Identification and Characterization of Ror2 as a Tumor Cancer Cell Specific Kinase in Renal Cell Carcinoma

Tricia M Wright

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Approved by:

W. Kimryn Rathmell, MD, PhD (Advisor)

Adrienne D. Cox, PhD

Ian J. Davis, MD, PhD

H. Shelton Earp, MD

William Y. Kim, MD

ABSTRACT

TRICIA M WRIGHT: Identification and Characterization of Ror2 as a Tumor Cancer Cell Specific Kinase in Renal Cell Carcinoma
(Under the direction of W. Kimryn Rathmell)

With the recent advent of molecularly targeted therapy, the potential for novel treatment options for carcinomas has been brought to the forefront. However, few tumor cell-specific targets exist for many cancers. Our goal was to identify and characterize cell specific kinases for the difficult to treat epithelial cancer renal cell carcinoma (RCC). Using a phospho-specific receptor tyrosine kinase screen, we identified Ror2, a developmentally regulated orphan receptor kinase, to be overexpressed in RCC tumors and cell lines. Ror2 is normally expressed in the heart, brain, kidney and lungs of developing mice and has also been implicated in the Wnt/β-catenin signaling pathway. Using archival RCC tumor specimens, we show that Ror2 was coordinately overexpressed with genes involved at the extracellular matrix (ECM), including MMP2. Consequently, when Ror2 was suppressed in cell lines, MMP2 expression was also suppressed, suggesting Ror2 may play a role in directing MMP2 expression. Ror2 suppression also supported cellular migration, without affecting doubling time or viability. Additionally, inhibition of Ror2 limited RCC growth in soft agar, a surrogate for invasive growth potential and anchorage independence, and produced fewer tumors *in vivo* in xenografts.

Since the discovery of Ror2 in RCC, Ror2 has since been implicated in several other cancers, where it is associated with invasive tumor characteristics. However, the regulatory or mechanistic events contributing to increased Ror2 expression had yet to be defined. We sought to place Ror2 in the most studied pathway in RCC, the inactivation of the tumor suppressor von Hippel-Lindau, *VHL*, leading to hypoxia induced factor (HIF)-1α and 2α dysregulation. We found that Ror2 expression was associated with pVHL loss and that VHL somatic mutations tightly coordinated with HIF-2α stabilization. Knockdown and rescue analysis of HIF expression suggests that Ror2 is dependent on the pathologic stabilization of both HIF1 and HIF2. However, induction of HIF by physiological hypoxia did not affect Ror2, suggesting a unique regulation of Ror2 dependent on non-physiologic HIF activation. This study identifies a novel putative kinase involved in key aspects of RCC tumorigenesis and also offers insight into how Ror2 is deregulated in RCC with potential application to other cancers.

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ABBREVIATIONS

ANOVA	Analysis of variance
aPKC	Atypical protein kinase C
ARNT	Aryl hydrocarbon receptor nuclear translocator or HIF-1β
BDB	Brachydactyl type B
bHLH	Basic helix-loop-helix
BLAST	Basic Local Alignment Search Tool
CAM-1	*C. elegans* ortholog of Ror2
CBP/p300	Creb-binding protein/ E1A binding protein p300
C. elegans	*Caenorhabditis elegans*
ChIP	Chromatin immunoprecipitation
CICBDD	Center for Integrative Chemical Biology and Drug Discovery
$CoCl_2$	Cobalt chloride
CRD	Cysteine-rich domain
cT	Cycle Threshold
CXCR4	Chemokine (C-X-C motif) receptor 4
Cyclin D1	cyclin family member D1
DAPI	4',6-diamidino-2-phenylindole
DMOG	dimethyloxalylglycine
Dsh	Dishevelled
EASE	Expression Analysis Systematic Explorer
ECM	Extracellular matrix

EGFR	Epithelial growth factor receptor
Egln3	Prolyl hydroxylase family member - egl nine homolog 3
EMT	Epithelial to mesenchymal transition
EPAS1	Endothelial PAS domain protein 1 or HIF-2α
EPO	Erythropoietin
FDA	US Food and Drug Administration
FDR	False discovery rate
FIH-1	Factor inhibiting HIF-1α
FLT3	FMS-like tyrosine kinase 3
Fz	Frizzled receptor
GFP	green flourescent protein
Glut1	Glucose transporter 1
GSK3β	Glycogen synthase kinase 3 beta
HIF-1α	Hypoxia inducible factor 1 alpha
HIF-2α	Hypoxia inducible factor 2 alpha or EPAS1
HIF-1β	Hypoxia inducible factor 2 beta or ARNT
H&E	Hematoxylin and eosin
HPRT	Hypoxanthine phosphoribosyltransferase 1
HRE	Hypoxia response element
HRP	Horse radish peroxidase
IB	Immunoblot
IF	Immunofluorescence
IgG	Immunoglobulin G

IHC	Immunohistochemistry
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinases
LC	Loading control
LOF	Loss-of-function
LRP	LDL-receptor-related protein
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MuSK	Muscle specific kinase
neoR	Neomycin resistance gene
NFκB	Nuclear factor κB
Oct-4	Octamer-4 or POU5F1 (POU class 5 homeobox 1)
ODD	Oxygen dependent degradation
PAS	PER-ARNT-SIM domain
PCP	Planar cell polarity
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PHD	Prolyl hydroxylase
pRS	pRetroSuper
PTEN	Phosphatase and tensin homolog
pVHL	von Hippel-Lindau tumor suppressor protein

pY	Phospho-tyrosine
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RCC	Renal cell carcinoma
Rbx1	Ring box protein 1
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SAM	Significance Analysis of Microarrays
SEM	Standard error of means
sFRP1	Secreted frizzled related protein 1
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SV40	Simian virus 40
TAD	Transcriptional activation domain
TIMPs	Tissue inhibitors of matrix metalloproteinases
TSC	Tuberous sclerosis complex family members
Twist1	Twist homolog 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	von Hippel-Lindau tumor suppressor
WT	Wildtype

CHAPTER 1

INTRODUCTION

Pathogenesis of Renal Cell Carcinoma

Sporadic renal cell carcinoma (RCC), one of the top 10 cancers diagnosed in the US per year (1), is the most common kidney malignancy. RCC, a notoriously hard to treat solid tumor malignancy, has minimal sensitivity to traditional chemotherapy and immune system modulation, the treatment of choice for the past 20 years. This devastating disease is diagnosed in over 40,000 new cases per year (1), with over half of those diagnosed developing metastatic disease. Alarmingly, the incidence of RCC is rising steadily at rate of 3% per year with men twice as likely as women to present with RCC. While the causes of renal cell carcinoma are unknown, certain risk factors including smoking and obesity are recognized as potential inducers of RCC, with those who smoke being twice as likely to acquire the disease. Additionally, exposures to certain occupational toxicants such as asbestos, gasoline and trichloroethylene have all been recognized as possible factors escalating the possibility of developing RCC.

Several different pathological types of RCC have been identified including papillary and chromophobe. However, it is clear cell histology of RCC that makes up approximately two-thirds of patients with this disease. Pathologically, clear cell RCC tumors have a highly glycolytic, vascular phenotype in which they accumulate lipids and glycogens in the cytoplasm. As a result, these tumor cells present with a clear cytoplasm and nuclei as compared to the two other histological subtypes, papillary and chromophobe (Figure 1.1) when stained with H&E. These distinct morphological characteristics give these tumors the classification of “clear cell”. This

body of work will focus specifically on the clear cell pathological subtype of RCC (either listed as ccRCC or simply RCC).

Clear cell RCC occurs sporadically and as part of the familial von Hippel-Lindau (*VHL*) disease. Biallelic mutations and functional inactivation of the *VHL* tumor suppressor protein (pVHL) have been identified as major contributory factors to both forms of the disease (3-6). The tumor suppressor gene *VHL* is mutated in over 70% of clear cell RCC histologies leading to overexpression of key growth factors, including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), among many others (7, 8).

The most current and effective therapies for renal cell carcinoma involve the use of kinase-specific inhibitors targeted against receptor tyrosine kinases (RTK) and ligands specific to angiogenesis signaling. However, though these agents have shown promise with a short extension in overall lifespan compared to the control agents, they have yet to be fully optimized. Currently, there are no reported cancer cell specific kinases expressed on RCC identified as targets for tumor cell directed therapy. Though there is an abundance of RTK inhibitors including sorafenib and sunitinib that have shown promise in RCC treatment (9-13), they have yet to be fully optimized providing a rationale for finding cell intrinsic kinase targets in RCC. Finding these kinase targets will in turn provide a rational pharmaceutical target for future therapeutic development.

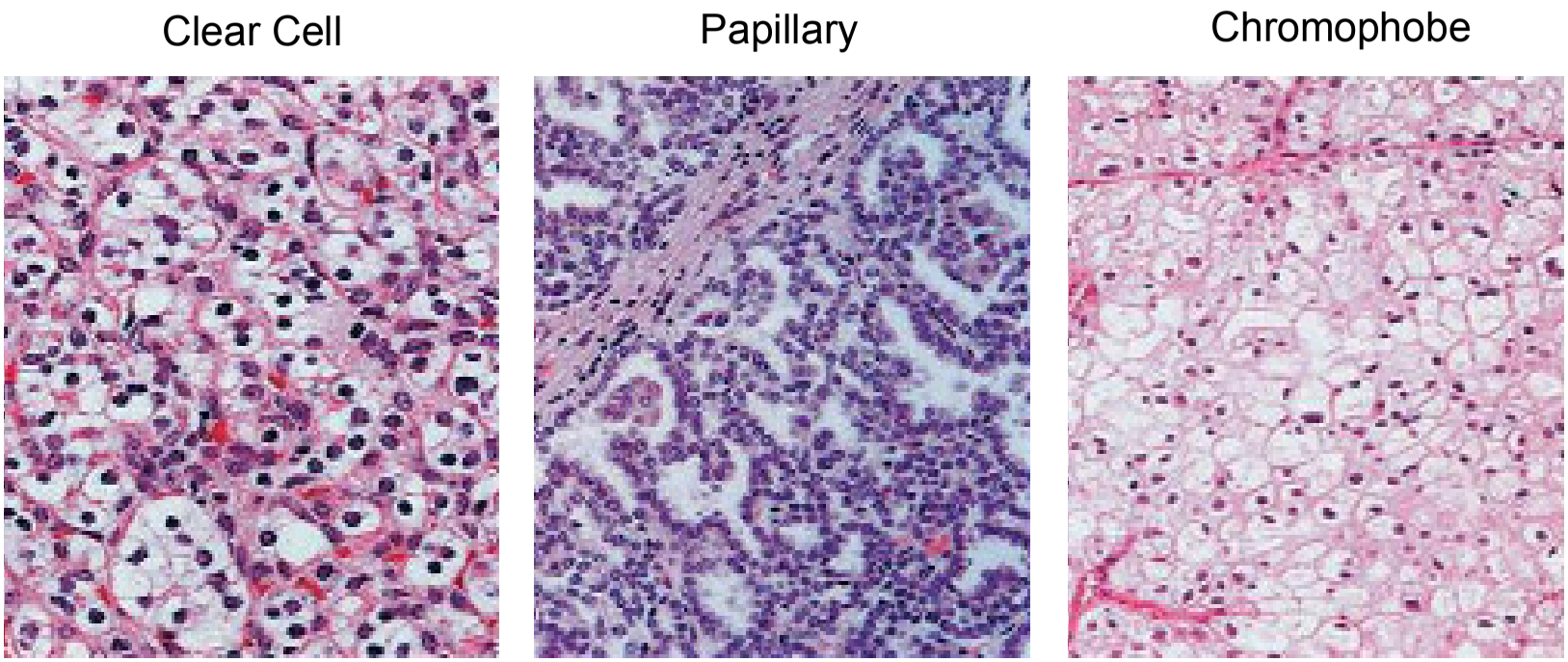

Figure 1.1: RCC histological subtypes. H&E representation of the different histological RCC subtypes. Clear cell RCC tumors represent approximately 70% of RCC cancers recognized in the clinic. The majority of cells is mutated for *VHL* and accumulates lipids and glycogens leading to the "clear cell" phenotype. Tumor cells morphologically show as having a clear cytoplasm devoid of cellular processes as only the nuclei is seen by staining as compared to the other histological subtypes. Figure modified from Linehan WM et al. *J Urol*. 2003 (2).

Molecular Biology of RCC

The von Hippel-Lindau tumor suppressor gene encodes a 213 amino acid (aa) protein, pVHL, whose mainly recognized role is to regulate the cellular response to changes in oxygen. The most well-documented function of the pVHL protein, which encompasses an α and β subunit, is to act as the substrate recognition component of an E3 ubiquitin ligase complex that includes Elongin C, Elongin B, Cullin 2, and ring box protein 1 (Rbx1 or Roc1) (14-17). The majority of clear cell RCC tumors are mutated for *VHL* leading to overexpression of hypoxia inducible transcription factors (HIF) including, but not limited to, VEGF and PDGF (18, 19).

Under normal oxygen conditions and when pVHL is functional, the HIFα subunits (HIF1, HIF2) are hydroxylated on specific prolyl residues by prolyl hydroxylases (known as PHD or Egln) in the oxygen dependent degradation (ODD) domain (20-22). The hydroxylated HIFα subunit is polyubiquitinated after being recognized by and recruited to the pVHL E3 ubiquitin ligase complex and then degraded by the proteasome (Figure 1.2A). Under low oxygen conditions (Figure 1.2B) or when *VHL* is mutated (Figure 1.2C) leading to a pseudohypoxic state, pVHL is unable to bind either HIFα subunit. This inability to bind leads to accumulation of the HIFα subunits which in turn forms a heterodimeric transcriptional complex with HIF1-β (also known as (ARNT) aryl hydrocarbon receptor nuclear translocator) that is translocated to the nucleus. This nuclear translocation leads to transcriptional activation via binding to conserved hypoxia response elements (HREs) and induction of hypoxia inducible target genes including VEGF, PDGF, glucose

transporter 1 (Glut1), prolyl hydroxylase family member Egln3 (also known as PHD3) and many other hypoxia factors (7) (Figure 1.2B, C).

Protein stabilization of the HIF subunits can also be achieved independently of *VHL* mutations through activation of the mammalian target of rapamycin (mTOR) pathway, another key regulator of tumor cell responses. mTOR is an intracellular serine/threonine kinase activated upstream of HIF and is part of the PI3K/Akt/mTOR signaling pathway. There are two distinct macromolecular complexes of mTOR, mTOR complex 1 (mTORC1), which is susceptible to rapamycin and contains raptor and mTORC2, which is rapamycin insensitive and contains rictor. Mutations disrupting mTORC1's proximal negative regulators, the tuberous sclerosis complex family members (TSC) 1 and 2 which is characterized by PTEN loss, predisposes to RCC development (23, 24). When the mTORC1 complex is mutated or otherwise inhibited, the PI3K/Akt pathway is activated leading to mTORC1-dependent protein stabilization (at the level of translational initiation) causing upregulation of the HIF subunits (25). Though more is known about the role of mTORC1, mTORC2's role in RCC is not as well-defined. However there is a difference in the mTOR subunits and the subsequent dependence of the HIF subunits. In fact, HIF-1α is dependent on both mTORC1 and mTORC2 whereas HIF-2α is dependent only on mTORC2 (25). Further, mTORC2 does not respond to rapamycin but appears to be generally associated with the cytoskeleton.

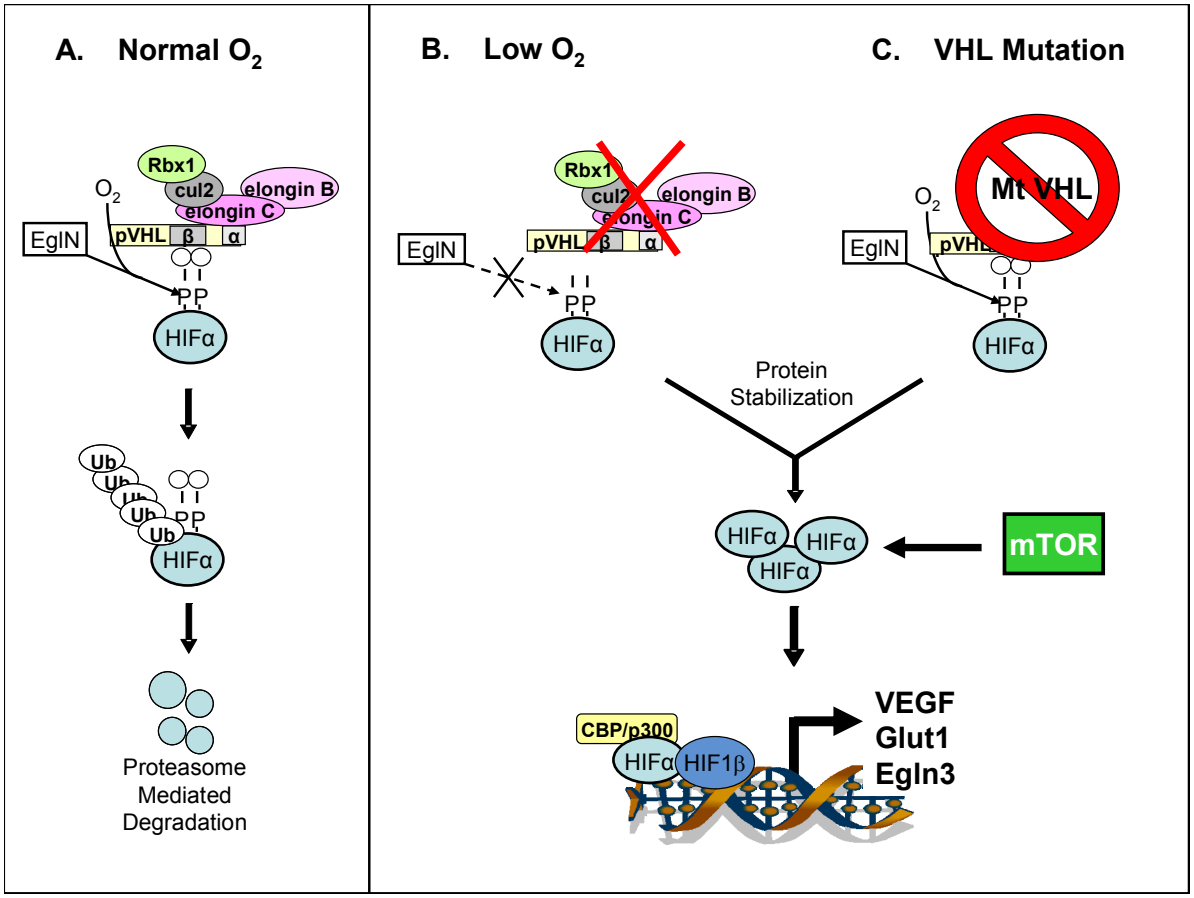

Figure 1.2: The control of O_2 regulated genes.
A. With normal O_2 levels and when pVHL is functional, *VHL* recognizes (specifically, the β subunit) and binds the hydroxylated HIFα subunit. The HIFα subunit is polyubiquitinated and subjected to proteasome mediated degradation.
B. Under low O_2 levels or if **C.** *VHL* is inactivated, *VHL* is no longer able to bind to the hydrolyxated HIFα subunit leading to an increase in binds to HIFα subunits. (mTOR activation also causes upregulation of the HIFα subunits, though this activation is independent of *VHL* inactivation). HIFβ then heterodimerizes with the HIFα subunit, is stabilized and translocates to the nucleus. The stabilized HIF subunit then leads to the transcriptional activation of many HIF target genes. Figure adapted from Rathmell WK, Wright TM & Rini B *Expert Rev Anticancer Ther* 2005 (19).

