Unraveling multifaceted roles of Grainyhead-like transcription factor-2 in breast cancer

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Chapter 7

Summary, Discussion and Future Perspectives

Breast cancer is the most prevalent cancer among women globally[1] and the therapeutic interventions remain limited due to the metastatic nature of the disease. Targeting metastasis is challenging due to the adaptive behavior of tumor cells.[2] Therefore, identifying the key regulators like epithelial-mesenchymal transition (EMT) is crucial.[3] Although EMT is not considered as the only prerequisite for the metastasis,[4,5] multiple studies have demonstrated its significant role in promoting metastasis and tumor progression.[6,7] EMT is controlled by a regulatory network of transcription factors; EMT transcription factors (EMT-TFs).[8,9] In this thesis, I focused on a master epithelial regulator of EMT, Grainyhead-like 2 transcription factor (GRHL2) and identified its diverse roles across breast cancer subtypes, providing novel mechanistic insights. Our initial literature study explored the interactions between molecular and physical cues that reshape tumor microenvironment and provide cellular plasticity, required for metastasis in **chapter 2**. We then examined distinct signaling networks and molecular processes regulated by GRHL2 in the luminal and basal A subtypes of breast cancer in **chapter 3-4**. Following these findings, we analyzed the function of GRHL2 in controlling kinase signaling, how central its role is in the EMT/mesenchymal-epithelial transition (MET) balance, and whether it regulates therapy response using a luminal and a basal-b model **(chapter 5)**. Lastly, a novel immune modulatory role was discovered for GRHL2 in breast cancer **(chapter 6).** Here, we explain the key findings of the thesis and their significance for cancer research. We also provide recent advancements for the future research.

Diverse mechanisms controlling cellular plasticity in cancer metastasis

Intratumor heterogeneity, characterized by tumor cells harboring distinct phenotypical and molecular features within the same tumor bulk, facilitates significant adaptability, often acquired through cellular plasticity.[10] This plasticity is further sustained by alterations in the genomic and phenotypic landscapes through EMT. EMT is influenced by various stimuli such as hypoxia and pH levels in the tumor microenvironment, as well as downstream signaling pathways including transforming growth factor-β (TGFβ), Wnt, and EGF.[9] In Chapter 1, we present a comprehensive investigation into the essential roles

played by the GRHL family, including GRHL1-3, both in embryogenesis and cancer.

It has been previously reported that mechanical stimuli also have an impact on maintaining the plasticity.[11] The tumor cells manipulate the interplay between several signaling pathways and mechanical cues, supporting their growth and survival to adapt to alterations within the tumor microenvironment. We discuss two distinct mechanisms; EMT and jamming/unjamming that remodel the tumor microenvironment to establish an optimal niche for the metastatic outgrowth (**chapter 2**). Additionally, we have outlined a roadmap for therapeutic interventions targeting these mechanisms.

Subtype-specific actions of GRHL2 across breast cancer subtypes

In **Chapter 3**, we analyzed GRHL2 binding sites and motifs in three luminal estrogen receptor (ER) (+) breast cancer cell lines using ChIP-seq.[12,13] Multiple studies have demonstrated a regulatory role for GRHL2 in ER-mediated transcriptional activity.[14–16] Hence, we examined the presence of GRHL2 binding regions in the binding sites for ER alpha and its regulators; FOXA1 and GATA3. However, only a surprisingly small subset of intersecting binding sites was found, consistent with the findings reported by Jozwik et al.[17]

While genome-wide distribution of GRHL2 motifs identified putative candidate target genes of GRHL2, we further investigated their transcriptional regulation by GRHL2 in luminal breast cancer. A conditional knock-out model in a luminal breast cancer cell model; MCF-7 was employed to measure dynamic changes in nascent mRNA using Bru-seq. Differential transcriptional changes were observed in response to GRHL2 deletion. We evaluated direct and indirect regulation of such genes by integrating ChIP-seq and Bru-seq results. A significant reduction was observed in the transcriptional activity of a set of genes associated with cell-cycle and DNA replication; EHF, E2F2 and CDCA7L.[18–20] Our study elucidated a direct transcriptional regulation of some of these genes facilitated by GRHL2 binding to their respective promoter regions. The loss of GRHL2 in MCF-7 cells also resulted in downregulation of cell growth, and our attempts to rescue this phenotype through EHF

upregulation were unsuccessful, suggesting the involvement of additional cell-cycle regulatory genes or factors.

