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PRINCIPAL INVESTIGATOR: Steven Frisch

CONTRACTING ORGANIZATION: West Virginia University Research Corporation

Morgantown, WV 26506-9142

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Steven Frisch

E-Mail: sfrisch@hsc.wvu.edu

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West Virginia University
Dept. Of Biochemistry and Molecular Pharmacology
PO Box 9142
Morgantown, WV 26506-9142

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14. ABSTRACT

The epithelial-mesenchymal transition (EMT) plays an important role in the genesis of renal cell carcinomas (RCC). Signaling through the Hepatocyte Growth Factor (HGF) – Met pathway plays a particularly crucial role in this EMT. We have now found that the expression of Grainyhead-like-2 (GRHL2) attenuates the HGF-Met pathway by suppressing the expression of Met target genes, including multiple matrix metalloproteases, The globally acting co-activator protein p300 interacted directly with GRHL2 protein, and GRHL2 inhibited activation of transcription through p300. This novel mechanism offers a unique opportunity to globally suppress pro-invasive/pro-metastatic gene expression in RCC.

15. SUBJECT TERMS
Grainyhead-like-2/kidney/collecting ducts/proximal tubules/distal tubules/clear cell renal carcinoma

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1. INTRODUCTION: The original intent of our project was to test the role of an epithelial master-programming transcription factor called Grainyhead-like-2 (GRHL2) in preventing the Epithelial-to-Mesenchymal Transition that was believed to be a characteristic of clear cell renal carcinoma (ccRCC). We were also planning to test the role of GRHL2 in regulating anoikis in normal and transformed renal cells. New and surprising results were obtained, as described below, that inform a new perspective on this problem.

2. KEYWORDS: Anoikis/Epithelial-Mesenchymal Transition/clear cell renal carcinoma/Grainyhead-like-2/Von Hippel-Lindau protein/proximal tubules/distal tubules/collecting ducts/kidney

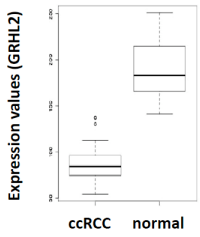
3. OVERALL PROJECT SUMMARY

Objective 1. To determine whether GRHL2 is a tumor suppressor for RCC.

In breast cancer, we had published previously that GRHL2 is a suppressor of the oncogenic epithelial-mesenchymal transition (EMT) that is down-regulated specifically in a subclass of breast cancer ("claudin-low") that is characterized by widespread EMT (*1, 2*).

With regard to clear cell renal carcinoma (ccRCC), analysis of published microarray data using the GEO database indicated a significantly decreased expression of GRHL2 in ccRCC compared to normal kidney (figure 1). Moreover, abundant literature has characterized ccRCC as an EMT-derived tumor type (e.g., (*3, 4*)). Combining these two concepts, we hypothesized that: a. the VHL defect in most ccRCC directly or indirectly down-regulates GHRL2 expression; b. the down-regulation of GRHL2, as in breast cancer, is a critical step for tumor cell commitment to EMT and anoikis-resistance.

Figure1. Decreased expression of GRHL2 in ccRCC compared to normal kidney (*1*)



With this in mind, we obtained a collection of normal and transformed kidney cell lines and examined the expression of GRHL2 as well as the effect of ectopically expressing GRHL2 on normal vs. tumor phenotypes. This panel included: RCC4 (ccRCC) cells and RCC4 plus VHL re-expression (*5*); 786-0 ccRCC, 786 plus VHL re-expression (*6*); HKC-8 (normal proximal tubule cells), HK-2 (normal proximal tubule cells), UMRC3 (ccRCC), UMRC3 with re-expressed Soluble Frizzled Receptor-3 (*7*), mIMCD3 (inner medullary collecting duct epithelial cells, mouse) and MDCK (distal tubule or collecting duct, canine). The results of these studies were as follows:

a. Initial preliminary data indicated that all these lines expressed GRHL2 to various extents on western blots, using a well-characterized "Atlas Project" GRHL2 antibody.
b. There were two problems with the identification of this western blot band as GRHL2, however. First, the band in HKC lysates migrated slightly slower than the known GRHL2 band in control (HMLE cell) lysates (figure 2a). Secondly, three different shRNAs directed against GRHL2 produced little or no knockdown of the band in HKC cells (figure 2b), while significantly knocking down the control GRHL2 band in HMLE cells (*1, 2*).
c. qRT-PCR data indicated lack of significant expression of GRHL2 mRNA in the cell lines listed above, except in the case of MDCK cells and mIMCD-3 cells –notably, both collecting duct-derived cell lines (although MDCK is sometimes cited as a distal tubule cell line).