HIFα independent functions of pVHL

Though *VHL* is widely known to function in response to cellular oxygen changes, it also has other roles within the cell (Figure 1.3). *VHL* has also been shown to have a role in the regulation of apoptosis, maintenance of primary cilia (26, 27) and extracellular matrix remodeling (28, 29).

Apoptotic regulation. RCC is notoriously resistant to cytotoxic treatments and this may be partially due to inactivation of apoptotic pathways or activation of prosurvival pathways. Emerging evidence suggests that pVHL plays a pivotal role in apoptotic regulation. pVHL has been shown to bind to, and inhibit, atypical protein kinase C (aPKC) (30, 31). aPKC has been implicated in cellular processes such as cellular proliferation, cellular survival, and in establishing and maintaining cellular polarity including cell-cell junctional maintenance. *VHL* expression and inactivation of aPKC leads to increased JunB expression which in turn antagonizes c-jun mediated neuronal apoptosis.

Additionally, other HIF-independent *VHL*-mediated factors of apoptosis have been identified including nuclear factor κB (NFκB) and p53. Kidney cancers have increased expression of NFκB (32), normally associated with survival and pVHL loss is shown to activate NFκB expression (33). *VHL* serves as a liaison by binding and phosphorylating the NFκB antagonist Card9 along with casein kinase 2 (CK2) leading to decreased NFκB activity. *VHL* also forms a complex with p53 along with ATM and p300, both of which promote p53 stabilization and activation (34, 35). When under genotoxic stress, there is interaction of p300 and p53 leading to p53

acetylation and increased expression in concert with p53 mediated cell cycle arrest and attenuated apoptosis (34, 35).

Regulation of Primary Cilia. The primary cilium is a microtubule based structure that protrudes from the surface of non proliferative cells and functions to sense molecular and environmental cues (36). Primary cilium is important in the kidney as loss of primary cilia leads to increased proliferation in tubular epithelial cells (reviewed in (37)). This results in the development of large renal cysts which eventually leads to kidney failure (26). Abhorrently large renal cysts are a prominent feature of *VHL* disease as the epithelial cells lining these cysts are *VHL* deficient and have increased HIF responsive gene expression (38). Further, the types of *VHL* disease that patients present with are associated with primary cilia formation as well. Those with Type 2B *VHL* disease are highly predisposed to developing ccRCC and retain the ability to maintain cilia while those with Type 2A *VHL* disease (not highly predisposed to ccRCC) and Type 1 *VHL* disease (highly predisposed to developing ccRCC) fail to maintain primary cilia (39, 40). This presents a paradox because though *VHL* patients frequently have loss of pVHL expression, renal cysts are so infrequent suggesting that pVHL inactivation alone may not be responsible for impaired primary cilium and other signals may play a prominent role as well. In other words, some *VHL* associated ccRCC cancers may not directly arise from renal cysts and though some *VHL* families may manifest renal cysts, they may be low risk and may not necessarily lead to RCC.

Consistent with these findings, several groups have shown that pVHL specifically localizes to primary cilia and is at least partially HIFα independent, both in *in vitro* and *in vivo* studies (26, 27, 40). This was further confirmed in primary cells as loss of pVHL does not specifically affect cilia formation *in vitro*. However, an indirect connection was made to loss of pVHL affecting cilia formation. GSK3β plays a key role in promoting microtubule stability as well as cilium maintenance and this role is *VHL* dependent (41). When GSK3β is inactive (such as when the PI3K-Akt pathway is activated), microtubule stability and cilium maintenance is now dependent on *VHL* expression. Without *VHL* expression, cells lose their cilia in response to GSK3β inactivation (40). Cellular disruptions of microtubules causing dysregulation of migration and polarity have also been implicated in tumor formation (41). As more *VHL* associated microtubule connections are discovered, it will be interesting to explore if *VHL* also plays a role, at least partially, in this microtubule associated function with cilia formation.

Extracellular Matrix Remodeling. One of the most clearly documented roles emerging in recent years is that of the role of *VHL* in extracellular matrix remodeling at both the organization level as well as with organelles located and associated with the ECM. An elegant study performed within the *C. elegans* world, where the simple nematode has a single *VHL* and single HIFα homolog, have opened up a wealth of information regarding pVHL independent of HIF activities and its role at ECM (28). When pVHL was deficient without the interference of HIFα expression, overlapping genes responsible for extracellular matrix (ECM) formation including those encoding

for procollagen hydroxylases (*dpy-18* and *let-268*) and those that encode for secreted metalloproteases (*gon-1* and *mig-17*) were found suggesting a commonality between the ECM and pVHL loss (28). Further, within mammalian cells, biochemical evidence suggests that pVHL directly has a role in the assembly of the extracellular fibronectic matrix (29). Molecular evidence directly links pVHL to ECM assembly *in vivo*, as cells lacking *VHL* expression fail to properly organize the extracellular matrix whereas when wild-type *VHL* was reintroduced into cells, pVHL was able to partially restore the ECM phenotype (29, 42). Additionally, cells mutated for *VHL* have increased expression of the matrix metalloproteinases, MMP2 and MMP9, enzymes important for degradation and remodeling of the ECM and also upregulate MMP inhibitors such as TIMPs (43). Extracellular matrix remodeling is an important aspect of cancer biology as it provides structural support for cells as metastasis has been associated with the destruction of the ECM and now, it is distinctly linked to *VHL*.

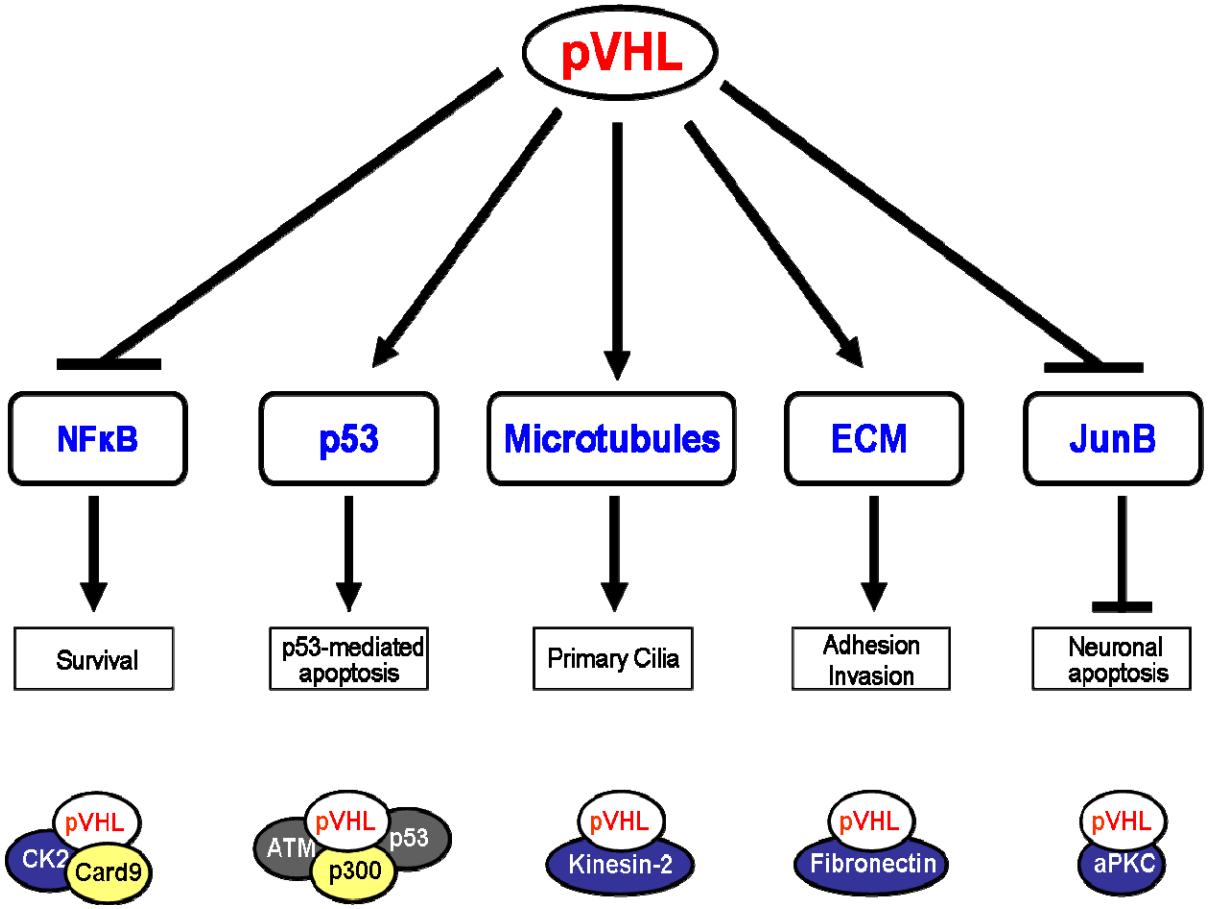

Figure 1.3: HIF independent functions of pVHL. pVHL has other activities and functions that are important for the features listed below, either activating (arrow) or blocking (block) that function. Below each is the cellular effect with the putative interactive partners. Figure adapted from Moch H, *Pathologe* 2008 (44).

Hypoxia Inducible Factors (HIFs)

Cellular adaptation to low oxygen conditions (hypoxia) is a survival mechanism that most mammalian cells have learned to adapt to for successful survival and development. Indeed, hypoxia is also a common feature of many cancers. Hypoxia in cancer occurs when the tumor outgrows the angiogenic regions leading to various areas of the budding tumor having hypoxic regions. The transcriptional response to physiological and pathological hypoxia is primarily mediated by two master regulatory hypoxia-inducible factors, HIF-1α and HIF-2α. Though these HIF factors are highly homologous in sequence (48%) and structure, they share both similar and divergent functions (18, 45, 46).

HIF regulation. Structurally HIF-1α has a basic helix-loop-helix (bHLH) and two tandem PAS domains (N-terminal PAS A and C-terminal PAS B). HIF-1β (also known as aryl hydrocarbon receptor nuclear transporter, ARNT1) also shares similar structural characteristics and encodes bHLH and PAS A and PAS B domains, key mediators of dimerization between the HIF-α and β subunits (47). In addition to containing bHLH and PAS domains, the HIF-1α subunit also has two transcriptional activation domains called TADs whose primary role is to act as recruiters of co-activators and also interacts with them. This recruiting function is crucial to the transcriptional activation of target genes. These domains are of additional importance as HIF-1α also undergoes post-translational regulation via other mechanisms including hydroxylation, phosphorylation and acetylation (48-50). The TADs are further segregated and named by where they are located, at the N-

terminus (N-TAD) or the C-terminus (C-TAD). HIF-1α contains an oxygen dependent degradation (ODD) domain that regulates protein stability based on oxygen levels. This domain encodes the N-TAD domain and has the two prolyls (P402, P577) that are subject to hydroxylation under normal oxygen conditions and recruit the pVHL E3 ubiquitin ligase. The C-TAD domain is responsible for recruiting the transcriptional co-activators, Creb-binding protein CBP/p300. This recruitment is oxygen dependent as under normoxia (or normal oxygen conditions); factor inhibiting HIF-1α (FIH-1) hydroxylates a conserved asparagine residue (N813) in the C-TAD domain preventing interaction with the transcriptional co-activators, CBP/p300 (Figure 1.4).

Similarly, HIF-2α (also known as endothelial PAS domain protein 1, EPAS1) also contains a bHLH, PAS (A & B), and TAD (N-TAD & C-TAD) domains. In fact, there is a 48% sequence similarity between HIF-1α and HIF-2α and within the specific domains, there is a high sequence homology; bHLH (85%), PAS (70%), PAS-A (68%), PAS-B (73%), N-TAD (40%) and C-TAD (69%) domains (51). Structurally, HIF-2α is similar to HIF-1α as described above (bHLH, PAS, N-TAD and C-TAD domains) with two specific prolyl residues (P405, P530) and a conserved asparagine residue (N851) that are subject to hydroxylation, the best characterized posttranslational modification.

However, within the C-terminus is where the major functional differences lie between the HIF-1α and HIF-2α subunits. Though both HIF-1α and HIF-2α can and do bind to hypoxic response elements (HREs) of hypoxia response genes, this binding is not sufficient for target gene activation (51, 52). For target gene

activation, the C-terminal region of HIF-1α and HIF-2α needs to cooperate and function with their specific N-terminal region and that cooperation determines specificity for gene activation (Figure 1.4).

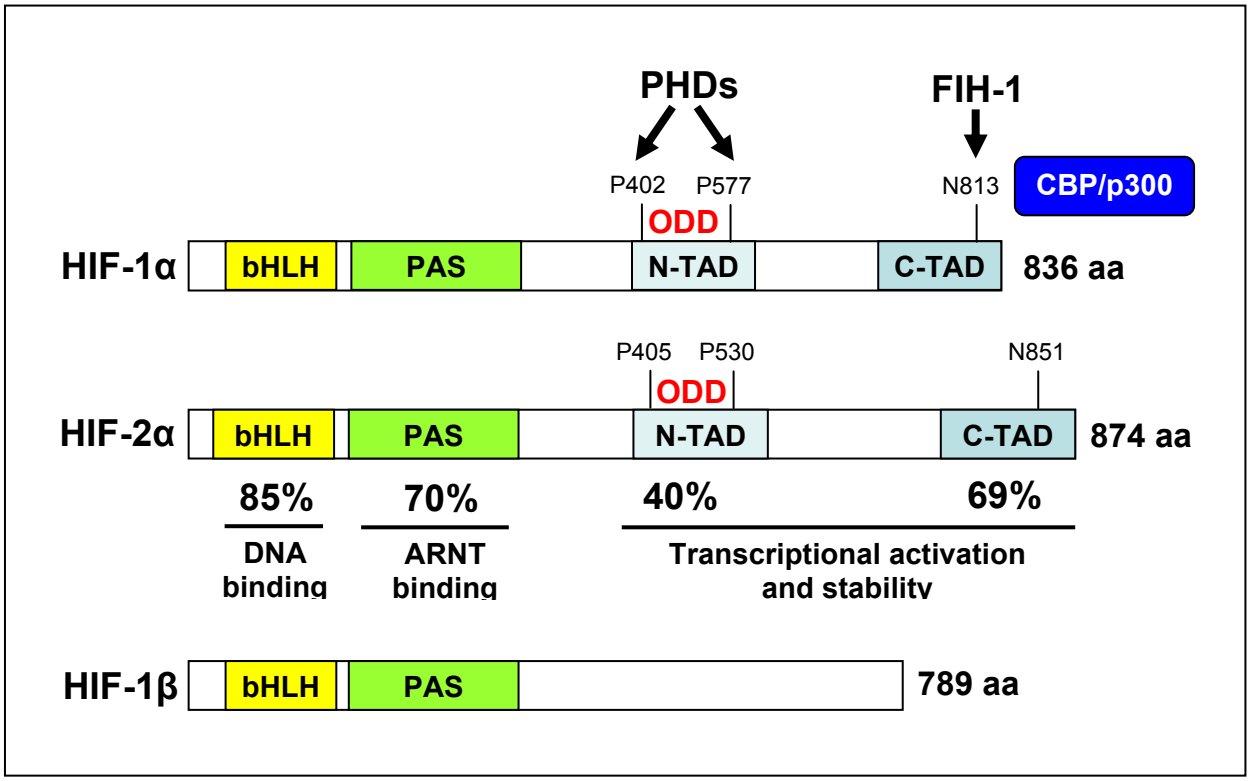

Figure 1.4: Structure of HIF subunits. HIF-1α and HIF-2α are highly similar in structure, particularly in their DNA and ARNT binding sites based on amino acid residue similarity. Likewise, HIF-1β is highly similar but only has DNA binding and ARNT binding sites that heterodimerize with the HIFα subunits. Figure adapted from Hu et al, *Mol Biol Cell* 2007 (51).

HIF targets. HIF-1α is more ubiquitously expressed whereas HIF-2α is expressed in a cell-type specific manner (18, 45, 46) specifically within regions important for oxygen tensions such as the lung, heart and endothelium. HIF-1α and HIF-2α are expressed in multiple malignant human tumors including breast, ovarian, prostate, pancreatic and renal cancers. Both HIF subunits are subjected to dimerization with HIF-1β/ARNT and bind the same sequence (5'-CGTG-3') with hypoxia response elements (HREs), oxygen dependent promoters/enhancers of target genes. Though highly homologous in structure and both bind similar sequences, HIF-1α and HIF-2α each transcriptionally activate analogous and divergent target genes (8).

Both HIF-1α and HIF-2α regulate genes important for angiogenesis including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), glucose transporter 1 (Glut1) as well as those having significant impact on invasion/metastasis including matrix metalloproteinase 2 (MMP2) and the chemokine (C-X-C motif) receptor 4 (CXCR4) (8). However, each subunit uniquely induces the upregulation of specific target genes. HIF-1α induces genes responsible for glycolysis including lactate dehydrogenase-A (LDA) and phospho-glycerase kinase (PKA) and genes responsible for apoptosis including the pro-apoptotic gene BNIP-3 (8, 53). Conversely, the embryonic transcription factor, Oct-4, shown to be important for de-differentiation and stem cell regulation, is the most widely agreed upon unique HIF-2α specific target gene (54). However, there are other genes induced predominantly by HIF-2α including erythropoietin (EPO) as well as genes responsible for cellular proliferation including Cyclin D1 and Twist1 (53, 55, 56). All

this evidence suggests that though the HIF subunits share similar structures, they also have differing functions that diversely affect cellular processes (Figure 1.5).