The interactions between GRHL2 and a set of EMT-TFs were also evaluated after GRHL2 deletion. Epithelial EMT-TFs; OVOL2 and CDLN4 were identified as direct targets of GRHL2 while only CLDN4 mRNA was altered in response to GRHL2 loss. A critical target of the EMT-TF; CDH1 which encodes the E-cadherin cell-cell adhesion receptor, is a known direct target of GRHL2. However, unlike other studies,[21] it remained unaffected by the loss of GRHL2 expression in our study. In addition, no GRHL2 binding in the promoter region of ZEB1 was found in contrast to the earlier reports showing direct negative regulatory feedback loop between GRHL2 and ZEB1.[13,22,23] These findings highlight the cell-type specific actions of GRHL2 and suggest the involvement of other mechanisms like post-transcriptional regulations.

Nest, a similar integrative approach was taken in three luminal and three basal-a breast cancer lines to unveil the different biological functions of GRHL2 in distinct breast cancer subtypes (**chapter 4**). Analysis of ChIP-seq data showed common changes in cell migration, epithelial proliferation and cell-cell junctions in both subtypes. Dual roles have been attributed to GRHL2 as a tumor suppressor and promoter in many cancers.[24–27] In agreement with our conclusion in chapter 3, cell-cycle arrest was the dominant response to GRL2 loss in luminal cell line, MCF-7. This effect was less pronounced in a basal A cell line, HCC1806. Indeed, this points to distinct roles for GRHL2 in different breast cancer subtypes. The differential response in growth might be explained by the enhanced activities of hormone receptors in luminal breast cancer. Elevated ER signaling in tumors is correlated with poor prognosis[28,29] and is linked to increased cell proliferation. ERα signaling supports the cell proliferation in MCF-7 cells by increasing the transcriptional activities of PCNA/E2F1 and inhibiting the induction of cell-cycle arrest via p53/p21 axis.[30]

In contrast to the findings in MCF-7, the deletion of GRHL2 resulted in increased cell migration in HCC1806 cells, accompanied by upregulation of N-

cadherin and Vimentin; mesenchymal genes.[31,32] Previous studies have linked the enhanced activation of mesenchymal markers to increased cell migration.[33,34] Although the downregulation of E-cadherin occurred as a common finding in both cell types, it didn't further induce a full EMT in luminal breast cancer, suggesting the necessity of other changes co-existing also in the mesenchymal spectrum of EMT.[35,36] Our findings indicate that such changes are already present in basal A breast cancer cells and here depletion of GRHL2 does activate an EMT. Altogether, these results point to a subtype-specific role for GRHL2 in breast cancer.

In vivo experiments supported the oncogenic role of GRHL2, given its location in the frequently amplified region (8q22) in many cancers.[36–38] The aspects of EMT induced by GRHL2 deletion in HCC1806 suggested that GRHL2 may have tumor- or metastasis suppressive roles in this model, as opposed to luminal cells where our findings pointed to a largely tumor promoting role. We investigated this in a basal A orthotopic transplantation model. However, these experiments indicated that despite the more obvious EMT upon GRHL2 depletion, also in basal-A cells tumor growth as well as metastasis are supported rather than suppressed by GRHL2.

Multifaceted roles of GRHL2 in cancer cell signaling and targeted therapies
Our findings in chapter 3 and 4 delineated the landscape of gene networks regulated by GRHL2 in luminal and basal A breast cancer subtypes. By using the data obtained in chapter 3, we evaluated the changes in a set of EMT-related genes after the induction of GRHL2 loss in MCF-7 cells (**chapter 5**). Our data revealed no changes in the expression patterns of epithelial markers and mesenchymal cell markers other than CLDN4. This indicated the absence of EMT-induction by GRHL2 deletion in luminal cancer, corroborating the results presented in chapter 4.