Figure 2a. Gel mobility of anti-GRHL2 reactive band differs in HMLE vs. HKC cells.

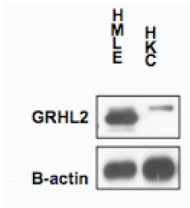


Figure 2b. GRHL2 shRNA only modestly reduces the 70 kDa band in HKC cells.

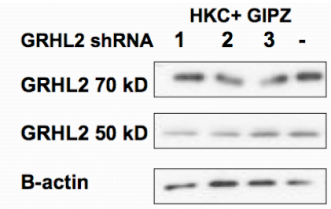


Figure 3. Lack of GRHL2 mRNA expression in HKC and 786 cells (qRT-PCR). Yellow bars: GRHL1, blue bars: GRHL2

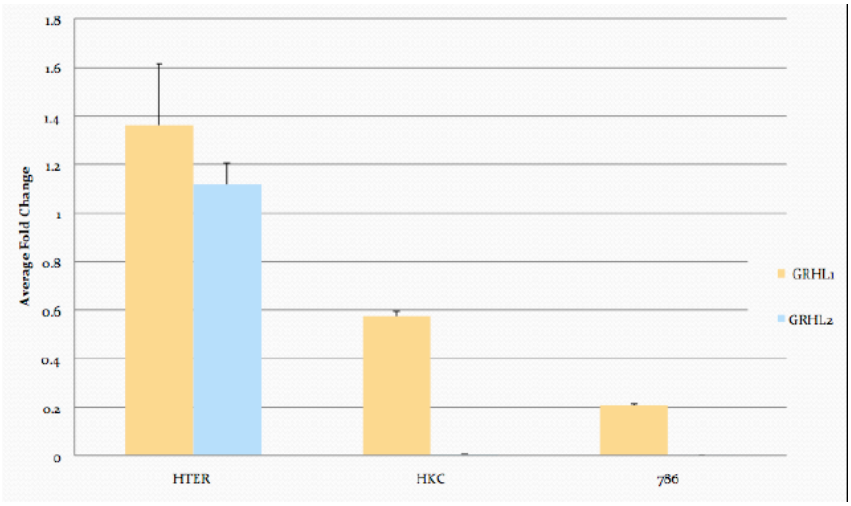
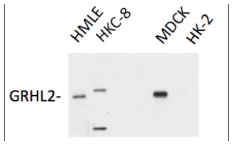


Figure 4a. MDCK cell GRHL2 is knocked down by GRHL2 shRNA

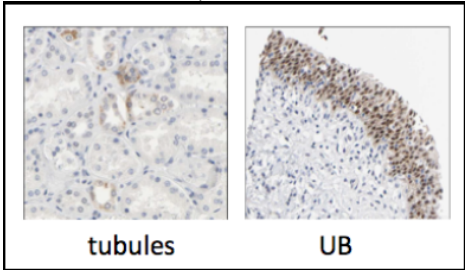
Figure 4b. MDCK GRHL2 co-migrates with HMLE GRHL2.



d. By contrast, in the two collecting duct-derived cell lines, mIMCD3 and MDCK, GRHL2 was expressed at high levels, co-migrated with HMLE-derived GRL2 on a western blot and could be knocked down efficiently with GRHL2 shRNA (figure 4a, 4b and data not shown).

e. Examination of the Atlas database for tissue specific expression using validated antibodies indicated only weak expression in tubules, that was mostly cytoplasmic (note that GRHL2 is a nuclear transcription factor). By contrast, the urinary bladder (UB) which is of the same tissue origin and cell type (urothelial cells) as the collecting ducts, was highly positive for nuclear GRHL2 (figure 5).

Figure 5. Expression of GRHL2 by IHC in kidney tubules and urinary bladder (data from http://www.proteinatlas.org/ENSG00000083307/normal)



In summary, these results indicate that renal cells derived from the metanephric mesenchyme – which include essentially all the cells within mature nephrons—are essentially negative for GRHL2 expression. These are the cells, however, that give rise to ccRCC. By contrast, the cells of the uretary tree/collecting duct (epithelial cells induced to form these structures by induction of GDNF/Ret signaling of the Wolffian duct during development) are GRHL2

positive and do not give rise to ccRCC. This raises the important question of whether GRHL2 expression is a potent protective factor against RCC, analogous to its tumor suppressor role in breast cancer.