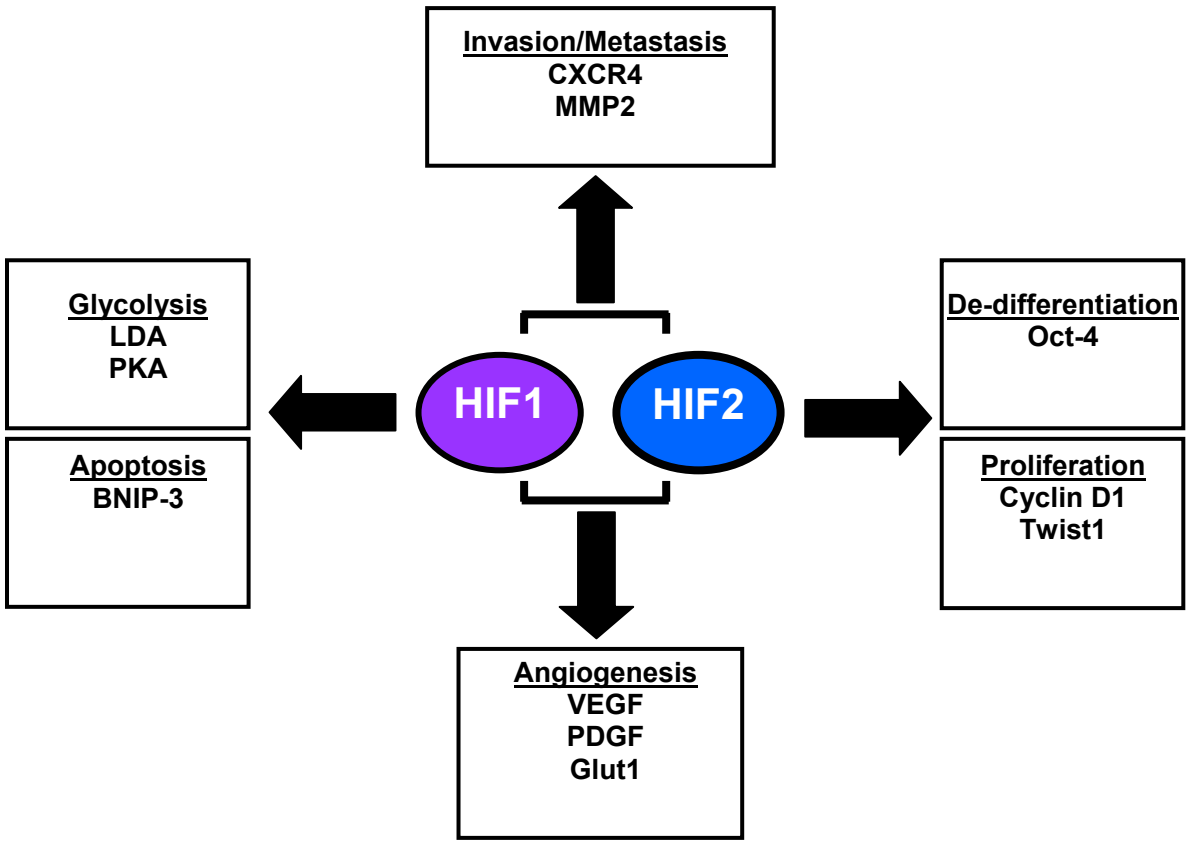

Figure 1.5: HIFs have both shared and unique targets. Though the HIF subunits share similar structures, they have divergent functions that diversely affect cellular processes**.** Figure adapted from Gordan JD & Simon MC, *Curr Opin Genet Dev* 2007 (57).

VHL Disease Subtypes Lead to ccRCC

Within the spectrum of *VHL* disease, there is a tight genotype-phenotype correlation seen among the subtypes corresponding with the severity of disease presentation. The von Hippel-Lindau (*VHL*) tumor suppressor gene encodes a 30kDa protein that is mutated in a majority of *VHL* disease patients. Those that present with *VHL* disease are predisposed to a variety of cancers including pheochromocytoma (adrenal gland tumors), hemangioblastoma (retinal tumors and tumors of the central nervous system) and the most lethal, clear cell RCC (58). *VHL* patients fall into distinct genotypic categories that can predict the spectrum of disease risk and phenotypic presentation (59, 60) (Table 1.1). Additionally, *VHL* disease subtypes can further be categorized by their mechanism of HIFα regulation (61) (Figure 1.6A). Type 1 *VHL* disease is characterized by nonsense mutations, frameshift mutations and truncations of *VHL* (null mutation) and have a high risk of ccRCC. Further, HIFα regulation is completely disrupted in this *VHL* subtype. Type 2 *VHL* diseases are characterized by missense mutations but can be further characterized based on the risk of ccRCC and predicted HIFα regulation. Type 2A disease subtype, designated by the representative mutation (Y112H), is predicted to have a low risk of developing ccRCC and disrupt the interaction with the HIFα subunit leading to a weak regulation by HIFα (62). The Type 2B disease subtype (R167Q) is predicted to have a high risk of developing ccRCC and has weak HIFα regulation as type 2Bs has impaired binding to Elongin C of the pVHL complex (62) (Figure 1.6B). *VHL* Type 2C disease (L188V) does not confer an advantage to developing ccRCC and its interaction with HIFα is predicted to be normally

regulated. Together, this suggests a definitive genotype-phenotype correlation predictive of ccRCC presentation.

VHL Disease Subtype	Likelihood of ccRCC	Representative Mutations	Predicted HIFα Regulation
Type 1	High	Null	Absent
Type 2A	Low	Tyr112His	Weak
Type 2B	High	Arg167Gln	Weak
Type 2C	None	Leu188Val	Normal

Table 1.1: Genotype-phenotype correlation of *VHL* disease subtypes. Table showing the predicted risk of developing ccRCC, the respective representative mutations and each subtype's predicted interaction with HIFα subunit. Table adapted from Rathmell et al *Cancer Res* 2004 (61).

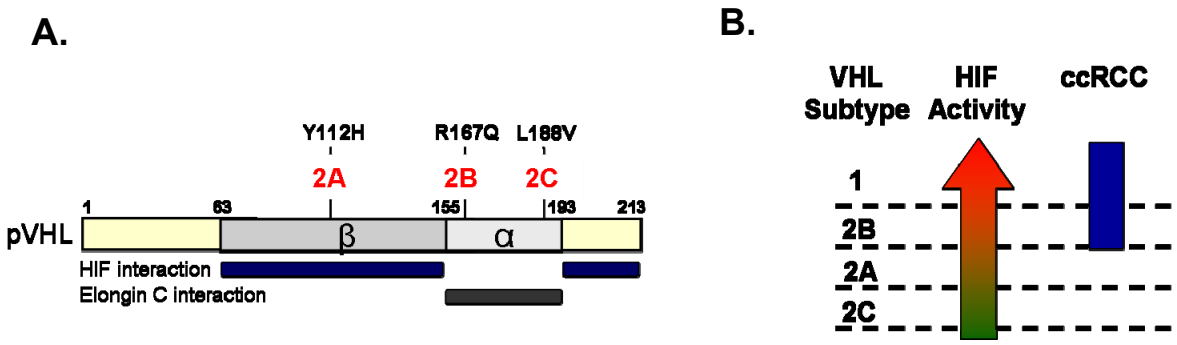

Figure 1.6: *VHL* gene structure and predicted HIFα regulation by *VHL* subtype.
A. *VHL* gene structure showing the amino acid positions of the representative mutations. Each corresponding *VHL* subtype is written in red. The region of interaction is shown for each *VHL* subtype: Type 2A to the HIF interaction domain, Type 2B to the Elongin C domain, Type 2C to a region of the pVHL α-domain not shown to interact with Elongin C. Figure modified from Rathmell et al *Cancer Res* 2004 (61).
B. Genotype-phenotype correlation based on predicted HIFα regulation. Arrow shows increasing levels of HIFα expression from green to red (bottom to top). With higher levels of HIFα expression, there is a higher predisposition to ccRCC (shown by the blue rectangle). Figure adapted from Kaelin WG *Nature Rev Cancer* 2008 (63).

Targeted Therapies for RCC

Targeted therapy has come into play for the treatment of many cancers. Recent developments in the molecular biology of RCC have identified several pathways associated with the development of this cancer. This includes the *VHL*/HIF pathway in which several HIF regulated targets such as PDGF and VEGF are upregulated and are key regulators of angiogenesis in RCC even though their respective receptors, PDGFR and VEGFR, are not present on RCC. RTK inhibitors targeted against many hypoxia target genes and receptors such as VEGFR, PDGFR and mTOR have been constructed and are in varying stages of clinical trials and approval by the US Food and Drug Administration (FDA).

Small molecule inhibitors targeted against the tyrosine kinase domains of intracellular receptors have burst onto the clinical landscape and are currently primarily utilized within clinics across the US. In fact, within the past 5 years, the FDA has approved 4 drugs for the treatment of RCC - two antiangiogenic agents, sorafenib and sunitinib and two mTOR inhibitors, temsirolimus and everolimus (13).

Sorafenib (Nexavar®, Bayer Pharmaceuticals and Onyx Pharmaceuticals) was originally developed as a Raf kinase inhibitor. It has since been shown to be a potent inhibitor of multiple kinases including VEGFR-2, VEGFR-3 and PDGFR-β (11) and is an orally available agent shown to significantly inhibit RCC tumor growth and prolong overall survival. In support of this, numerous clinical trials have been conducted and show that treatment with sorafenib has significant clinical benefit and improves patient survival by several months when compared to placebo (9, 10). This is significant for the treatment of RCC as this disease is notoriously difficult to treat.

Additionally, sunitinib (Sutent®, Pfizer Inc), an antiangiogenic and antitumor drug, has also shown efficacy in RCC. Sunitinib inhibits multiple kinases including the VEGFRs, FMS-like tyrosine kinase 3 (FLT3), PDGFR and c-KIT. In various clinical trials, RCC patients treated with sunitinib have both reduced tumor mass as well as tumor growth inhibition compared to the control treatment interferon alpha (11, 12).

A third inhibitor approved for the treatment of RCC is the mTOR inhibitor, temsirolimus, a water-soluble ester of rapamycin. A survival advantage has been shown with the use of temsirolimus in comparison with interferon-alpha in advanced stage, poor-prognosis RCC patients (64). Another mTOR inhibitor recently approved for RCC treatment in March 2009 is everolimus (RAD001, Novartis). Everolimus binds to FKBP12 with high affinity and the everolimus-FKBP12 complex inhibits downstream mTOR signaling (65) including genes regulated by HIF-1α. Everolimus has been shown to improve progress-free survival in clinical trials when compared to the placebo (12).

With the advent of RCC treatments, several guidelines have been recommended in support of when to administer the drug to RCC patients. Sunitinib is recommended as a first line of treatment for patients who are at low or intermediate risk for metastatic RCC. Sorafenib is suggested as a second line of treatment while temsirolimus is suggested as a first line of treatment for patients who are at high risk for metastatic RCC (66). Additionally, other inhibitory agents have also shown efficacy for the treatment of RCC and are currently being investigated for their efficacy in treating RCC. An example of this is Bevacizumab (Avastin®, Genentech/Roche) which is a humanized monoclonal antibody that targets VEGF

and improves progression-free survival in RCC patients and is suggested for use as a second line of treatment (67).

Although inhibitor treatment options exist for RCC (Figure 1.7), there is still room for drug target optimization for clinical relevance and further patient benefit. Within the last several years, certain targeted therapies have been shown to have a better end effect depending on the kinase targeted. This could be because cancers have developed an “oncogenic addiction” in which tumors come to depend on certain cell signaling pathways. Some examples of targeting the tumor kinase rather than the tumor supporting cells include the use of gefitinib to target the mutated form of the EGFR for non-small cell lung cancer treatment which causes durable disease remissions in patients with EGFR kinase domain mutations (68). Another example of targeting a cell intrinsic kinase rather than tumor supporting cells is the use of sunitinib, which targets the mutated form of c-kit in the gastrointestinal stromal tumor (GIST) (69). When this cell-intrinsic mutant kinase is targeted, a dramatic and durable reduction in tumor burden is seen. However, this illustrates a gap in the RCC field as there are no known reported tumor cell intrinsic kinase targets expressed on RCC.

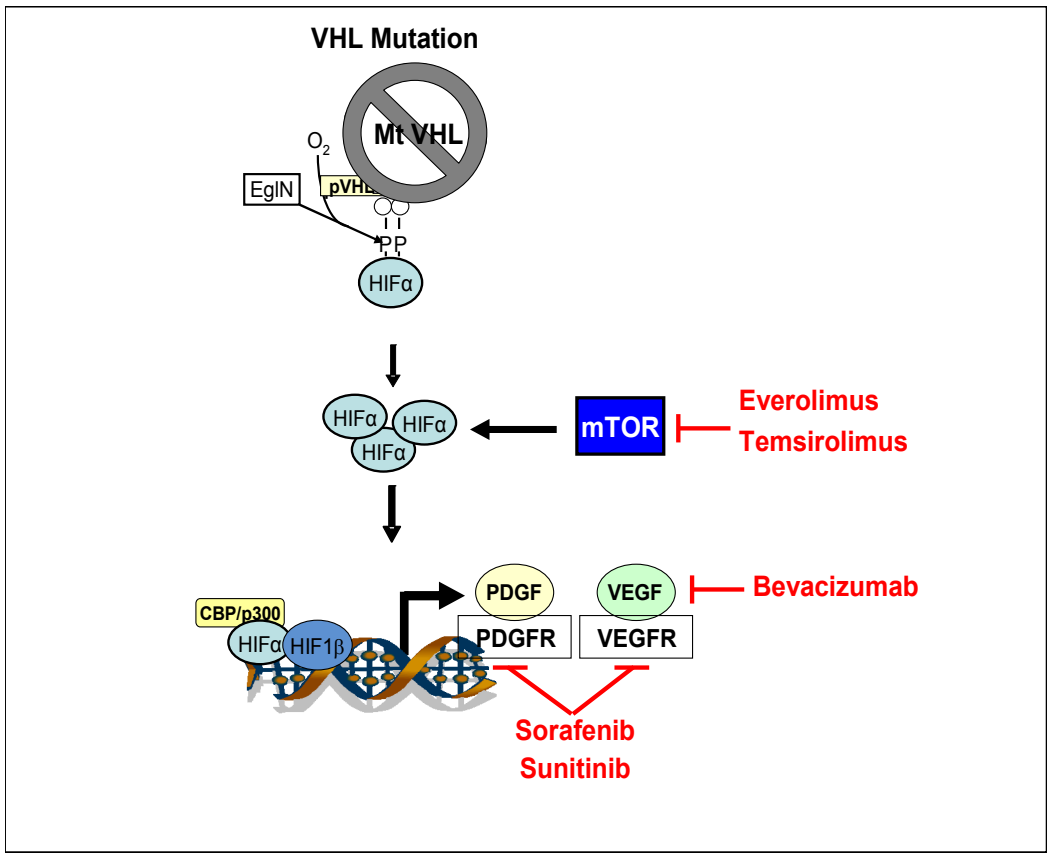

Figure 1.7: Targeted therapies currently in use for treating RCC. Figure adapted from Rathmell WK, Wright TM & Rini B *Expert Rev Anticancer Ther* 2005 (19).

Ror2

The receptor tyrosine kinase-like orphan receptor 2 gene is part of a family of orphan RTKs that includes Ror1 and Ror2 and contains 9 exons (9q22) (70, 71). Ror2 is evolutionary conserved across several species including *C. elegans*, *Drosophila*, Xenopus, mice and humans. In fact, there is a 92% amino acid identity between mouse and human Ror2. Ror2 is characterized by an intracellular tyrosine kinase domain, which is highly related to the Trk-family RTKs and muscle specific kinase (MuSK) (72). Ror2 also contains a proline rich domain flanked by serine-threonine rich domains. Proline rich domains have previously been shown to be important for phosphorylation and protein interactions (73), however the role of proline rich domains in Ror2 have not been fully elucidated. The extracellular domain contains an Ig-like domain, a Frizzled-like cysteine-rich (CRD) domain, which has been shown to act as receptor for Wnt, and a Kringle domain (Figure 1.8) (74). During mouse development, Ror2 expression is seen in the face, limbs, heart, brain and lungs (75-78).

In *C. elegans*, only one Ror gene exists as compared to the two normally found in mammals. Through mutational analysis, the *C. elegans* Ror2 ortholog CAM-1 has been shown to direct correct cellular orientation and migration (79). Mouse Ror2 (*mRor2*) knockout mice have also been engineered by two independent labs. The neomycin resistance gene (*neoR*) was used to replace the Ig domain in one group (77), while the other group used neoR and lacZ to replace the tyrosine kinase domain (71). Similar phenotypes were observed from these two groups. Heterozygote *mRor2* were phenotypically wildtype (WT) while homozygote *mRor2*

knockouts died shortly after birth. Further, *mRor2* deficient mice had atypically short limbs and tails, malformed facial and vertebrae features and respiratory defects (71, 77).

Two human genetic disorders also arise from mutations in human Ror2 (*hRor2*). Short-limbed dwarfism and abnormal facial features are characteristic of autosomal recessive Robinow syndrome, which arises from missense, nonsense and frameshift mutations in the CRD, kringle and kinase domains of *hRor2.* This is similar to phenotypes observed in Ror2 mutant mice suggesting that Robinow syndrome may be caused by a loss-of-function (LOF) mutation of Ror2 (80-82). The autosomal dominant inherited disorder Brachydactyl type B (BDB) arises from mutations resulting in the truncation of *hRor2* by either (1) loss of the entire kinase domain after the membrane spanning region or (2) loss of the serine/threonine and proline-rich domain just below the tyrosine kinase domain. In this disorder, individuals' bones are shortened or misshapen because of endochondral bone ossification (71, 80, 83).