To understand the mechanisms underlying differential regulation of cellular processes controlled by GRHL2, we next profiled the kinase activities associated with the breast cancer signaling in luminal breast cancer. Several signaling pathways; estrogen receptor (ER), PI3K, Hedgehog (HH), TGFβ and

androgen receptor (AR) were analyzed with a qPCR-based platform, designed for its use in the clinic to determine personalized therapies for breast cancer patients.[39] GRHL2 exerts its diverse functions by rewiring signaling pathways in many cancer types.[15,40] The elevated GRHL2 expression was shown to induce MAPK activity, resulting in suppression of TGFβ mediated epithelial plasticity and carcinogenesis in oral cancer.[41] However, we have not observed any changes in MAPK activity in GRHL2 deleted cells. This pattern was also observed across other pathways except for PI3K and TGFβ, being upregulated upon GRHL2 loss. The importance of TGFβ signaling and its function in inducing EMT to sustain tumorigenesis have been implicated by several studies.[42,43] The tumor suppressing function of GRHL2 is often linked to the downregulation of TGFβ signaling,[44,45] supporting our analysis in luminal breast cancer. The potential rationale for the pathways remaining unaffected by GRHL2 deletion could be attributed to the utilization of a conditional CRISPR-Cas9 knockout system. The analyzed samples for the pathway analysis were originated from a knockout study conducted for 8 days in MCF-7 cells. It is possible that a longer duration for GRHL2 knock-out is necessary for the modifications of the post transcriptional machinery and signaling pathways.

EMT is defined by the balance between epithelial and mesenchymal states, and its progression is characterized by the gain of mesenchymal characteristics which is associated with therapy resistance.[5,46] As elucidated in chapter 3 and 4, GRHL2 plays a pivotal role in determining the balance between EMT and MET in breast cancer but, its deletion in luminal cells is not sufficient to drive an EMT. The basal B subtype of breast cancer is characterized by its enhanced mesenchymal features, limiting the response to the therapies.[47] Consequently, our investigation centered on understanding whether expression of GRHL2 would be sufficient to suppress the mesenchymal phenotype and affect the therapy response in basal B breast cancer.

Overexpression of GRHL2 in the basal B subtype breast cancer cell line, MDA-MB-231 did not induce alterations in the expression patterns of any epithelial markers (Occludin, CLDN4, E-cadherin, ZO-1) or mesenchymal markers (Vimentin and Zeb1). Differing from our findings, overexpression of GRHL2 has

been shown to induce MET-like changes, both phenotypically and genotypically, including increased E-cadherin expression.[22] However, both studies showed that cell growth remained unaffected, unlike the changes observed in Chapter 4 in the luminal and basal A subtypes of breast cancer. This indicates that GRHL2 manipulation on its own, does not suffice to drive an EMT in a basal B model suggesting that other, critical regulators of the epithelial/mesenchymal balance must be altered.

We next assessed the drug responses facilitated by GRHL2 in absence of confounding influences of changes in the EMT/MET balance. MDA-MB-231 cells, with and without GRHL2 overexpression, were treated with a small kinase inhibitor library, and drug responses were evaluated based on changes in cell growth. Similar to a previous study showing the co-operation of GRHL2 with PI3K/Akt pathway in colorectal cancer,[48] we also found two candidate kinases targeting PI3K pathway, exhibiting GRHL2 mediated sensitivity. However, this vulnerability wasn't further validated. Altogether, the findings in chapter 5 indicate that GRHL2 loss in basal B cells is not sufficient to drive an EMT and in absence of such an effect, the impact on therapy response is limited or absent.

Studying tumor-immune cell interactions in the context of GRHL2-mediated immune evasion

The interaction between GRHL2 and immune regulatory mechanisms has been only minimally addressed by studies thus far. By integrating the data from breast cancer cell lines and breast adenocarcinoma patient tumors, we detected a significant negative correlation of GRHL2 with expression of the ecto-enzyme, NT5E/CD73 **(chapter 6)**. Based on our findings in chapter 3 and 4, we identified the CD73 encoding gene, NT5E as one of the direct targets of GRHL2 in luminal breast cancer.

