Exploring Grainyhead-like 2 target genes in breast cancer

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Chapter 6

General summary and discussion

Breast cancer

Among women, breast cancer is the most prevalent cancer type, accounting for 25% of all cancers [1]. According to gene expression profiling, breast cancer is mainly categorized into 5 major subtypes, including basal-like, luminal-like, human epidermal growth factor 2 (HER2)-enriched, claudin-low, and normal-like subtypes. Luminal- and basal-like subtypes have the highest prevalence and this dissertation focuses on these subtypes.

Existing therapies for basal-like breast cancers

At least 60% of basal-like breast cancers are negative for estrogen receptor (ER), progesterone receptor (PR) and HER2 [2], and are named triple negative breast cancer (TNBC). Basal-like breast cancer cell lines are further divided into basal A, being more luminal-like and basal B exhibiting a cancer stem cell like profile [3,4]. The cell lines generally reflect the genomic heterogeneity and gene copy number observed in the primary tumors [3] Due to the triple negative phenotype, TNBC responds poorly to existing targeted drugs. Multiple new potential therapeutic targets for TNBC are emerging such as poly(ADP-ribose) polymerase (PARP) 1, immune-checkpoints, androgen receptor, and epigenetic proteins [5]. PARP inhibitors (PARPi's) target PARP1, which is involved in cell proliferation, tumor transformation and DNA damage response. At present, several PARPi's (e.g., Olaparib, Veloparib, Niraparib, Talazoparib, and CEP7722) are under evaluation for clinical management. Among these candidate inhibitors, a monotherapy of olaparib induces partial responses in a cohort of mostly breast cancer patients with *BRCA1/2* mutations [6], suggesting it is a promising targeted drug. Cytotoxic chemotherapy still plays an important role for TNBC such as anthracyclines and taxanes. The effectiveness of chemotherapy strategies with dose dense (i.e. increasing dose intensity by short interval admission of standard-dose chemotherapy) or metronomic polychemotherapy has been validated [7]. Platinum agents (e.g. Cisplatin) have gained focus in the TNBC treatment, based on promising preclinical and clinical findings. Single cisplatin treatment allows patients with TNBC to achieve complete or near-complete responses in preoperative therapy [7]. On the other hand, it is well documented that TNBC responds to chemotherapy initially, but complete response at the early stage is not correlated with overall survival [8]. There is

no standard chemotherapy strategy for TNBC patients with specific tumor size, grade and lymph node [8]. The contribution of chemotherapy is still debated. A small proportion of TNBC are positive for androgen receptor (AR) providing an opportunity for targeted therapy. It has been shown that single AR antagonist bicalutamide treatment leads to a 19% benefit rate with a median progression-free survival of 12 weeks, with well-tolerated adverse side effects [9].

Existing therapies for luminal-like breast cancers

Luminal-like breast cancer is divided into two subgroups: luminal A and luminal B subtypes. The major difference between them is that luminal B has relatively lower expression of ER-related genes and higher expression of genes associated with cell growth (e.g., *CCND1*, *MKI67* and *MYBL2*) [10]. Luminal breast cancer cell lines are typically not further subdivided. Luminal A breast cancer is positive for ER and PR, with lower expression of proliferation-related genes (e.g., *MKI67*). Estrogen functions as a stimulus for growth and development of human mammary tissue by binding to ER. Selective ER modulators (SERMs) have high affinity for ER and inhibit activation of estrogen-mediated signaling in ER positive breast cancer. Furthermore, SERMs possess the capability to reduce expression of ER, further reducing the response to endogenous estrogen [11]. Tamoxifen is widely used in first-line clinical practice to repress growth of ER positive breast cancer by binding to the ER alpha [12,13]. In recent years, aromatase inhibitors (AIs) such as arimidex and aromasin, that inhibit estrogen biosynthesis, are replacing tamoxifen as hormone therapy. AIs are characterized by their capability to reduce estrogen biosynthesis as much as 98%, together with superiority over tamoxifen in terms of adverse side effects [14].