Ror2 has also been implicated in the Wnt signaling pathway. In both canonical and noncanonical Wnt signaling, the Wnt proteins bind to the Frizzled (Fz) family of cell-surface receptors along with the co-receptor protein LDL-receptor-related protein (LRP5 and 6). However, in classic canonical Wnt signaling, binding of the Wnt protein to Fz and LRP leads to LRP phosphorylation, activates the cytoplasmic signaling protein Dishevelled (Dsh) and blocks GSK3β phosphorylation, which is now incapable of phosphorylating β-catenin. Unphosphorylated cytosolic β-catenin accumulates and translocates to the nucleus, driving the transcription of

numerous target genes by displacing the co-repressor protein *Groucho* from TCF. Without Wnt signaling, Dsh is inactivated and no longer able to block GSK3β phosphorylation. GSK3β phosphorylates cytosolic β-catenin, tagging it for ubiquitination and is then degraded by the proteasome. In planar cell polarity (PCP) noncanonical Wnt signaling, after Fz is activated, Dsh linked to disheveled-associated activator of morphogeneis 1 (Daam1) activates JNK via Rac to regulate cellular orientation.

Recent work shows that the CRD and proline rich domain of *mRor2* are required to induce noncanonical Wnt5a mediated cellular migration (84). Along the same line, there is evidence suggesting that *mRor2* can also activate the Wnt/JNK pathway and inhibit *Xenopus* convergent extension movements via the noncanonical Wnt5a pathway (85). One proposed mechanism suggests that when the representative noncanonical Wnt5a ligand interacts with *mRor2*, canonical Wnt/β-catenin pathway is inhibited downstream of β-catenin stabilization at the level of TCF-mediated transcription (86). Another proposed mechanism hypothesizes that *hRor2* inhibits the ability of the canonical Wnt1 and Wnt3 ligands to stabilize β-catenin (Figure 1.9). Wnt1 activates an unidentified signaling cascade which in turn activates Wnt-responsive promoter activity. On the other hand, Wnt3 activates other Tyr kinase dependent events and causes the inhibition of Wnt-responsive promoters (87). Although the above information is available in current literature, how Ror2 functions in the context of RCC had yet to be determined.

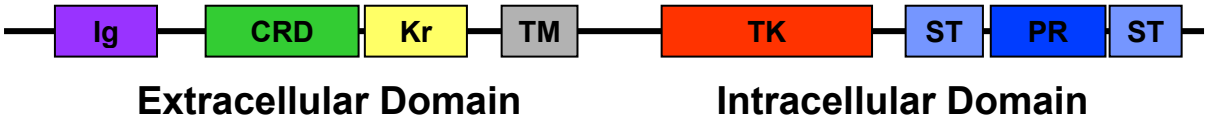

Figure 1.8: Structural Representation of Ror2. Ror2 is characterized by an intracellular tyrosine kinase (TK) domain which is highly related to the Trk-family RTKs and muscle specific kinase (MuSK) and a proline-rich (PR) domain flanked by serine-threonine (SR) domains. The extracellular and intracellular domain are separated by the transmembrane domain (TM) and contains an immunoglobin-like (Ig) domain, a Frizzled-like cysteine-rich (CRD) domain, which has been shown to act as receptor for Wnt in other proteins, and a Kringle domain (Kr).

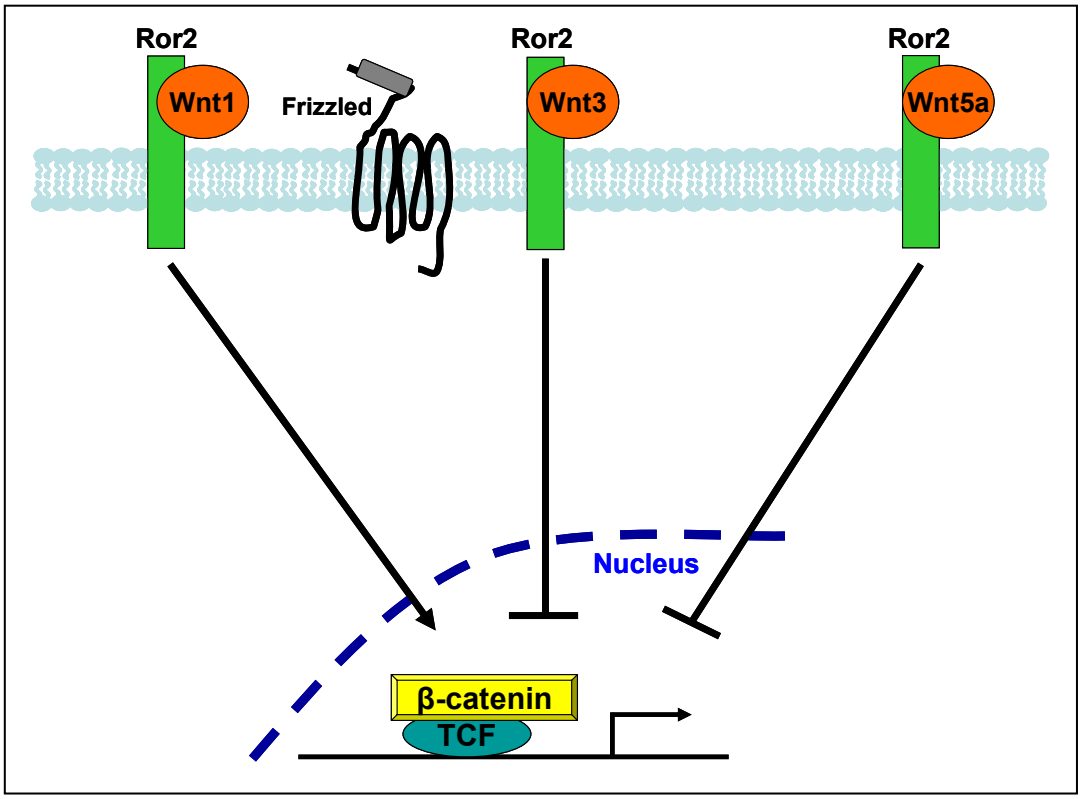

Figure 1.9: Proposed Mechanisms of Ror2 actions. Several mechanisms have been proposed for the actions of Ror2. Ror2 binds Wnt and sequesters them away from frizzled receptors inhibiting their ability to stabilize ß-catenin. Wnt1 binding to Ror2 activates an unidentified signaling cascade requiring Tyr Kinase activity and activates Wnt-responsive promoter activity. Wnt3 binding to Ror2 activates other Tyr kinase dependent events that inhibits Wnt-responsive promoters. When Wnt5a interacts with *mRor2*, the canonical Wnt/β-catenin pathway is inhibited downstream of β-catenin stabilization at the level of TCF-mediated transcription. Figure adapted from Gordon and Nusse *J Biol Chem* 2006 (88) and Billiard et al *Mol Endocrinology* 2005 (87).

Ror2 and Cancer

At the time of discovery of Ror2 in RCC in 2006, aberrantly expressed Ror2 had not been described in any cancer. Since then, the landscape of Ror2 in cancer has changed dramatically. To date of writing this thesis, the developmentally regulated kinase Ror2 has been described as overexpressed in a variety of cancers including melanoma, squamous cell carcinoma and gastric cancer (Table 1.2).

Via cDNA microarray analysis, O'Connell et al (89) recently found that Ror2 is expressed in a majority of metastatic melanomas. Previous data has shown that Wnt5 has a role in Ror2 signaling and, in fact, O'Connell and colleague's previous data suggests that the noncanonical Wnt ligand Wnt5a may be an important aspect of melanoma tumorigenesis. Indeed, Wnt5a expression correlated with Ror2 expression – when Wnt5a was increased so was Ror2 expression and when Wnt5a levels were decreased, it also led to a decrease in Ror2 expression. However, when Ror2 levels were decreased, Wnt5a levels remained constant but there was a decrease in Wnt5a downstream signals. When Ror2 expression was suppressed, there was a drastic reduction in cell motility, cellular invasion and metastasis in the melanoma cell lines examined. This work clearly suggests that Ror2 has a role in Wnt5a mediated invasion in metastatic melanoma.

Ror2 has also been implicated in osteosarcoma, a malignant adolescent bone cancer (90). Utilizing cDNA microarrays, Morioka et al (90) found Ror2 to be transactivated in a majority of osteosarcoma malignancies. Upon further analysis of Ror2 expression in osteosarcoma, Ror2 suppression exhibited reduced cellular migration and proliferation. Additionally, using two osteosarcoma cell lines (SaOS-2

and U2OS cells), Enomoto et al (91) show that cellular migration is mediated by the expression of Ror2 and its potential receptor/co-receptor, Wnt5a. With the suppression of either Ror2 or Wnt5a, cellular invasion was inhibited. Further, with the suppression of Ror2, they identified a matrix metalloproteinase, MMP13 - shown to be important for extracellular matrix (ECM) regulation and invadopodia (actin-rich protrusions that degrade and extend into the ECM) formation. Their data emphatically suggests that MMP-13 expression induced by Wnt5a/Ror2-mediated expression is important for cell migration, ECM degradation and invadopodia formation. Together, these data place Ror2 as having an important role in malignant osteosarcoma.

Ror2 was also identified in squamous cell carcinoma by Kobayashi and colleagues (92) as being more highly expressed in oral malignant cancer epithelial cells compared to normal mucosa. In squamous cell carcinoma, Ror2 expression paralleled the development of invasive features of these tumors. Further, this study indicates that in squamous cell carcinoma, Ror2 expression is associated with cell motility and cell polarity.

Additionally, Ror2 has also been identified in poorly differentiated invasive gastric cancers by a tumor genomic analysis as a frequent target of non synonymous mutagenesis (93). An additional study by Ohta and colleagues (94) identified Ror2 as one of the genes upregulated downstream of Hedgehog (Hh) and epithelial to mesenchymal transition (EMT) signaling in their search for potential gastric cancer molecular targets. In the large majority of intestinal-type and diffuse-type gastric cancers tested, Ohta et al identified the EMT regulator, SIP1, as a target

of Hh signaling and as a regulator of downstream Ror2 expression. Using siRNA, they show that SIP1 directly reduces gastric cancer cell invasion. Additionally, they show distinctly that Ror2 is a cancer-specific target of Hh and EMT signaling tying the expression of Ror2 to another malignant cancer.

Group	Cancer	Role of Ror2
O' Connell et al (89)	Metastatic Melanoma	* Metastasis * Cell Migration * Wnt5a mediated signaling
Morioka et al (90)	Osteosarcoma	* Cell Migration * Cell Proliferation
Enomoto et al (91)	Osteosarcoma	* Cell Migration * Invadopia Formation * ECM Degradation
Kobayashi et al (92)	Squamous Cell Carcinoma	* Cell Polarity * Cell Motility * Malignancy
Kubo et al (93)	Gastric Cancer	* Ror2 mutated in activation domain
Ohta et al (94)	Gastric Cancer	* Cell invasion * Downstream of crosstalk between Hh and EMT signaling

Table 1.2: Role of Ror2 in a variety of cancers.

Significance

Treatment alternatives for RCC are sought as this is a complex disease with limited traditional management options. Targeted therapy for RCC has recently come into play as targeted therapeutics have been shown to cause disease stabilization and prolong survival by several months. However, tumor cell intrinsic targets for RTK inhibition remain elusive. In studying the role of the novel kinase Ror2 in RCC, I hope to provide a new potential target for pharmacological inhibition in RCC. This thesis provides the characterization tools necessary to shed light on a target that has the additional potential to be used as a pharmaceutical biomarker for clinical treatment of RCC.

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CHAPTER 2

DISCOVERY AND VALIDATION OF ROR2 AS A RCC CANCER CELL SPECIFIC KINASE

This work is modified from: Wright et al (2009) Oncogene (1)

ABSTRACT

Abhorrent kinase activity is a defining feature of many solid malignancies, and the recent widespread introduction of receptor tyrosine kinase (RTK) inhibitors into the mainstream treatment of cancer has revolutionized the practice of oncology. However, the potency of a kinase-directed treatment can only be as effective as the activity of the target. Thus, the identification of novel target molecules has the potential to rapidly and durably impact tumor growth for the treatment of patients with cancer. Here we identify Ror2, an orphan receptor tyrosine kinase previously unrecognized as an epithelial cancer-related kinase, to be upregulated in renal cell carcinoma (RCC) cell lines and tumor samples. Ror2 is normally repressed in adult tissue; however this study identifies, Ror2, a developmentally regulated kinase, to be overexpressed in RCC for the first time.

INTRODUCTION

Sporadic renal cell carcinoma (RCC) is a notoriously difficult to treat solid tumor malignancy that has minimal sensitivity to traditional chemotherapy and immune system modulation. Affecting over 40,000 individuals in the US each year (2), this disease is highly refractory to conventional cytotoxic therapies. The majority of clear cell histology RCC tumors are characterized by mutation, methylation, or loss of the *VHL* gene rendering them deficient in the pVHL protein (3-6). One well-documented function of pVHL is to act as the substrate recognition component of an E3 ubiquitin ligase complex (7-10). The substrates of pVHL E3 ligase activity most tightly associated with RCC are the hypoxia inducible factor (HIF)-α subunits (HIF-1α and HIF-2α) (11-13). As a result of *VHL* inactivation in RCC, one or both of these factors are stabilized and transcriptionally activated in the tumor cells, regulating a large panel of hypoxia responsive genes (14, 15). This transcriptional response leads to the phenotypic characteristics of RCC and at least a component of the transformed phenotype of the cells (16).

As RCC is relatively refractory to conventional therapies, recent advances for the treatment of RCC have come in the form of multitargeted kinase inhibitors implicating kinase signaling as a potential viable area for further development. In this study, we sought to identify activated tumor specific kinases expressed on RCC cells in order to discern active signal transduction pathways available for targeted therapy development. To do so, we utilized a phospho-RTK array with RCC cell lysates to identify tumor cell specific activated in RCC.

We report the identification of a novel Wnt receptor of embryonic mesenchymal origin expressed in RCC cell lines and tumors. The receptor tyrosine kinase-like orphan receptor 2 (Ror2) gene is part of a family of orphan RTKs that includes Ror1 and Ror2 (17). Ror2 is characterized by an intracellular tyrosine kinase domain (18) and an extracellular Frizzled-like cysteine-rich domain (CRD), shown to act as receptor for Wnt (19). Mouse Ror2 (mRor2) expression is normally observed in the developing heart, brain and lungs (20-23), with the greatest expression seen in the migrating neural crest and mesenchymal tissues (23).

By immunoblot and qRT-PCR, we show that Ror2 is expressed in multiple renal carcinoma cell lines. In addition, we detected Ror2 transcripts in more than 55% of a set of 19 archival human RCC tumor specimens. Further, we were able to detect protein expression in primary RCC tumors. This definitively identifies and confirms the expression of Ror2 in RCC validating the need for further characterization of this kinase in RCC.

RESULTS

Identification of a novel kinase in RCC

As inappropriate kinase expression is a hallmark of many cancers and offers insight into essential signaling pathways of tumor pathogenesis, we sought to identify profiles of activated kinases in RCC cell lines. Kinases that facilitate essential activities of the tumor can provide important and effective targets for inhibitor therapy; therefore, a phosphorylated-tyrosine kinase array was used as a screen to identify kinases with a basal level of tyrosine phosphorylation in unstimulated RCC lysates.

Currently, there are no known disease-associated cell intrinsic kinases for RCC. Our initial goal was to identify cancer cell specific kinases expressed on RCC as rational targets for pharmaceutical development. Since the majority of RCC tumors are characterized by *VHL* mutation rendering them *VHL* deficient, we utilized the well-established RCC tumorigenic cell line 786-0 RC3, a derivative of the parental 786-0 RCC cell line created by introduction of an empty vector for the initial studies. The parental 786-0 cell line contains a mutation in the *VHL* gene so pVHL is not produced (Table 2.1). In this cell line, only HIF2α is deregulated. HIF2α is considered to be the more tumorigenic of the two HIFα subunits as downregulation of HIF2α is necessary for tumor suppression by pVHL (16, 24, 25). We utilized the human phospho-receptor tyrosine kinase (RTK) array (RND Systems - Minneapolis, MN) to assess the relative phosphorylation changes in our cells as this offers a more sensitive measure of relative phosphorylation levels. In this assay, antibodies targeted against 42 RTKs are spotted in duplicate on nitrocellulose membranes.

786-0 RC3 cell lysates were diluted and placed on the nitrocellulose membranes. After the cell lysates were bound to the extracellular domain of both phosphorylated and unphosphorylated antibodies, phosphorylated RTKs were visualized using an anti-phosphorylation antibody conjugated to horse radish peroxidase. Chemiluminescence was used to detect the activated RTK antibodies according to the manufacturer's instructions.

With this assay, we observed the presence of activated kinases known to be present on RCC such as epithelial growth factor receptor (EGFR). Additionally, we were able to confirm the absence of active vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) on RCC cells. Though several potential kinases were identified from this phospho-RTK screen, we chose to move forward with Ror2, a receptor tyrosine kinase-like kinase previously unknown in renal carcinoma cells (Figure 2.1) as it was the most novel at the time and had not been identified in any cancer.

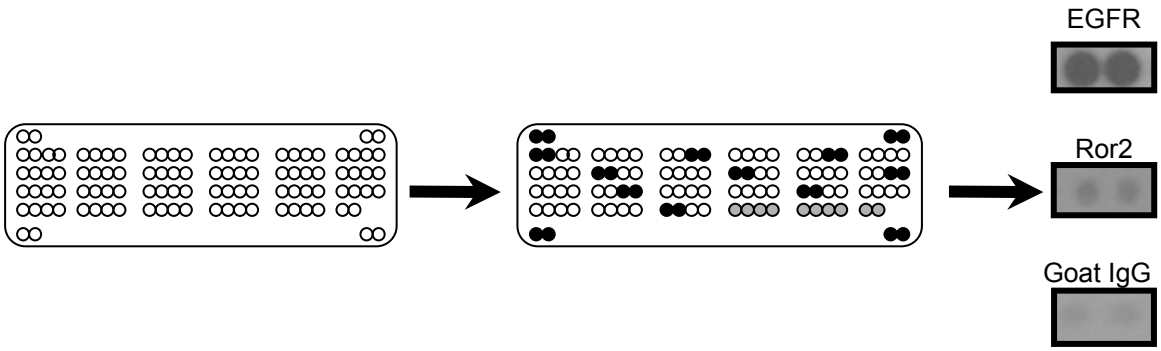


Figure 2.1: Human Phospho-RTK array screen for activated kinases in RCC.
Human Phospho-RTK array screen for activated kinases in RCC. Utilizing a phospho-RTK array, 42 RTKs were screened for phosphorylation in RCC using the 786-0 RC3 cell line. Activated kinases were identified using an HRP-conjugated pan-phosphotyrosine antibody. From this screen, one novel RTK identified was Receptor Tyrosine Kinase-like Orphan Receptor 2 (Ror2). Additional spots demonstrating positive identification include a kinase known to be activated in RCC cells (EGFR) and negative control spots (Goat IgG).

