α/β, FOXM1 and p73 [31,37–39]. The outcome of binding can be either oncogenic or tumor suppressive

depending on the transcription factor and cellular context. TEAD transcription factors play vital roles in cell growth and the upregulation of their target genes including CTGF, CYR61, and AXL, is associated with tumor progression [40–43]. In addition to dysregulation of YAP and TAZ, TEAD expression is often elevated in triple negative breast cancer (TNBC) [44], which could result in even higher expression of YAP/TAZ-TEAD target genes and further tumor progression.

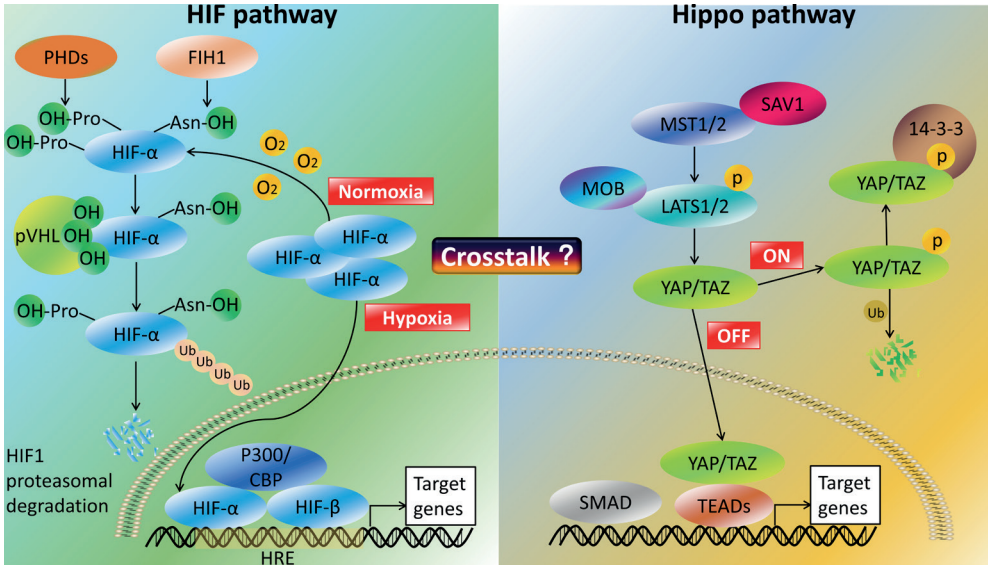

Figure 2. HIF pathway and Hippo pathway. In normoxia, HIF-1α is hydroxylated at the oxygen dependent domains (ODD) by PHDs. Later, HIF-α is ubiquitinated by binding to pVHL and subsequently degraded. In hypoxia, HIF-α ubiquitylation is inhibited, HIF-α accumulates and forms a heterodimer with HIF-β. HIF-1 translocates to the nucleus and binds to HREs in the genome regulating the hypoxic response. When the Hippo pathway is on, MST1/2 are phosphorylated and bind to SAV1 to form an enzymatic complex phosphorylating and activating LATS1/2 and the scaffold proteins MOB1, which in turn together phosphorylate YAP and TAZ. YAP/TAZ transcriptional activity is suppressed by 14-3-3 mediated cytoplasmic retention and ubiquitination by the SCFβ-TrCP E3 ligase and subsequent proteasomal degradation. The process prevents nuclear entry of YAP and TAZ and their binding to multiple transcription modulators including TEAD1-4 and others such as SMAD. When the Hippo pathway is off, YAP and TAZ will reside in the nucleus and bind to transcription factors. Recent research has pointed to crosstalk between the HIF pathway and the Hippo pathway, but the underlying mechanism has not been unraveled. (adapted from Zhao C, *et al.* 2020)

Crosstalk between hypoxia and Hippo pathway in breast cancer

There are a few studies showing a correlation between hypoxia and the Hippo pathway [45]. Ma B *et al*. showed that hypoxia suppressed the activity of the Hippo pathway [46]. Hypoxia exerted this effect by upregulating the E3 ubiquitin ligase SIAH2 which resulted in destabilization of LATS2. YAP was further stabilized by forming a complex with HIF-1α. Xiang L *et al.* showed that TAZ mRNA expression was increased under hypoxic conditions in multiple breast cancer cell types [47]. In this study, HIF-1 bound to HRE elements of the TAZ gene, encoded WWRT1. Moreover, based on a database of human breast cancer, they showed that only simultaneous high expression of HIF-1α

and TAZ correlated with poor survival. A follow-up study further showed that TAZ and HIF-1α acted as each other's transcriptional co-activators [48]. HIF-1α interacted with TAZ and served as a co-activator for target genes such as CTGF, and TAZ interacted with HIF-1α to serve as a co-activator for target genes such as PDK1 or LDHA. Other scenarios in which hypoxia can influence YAP and TAZ activity and their phosphorylation remain to be elucidated. Bendinelli P *et al*. investigated whether Hippo-pathway effectors (Wwox and TAZ) have effects on the bone-metastatic phenotype through HIF-1 activity [49]. They found that hypoxia led to nuclear co-localization of increased levels of HIF-1α and TAZ and co-immunoprecipitation showed an interaction between these proteins, resulting in enhanced HIF-1-DNA binding and transactivation, which might be important for bone metastasis from breast cancer. Above all, it provides new thoughts concerning therapeutic targeting of HIF-dependent signalling pathways based on YAP/TAZ in breast cancer. The mechanism(s) of crosstalk between the HIF pathway and the Hippo pathway need further unraveling.

