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Leiden
The Netherlands

Regulation of signal transduction pathways by hypoxia in breast cancer subtypes

Liu, Q.

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Chapter 7

General summary and discussion

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Breast cancer has a high mortality in women worldwide, and typically metastasis rather than the primary tumor is the cause of death. Tumor cells experience hypoxia, which is accompanied by alterations in cell metabolism and can drive metastasis by triggering an epithelial–mesenchymal transition (EMT) in the tumor cells. Yes-associated protein (YAP) and a transcriptional co-activator with PDZ-binding (TAZ) are two transcriptional co-activators involved in growth, metabolism, and metastasis in cancer [1,2]. Breast cancer can be divided into different subtypes. One criterium underlying such subtypes is based on the levels of Human Epidermal growth factor Receptor 2 (HER-2), Estrogen Receptor (ER) and Progesterone Receptor (PR). The subtypes include luminal-like (luminal A and luminal B), HER-2 enriched and basal-like (often “triple negative”) [3]. Triple negative breast cancer (TNBC; ER and PR negative and no enrichment of Her-2) has a lower survival rate due to the lack of therapeutic targets. Fundamental research exploring the molecular mechanisms at work in cancer cells and their response to a hypoxic environment may contribute to insights for future clinical treatment. This thesis focused on profiling breast cancer cells belonging to distinct subtypes under acute and chronic hypoxia, investigating the crosstalk between hypoxia regulated pathways and YAP/TAZ signaling in luminal breast cancer versus TNBC cells, and identification of the potential targets of TAZ in breast cancer cells.

1. Distinct features of luminal and basal breast cancer subtypes in breast cancer

Luminal-like and basal-like breast cancer different in molecular subtype, prognosis, metabolism, migration and proliferation. Luminal-like breast cancer can be divided into luminal A (PR and/or ER positive, and HER2/neu negative) and luminal B (PR and/or ER positive, HER2/neu positive) with less aggressiveness, favorable prognosis, and better sensitivity to targeted therapies such as inhibitors of CDK4/6 or PIK3CA [3–5]. Luminal-like cell lines (not further differentiated) used in this thesis were MCF-7, T47D and BT474 (**chapter 2, 3, 5**). Basal-like breast cancer can be further divided into basal, claudin-low and/or metaplastic breast cancer (MBC) with more aggressiveness, poor prognosis, better sensitivity to chemotherapy and PARP inhibitors, and less sensitivity to hormone therapy [3–5]. Basal-like breast cancer cell lines can be further divided into basal A-like (more epithelial characteristics) and basal B-like (more mesenchymal with for instance a loss of E-cadherin). HCC1143, HCC1806 and SUM149PT were basal A-like cell lines (**chapter 3, 5**), while MDA-MB-231, HCC38 and Hs578t cell lines (**chapter 6**) were basal B-like cell lines used in the thesis.

Metabolic heterogeneity is shown in breast cancer progression and cell subtypes. Tumor cells can generate energy by aerobic glycolysis instead of mitochondrial oxidative phosphorylation (OXPHO) regardless of the lower ATP production. It has been shown that luminal-like breast cancer cells prefer OXPHO, whereas basal-like cells prefer glycolysis [6,7]. Indeed, in **chapter 3**, we measured glycolytic status in three luminal (MCF-7, T47D and BT474) and three basal A (HCC1143, HCC1806 and SUM149PT) breast cancer cell lines. Lactate production levels in luminal cells is lower than basal A (except HCC1806) breast cancer cells, which means that luminal cells are less glycolytic than basal A cells. Luminal-like and basal-like cells also respond differently to hypoxia. Luminal breast cancer cell lines displayed a metabolic switch: they produced more lactate under chronic hypoxia indicating a shift to glycolysis (**chapter 3**). Basal A cells showed a different response (HCC1143), whereby their migration, which was already faster than luminal