Verification of Ror2 activity in RCC

Physiological expression of Ror2 in adult tissues had not previously been shown at the time of Ror2 discovery in RCC, with the exception of osteoblasts (26). Abundant expression of the endogenous protein in 786-0 RC3 cells was confirmed by immunoblot analysis and immunoprecipitation of Ror2 with independent antibodies (Figure 2.2). Detecting phosphotyrosine from the immunoprecipitation using a nonspecific phospho-tyrosine antibody (pY99), further confirmed that the kinase is phosphorylated under basal conditions. Similarly, Ror2 protein was not detectable in lanes controlling for antibody isotype and nonspecific activity of the resin. These data substantiate the detection of Ror2 protein, the activation of Ror2 kinase in RCC and merits further analysis.

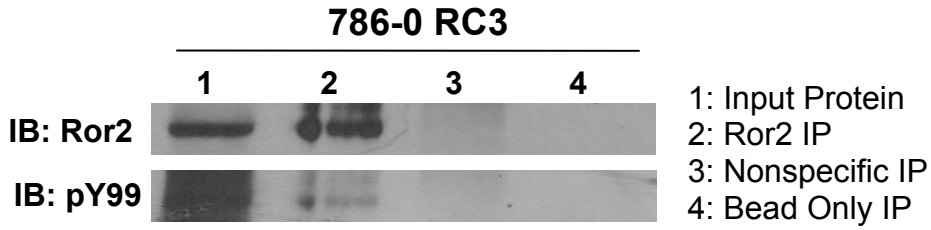


Figure 2.2: Verification of Ror2 activity in RCC cells.
Expression and phosphotyrosine activation of Ror2 is confirmed by immunoprecipitation from RCC cells. Ror2 protein was immunoprecipitated from 786-0 RC3 cells using biotin-conjugated goat Ror2 antibody for antigen retrieval and streptavidin conjugated agarose beads. Immunoblot was performed with an alternate isotype-specific Ror2 antibody to confirm the capture of Ror2 and the nonspecific pY99 antibody used to assay for phosphotyrosine activity. Individual lanes show protein detected by immunoblot on (1) whole cell extract (input protein), (2) Ror2 immunoprecipitate, (3) isotype-matched nonspecific antibody immunoprecipitate and (4) no antibody control. Ror2 protein was not detectable in lanes controlling for antibody isotype and nonspecific activity of the resin. Detecting phosphotyrosine from the immunoprecipitation using a nonspecific phospho-tyrosine antibody (pY99), further confirms that the kinase is autophosphorylated under basal cell culture conditions.

	VHL	HIF1α	HIF2α	Glut1
786-0	-	N/A	+	+
786-0 RC3	-	N/A	+	+
786-0 WT8	+	N/A	-	-
RCC4 2-1	-	+	+	+
RCC4 3-14	+	-	-	-
HKC	+	-	-	-

Table 2.1: RCC tumor cell lines used for Ror2 analysis (expression under normal oxygen conditions).

Ror2 expression is seen in multiple RCC cell lines

Since Ror2 was expressed in the 786-0 RC3 cell line, we also wanted to assay for expression in other RCC cell lines. To validate the expression of Ror2 in RCC, we utilized two independent antibodies targeting the extracellular domain of Ror2: 1) a monoclonal antibody that works under nonreducing conditions and; 2) a polyclonal antibody that works under both nonreducing and reducing conditions (RND Systems - Minneapolis, MN). We used the parental 786-0 cell line as well as its empty vector containing derivate 786-0 RC3, both of which overexpress HIF2 and lack functional pVHL (27, 28). We also utilized another RCC cell line, RCC4 2-1 which overexpresses both HIF1α and HIF2α and lacks functional pVHL under both normal and low oxygen conditions. The HKC cell line is a control SV40 transformed proximal tubule epithelial kidney cell line which has functional pVHL (Table 2.1). Our results show that the expression of Ror2 is seen in all the RCC cell lines examined except the human proximal tubule cell line HKC suggesting that Ror2 is expressed in multiple RCC cell lines but not in the normal control. This result is seen with both the monoclonal and polyclonal antibodies targeting Ror2 (Figure 2.3). The expression of Ror2 was further confirmed by qRT-PCR as elevated mRNA levels were also detected in these cell lines (data not shown).

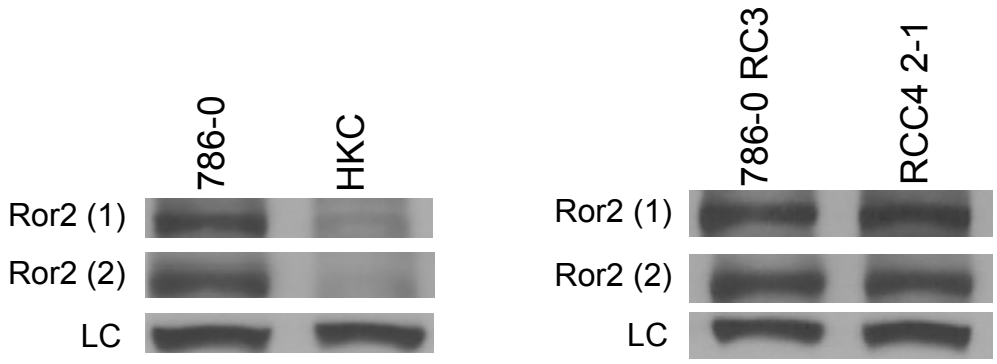


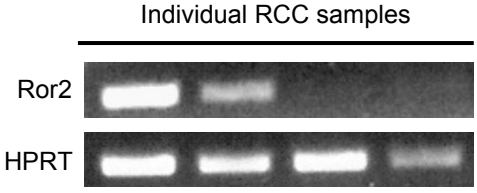
Figure 2.3: Confirmation of Ror2 protein expression in RCC cell lines.
Ror2 is expressed in RCC cells but not in a normal kidney cell line. Whole cell extracts were immunoblotted with monoclonal Ror2, *top*, polyclonal Ror2, *middle*, and loading control (LC), *bottom*. Ror2 expression is seen predominantly in the RCC cell lines, 786-0, 786-0 RC3 and RCC4 2-1, but not in the proximal human tubule cell line, HKC, using 2 different antibodies targeting the extracellular domain of Ror2.

Ror2 expression in human RCC tumors

Ror2 is a developmentally regulated kinase, with expression primarily relegated to embryonic mesenchymal tissues (23). To examine a possible role in malignant tissue, Ror2 mRNA expression was analyzed by RT-PCR in 19 primary human RCC tumors. Four representative tumor specimens are demonstrated in Figure 2.4A with the HPRT gene used as a control for RNA abundance and integrity. The band detected as Ror2 in Figure 2.4A was purified and sequenced, confirming identity to the Ror2 CRD domain as predicted (data not shown). Overall, Ror2 transcript was detected in more than 55% of an unselected set of archival renal tumors (Figure 2.4A). Ror2 mRNA was detected even in some tumors of non-clear cell histology, suggesting that various genetic mechanisms may have the potential to promote the induction of Ror2 in renal cancers.

Detecting Ror2 expression in a malignant context prompted an evaluation of Ror2 expression in the normal human kidney. Ror2 is well known to be expressed in developing tissues with expression often restricted to mesenchymal cells (23). Consistent with this profile, Ror2 protein was detected in human fetal kidneys in the migrating nests of mesenchymal cells that give rise to the bulk of the cell types of the developing kidney (Figure 2.4B).

A



Total tumors expressing Ror2 (11/19)	57.9%
Clear Cell RCC expressing Ror2 (6/10)	60.0%
Papillary RCC expressing Ror2 (4/6)	66.7%
Other RCC expressing Ror2 (1/3)	33.3%

B

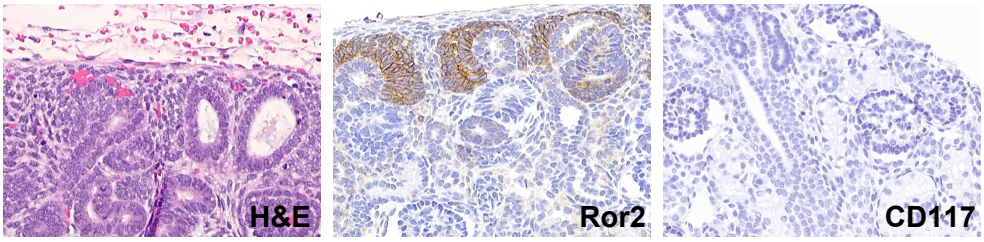


Figure 2.4: Ror2 expression in human RCC tumors.
A. RT-PCR of archival human tumor samples shows mRNA expression of Ror2. Nineteen (19) archival tumor samples from the UNC Tumor Bank (1994-2004) were amplified with primers against the CRD domain of Ror2 (HPRT gene used as a control to assess quality of RNA). Direct sequencing of the PCR product confirmed the product as Ror2. Representative examples of Ror2 expression seen in RCC tumors are shown. A tabular distribution of tumors expressing a Ror2 transcript is shown for the individual RCC histological subtypes.
B. Ror2 is expressed in fetal kidney tissues. Left panel, H&E stain of fetal kidney, Middle panel, Ror2 expression detected by immunohistochemistry on primitive migrating mesenchymal cells, Right panel, CD117 is used as a negative control for staining specificity in the fetal kidney and (expected to be negative as shown) is a control for staining specificity for the secondary antibody.

Ror2 protein expression in human RCC tumors

As Ror2 mRNA expression was found in RCC tumors, we asked if Ror2 could also be identified at the protein level. Protein expression of Ror2 was analyzed by immunostaining two tumors demonstrating high levels of Ror2 transcript and two Ror2 transcript-negative tumors (one representative Ror2 negative tumor shown). Hematoxylin and eosin (H&E) serial sections demonstrate the clear cell histology of each of these tumors and lack of significant non-tumor cell infiltration. Immunofluorescent detection of Ror2 demonstrates tumor cell specific expression of Ror2 protein (Figure 2.5). The immunofluorescence signal was undetectable in tumors identified as lacking Ror2 mRNA expression based on the RT-PCR evaluation, or in the absence of Ror2 primary antibody. Nuclear stain shown from the same field demonstrates the presence of cellular constituents. Ror2 protein is, therefore, present in a subset of primary RCC tumors and can be detected specifically on the tumor cells of the specimen.

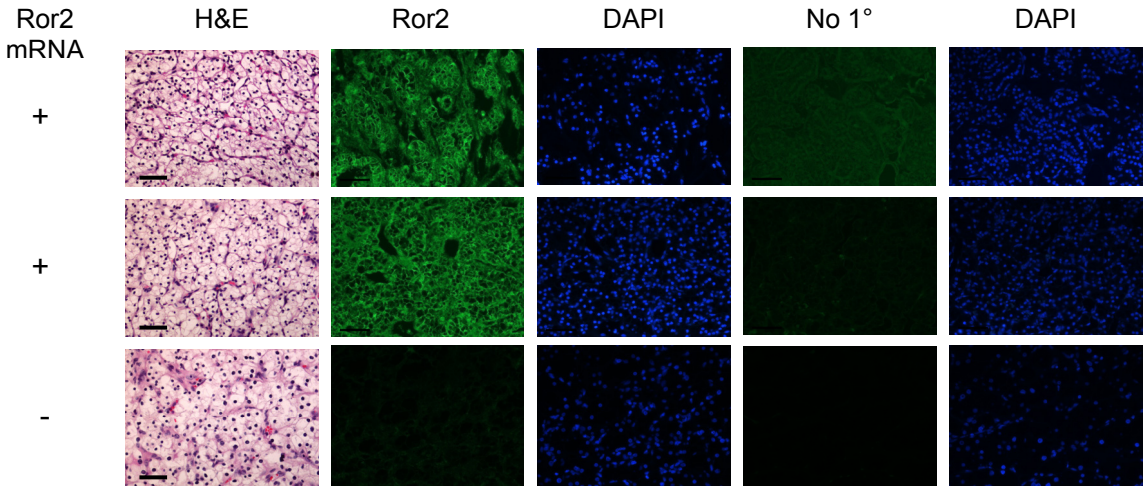


Figure 2.5: Protein expression of Ror2 in human RCC tumors.
Three individual RCC tumors were stained to determine the cellular expression of Ror2. H&E of the 3 tumors verifies clear cell histology. Two tumors were selected having high expression of Ror2 transcript (+), and a third tumor identified as having undetectable levels of Ror2 transcript (-) was used to verify antibody specificity. An Ror2 antibody was used to assay for Ror2 expression in the RCC tumors. Top and middle panels show that Ror2, a transmembrane cell surface receptor, is mainly expressed in the cell surface or cytoplasm, with no detectable protein in the control tumor in the bottom panel. Control panels show negative staining in the no primary control. DAPI is included for both primary and no primary antibody stained sections to demonstrate nuclear staining. All pictures were taken at 20X and the black bar represents 34 uM.

DISCUSSION

In this study, we were interested in identifying activated tumor specific kinases expressed on RCC cells. Using this information, we hoped to identify active signal transduction pathways that could be used for targeted therapy development in the future.

We exploited the properties of RCC where the majority of clear cell histology RCC tumors are mutated for *VHL* by utilizing a transformed RCC cell line with mutated *VHL* for our initial studies. We identified Ror2, a developmentally regulated orphan kinase. Ror2 is physiologically expressed primarily on embryonic mesenchymal cells, and is developmentally expressed on migrating mesenchymal cells of the fetal kidney. This kinase was recognized as an activated tyrosine kinase in RCC cancer cell lines and renal tumors. The aberrant expression of Ror2 in human tumors had not been specifically described at the time of Ror2's discovery in RCC, although evidence of active Ror2 has been observed on kinase arrays from glioblastoma cell lines (29). In this study, however, we show that Ror2 expression was detected in the majority of human RCC tumors, and protein expression was confirmed at the tumor cell level. These data support the identification of a tumor cell intrinsic receptor tyrosine kinase, which is usually limited to developing mesenchymal cell subsets, that is detectable on the majority of renal cell carcinomas.

As a protein not expressed in other adult tissues, Ror2 has the potential to be used as a molecular biomarker for RCC to provide a target for tumor cell identification, tumor imaging, or tumor cell-directed systemic therapy. Ror2 provides

another signaling event in the development of RCC and may represent a viable target for tumor-directed treatment for patients with RCC suggesting that further characterization is warranted.

MATERIALS AND METHODS

Human Phospho-Receptor Tyrosine Kinase (RTK) array

A human phospho-RTK array kit (RND Systems, Minneapolis, MN) was utilized to determine the relative phosphorylation levels of RTKs according to the manufacturer's instruction. The array consists of antibodies targeted against forty-two different RTKs spotted in duplicate on nitrocellulose membranes. 786-0 RC3 (a control vector-transfected derivative of the *VHL* mutant RCC cell line 786-0) cell lysates were diluted and incubated with the Phospho-RTK array. Phosphorylated RTKs were identified with a pan anti-phospho-tyrosine horseradish peroxidase conjugated antibody and activated receptors detected using ECL-Plus chemiluminescence reagents (GE, Pittsburg, PA).

Cell Culture

786-0 and RCC4 cell lines and their derivatives, including the paired cell lines 786-0 WT8 and 786-0 RC3 (kindly provided by Dr. W. Kaelin, Boston, MA), RCC4 2-1 and RCC4 3-14 (kindly provided by Dr. M.C. Simon and Dr. B. Keith, Philadelphia, PA), were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), L-glutamine and non essential amino acids. The HKC cell line (kindly provided by Dr. L. Racusen, Baltimore, MD (30)) was maintained in DMEM/F12 supplemented with 10% FBS.

Immunoblotting

Cells were lysed in whole cell extraction buffer containing 20mM Tris, 100mM NaCl, 1 mM EDTA, 1% NP-40 and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Bradford reagent was used to assess the concentration using standard absorbance. Protein samples were size separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (GE, Pittsburg, PA). The following antibodies were used to assess for expression: Monoclonal Ror2 at 1:250 (RND Systems, Minneapolis, MN), Polyclonal Ror2 at 1:3000 (RND Systems, Minneapolis, MN) and Ku80 at 1:2000 (Genetex Inc, San Antonio, TX) as a loading control (LC). The appropriate horseradish peroxidase conjugated secondary antibodies were used and proteins detected using ECL-Plus chemiluminescence reagents (GE, Pittsburg, PA).

Immunoprecipitation

500µg of cell lysates were immunoprecipitated using a polyclonal biotinylated Ror2 antibody (RND Systems, Minneapolis, MN), Epo (N-19) nonspecific IgG goat antibody (Santa Cruz, Santa Cruz, CA) and a bead only control per standard protocol. Immunoblotting was then performed as described above using monoclonal Ror2 at 1:250 (RND Systems, Minneapolis, MN) and pY99 anti-phosphotyrosine at 1:1000 (Santa Cruz, Santa Cruz, CA) with 50µg whole cell extract control as input.

RT-PCR Analysis

Primers targeted against the CRD domain of Ror2 (Left primer: 5' tttcaggatgattaccacgag 3', Right Primer: 5' ctcacacttgggcagctgaa 3') were generated.

The following protocol was used to generate the PCR fragment: 94°C for 2 minutes (1x), 94°C for 30 seconds, 61.5°C for 30 seconds, 72°C for 30 seconds (34x) and 72°C for 5 minutes (1x). The Ror2 CRD domain PCR fragment was confirmed by bidirectional DNA sequencing in RCC cells and by BLAST sequence homology analysis (data not shown). Standard HPRT primers (Left primer: 5' cctgctggattacatataagcactg 3', Right primer: 5' gtcaagggcatatccaacaacaaac 3') were used to analyze the integrity of the RNA.