Luminal B breast cancer is less sensitive to endocrine therapy relative to the luminal A subtype, and to chemotherapy in comparison with basal-like and HER2 enriched breast cancer. Based on the aberrant activation of insulin-like growth factor 1 (IGF-1) signaling in luminal B tumors, IGF-1 inhibitors (e.g., OSI-906) and antibodies against IGF-1 receptor (MIK-0646) are developed and may lead to new candidate drugs [15]. Luminal B tumors are characterized by overexpression of fibroblast growth factor receptor 1 (FGFR1), indicating that antibodies and inhibitors against FGFR1 may also

represent potential drugs. Indeed, small molecular inhibitors such as TKI-258 and AZD-4547 are in phase 2 clinical testing [15]. Furthermore, the addition of PI3K inhibitors to endocrine therapy results in increased inhibition of growth in luminal B breast cancer cell lines, suggesting PI3K inhibitors may contribute to the treatment of luminal B breast cancers [16]. A negative feedback loop between mTOR and IGF-1 signaling has been described by which inhibition of mTOR signaling induces increased expression of IRS1, which in turn activates AKT signaling. A preclinical study shows that the combination of ridaforolimus (mTOR inhibitor) and dalotuzumab (antibody against IGF-1R) is a potential effective treatment against luminal B breast cancer [15]**.**

Distinct modes of E-cadherin regulation in normal epithelia and cancer cells

GRHL2 is a transcription factor that activates or represses gene expression through indirect and direct binding to the promoter of the genes and histone modification [17,18]. GRHL2 is specifically expressed in cells with epithelial features. Several studies show that reduction of GRHL2 expression triggers epithelial to mesenchymal transition (EMT) with upregulation of mesenchymal markers (e.g., Vimentin and ZEB1) and downregulation of epithelial genes (e.g., *CDH1*) in breast cancer [17,19,20]. In **Chapter 2**, our results show that GRHL2 loss results in downregulation of E-cadherin (gene name: *CDH1*) in basal A (HCC1806 cells) and luminal-like (MCF7 cells) subtype breast cancer. However, our ChIP-seq data in **Chapter 3** reveal that GRHL2 binding is not observed at the promoter region of *CDH1* in any of the basal A (HCC1806, BT20 and MDA-MB-468 cells) and luminal-like (MCF7, T47D and BT474 cells) subtype breast cancer cell lines tested. In addition, our Bru-seq data in **Chapter 4** demonstrate that RNA synthesis of *CDH1* is not altered in response to GRHL2 deletion in MCF7 luminal-like breast cancer cells. This indicates that GRHL2 loss leads to E-cadherin downregulation while *CDH1* RNA synthesis is maintained, suggesting that GRHL2 regulates *CDH1* post-transcriptionally. This is in contrast to an earlier report showing that GRHL2 directly regulates transcription of *CDH1* in mouse inner medullary collecting duct cells by contacting the *CDH1* promoter through a chromatin loop [18]. This may suggest that GRHL2-mediated regulation of E-cadherin (and perhaps other epithelial genes) is markedly different in non-transformed epithelia versus epithelial cancer cells, such as the breast cancer cell lines tested by us.

Biological functions of GRHL2 in cancer – from proliferation to invasion

In **Chapter 2**, the effect of GRHL2 loss on EMT, cell migration (2D random migration), and cell invasion capacity (3D collagen gel invasion) are investigated. Interestingly, E-cadherin downregulation is seen in MCF7 as well as HCC1806 cells, while other effects appear to be subtype specific. GRHL2 loss triggers enhanced cell migration and invasion in HCC1806 cells, concomitantly with enhanced expression of mesenchymal markers i.e., N-cadherin and Vimentin [21,22], which are associated with cell migration. By contrast, in MCF7 cells GRHL2 loss does not lead to expression of mesenchymal markers and does not enhance the ability to invade. Others have shown *in vivo* and *in vitro* that exogenous expression N-cadherin allows MCF7 cells to invade more efficiently [23]. MCF7 cells expressing both N-cadherin and E-cadherin were also found to have the ability to invade, indicating that the mesenchymal marker, N-cadherin, may be a determinant of cell invasion, rather than loss of E-cadherin [23]. Taken together, these findings suggest that cell invasion triggered by GRHL2 deletion is associated with the emergence of mesenchymal markers and may be subtype specific.