Several studies have emphasized the role of elevated adenosine levels, facilitated by CD73 in tumor cells, in immune evasion.[49,50] Our investigation revealed that the loss of GRHL2 in luminal breast cancer increases CD73-mediated extracellular adenosine production. However, tumor cells are not the

sole contributors to the adenosine production. Studies have demonstrated that immune cells with pro and anti-tumor capabilities including NK cells,[51] macrophages,[52] and cytotoxic (CD8+) T cells,[53] also significantly contribute to elevated adenosine levels within the tumor microenvironment.

To delineate the impact of GRHL2-controlled adenosine production on luminal breast cancer, we employed a trans-well migration model to study tumor-immune cell interactions in response to GRHL2 loss. Surprisingly, we found that the absence of GRHL2 increased the CD8+ T cell migration, which could be reverted by a CD73 inhibitor. The finding that CD73-mediated adenosine production in tumors may actually increase, rather than decrease immune infiltration was supported in clinical samples showing a positive relation between CD73 and Cd8+ T cell presence although the correlation was weak. This unveiled a novel role for GRHL2 in shaping the immune response within luminal breast cancer. Other studies have focused on the impact of extracellular adenosine on the cytotoxic activity of CD8+ T cells.[54,55] As previously displayed in chapter 4, GRHL2 deletion induces cell-cycle arrest in luminal breast cancer. Therefore, we were unable to investigate the cytotoxic effects mediated by adenosine using our conditional knockout model.

Studying the immune evasion related mechanisms in 2D might underestimate the complexity of the tumor microenvironment. During tumor progression, remodulation of the tumor microenvironment, including the formation of a collagen-rich, stiff extracellular matrix (ECM) occurs.[56] It has been reported that the highly dense ECM had an impact on the cytotoxic activity of immune cells[56,57] and the profile of T cells,[58] representing a mechanism of tumor immune evasion. Hence, it will be interesting to further explore the impact of GRHL2 loss on interactions with the immune system in 3D co-culture systems and using *in vivo* models.

Conclusion and future perspectives

In conclusion, this thesis examines multifaceted roles of GRHL2 across breast cancer subtypes. We explore the underlying mechanisms that support cellular plasticity and their implication for the cancer therapy. We outline the

signaling networks orchestrated by GRHL2 in luminal breast cancer and discern differential roles of GRHL2 in cell growth and cell migration between luminal vs. basal A subtypes of breast cancer. Our research highlights that altering GRHL2 expression is, by itself, not sufficient to drive EMT in luminal, or MET in basal B subtype breast cancers. This may also explain that in our studies, no significant correlation is identified between GRHL2 expression and therapy responses tested. Moreover, a novel immunomodulatory function via the NT5E/CD73-extracellualr adenosine axis is identified in luminal breast cancer.

It will be important to unravel the interaction between co-factors and GRHL2 in different breast cancer subtypes using co-immunoprecipitation and other approaches, to understand to mechanisms underlying context-dependent GRHL2 controlled cell functions. The use of patient derived xenografts or organoid models will provide more insights for the tumor heterogeneity in different breast cancer subtypes. This will allow the identification of more clinically relevant GRHL2-regulated mechanisms underlying its role in tumor growth, metastasis, and therapy response. 2D tumor models lack the complexity of the tumor microenvironment and lack the abundance of metabolites and cytokines, secreted by numerous cell types in the tumor microenvironment. With respect to the novel GRHL2-regulated interaction with CD8+ T cells we discover, further exploration of this mechanism in complex 3D or *in vivo* models are warranted to place in context of the diverse tumor microenvironment components that also contribute to extracellular adenosine production. Overall, this thesis illuminates novel insights into the context and subtype-specific roles of GRHL2 in breast cancer subtypes and offers opportunities for targeted vulnerabilities in breast cancer therapy.

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