Immunohistochemistry and Immunofluorescence

Formalin fixed and paraffin embedded clear cell RCC tumor blocks were obtained from the UNC Tissue Procurement Facility tumor bank (1994-2007) following approval of this research by the Institutional Ethics Review Board. Tissue blocks were serially sectioned at 5um thickness onto unprepared glass slides. The first section was stained with hematoxylin and eosin (H&E) for tumor verification. The slides were deparaffinized, rehydrated in graded ethanol, and incubated with the primary mouse anti-human Ror2 monoclonal antibody (generated by Drs. Mikels and Nusse). A biotin conjugated secondary anti-mouse antibody, avidin biotin conjugated horseradish peroxidase antibody, and Fluorophore Tyramide were used for human RCC tumor staining according to the instructions of the TSA^{TM}-plus Fluorescien System Kit (NEL 741001 KT from PerkinElmer Labs, Inc, Waltham, MA). Slides were then mounted with Vectashield (H-1200, Vector Laboratories, Burlingame, CA) and assayed using fluorescence microscopy. Immunofluorescent

images were taken at 20X magnification at the exposure specified (100 or 200ms). Black bar represents 34uM.

Immunohistochemical stain on human fetal kidney sections acquired through an IRB-approved protocol of discarded specimens was performed using the same Ror2 monoclonal antibody as described above. Standard antigen unmasking procedures were used and protein was detected using di-amino benzidine.

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CHAPTER 3

ROR2, A DEVELOPMENTALLY REGULATED KINASE, PROMOTES TUMOR GROWTH POTENTIAL IN RENAL CELL CARCINOMA

This work is modified from: Wright et al (2009) Oncogene (1)

ABSTRACT

Inappropriate kinase expression and subsequent promiscuous activity defines the transformation of many solid tumors including renal cell carcinoma (RCC). Thus, the expression of novel tumor-associated kinases has the potential to dramatically shape tumor cell behavior. Further, identifying tumor-associated kinases can lend insight into patterns of tumor growth and characteristics. Here, we report the identification of Ror2, a new tumor-associated kinase in RCC cell lines and primary tumors. Ror2 is an orphan receptor tyrosine kinase with physiological expression normally seen in the embryonic kidney. However, in RCC, Ror2 expression correlated with expression of genes involved at the extracellular matrix, including Twist and matrix metalloprotease-2 (MMP2). Expression of MMP2 in RCC cells was suppressed by Ror2 knockdown, placing Ror2 as a mediator of MMP2 regulation in RCC and a potential regulator of extracellular matrix remodeling. The suppression of Ror2 not only inhibited cell migration, but also inhibited anchorage independent growth in soft agar and growth in an orthotopic xenograft model. These findings suggest a novel pathway of tumor-promoting activity by Ror2 within a subset of renal carcinomas, with significant implications for unraveling the tumorigenesis of RCC.

INTRODUCTION

Although tumor biology is tightly linked in many ways to the processes of embryonic development, a largely untapped area of solid tumor oncology is the specific examination of growth promoting pathways that are unique and critical to early developmental patterning. One such signaling pathway, Wnt, is a process common to both developmental and malignant states (2). The identification of these signaling molecules that play a physiologic role in early developmental processes, with potential to promote tumor growth or other pathologic characteristics in the adult, provides an opportunity to define biological cause for various tumor attributes (3). Further, identifying such molecules with restricted tissue expression in the adult organism provides a valuable chance for diagnostic or therapeutic implementation.

Epithelial cells require modulations in diverse signaling pathways to support the numerous cellular changes necessary to attain metastatic potential. Specifically, receptor tyrosine kinase (RTK) activation causes deregulated signaling and can define tumor subsets across a broad spectrum of tumors (4). Such variations in kinase activation have the potential to facilitate the cellular adaptations that determine patterns of tumor growth and can provide tremendous insight into the cell biology of that transformative process (5, 6).

Among the cellular changes associated with tumor progression is tissue remodeling compatible with the extensive migration necessary for the progression of metastatic disease. This cellular transition is shared by the developmentally regulated processes, which direct limb formation or other tissue patterning events. Tissue remodeling is driven by matrix metalloproteases (MMPs), a family of

enzymes that degrade the extracellular matrix (ECM), and is integral to both normal tissue development and cancer progression (7). These processes signal cellular alterations that allow single cells to detach from the epithelial tumor collective, interrupting cell-cell junctions and increasing cell motility. Taken together, this highly coordinated process is critical for the lethality of most epithelial malignancies.

One such lethal epithelial malignancy, renal cell carcinoma (RCC), affects over 40,000 individuals in the US annually (8). Nearly a third of these patients present with unresectable or metastatic disease, for which antiangiogenic therapies are the mainstay of therapy (9, 10). RCC describes a group of histologically and genetically distinct neoplasms, the most common being clear cell RCC, with the remainder being papillary and chromophobe RCCs (11). The mechanisms that promote tumor invasiveness or the acquisition of metastatic potential for the subtypes of RCC remain undefined.

ECM remodeling, a common step towards invasiveness, is promoted by tumor cells through changes in intracellular signaling and commonly acquiring characteristics of less differentiated cellular precursors (7). One developmentally-regulated pathway inappropriately activated in many epithelial tumors is Wnt protein signaling via the frizzled and LRP co-receptors (12, 13). Here we report the identification of a Wnt receptor of embryonic mesenchymal origin expressed in RCC cell lines and tumors that define a subgroup of human tumors with an invasive growth profile of gene expression. The novel receptor tyrosine kinase-like orphan receptor 2 (Ror2) is part of a family of orphan RTKs (14, 15). Ror2 is characterized by an intracellular tyrosine kinase domain (16) and an extracellular Frizzled-like

cysteine-rich domain (CRD), shown to act as a receptor for Wnt ligands (17). Mouse Ror2 (mRor2) expression is normally observed in the developing heart, brain and lungs (18-21), with the greatest expression seen in migrating neural crest and mesenchymal tissues (21). In the developing kidney, we have observed Ror2 expression in the migrating nests of mesenchymal cells. Further, Ror2 expression was identified in RCC tumors and is coordinated with invasive growth in culture. Coupled with the observations that Ror2 impacts expression of MMP2 and tumor cell growth *in vitro* and *in vivo*, this study identifies a novel developmentally regulated receptor that enhances RCC tumor cell growth capabilities.

RESULTS

Ror2 expression in RCC tumors defines a tumor genetic profile of ECM genes

To analyze the role of Ror2 signaling in human RCC, an initial set of 13 human tumors were examined for gene expression using the 44K Agilent array platform. Tumors were classified as having undetectable, low, or high expression of Ror2 based on absolute Ror2 probe values on the array. Ror2 expression on the array was congruent with expression patterns observed by conventional RT-PCR. Based on these designations, a three-way analysis for differentially expressed targets was performed. When the 250 genes that associate most significantly with Ror2 expression (False Discovery Rate (FDR) < 0.07) were analyzed by hierarchical clustering, a subset of genes including extracellular matrix (ECM) candidates Twist1 and Twist2 were identified (Figure 3.1). In a larger, but still highly significant gene cluster for association with Ror2 (FDR < 0.15), the ECM factor MMP2 was also identified (Figure 3.1). Other genes identified in this significant association included those linked with Wnt signaling such as catenin δ2, frizzled related protein and cathepsin K.

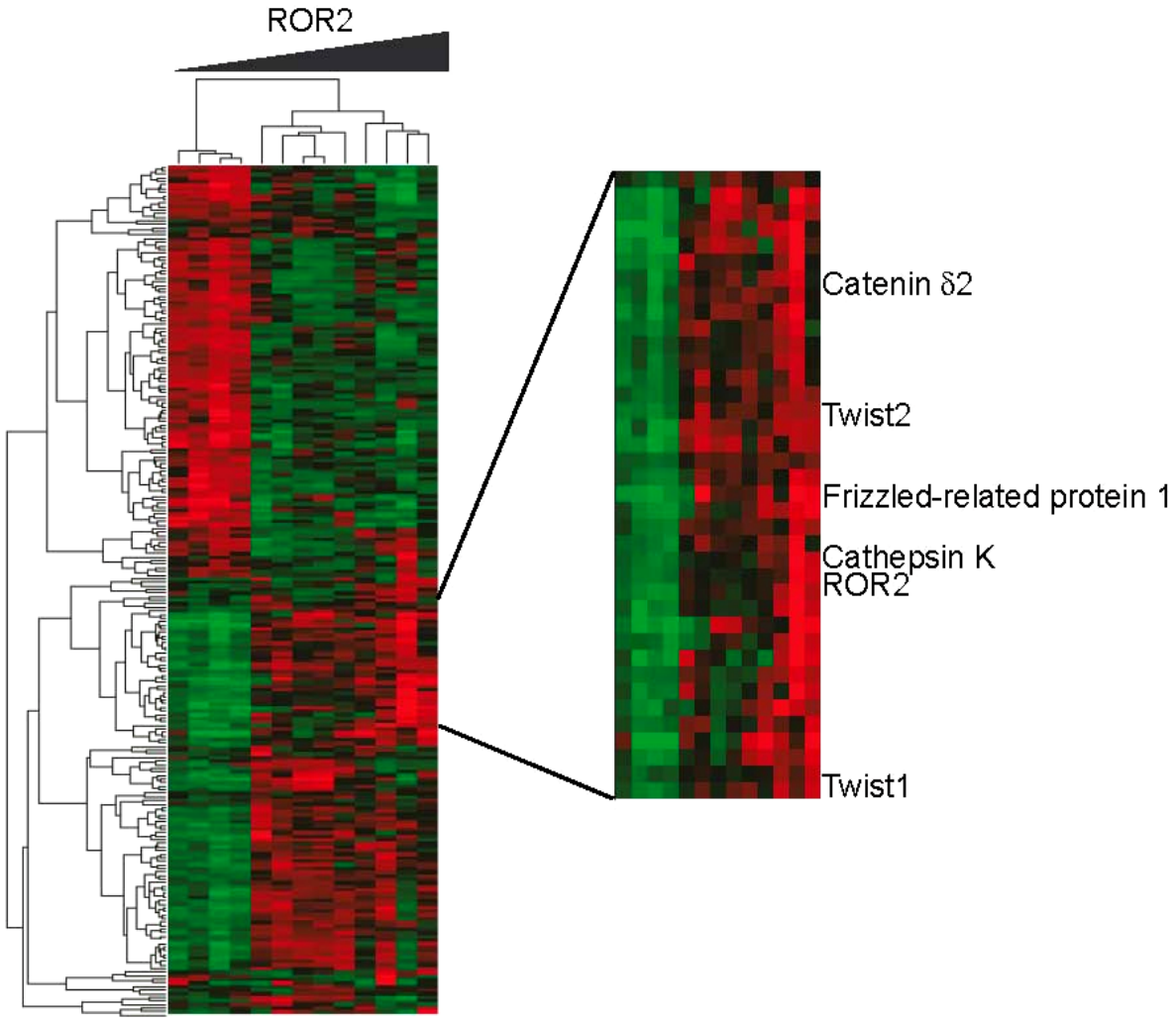
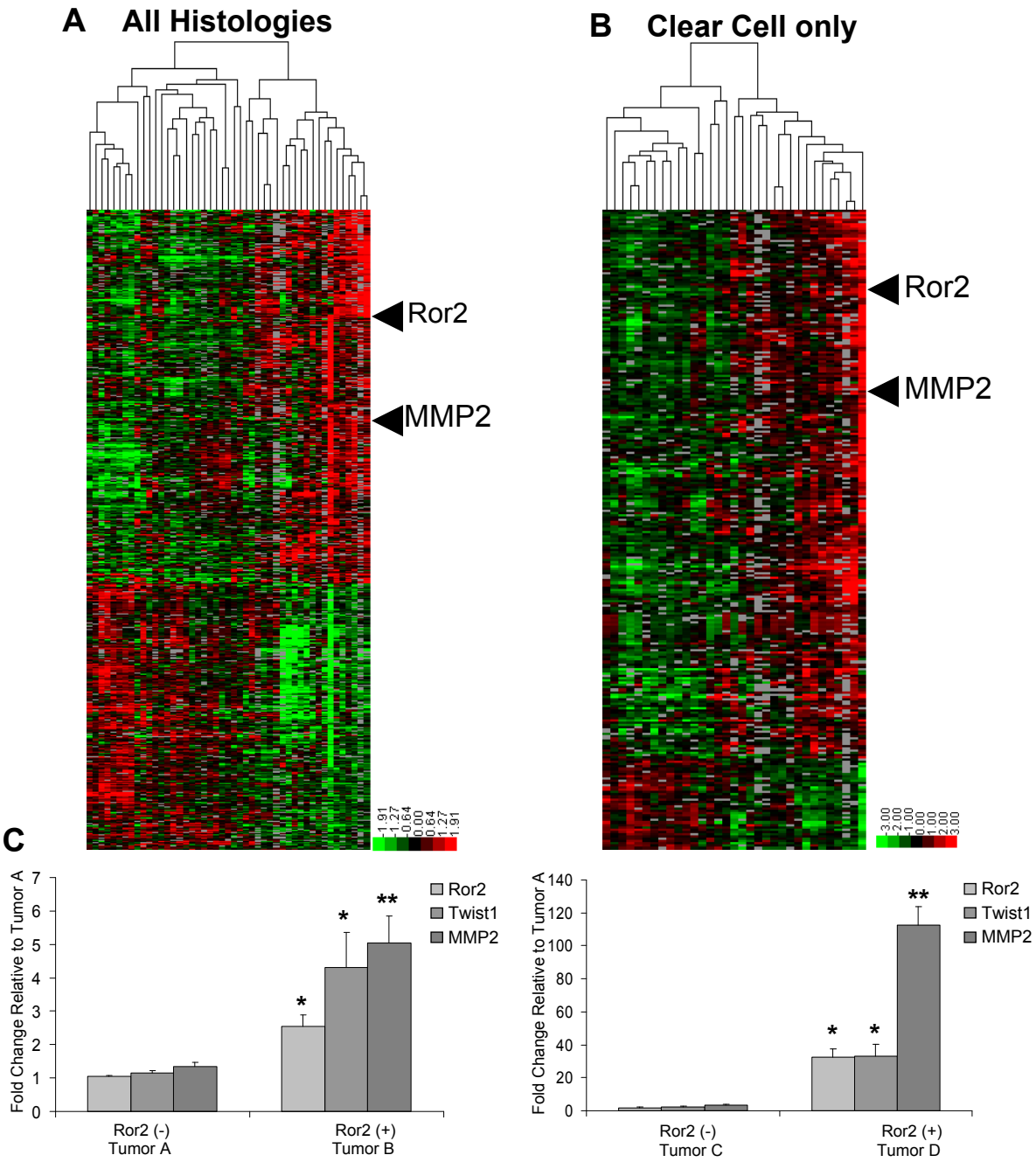


Figure 3.1: Microarray analysis defines a tumor genetic profile of ECM genes. Human RCC tumor microarray analysis. Tumor mRNA was analyzed by Agilent 4x44K gene expression microarray and separated into groups with low (n=4), intermediate (n=5) or high (n=4) Ror2 expression. SAM analysis was performed to identify differentially expressed genes, and the most significant (FDR <0.07) were analyzed by two-way hierarchical clustering, validating the observations in Figure 3 on an independent set of arrays.

In support of these data, additional RCC tumors were analyzed and clustered based on Ror2 expression. Independent microarray analysis was performed using the same 44K Agilent array platform revealing a similar set of highly coordinately regulated genes which were identified in all RCC histologies with a FDR < 0.045 (Figure 3.2A) and in clear cell with a FDR < 0.039 (Figure 3.2B). Again, Twist1 and MMP2 were found to be tightly associated with Ror2 expression. Altered expression for these transcripts (Twist1 and MMP2) was confirmed by quantitative RT-PCR of four representative tumors (two Ror2 + and two Ror2 -), confirming co-regulation (Figure 3.2C). Gene set analysis further supports an association between Ror2 expression and MMP2, identifying gene associations with the extended list of MMP family members and members of the Reck pathway. Additionally, in its physiologic role, Ror2 is tightly linked to skeletal development, thus it is intriguing to see a gene set reminiscent of that process associated with high statistical significance with a subset of human tumors of epithelial origin. An expanded listing of the members of the associated gene sets is shown in Figure 3.2D. These gene sets validate the initial gene expression study and support a thematic program of gene expression in these tumors associated with extracellular matrix remodeling. Together, these data extend a potential role for Ror2 as a mediator of tumor characteristics normally reserved for early and highly regulated developmental processes.



D

Gene Set	Genes
MMPs	MMP2, ADAM12, TIMP2, TIMP1, BMP1, MMP11, CHIT1, MMP7, MMP3, MMP12, MMP9, ADAM9, TNF, MMP14, MMP15, PEPD
RECK Pathway	MMP2, TIMP1, TIMP2, TIMP3, TIMP4, MMP9, RECK, MMP14
Skeletal Development	GDF10, POSTN, FBN1, DMP1, COL1A2, GLI2, MSX2, INHBA, MGP, EN1, GLI1, COL11A1, COMP, IGF1, TGFB1, ALX1, TWIST1, COL13A1, UFD1L, GDF11, PRELP, ANKH, TWIST2, SHOX, INHA, COL9A2, SPARC, PAX1, ZNF675, COL12

Figure 3.2: Microarray analysis of RCC tumors defines a tumor genetic phenotype. Tumor mRNA from 40 RCC tumors were analyzed by Agilent 4x44K gene expression microarray and separated into groups based on their Ror2 expression. SAM analysis was performed to identify differentially expressed genes, and the most significant in **(A)** all histologies (FDR <0.045) and **(B)** clear cell only histology (FDR<0.0388) were analyzed by two-way clustering.

C. Ror2 (+) RCC tumors have increased mRNA expression of genes involved at the ECM. Quantitative RT-PCR analysis of 2 Ror2 (-) and 2 Ror2 (+) human RCC tumors were analyzed to verify microarray expression. Transcript values are normalized to β-actin RNA internal standard and are shown relative to an Ror2 (-) tumor (Tumor A). Error bars represent SEM. Significant differences were observed in Twist1 (*p<0.001) and MMP2 (**p<0.0001) in the Ror2 (+) tumors (*p<0.001) compared to Ror2 (-) tumor (Tumor A), p-values are based on cT values.

D. Table showing the significant gene set list with corresponding genes involved at the ECM that clustered with Ror2 expression. Tumor mRNA were analyzed by multiclass comparison using SAM, and the resulting Ror2 cluster was further analyzed for over-represented gene categories using the Expression Analysis Systematic Explorer (EASE) (22). Genes from the three most tightly associated ontogenies are listed in the table.