In luminal cells GRHL2 may support proliferation and invasion through steroid hormone signaling. Activation of ERβ signaling contributes to inhibition of IMP3, which is an mRNA-binding protein that influences expression or localization of invasion-related mRNAs [24]. MCF7 cells are reported to express ERβ modestly [24,25] and ERβ signaling is regulated by GRHL2 that acts as a part of ER transcriptional complex to stimulate transcription of ER target genes [26]. Our Bru-seq data show that following GRHL2 deletion, *IMP3* RNA synthesis is upregulated at day 16, indicating that GRHL2 is involved in crosstalk between ERβ and IMP3. Hence, GRHL2 deletion may be concomitant with inactivation of ERβ signaling, which results in cell invasion through upregulation of IMP3 in HCC1806 cells.

Our study indicates that GRHL2 may have differential biological functions for basal A and luminal-like breast cancer. In **Chapter 3**, GRHL2 binding sites across breast cancer subtypes are profiled by Chip-seq to further investigate the differences in

molecular mechanisms. GRHL2 binding motifs are found, the landscape of GRHL2 binding sites is mapped, and, based on overlap between three luminal and three basal breast cancer cells lines, subtype-specific and common binding sites and candidate GRHL2-regulated genes are identified. Our findings to some extent confirm and extend previous findings in other cell types, such as the distribution of GRHL2 binding sites and GRHL2 binding motifs.

We also find novel GRHL2-interacting genes. However, ChIP-seq alone is insufficient to identify GRHL2 target genes, due to the fact that binding sites identified by ChIP-seq do not necessarily imply GRHL2-mediated gene expression. Therefore, to identify direct target genes of GRHL2 in breast cancer, Bru-seq is carried out in a conditional CRISPR/Cas9 MCF7 model in **Chapter 4**. Bru-seq is an innovative method to capture changes in initial transcription based on labeling and isolation of nascent RNA using bromouridine (Bru) [27]. Bru-seq measures transcription near transcription start sites (TSSs) and can capture initial transcription rapidly, by which the effects of post-transcriptional regulation on gene expression are eliminated such as RNA binding proteins and microRNAs. This chapter reveals dynamic changes in nascent RNA in response to GRHL2 loss. Through subsequent bioinformatics analysis such as Ingenuity Pathway analysis (IPA) and Gene Ontology terms analysis we provide new insights into molecular mechanisms that may underlie GRHL2 biological functions. As the generation of a conditional basal-A model was unsuccessful, we could so far only perform this analysis for the luminal MCF7 model system.

In **Chapter 2,** cell cycle analysis shows a rapid G0/1 arrest triggered by GRHL2 loss in HCC1806 and MCF7 cells, which is correlated with the findings in **Chapter 4** that demonstrates inhibition of genes involved in cell cycle/DNA replication signaling (*E2F1*, *E2F2, MCM7, CDC20, ESPL1, MCM2, PTTG1, SFN, RNASEH2A, FEN1*) by Bru-seq. In order to identify direct target genes of GRHL2, ChIP-seq and Bru-seq data are integrated in **Chapter 4**. In this way, 48 novel direct target genes of GRHL2 are identified, some of which are involved in cell cycle/DNA replication signaling. Integrating the data from **Chapters 2-4** provide novel insights into the molecular mechanism of GRHL2-mediated cell cycle/DNA replication regulation. It is well

documented that downregulation of GRHL2 inhibits cell proliferation in multiple human cell lines [20,28,29]. However, little is known about the molecular mechanism of GRHL2 loss-mediated inhibition of cell growth. Several direct target genes of GRHL2 have been previously implicated in cell growth such as *EHF* and *E2F2* [30,31]. Downregulation of EHF is associated with inhibition of cell growth in ovarian cancer cells [31]. However, our data shows that ectopic overexpression of EHF did not rescue GRHL2 loss-triggered inhibition of cell growth in MCF7 cells. One possible explanation is that EHF is not associated with cell proliferation in MCF7 cells, indicating that the GRHL2-EHF biological function is cell context dependent. Another possibility is that GRHL2 loss inhibits cell growth through multiple signaling pathways, which include several target genes. Rescue of one arm of the signaling network in this case is not sufficient to rescue GRHL2 loss-mediated cell proliferation. Indeed, there is currently no single target gene described that can rescue proliferation upon GRHL2 loss on its own, further pointing to cooperative roles of GRHL2 direct target genes (e.g., *E2F2* and *CDCA7L*) in cell proliferation.