Objective 2. To determine how GRHL2 suppresses EMT. As explained above, the precursor cells for ccRCC are GRHL2-negative. Plausibly, it is this property that makes them susceptible to the partial EMT accompanying oncogenic transformation. We therefore examined the effects of GRHL2 expression on the responses of renal epithelial cells to TGF-b and HGF. The rationale for this was that both of these factors contribute significantly to ccRCC development, although TGF-b is a potent pro-fibrotic agent in the kidney while HGF suppresses fibrosis (*8-10*).

Our results are summarized as follows:

We showed in our previous progress report that constitutive GRHL2 expression (by infection with a GRHL2 retroviral vector) suppressed the HGF- induced collagen gel invasion of 786-0 RCC cells. Subsequently, we profiled gene expression in MDCK cells with GRHL2 shRNA vs. GRHL2 stable over-expression, either treated with HGF or untreated.

The overall results are summarized in table 1. They indicate that the expression of GRHL2 attenuated the expression of several HGF/Met target genes.

In light of the crucial role of MMPs in the invasion of RCC tumors, we investigated this gene family further. First, the suppression of MMP-1 and MMP-13 expression by GRHL2 was confirmed by qRT-PCR (figure 6).

Table 1. RNA-seq summary, comparing MDCK (renal collecting duct) cells +/- HGF and with GRHL2 shRNA vs. GRHL2 over-expression.

Genes regulated by GRHL2		
Gene ID	Fold Change (shGRHL2/ GRHL2)	Significance in kidney development
MMP-13	15.1	Necessary for MDCK tubulogenesis (33)
MMP-1	5.0	Necessary for MDCK tubulogenesis (34), regulated only in the presence of HGF
BMP-4	3.5	Inhibits ureteric branching morphogenesis (31)
TIMP1	3.3	Necessary for MDCK tubulogenesis (33)
GFRα	-2.7	RET ligand-binding co-receptor [1] (38), regulated only in the absence of HGF
Genes regulated by HGF in the absence of GRHL2		
Gene ID	Fold Change (shGRHL2+hgf/ shGRHL2)	Significance in kidney development
FGF9	-23.6	Maintains stemness of nephron progenitors (27)
DUSP9	-22.6	Controls Erk activity in mouse embryonic stem cells (28)
SLIT2	-9.8	Restricts kidney induction to a single site (29)
Wnt11	-7.6	Cooperates with GDNF/RET in regulating ureteric branching (30, 31)
Wnt8a	6.4	Interacts with the BMP-4 pathway (32)

The promoter of the MMP1 gene was then found to be repressed by GRHL2 in co-transfection assays (figure 7.)

In light of the important role of AP1 transcription factor in activating the expression of multiple MMP genes, we then tested the effect of GRHL2 on AP1 activity in co-transfection assays. AP1 activity was inhibited strongly by the co-expression of GRHL2 (figure 8).

The crucial role of p300/CBP co-activator proteins in AP1 function is well-characterized. This motivated us to test the effect of GRHL2 upon the ability of p300 to activate transcription (using an assay that was independent of AP1 activity per se, figure 9).

These data suggested that GRHL2 protein directly or indirectly inhibited the transcriptional activation function of p300, thereby preventing MMP expression and tumor cell invasion. To determine whether GRHL2 and p300 proteins interact, an endogenous co-immunoprecipitation was performed, in which p300 was immunoprecipitated (from HMLE cells) and the presence of GRHL2 was determined by western blotting (figure 10).

Figure 6. GRHL2 represses MMP expression in MDCK renal collecting duct cells.