cells (MCF-7) under normoxia, was further enhanced in response to chronic hypoxia. This was accompanied by an increase in stress fibres under chronic hypoxia (**chapter 3**). Basal A cells (HCC1143, HCC1806 and SUM149PT) showed reduced proliferation under chronic hypoxia (**chapter 5**), and HCC1143 cells were arrested in G1 phase (**chapter 3**). Thus, while luminal cells show a predominantly metabolic response to hypoxia, basal A cells seem to reduce growth and enhance migration. Clearly, the response to hypoxia is heterogeneous in different breast cancer subtypes. Interestingly, Fan M *et al.* found that a natural product small molecule, diptoindonesin G can induce a phenotype switch in breast cancer cells from basal to luminal, pointing to a therapeutic strategy to reduce aggressiveness in the clinic [8]. Our findings indicate distinct responses of luminal-like and basal-like breast cancer cells to hypoxia, which could provide further insights for distinct responses to therapy of breast cancer subtypes.

2. Mimicking hypoxia in cell culture models

Hypoxia, a common feature in tumors lacking oxygen, leads to treatment resistance in patients and contributes to tumorigenesis. Hypoxia-inducible factor α (HIF α) is the most prominent of the HIFs involved in hypoxia, accumulates and forms a heterodimer with HIF- β [9]. HIFs translocate to the nucleus, bind to hypoxia response elements (HREs) in the genome regulating a transcriptional response to hypoxia [9]. Chemical reagents (dimethylxalylglycine (DMOG) and cobalt(II) chloride (CoCl₂)) [10–12] and physiological ranges of oxygen (0.02% - 1%) [13–15] are typically used to mimic hypoxia. However, chemical reagents sometimes have side effects. For example, the HIF α stabilizing compound, DMOG influenced cell metabolism by decreasing NADH levels apart from HIF accumulation [16]. Besides, chemical reagents can be toxic and the concentration should be carefully tested before use. In **chapter 3**, 1% oxygen and 1 mM DMOG were used in our studies to mimic hypoxia and their effects on TAZ phosphorylation were identical. This indicates that the effect of hypoxia in these studies likely involves the canonical HIF-mediated response. We explicitly tested concentration ranges of DMOG for effects on cell growth in our cell models and 1mM DMOG was selected.

Hypoxia has become a hot spot in cancer research and discoveries revealing how cells respond and adapt to changes in oxygen availability won the 2019 Nobel Prize [17]. More and more hypoxia in vitro research was published but there is no standard guideline on exposure time and oxygen percentage to determine hypoxia. We discussed this topic based on existing literature in **chapter 2**, according to HIF activation and cellular responses. We define i) acute hypoxia when the cells are exposed for no more than 24 hours to an environment with 1% O₂ or less; ii) chronic hypoxia when the cells are exposed for more than 48 hours to an environment with 1% O₂ or less and iii) intermittent hypoxia when the cells are exposed to at least two rounds of hypoxia (1% O₂ or less) separated by at least one period of reoxygenation by exposure to normoxia (8.5% O₂ or higher). However, it should be noted that depending on the cell or tissue type, distinct percentages of oxygen may be physiologically relevant. Oxygen percentages vary in different tumor and normal tissues. For example, O₂ is 1.7% in brain tumor and 2.0% in normal brain tissue, 0.8% in liver tumor and 5.0% in liver normal tissue, 1.9% - 2.2% in lung tumor and 2.6% - 3.0% in lung normal tissue, 1.3% in breast tumor and 5.2% in breast normal tissue [18]. For hypoxic conditions, O₂ around 1% is widely used in current research and 21% O₂ is used for normoxia, as we have used in our studies.

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However, based on the above tissue/disease-specific measurements, adaptations and standardizations may be needed to experimentally model normoxia/hypoxia in human physiology and pathology and facilitate comparisons between different studies.