Ror2 expression is significantly expressed in ccRCC tumors

A statistical analysis of Ror2 gene expression as detected by a single randomly selected probe on a gene expression microarray demonstrates that Ror2 expression is more highly expressed in tumors of clear cell histology than in papillary RCC tumors with high significance (Figure 3.3). Also, those few tumors demonstrating chromophobe or mixed histology (data not shown) were not significantly distinct. Overall, these data support Ror2 gene expression in human primary RCC tumors detectable in a high proportion of samples, particularly of the conventional clear cell type.

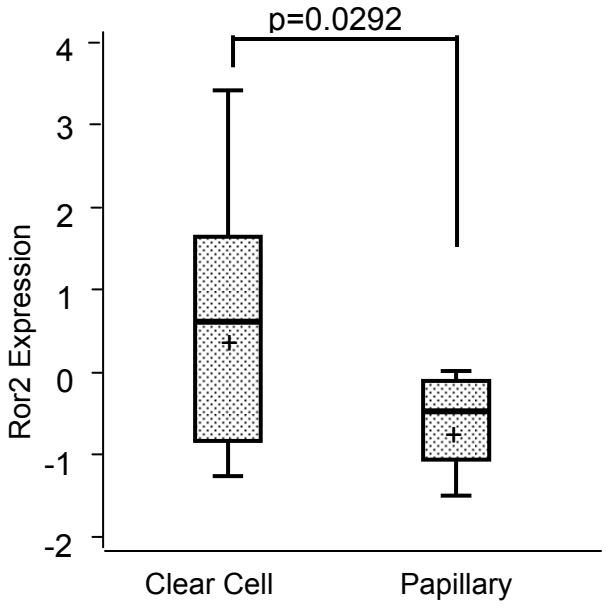


Figure 3.3: Box plot of Ror2 transcript expression in RCC.
Ror2 transcript expression comparing the primary histology of clear cell and papillary RCC tumors demonstrates a significantly higher expression of Ror2 among clear cell tumors compared to papillary RCC.

Ror2 expression directs MMP2 gene expression in RCC

Based on the expression array findings, we sought to explore a direct relationship between Ror2 expression and the expression of genes involved in ECM remodeling. Suppression of Ror2 protein in the 786-0 RCC cell line with two independent shRNAs targeting distinct regions of Ror2, in comparison to the scramble shRNA and control empty vector pRS, is demonstrated by immunoblot in Figure 3.4A. The levels of Twist1 protein were also examined and no change in expression was observed by immunoblot analysis (Figure 3.4A). This result suggests that Ror2 may be part of a larger program of gene expression but does not play a direct role in regulating Twist1 expression. Ror2-deficient cells were examined by quantitative RT-PCR for effects on other genes expressed as a part of the ECM phenotype. Transcript levels of MMP2 were highly suppressed by Ror2 shRNA (Figure 3.4B). Thus, Ror2 may participate in the coordinated events involved in renal epithelial ECM remodeling by regulating the expression of MMP2 in tumor cells derived from this tissue.

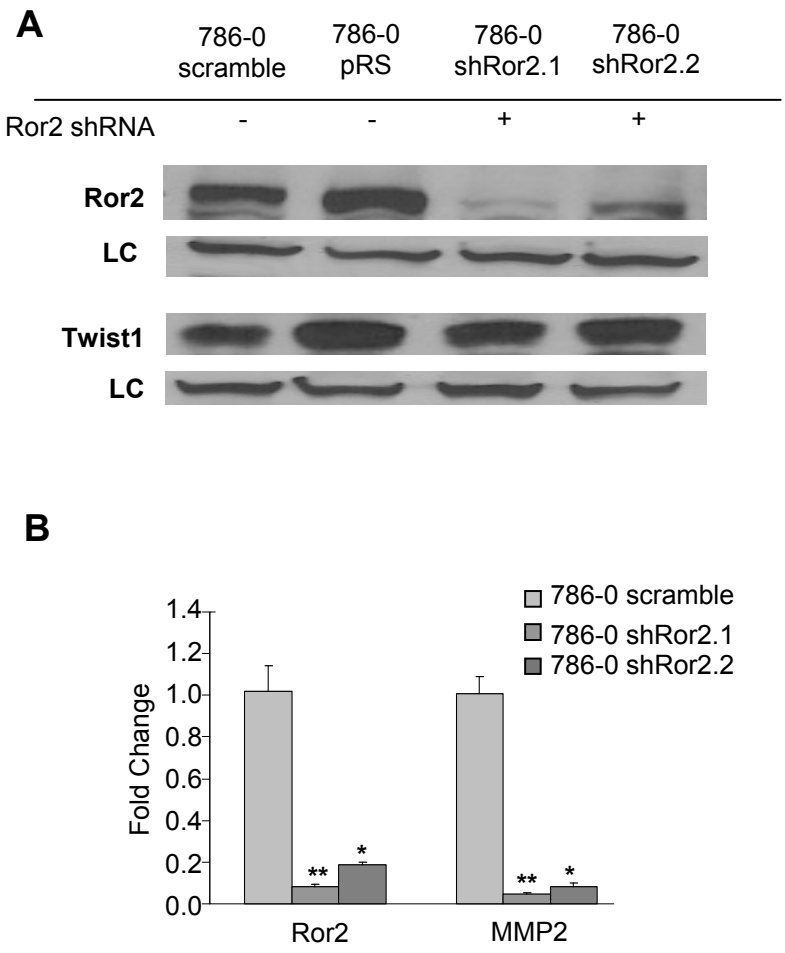


Figure 3.4: Ror2 expression directs MMP2 gene expression.
A. Ror2 expression is suppressed in 786-0 cells by shRNA. Whole cell protein extracts from RCC cells (786-0) were infected with a scramble short hairpin retrovirus, a pRS control virus or Ror2 short hairpin retroviruses and immunoblotted with polyclonal Ror2 antibody, Twist1 antibody or Ku80 antibody as a loading control (LC).
B. MMP2 expression is suppressed when Ror2 levels are knocked down. Quantitative RT-PCR analysis of two 786-0 Ror2 knockdown cell lines demonstrates MMP2 suppression coordinate with the degree of suppression of Ror2. Significant differences were observed in MMP2 (**$p<0.001$ for both comparisons) in the 786-0 Ror2 suppressed cell lines compared to the Ror2 expressing cell lines (**$p<0.001$ for both comparisons). Transcript values are normalized to β-actin RNA internal standard and are shown relative to 786-0 scramble RNA. Error bars represent SEM.

Ror2 expression directs cell migration and anchorage independent growth in RCC

The functional consequence of Ror2 suppression was examined in assays of tumor cell invasive properties, beginning with cell migration. Wound healing was measured as a means to quantify cellular migration because suppression of Ror2 by shRNA did not directly impact doubling time or viability of cultured cells (Figure 3.5). We examined wound healing in two independent stable shRNA Ror2 knockdowns generated in 786-0 subclones. Healing was assayed in triplicate at 0, 8, 16, and 24 hour timepoints. Cells lacking Ror2 expression failed to close the wound as efficiently as their isogenic counterparts, scramble shRNA (data not shown) or pRS (Figure 3.6A), suggesting that Ror2 may play an important role in promoting cell migration.

Another tumor cell characteristic of metastatic RCC and tumor invasiveness associated with the ECM is anchorage independent growth. To determine whether Ror2 suppression also impacts this mode of cell growth, we assessed the growth of stable shRNA Ror2 knockdowns in soft agar (Figure 3.6B). Colonies greater than 150 uM in diameter were counted from triplicate plates in duplicate assays. Cells lacking Ror2 expression retained the ability to survive as single cells in soft agar, but failed to proliferate. These cells, therefore, failed to exhibit the anchorage independent growth of their Ror2-expressing isogenic counterparts, suggesting that Ror2 may play an important role in supporting cellular invasion in RCC tumorigenesis.

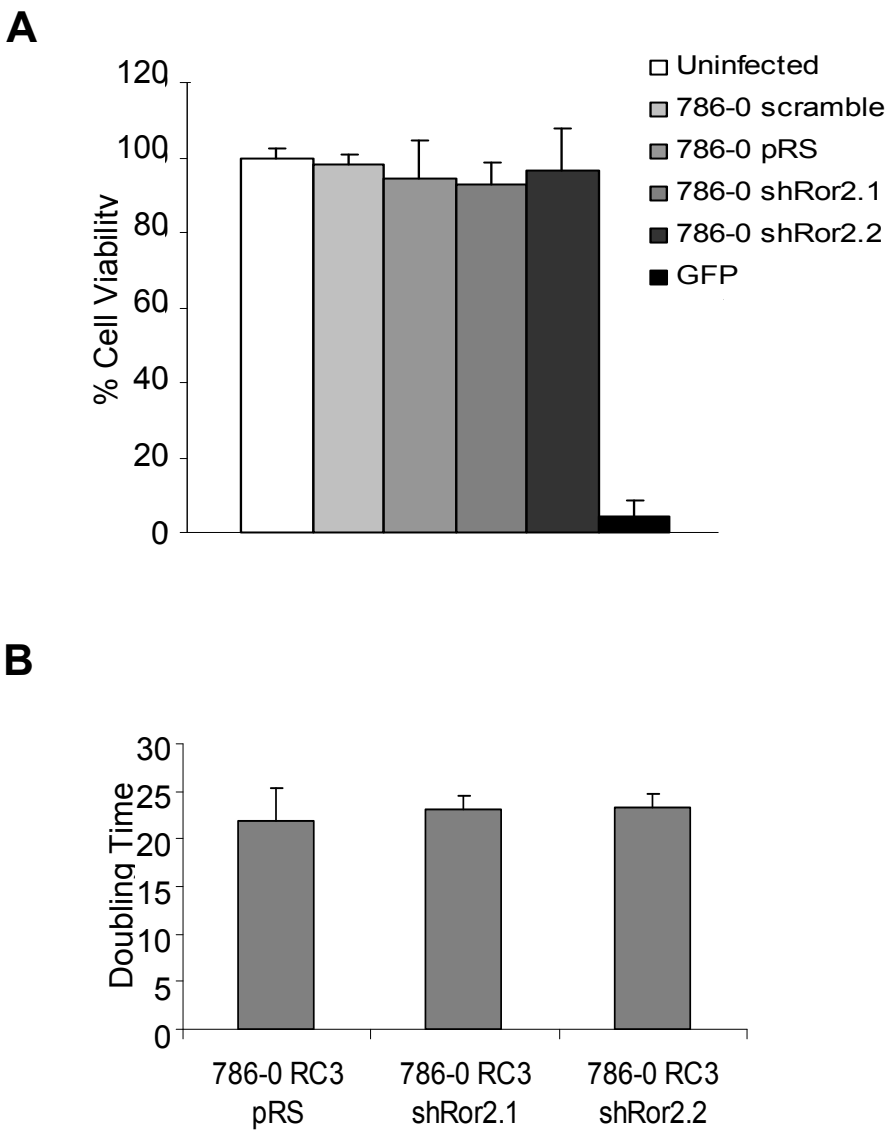


Figure 3.5: Ror2 suppression does not affect RCC cellular viability or doubling time.
A. Ror2 suppression does not inhibit RCC cell viability. RCC cells (786-0) were infected with a scramble short hairpin RNA retrovirus, a control virus pRS or Ror2 short hairpin RNA retroviruses or a GFP control (without a selection marker). Cells were plated on a 96-well plate and selected with puromycin for maintenance of the plasmid. Cellular viability was examined via an MTT assay with comparisons made to uninfected/unselected 786-0 cells. There is no significant difference in cellular viability between the infected shRNA cell lines and the uninfected and unselected cell lines. GFP infected cells confirm the selection activity.
B. Ror2 knockdown does not change doubling time in 786-0 RC3 cells. RCC cells (786-0 RC3) were infected with a control virus pRS or Ror2 short hairpin RNA retroviruses, they were then plated and doubling time was observed over multiple passages. No significant difference was observed in the doubling time of the control cell line as compared with the two independent shRor2 cell lines.

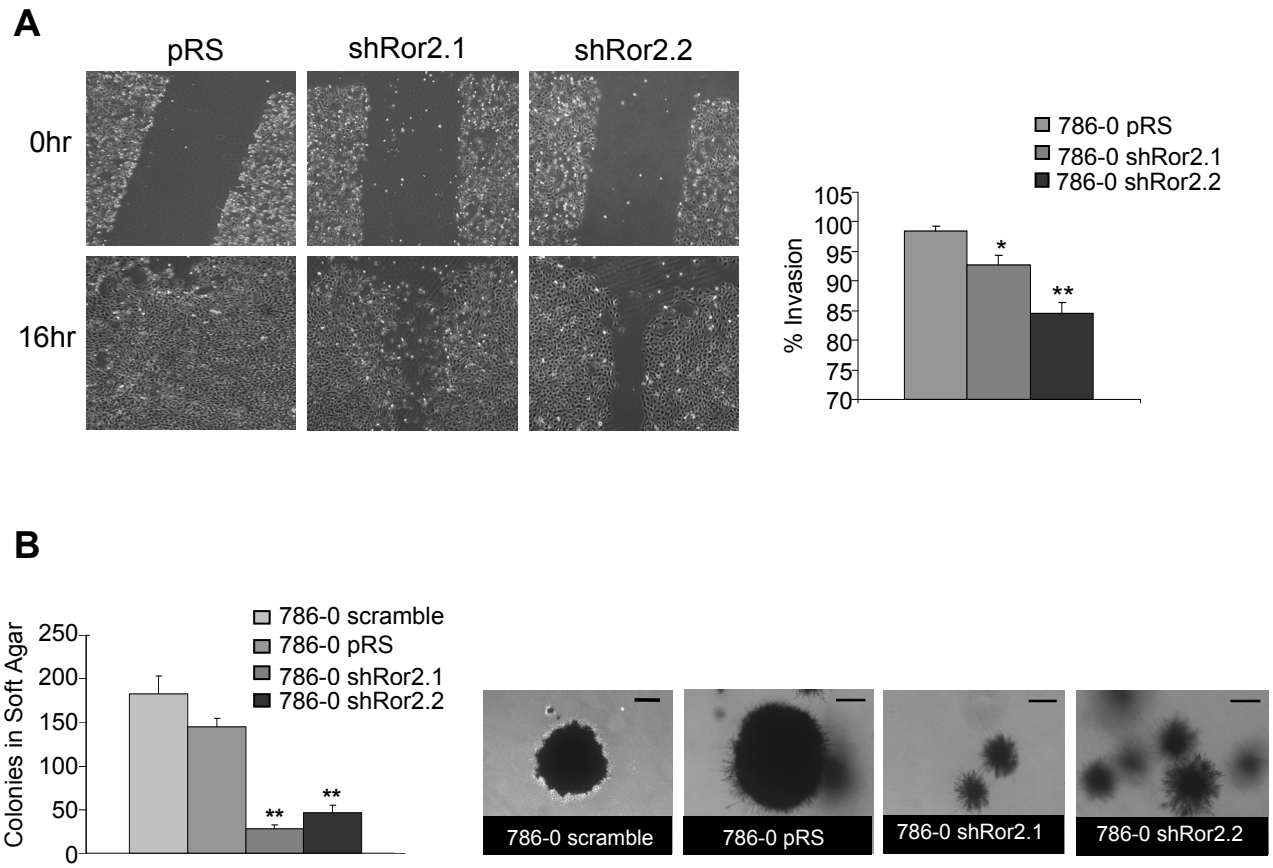


Figure 3.6: Ror2 expression directs anchorage independent growth and invasive potential *in vitro*.
A. Ror2 knockdown decreases cell migration. RCC cells (786-0) infected with a control virus pRS or Ror2 short hairpin RNA retroviruses were plated and allowed to grow overnight. Cells were scratched and the length of the scratch was observed for both 0 hr and 16hr timepoints (left). Significant differences were observed when comparing the % invasion of the Ror2 knockdown cell lines compared to the empty vector pRS control (right) - (**p<0.0001, *p=0.009). Error bars represent SEM.
B. Ror2 knockdown inhibits anchorage independent growth. RCC cells (786-0) infected with a scramble short hairpin RNA retrovirus, a control virus pRS or Ror2 short hairpin RNA retroviruses were allowed to grow in soft agar over a period of 3-4 weeks. Ror2 knockdown cells have inhibited growth in comparison to the scramble short hairpin retrovirus and the empty vector pRS control. Multicellular colonies >150 uM were counted from triplicate plates. Data shown is from the combination of two representative experiments (**p<0.0001). Error bars represent SEM. Inset - Cells were stained with MTT dye, and pictures taken at 10x magnification. Black bar represents 67.3 um.

Ror2 overexpression enhances anchorage independent growth

As Ror2 suppression is shown to be necessary for anchorage independent growth, we also asked if Ror2 expression was sufficient to induce growth. Human Ror2 (hRor2) expression plasmids were generated for expression in two RCC cell lines – 786-0 where Ror2 is highly expressed (Figure 3.7A) and a derivative line, WT8, where Ror2 expression is negligible (Figure 3.7B). Adding more hRor2 to the 786-0 cell line did not change anchorage independent growth (Figure 3.7C) suggesting that increasing the level of Ror2 beyond a threshold fails to impact this feature of tumorigenicity. However, when hRor2 was added to an RCC cell line that lacks robust Ror2 expression, an increase in anchorage independent growth was observed (Figure 3.7D), suggesting that Ror2 is sufficient to enhance anchorage independent growth. Taken together, these data suggest that Ror2 expression is sufficient for the anchorage independent growth characteristic of RCC.