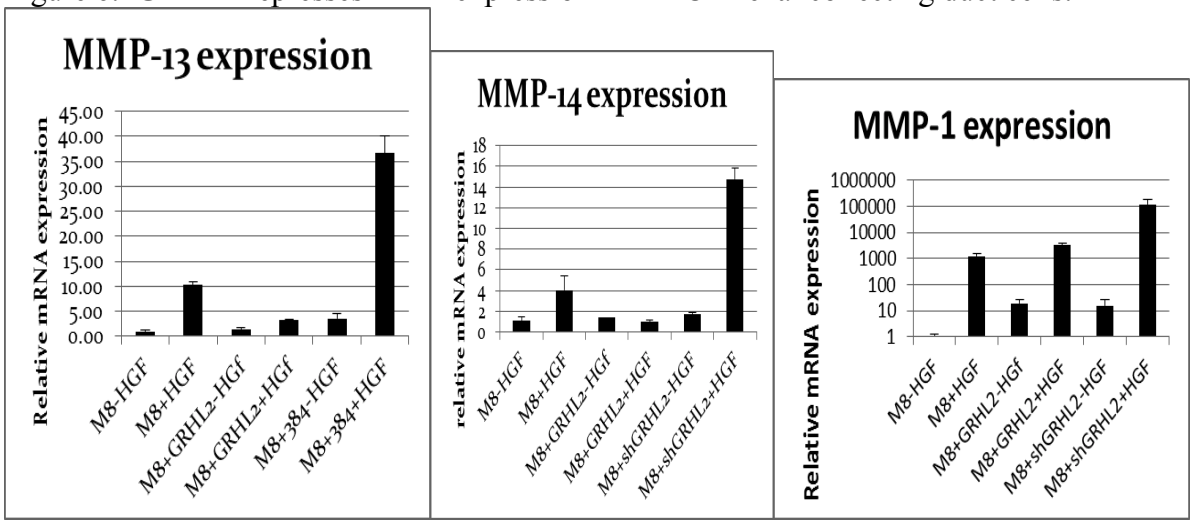


Figure 7. GRHL2 represses the MMP1 and MMP14 promoters (co-transfection assays)

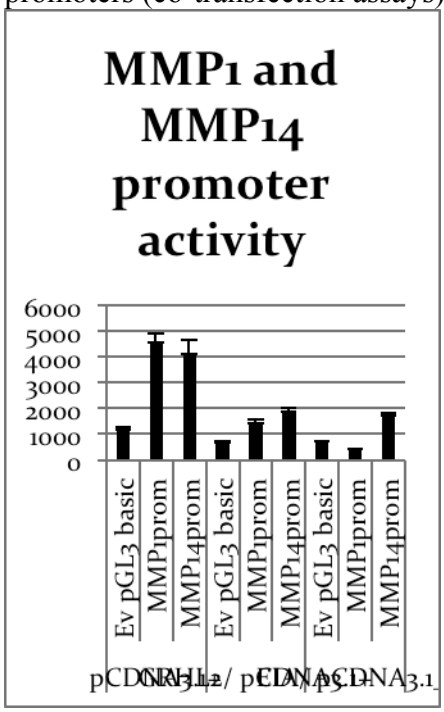


Figure 8. GRHL2 represses transcription that is mediated by AP-1.

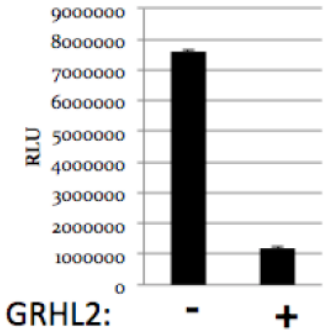


Figure 9. GRHL2 inhibits the transcriptional activity of p300. The principle of the assay is diagrammed below the graph.

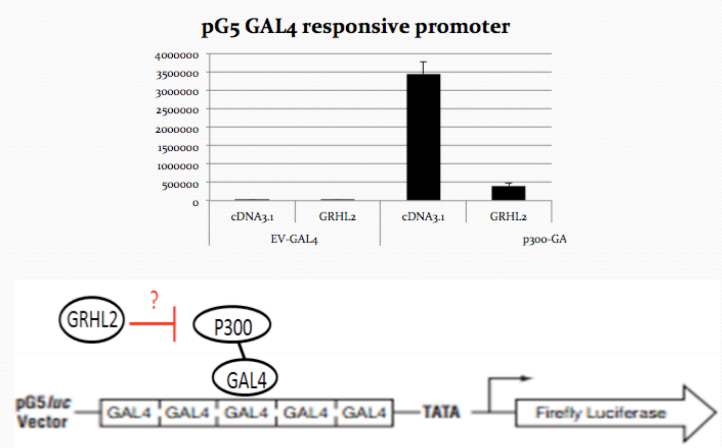
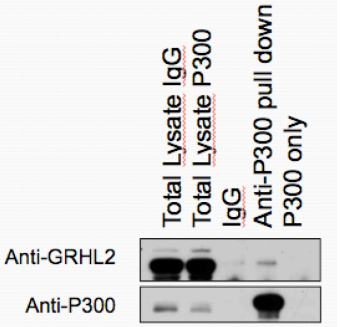


Figure 10. GRHL2 interacts with p300. Endogenous co-immunoprecipitation; IP: p300, blot: GRHL2.