Tumor cell proliferation and metastasis

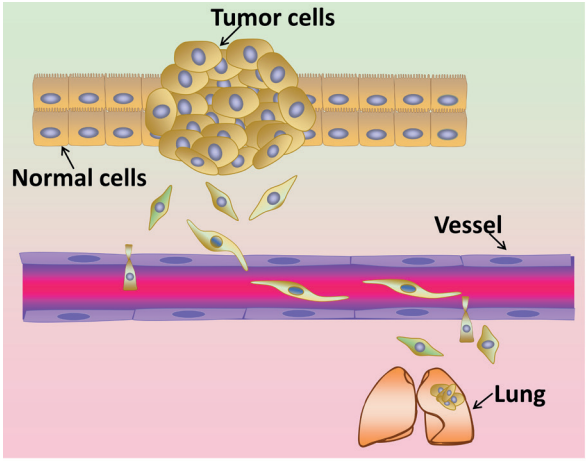

Figure 3. Tumor proliferation and metastasis. Tumor cells overgrowth leads to hypoxia activating pro-motility genes and promotes cell migration and invasion resulting in metastasis. Tumor metastasis includes several steps: invasion, intravasation (into vessel), systemic transport (in vessel), extravasation, and secondary site colonization site (e.g., lung).

Proliferation and metastasis are two hallmarks of cancer and that accompany each other [50]. Sustained proliferation in cancer is controlled by cell cycle proteins such as cyclin-dependent kinases (CDKs) and cyclins [51]. Tumor cells outgrow by proliferating more rapidly than normal cells leading to a hypoxic microenvironment, which induces HIFs activating key pro-motility genes leading to cell intravasation, the primary step of metastasis [52]. Tumor metastasis includes several steps: invasion, intravasation, systemic transport, and extravasation resulting in the secondary metastatic site [52]. EMT, the sign of migratory and invasive capacity, means that cells acquire mesenchymal features enabling them to migrate and invade as small clusters or single cells [53], which promotes tumor metastasis. EMT is divided into three subtypes: 1) type 1 EMT is involved in implantation, embryogenesis, and organ development; 2) type 2 EMT is related to tissue regeneration and organ fibrosis; 3) type 3 EMT is associated with metastasis in cancer progression [54]. During EMT, gene expression and mechanisms of post-translational modification are changed, and identifying molecular regulators of EMT such as E-cadherin, N-cadherin, Vimentin, Snail, Slug, β-catenin and ZEB1 is important for exploring the process of tumor progression [55]. EMT promotes cell migration, which

is the key function of cells depending on actin assembly and disassembly dynamics [56]. Actin polymerization forms actin filaments, of which assembly and disassembly are regulated by actin-binding proteins [57]. The fibers of the actin cytoskeleton generate pushing or pulling forces by actin polymerization or mutual sliding of actin and myosin II filaments to realize cell migration and morphogenesis [58–60]. Even though enhanced migration of tumor cells during tumor progression has been studied for decades, there is still a challenge in translating the theory into clinical practice. Combating progression of breast cancer towards a metastasic disease is a main challenge in the clinic.

Aim and outline of the thesis

In this thesis, I aim to investigate hypoxia, YAP/TAZ signaling, and their crosstalk in breast cancer to have a better understanding on underlying mechanisms. We summarize the current situation of hypoxia *in vitro* research in breast cancer in **chapter 2**. We discuss current methods of hypoxia research, to explore how exposure regimes used in experiments are connected to signalling by different HIFs and to distinct cellular responses in the context of hallmarks of cancer. We describe discrepancies in the existing literature on hypoxia research within the field of breast cancer and propose a clear definition of acute, chronic, and intermittent hypoxia based on HIF activation and cellular responses. It is currently not known to what extent signalling responses to hypoxia differ between different breast cancer subtypes. In **chapter 3**, we compare luminal and basal TNBC breast cancer cells in acute and chronic hypoxia by TempO-Seq targeted RNA sequencing followed by data analysis and wet lab experiments. We find that chronic hypoxia affects cytoskeletal organization in basal cells whereas it mainly regulates metabolism in luminal cells. This is further confirmed by higher cell migration speed and more F-actin stress fibres in basal cells versus more lactate production and increased GADPH expression in luminal cells. We also identify hub genes in the hypoxia regulated pathways and explore their association with disease progression in clinical datasets. In **chapter 4**, we review existing literature for evidence for crosstalk between hypoxia and mechanical aspects of the TME, including the ECM and the impact on breast cancer progression. In **chapter 5**, we investigate the response to hypoxia in a series of luminal breast cancer and TNBC cells. HIF1 mediated phosphorylation of TAZ, but not YAP, at a site known to promote its cytoplasmic sequestration and proteasomal degradation, was observed in all basal A TNBC cell lines tested but not in any of the luminal breast cancer cell lines. Such TNBC specific phosphorylation did not involve activation of the Hippo signaling cascade. Instead, we identified CDK3 as a kinase responsible for phosphorylation of TAZ in TNBC cells under hypoxia. In **chapter 6**, we explore the role of CSE1L, a candidate target of YAP/TAZ identified in the TempO-Seq experiment from chapter 3, in a panel of different breast cancer cell lines with various subtypes. We find that hypoxia regulates CSE1L on the transcriptional level and investigate CSE1L nuclear/cytoplasmic distribution. We find that CSE1L is involved in cell migration and targets EMT markers, N-cadherin, Slug and Snail in TNBC cell lines under hypoxia. In **chapter 7**, I discuss and conclude the overall research of this thesis and future perspectives.

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