3. Uncovering the signaling pathways regulated by hypoxia

HIFs are the core components of the transcriptional response to hypoxic stress, Depending on the duration of hypoxia HIFs regulate cell proliferation, apoptosis, migration and invasion, metabolism and immune activation in tumors [19–21]. In this thesis, we show that both the duration of hypoxia and the breast cancer subtype influence the signaling pathways that are activated. Throughout, we use for acute hypoxia 24 hrs exposure to 1% O₂ and for chronic hypoxia 5 days exposure to 1% O₂. We performed targeted RNA sequencing for luminal (MCF-7) and basal (HCC1143) breast cancer cells under acute or chronic hypoxia in **chapter 2, 3, 5** to uncover pathways regulated by hypoxia. Under acute hypoxia, both luminal and basal cells activated HIF1 α signaling. Both subtypes stabilized HIF1 α and induced expression of a typical HIF1 α target, CA9. However, luminal cells in addition to pathways associated with HIF signaling also activated glycolysis and AMPK signaling, which may be related to the fact that they are known to be less glycolytic than basal cells and have lower HIF1A gene expression (**chapter 3**), which has also been found in other studies [6,22]. Additionally, the distinct gene expression profiles in luminal and basal breast cancer cells will further affect their response to hypoxia [23,24]. Under chronic hypoxia both subtypes showed pathways associated with cell cycle regulation and luminal cells maintained activation of metabolic signaling pathways including glycolysis and cholesterol metabolism, while basal cells activated pathways associated with cancer, migration and cytoskeleton, such as the ILK pathway (**chapter 3, 5**).

YAP/TAZ, transcriptional co-activators in the Hippo pathway, regulate cell metabolism and mechanotransduction, and have been implicated in crosstalk between the extracellular matrix (ECM) and hypoxia in the tumor microenvironment [25–28], which is reviewed in **chapter 4**. In **chapter 5**, we focused on crosstalk between hypoxia and YAP/TAZ signaling in luminal and basal breast cancer cells. Under chronic hypoxia, phosphorylation of TAZ but not YAP was strikingly increased in 3 basal cell lines. Such a response was not observed in 3 luminal cell lines. Yan L *et al.* also found an increase in p-TAZ without an effect on YAP/p-YAP, after 24 hrs and 48 hrs exposure under hypoxia [27]. It indicates that TAZ rather than YAP may be regulated by hypoxia. Shreberk-Shaked M *et al.* found that YAP is mainly involved in cell growth and TAZ is mainly involved in cell migration in lung cancer cells [29]. Plouffe SW *et al.* found that YAP governs multiple cellular processes not affected by TAZ, by comparing YAP and TAZ knockouts in HEK293A cells [30]. These studies show that the function of YAP and TAZ is distinct. Thus, YAP and TAZ should be distinguished instead of regarding them as a complex [29–31]. The localization of YAP/TAZ regulates their activity and role in signal transduction. When the Hippo pathway is on, YAP/TAZ are phosphorylated and bind to 14-3-3 proteins to be sequestered in the cytoplasm or processed further for degradation. In this way, YAP/TAZ activity is suppressed [32]. When the Hippo pathway is off, YAP/TAZ are in the nucleus and bind to transcription factors (TEADs, SMADs) leading to cell proliferation [33–35]. Targeting YAP/TAZ in the nucleus is considered as a potential anticancer therapeutic strategy in the clinic. The functional impact of enhanced phosphorylation of TAZ in hypoxic basal cells that we identify remains enigmatic. Expression of a non-

phosphorylatable TAZ mutant did not affect proliferation of basal A cells under hypoxia indicating that TAZ phosphorylation does not represent a major (mal)adaptive response to hypoxia (**chapter 5**). We find that the majority of p-TAZ is located in the cytoplasm where it is usually regarded as non-functional and this is in line with the notion that TAZ phosphorylation prevents its nuclear translocation. However, the nucleo-cytoplasmic shuttling of YAP/TAZ and the role of phosphorylation is still not a well understood area which needs further studies in the future.