GRHL2-regulated functions as therapeutic targets in breast cancer

Findings from us and others demonstrate that GRHL2 is involved in cell proliferation and cell cycle progression. Our data suggest that GRHL2 loss-induced cell invasion and multiple aspects of EMT occur in basal A but not luminal-like breast cancers. This implies that GRHL2 may represent a promising therapeutic target especially for luminal-like breast cancer. Although there is no molecular inhibitor targeting GRHL2 to date, inhibitors targeting downstream effectors of GRHL2-regulated proliferation signaling could be identified.

Miniature chromosome maintenance 7 (*MCM7*), a direct target gene of GRHL2 based on our ChIP-seq and Bru-seq data **(Chapter 3 and 4)**, is involved in the initiation of DNA replication. Reduced expression of MCM7 results in apoptosis in RB deficient cells and overexpression of MCM7 is associated with chemotherapy resistance [32]. Our findings show that GRHL2 loss-induced inhibition of cell proliferation is concomitant

with a downregulation of *MCM7* RNA synthesis, suggesting that targeting MCM7 may be a potential strategy for luminal breast cancer treatment.

As a widely used molecular inhibitor against 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA), simvastatin (SVA) that belongs to the statins may have anti-tumor effects. This was demonstrated by a*n in vivo* mouse model study showing SVA treatment inhibits breast cancer cell growth, through inhibiting MCM7 expression [32]. Additionally, SVA was reported to re-sensitize tamoxifen resistant breast cancer to chemotherapy [32,33]. Thus, the small molecular inhibitor SVA against HMGCoA represents a candidate drug for anti-breast cancer therapy that inhibits a GRHL2 target MCM7. The hexameric protein MCM2-7 complex is composed of six distinct subunits (named MCM2 to MCM7) with an AAA^+ ATPase that is targeted by the drug ciprofloxacin. MCM2 is identified as a direct GRHL2 target in our studies **(Chapter 3 and 4)**. Ciprofloxacin, a common and approved human fluoroquinolone inhibitor, was reported to inhibit cell growth by repressing the DNA helicase activity of MCM2-7 [34]. Other quinolone inhibitors such as 271327 and 314850 can selectively target MCM2-7 and may also be further developed for anti-breast cancer therapy. Targeting the enzymes involved in cancer metabolism, which facilitates tumorigenesis and metastasis, may be an alternative strategy for anti-cancer treatment. We identify an interesting candidate in this respect, the glycolytic enzyme aldolase A (ALDOA). ALDOA is a critical enzyme associated with cancer metabolic reprogramming and metastasis [35] that we find to be regulated by GRHL2 **(Chapter 3 and 4)**. The interaction of ALDOA with γ-actin is linked to enhanced metastatic potential of cancer cells and disruption of this interaction by the small molecular inhibitor raltegravir may represent a potential therapeutic strategy [35].

In the set of genes whose expression is attenuated in response to GRHL2 loss, involvement in cell cycle signaling is significantly enriched. A typical hallmark of cancer progression is dysregulation of the cell cycle, in which Cyclin D-dependent kinase 4/6 (CDK4/6) activity is enhanced by activated mitogenic pathways such as PI3K-AKT-mTOR and RAS-RAF-MEK-ERK signaling. CDK4/6 inhibitors, including abemaciclib, palbociclib and ribociclib interfere directly in the cell cycle and synergize with endocrine therapy [36]. As the first drug of CDK4/6 inhibitors, palbociclib benefits luminal-like breast