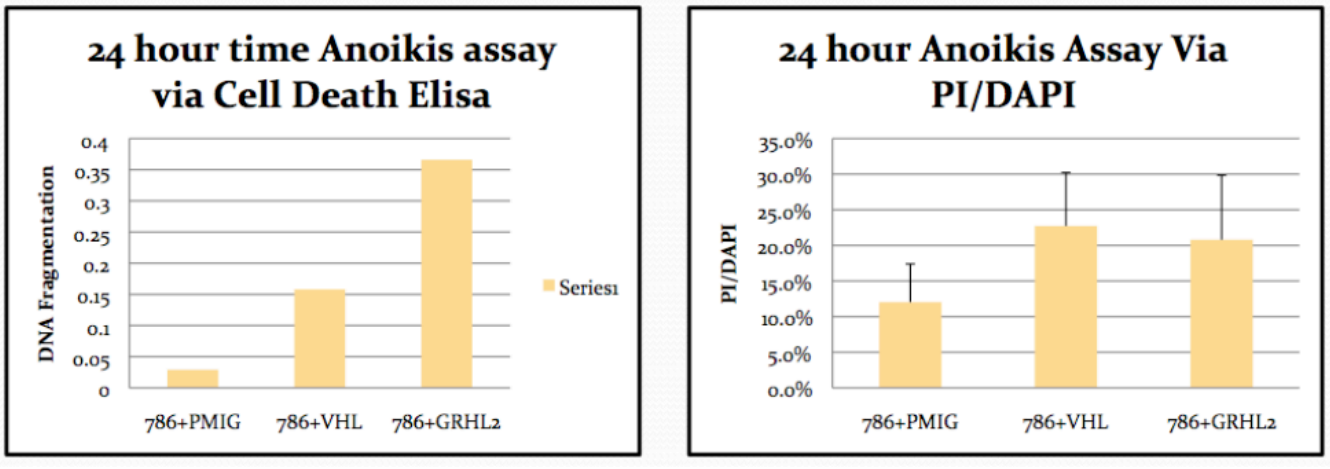


Objective 3. To determine how GRHL2 suppresses EMT-mediated anoikis-resistance.

We are currently testing the hypothesis that GRHL2 confers anoikis-sensitivity by reprogramming gene expression in tumor cells, altering intracellular metabolism such that anoikis is augmented.

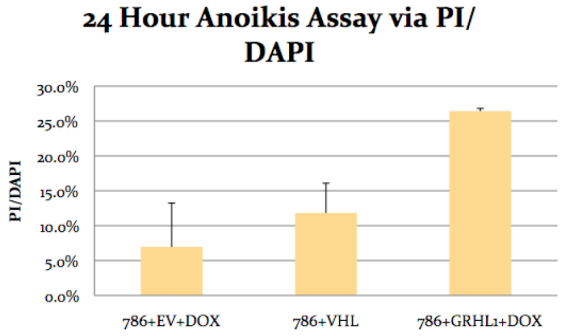
To determine how GRHL2 suppresses EMT-mediated anoikis-resistance, thus preventing RCC progression. The initial phase of this aim was to test the effect of GRHL2 expression on anoikis sensitivity in normal or ccRCC cell lines. To initiate this study, we expressed GRHL2 in the (GRHL2-negative, VHL-negative) ccRCC cell line, 786-0 and assayed this pair of isogenic cell lines, together with the 786-0+VHL cell line. Interestingly, either re-expression of VHL or ectopic GRHL2 expression sensitized 786-0 cells to anoikis (figure 11).

Figure 11. GRHL2 and VHL sensitize 786-0 RCC cells to anoikis. Assayed by Cell Death ELISA (left) or % PI positive (apoptotic) cells (right).



Additionally, the constitutive expression of GRHL1, which was expressed endogenously in 786-0 cells at low levels (data not show), also sensitized the cells to anoikis (figure 12).

Figure 12. Constitutive expression of GRHL1 sensitizes 786-0 cells to anoikis.



KEY RESEARCH ACCOMPLISHMENTS:

--Discovery that GRHL2 suppresses MDCK cell collagen gel invasion in response to HGF
--Discovery that GRHL2 suppresses matrix metalloprotease gene expression
--Discovery that GRHL2 inactivates p300, thereby preventing transcription factors such as AP1 and others from inducing a pro-invasive gene expression program

4. CONCLUSION

The results of this project have proven significant beyond our best expectations. P300 protein is universally required for EMT of renal cell carcinoma and other cancer types. In this project, we have identified GRHL2 as a novel suppressor of p300 function. This has important implications for both basic tumor biology and for translational value. In particular, the use of existing or newly developed p300 inhibitors may prove clinically useful. This will be explored in the near future.

5. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

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The use of existing or newly developed p300 inhibitors may prove clinically useful. This will be explored in the near future.

8. OTHER ACHIEVEMENTS:

9. REFERENCES: List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

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10. APPENDICES

N/A