4. Targets regulated by YAP/TAZ

TAZ was first reported as a novel transcriptional co-activator interacting with 14-3-3 and sharing homology with YAP by Kanai F *et al.* in 2000 [36]. TAZ is often mentioned with together YAP where YAP is typically the major protein to be investigated [37,38]. In fact, besides overlapping functions YAP and TAZ may have important distinct functions as mentioned above and the importance of TAZ may be no less than YAP in cellular processes. To find TAZ targets, siRNA-mediated knockdown in HCC1143 was performed under normoxia and hypoxia. We identify TAZ regulated genes and further show that the impact of TAZ depletion on the target gene expression differs between normoxia and hypoxia. 26 differentially expressed genes (DEGs) were found in siWWTR1 NX and siWWTR1 HX genesets (not shown in the thesis), 107 DEGs in siWWTR1 HX and HX/ NX genesets (**chapter 6**), 19 DEGs were found in HX/NX, siWWTR1 NX and siWWTR1 HX genesets (not shown in the thesis). It indicates that TAZ regulated genes in different ways when comparing normoxia and hypoxia. Shreberk-Shaked M *et al.* listed genes regulated by YAP and TAZ separately, and drugs targeting YAP and TAZ individually [29]. Expression of a set of YAP/TAZ target genes (e.g., CTGF, AXL and CYR61) is usually used to detect YAP/TAZ activity [39,40]. However, our work shows that this can in fact not be generalized. In **chapter 5**, TAZ activity is decreased based on its phosphorylation and cellular localization while YAP is not affected in basal cells under hypoxia, but CTGF mRNA levels were increased both in the targeted RNA sequencing data and RT-qPCR results in HCC1143 cells. The fact that other YAP/TAZ targets, such as AXL, CYR61, AMOT and AMOTL1/2, did not show decreased gene expression under hypoxia (even though TAZ activity was suppressed) and their expression was not affected by siRNA-mediated TAZ depletion (**chapter 6**), may be explained by regulation of these targets mainly by YAP or by both YAP and TAZ. Literature is still confusing in this area: e.g., CTGF is regulated by TAZ but not by YAP in one study [29], whereas CTGF was reported to be regulated by YAP during angiogenesis in another study [41]. These aspects indicate that it remains impossible to predict changes of YAP/TAZ activity based on a decreased/increased expression of a set of YAP/TAZ target genes.

5. Future perspectives

In this thesis, experiments were performed using 2D cell cultures. Experiments using 3D cultures will be a good next step to better reflect the situation in tumors in the human body and to better understand the response in that situation to hypoxia. ECM components are expressed differently between 2D and 3D cultures [42,43]. It is already known that YAP/TAZ proteins are involved in ECM remodelling and it will be interesting to test whether the effect of hypoxia on phosphorylation of TAZ affects growth and ECM interactions in 3D. Notably, while the cells in 2D, as used in our studies, are exposed to normoxia/hypoxia conditions as tested, mimicking hypoxia in 3D is more of a challenge as the exact oxygen percentage the cells will sense may be affected by their location

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in the 3D tumoroid and may be influenced by the 3D ECM environment [44,45]. YAP/TAZ expression is used as a prognostic predictor in cancers, for example in lung and colorectal cancer, and also as therapy biomarker in HER2-positive breast cancer patients in the clinic [46–48]. Models using patient-derived cultures rather than cell lines will represent another step to get closer to the clinical situation.

6. Conclusion

Overall, the work in this thesis contributes to our understanding of the response of tumor cells to hypoxia and identifies a new mechanism of cross talk between hypoxia and TAZ, which occurs in basal but not luminal breast cancer cells. We compare the response to acute and chronic hypoxia (**chapter 2, 3**), we show that TAZ rather than YAP undergoes a post translational response to hypoxia in basal-A TNBC cells (**chapter 5**), and we identify YAP/TAZ target genes affected by hypoxia (**chapter 6**). The work provides novel insights into cross talk between hypoxia and YAP/TAZ signaling in breast cancer cells that may occur in breast cancer patients and affect tumor development and progression.

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