cancer patients when combined with letrozole or fulvestrant, leading to over 10-months improvement in median progression-free survival (mPFS) [37]. This approach has manageable adverse side effects such as neutropenia [37]. As the latest CDK4/6 inhibitor, abemaciclib can act as a monotherapy for luminal-like advanced/metastatic breast cancer, through inhibition of phosphorylation of RB and induction of G1-phase cell cycle arrest in RB-proficient cancer [36]. Meanwhile, abemaciclib induces breast cancer cell senescence with accumulated β- galactosidase, instead of cell apoptosis [38]. An *in vivo* study shows that abemaciclib can penetrate the blood-brain barrier and functions at lower does relative to palbociclib [39,40]. Compared with palbociclib and ribociclin, abemaciclib has higher selectivity for the CDK4/Cyclin D1 complex but there is no evidence for differences in antitumor effects between these drugs for patients with HR positive and HER2 negative breast cancer [36,38]. Unexpectedly, abemaciclib is also reported to facilitate antitumor immunity through enhancing tumor antigen presentation and inhibiting regulatory T cell proliferation. Moreover, type III interferons and interferon-related genes (e.g., *STAT1, IRF9* and *NLRC5*) are upregulated in the presence of abemaciclib in *in vivo* breast cancer models [38,41]. These observations suggest that abemaciclib triggers breast cancer cytostasis, rather than cell apoptosis, and consolidates antigen presentation to stimulate cytotoxic T cells. Collectively, GRHL2 and the components of signaling pathways regulated by GRHL2 may be potential targets for anti-breast cancer treatment.

Strengths and limitations of the integrated approaches in this study

Our study is the first to map the genome-wide landscape of GRHL2 binding sites across breast cancer subtypes (i.e., basal A and luminal-like). This is essential to understand potential subtype specific biological roles of GRHL2. In comparison to ChIP-chip (ChIP-microarray), ChIP–seq generates higher resolution, greater coverage and less noise data [42]. More replications of the ChIP-seq experiments would enhance confidence. On the other hand, by focusing only on candidate targets that are shared within 3 cell lines for each subtype or across all 6 cell lines, robustness of the data is ensured. Moreover, ChIP-PCR is used to validate key targets. Another aspect is the processing of the data to identify bona fide binding sites as well as deciding on the

most promising target genes with binding sites spanning across the genes rather than clustering at promoter regions.

The integration of ChIP-seq with our Bru-seq experiment significantly enhances the ability to find direct GRHL2 target genes. Bromouridine sequencing (Bru-seq) is an innovative method to measure RNA synthesis, based on metabolic labeling using bromouridine [43]. The expression level of an individual mRNA is determined by RNA production and degradation [44]. In contrast to standard RNA-seq, Bru-seq monitors changes in transcription and eliminates effects of post-transcriptional regulation/RNA stability on mRNA expression level [45]. Here, we make use of a conditional CRISPR/Cas9 system to delete GRHL2 gene and study the impact on genome-wide RNA synthesis. The CRISPR/Cas9 system has some intrinsic limitations including off-target effects. The fact that we could so far only design the model for MCF7 precludes a strategy such as used for ChIP-seq where overlay of data in different cell lines enhances robustness. Nevertheless, the overlap between ChIP-seq and Bru-seq represents a powerful approach to the identification of GRHL2 direct target genes and is a starting point for unraveling of GRHL2-regulated signaling networks as we do in **Chapter 4**.

A limitation of our study is the use of two-dimensional (2D) cultures where the cell environment differs significantly from the microenvironment of intact living tissues such as the interaction with extracellular matrix (ECM), the concentrations of essential nutrients, and tissue architecture [46]. While three-dimensional (3D) cultures are not used to collect samples for ChIP-seq or Bru-seq, we make use of a 3D collagen model to study the effect of GRHL2 deletion on invasion. Here, a collagen concentration is used that has been reported to mimic physiological and pathological tissue stiffness [47-49]. We show that effects of GRHL2 deletion on migration in 2D culture are recapitulated in 3D **(Chapter 2)**. Importantly, our results point to differential effects of GRHL2 loss on cell migration in 2D and 3D in basal A versus luminal-like breast cancer. Moreover, we identify novel GRHL2-regulated genes and signaling networks, we find GRHL2 targets that are conserved across epithelial tissues (other reports) and cancer cells (our work), and we discover an alternative relation between GRHL2 and the *CDH1*

gene, by which *CDH1* is not a direct target gene suggesting that mechanisms identified in non-transformed epithelial tissues (i.e. direct GRHL2 binding and regulation of *CDH1*) appear to be altered in cancer cells.

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