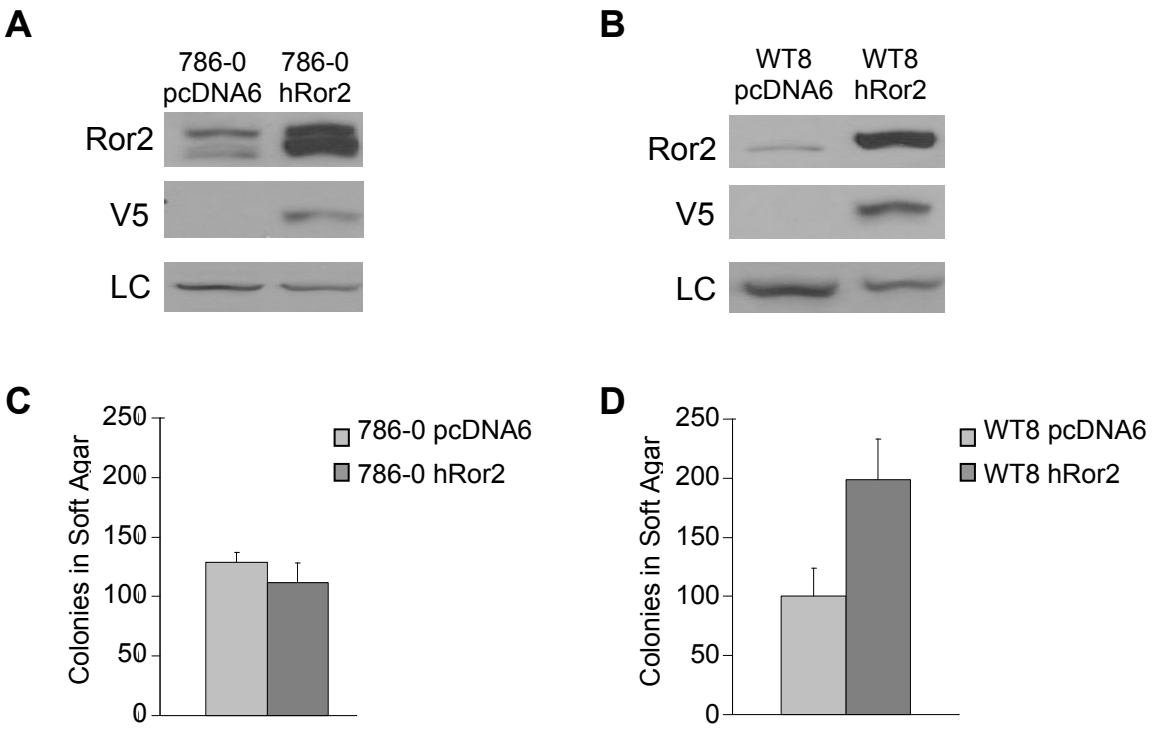


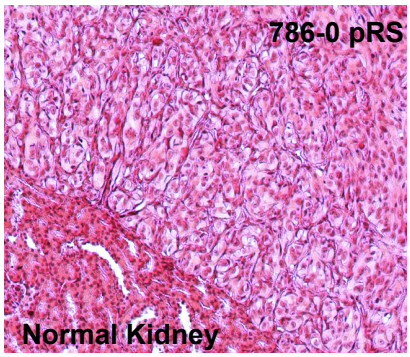
Figure 3.7: Ror2 overexpression enhances anchorage independent growth *in vitro*.
A. Ror2 expression is increased in 786-0 cells with an overexpression plasmid. RCC cells (786-0) were transfected with control plasmid pcDNA6 or hRor2 cDNA and immunoblotted with polyclonal Ror2 antibody, a V5 antibody or Ku80 antibody as a loading control (LC).
B. Ror2 expression is amplified in the 786-0 derivative cell line WT8, a clone expressing a VHL cDNA which expresses reduced levels of Ror2. The RCC cell line WT8 was transfected with a control plasmid pcDNA6 or hRor2 cDNA and immunoblotted with polyclonal Ror2 antibody, a V5 antibody or Ku80 antibody as a loading control (LC). Panel A and B represent a contiguous blot with intervening lanes removed.
C. An increase in Ror2 expression in Ror2 expressing cells does not affect anchorage independent growth. RCC cells with high levels of Ror2 already expressed (786-0) transfected with a control plasmid pcDNA6 or hRor2 cDNA were allowed to grow in soft agar over a period of 3-4 weeks. Multicellular colonies >150 uM were counted from triplicate plates. An increase in Ror2 expression did not change colony growth. Data shown is from two representative experiments.
D. Ror2 overexpression increases anchorage independent growth in cells lacking Ror2 expression. RCC cells lacking high level of expression of Ror2 (WT8) transfected with a control plasmid pcDNA6 or hRor2 cDNA were allowed to grow in soft agar over a period of 3-4 weeks. Multicellular colonies >150 uM were counted from triplicate plates. Ror2 overexpression cells displayed enhanced growth in comparison to the control empty vector pcDNA6. Data shown is from one representative experiment (p=0.079).

Ror2 suppression reduces tumor growth *in vivo*

As our data showed that 786-0 Ror2 shRNA suppressed anchorage independent growth *in vitro* in RCC cells, we also sought to assess tumor growth *in vivo*. Two independently derived Ror2 deficient cell lines and an isogenic control shRNA cell line were injected orthotopically in a cohort of athymic nude (*nu/nu*) female mice. Macroscopically visible tumors were only detected in mice injected with 786-0 isogenic control cells. Additionally, the control cell line had significantly more tumors than the two shRNA cell lines combined. The histology of a control engrafted tumor with adjacent normal kidney tissue is shown in Figure 3.8A. By comparison, tumor cells derived from the Ror2 suppressed cells were present on microscopic evaluation of two kidneys in the vicinity of the kidney subcapsule (Figure 3.8B, arrows), but failed to form clearly discrete masses. The extent of tumor growth, including tumors which implanted outside the area of injection, is quantified in the panel of Figure 3.8C and the average of all ten implants (in cm) was larger in the isogenic control cell line than in the two shRNA cell lines (Figure 3.8D). This experiment was replicated independently with xenografts grown longer than 3 months, and the results mirrored these findings (data not shown). MMP2 protein levels were also examined in these engrafted tumors. As previously shown at the transcription level, when Ror2 is expressed there is an abundance of MMP2 protein expression (Figure 3.8E, F). In tumors where Ror2 expression is suppressed, MMP2 protein levels are decreased (Figure 3.8G, H). This data confirms *in vivo* the findings of the experiments examining anchorage independent growth, validating a role for Ror2 in promoting tumor cell growth independent of tumor cell survival in

RCC. Further, this data provides concrete evidence that, for the first time, links Ror2 to RCC tumor development and places this signaling pathway as an immediate facilitator of tumor growth.

A



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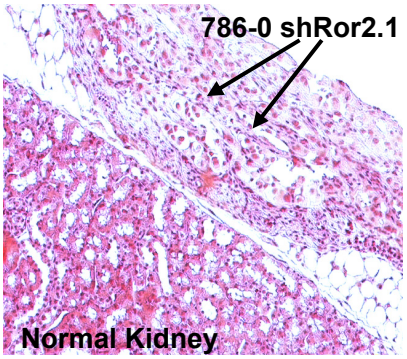
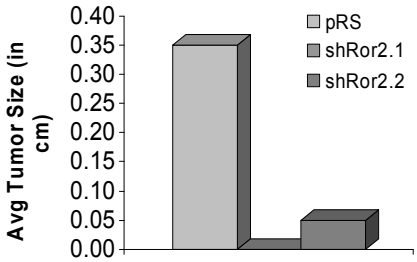


C

	786-0 pRS	786-0 shRor2.1	786-0 shRor2.2
Tumors/ implant	7/10 (70%)	1/10 (10%)	1/10 (10%)

D



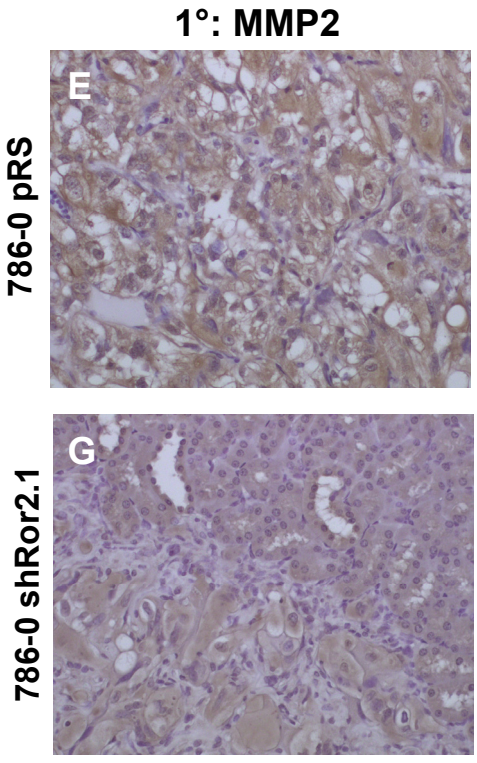


Figure 3.8: Ror2 suppression reduces tumor growth *in vivo*.
Two independent shRNA cell lines were injected orthotopically under the kidney capsule of athymic nude mice, compared to 786-0 pRS control cells.
A. Histology of control engrafted tumor with adjacent normal kidney tissue is shown.
B. H&E of the kidney surface demonstrated that Ror2-suppressed engrafted tumor cells were present in the vicinity of the subcapsule microscopically but failed to form distinct tumors.
C. Macroscopically visible tumors were only detected in mice injected with 786-0 pRS control cells. Macroscopic and microscopic evidence of tumor cells was tabulated, demonstrating significantly more tumors identified in the control cell implanted mice than those implanted with either of the two Ror2 shRNA cell lines.
D. Average tumor diameter of each implant from the 786-0 pRS control cell line and the two Ror2 shRNA cell lines (in cm).
E. The control engrafted tumor, 786-0 pRS, displays increased levels of MMP2 as determined by immunohistochemistry.
F. Control panel for 786-0 pRS shows negative staining in the no primary control.
G. The Ror2-suppressed engrafted tumor, 786-0 shRor2.1, has diminished levels of MMP2 expression compared to the empty vector control, 786-0 pRS.
H. Control panel for 786-0 shRor2.1 shows insignificant background staining in the no primary control.

DISCUSSION

In a strategy to uncover potential signaling kinases for RCC, we have identified the developmentally regulated orphan kinase Ror2 as an activated tyrosine kinase in RCC cancer cell lines and renal tumors. Ror2 is physiologically expressed primarily on embryonic mesenchymal cells and developmentally expressed on migrating mesenchymal cells of the fetal kidney. The aberrant expression of Ror2 in human RCC tumors has not been specifically described to date, although evidence of active Ror2 has been observed on kinase arrays from glioblastoma cell lines (4). In this study, however, for the first time we show that Ror2 expression was detected in the majority of human RCC tumors, and protein expression was confirmed at the tumor cell level. These data support the identification of a tumor cell intrinsic receptor tyrosine kinase, usually limited to developing mesenchymal cell subsets, that is detectable on the majority of renal cell carcinomas.

Ror2 expression in RCC may herald the activation of many signalling events, particularly in the aberrant context of a tumor cell environment. For epithelial cells to metastasize, they must acquire mesenchymal characteristics that permit proliferation outside of the usual niche. Ror2 is primarily expressed in mesenchymal cells, immediately suggesting a possible role for this kinase as part of the ECM remodeling process in tumor development. Expression of the Ror2 kinase in human tumors was found to be tightly correlated with genes involved at the ECM, in particular MMP2, linking this epithelially derived tumor with mesenchymal phenotypic markers and a potentially important transitioning event in the development of invasive renal cell carcinoma. Matrix metalloprotease-2 (MMP2) has previously been shown to be

overexpressed in many metastatic cancers including RCC (23, 24) and Ror2 may define an important and unique pathway for this process in kidney cancers (23). MMP2, in particular among the MMP family, is essential for the renal tubular cell epithelial to mesenchymal transformation (EMT) specific to the recovery from renal tubular injury (25). This process requires adaptations of the extracellular matrix as well as mechanisms of promoting cellular migration. Using wound healing as a surrogate for cell migration, we found that Ror2-suppressed cells migrated at a slower rate than their isogenic partners. As a critical test of tumor cell growth in a three-dimensional environment, Ror2 expression supported anchorage independent cell growth, an indicator of tumor cell growth and a critical quality of metastatic tumors. We further demonstrated that suppression of Ror2 was sufficient to inhibit tumor growth in xenografts, suggesting a key role in permitting the 3-dimensional growth essential for tumor formation.

It is additionally intriguing to consider the unique embryonic origins of the kidney, in which the epithelial tissues are entirely derived from a mesenchymal source. The process of developing the excretory tubules has in fact been termed a "mesenchymal to epithelial transition" (26). The kidney is derived from the ureteric bud and the metanephric mesoderm (27), thus the emergence of Ror2 as a part of a dedifferentiation profile may provide the embryonic signal necessary to reinact a mesenchymal program in malignant epithelial cells derived from the kidney. Taken together, these data suggest that Ror2 can play an important role in renal cell carcinoma growth patterns by providing tumor cells with an immature phenotype primed for invasive growth.

Further, the regulation of Wnt signaling via Wnt receptors such as Ror2 can be greatly influenced by the availability of Wnt ligands, Frizzled family members, and factors such as the secreted frizzled related protein 1, which may influence the activity of Ror2 in tumorigenicity (28). It remains uncertain which specific Wnt signaling pathway Ror2 engages as a cancer promoting factor; thus, the identification of Ror2 as a feature of RCC presents a novel avenue for future integrated studies of developmental and cancer biology.

Ror2 activity thus defines a novel component of the signaling machinery in place to promote the growth of many renal tumors and may define a unique tumor subset with distinct pathologic or physiologic characteristics. Finally, this newly identified kinase in RCC provides an ideal candidate to explore as a marker of tumors with aggressive growth potential or as a putative target to disable tumor growth.

MATERIALS AND METHODS

Cell Culture

786-0 and RCC4 cell lines and their derivatives, including the paired cell lines 786-0 WT8 and 786-0 RC3 (kindly provided by Dr. W. Kaelin, Boston, MA), RCC4 2-1 and RCC4 3-14 (kindly provided by Dr. M.C. Simon and Dr. B. Keith, Philadelphia, PA), were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), L-glutamine and non essential amino acids. The shRNA knockdown cells were generated by infecting the cells with commercial shRNAs for Ror2 (Origene Technologies Inc, Rockville, MD). Two shRNAs targeting separate portions of the gene were used in these studies. Selection to maintain the shRNA was achieved with puromycin supplementation of the media for these cells. Cell lines overexpressing Ror2 were generated by transferring hRor2 cDNA (Origene Technologies Inc, Rockville, MD) into the pcDNA6/V5-HisA plasmid (Invitrogen, Carlsbad, CA) via SacI/XhoI restriction enzyme sites (New England BioLabs Inc, Ipswich, MA). The HKC line (kindly provided by Dr. L. Racusen, Baltimore, MD (29)) was maintained in DMEM/F12 supplemented with 10% FBS.

Immunoblotting

Cells were lysed in whole cell extraction buffer containing 20mM Tris, 100mM NaCl, 1 mM EDTA, 1% NP-40 and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Bradford reagent was used to assess the concentration. Protein samples were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose (GE, Pittsburg, PA). The following antibodies were used to assess for

expression: Monoclonal Ror2 at 1:250 (RND Systems, Minneapolis, MN), Polyclonal Ror2 at 1:3000 (RND Systems, Minneapolis, MN), Twist1 at 1:1000 (Cell Signaling Technology Inc, Danvers, MA), V5-HRP at 1:2500 (Invitrogen, Carlsbad, CA) and Ku80 at 1:2000 (Genetex Inc, San Antonio, TX) as a loading control (LC). Appropriate horseradish peroxidase conjugated secondary antibodies were used and proteins detected using ECL-Plus chemiluminescence reagents (GE, Pittsburg, PA).

Wound Healing Assay

35,000 cells were plated in triplicate on 6-well plates and allowed to adhere overnight at 37°C in 10% FBS containing RCC media. The monolayer was then "wounded" in perpendicular directions with a 200ul pipet tip (forming a cross) and 2% FBS containing RCC media was added. Pictures were taken at each of the cross section junctions at 4x magnification at 0, 8, 16, and 24 hours.

Soft Agar Colony Formation Assay

0.6% bottom agar was plated onto 6-well plates. 10,000 cells were then plated in triplicate, placed at 37°C overnight and covered with 0.2mL RCC media. Colonies were allowed to form over 3-4 weeks and then stained with 2mg/mL MTT (Sigma-Aldrich, St. Louis, MO) for 1-4hrs. Images were taken at 10x magnification. Black bar represents 67.3μm.

Cell Viability Assay

A modified MTT assay (Promega, Madison, WI) was used to assess cell viability. 5,000 cells were plated with 50uL selection media for 5 days. The MTT dye containing the tetrazolium salt was added according to manufacturer instructions and absorbance measured at 570nm.

Doubling Time Assay

300,000 cells were plated and after 96hrs, the total number of cells was counted. This process was repeated 10 times and the doubling time calculated.

qRT-PCR Analysis

Total RNA was prepared from 80% confluent plates using Gentra Systems RNA Extraction Cell Kit (Minneapolis, MN). cDNA was made from 0.5µg total RNA by reverse transcriptase (RT) using oligo dT primers and the Superscript II RT-PCR reagents (Invitrogen, Carlsbad, CA). For automated TaqMan qRT-PCR, proprietary commercial FAM labeled primers were used targeting Ror2, MMP2, Twist1 and β-actin (Applied Biosystems, Foster City, CA) and analyzed on the 7900H Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

Microarray Analysis

Fresh frozen tumors from the UNC Tissue Procurement Facility tumor bank (1994-2007) were used for these studies. Total mRNA was prepared as described above, incorporating a Rotor-Stator for tissue homogenization and quantified with the NanoDrop (NanoDrop Technologies, Wilmington, DE). RNA quality check,

amplification, labeling, and hybridization were performed by the UNC Genomics Core Facility. Two-color hybridization was performed, with a modified reference human mRNA derived from the Stratagene Universal Human Tumor Reference RNA (kindly provided by Dr. C. Perou, University of North Carolina at Chapel Hill) and compared to each tumor.

Raw data was derived using Agilent feature extraction software, and results were expressed as a Log (2)-transformed ratio between tumor and control mRNA. Three-way comparison was performed using Significance Analysis of Microarrays (SAM) version 3.01 (30) for Figure 3.1. The 250 most significantly altered genes were then used for clustering analysis. After duplicate genes were removed, targets were median centered and complete linkage analysis was performed with Pearson's Correlation with Cluster 3.0. For Figure 3.2, quantitative analysis of data according to Ror2 gene expression was performed using SAM. Genes with a false discovery rate <5% were then used for average linkage analysis with Cluster 3.0. Gene set analysis was also performed in SAM according to Ror2 gene expression values, using a cutoff of FDR=0. Gene sets analyzed were from Erin Segal or the MSigDB collection at the Broad Institute.

Xenograft Analysis

500,000 cells were injected orthotopically under the kidney capsule in a cohort of athymic nude (*nu/nu*) female mice and aged 2.5 or 3 months. The xenografted tumors were formalin fixed, paraffin embedded and tissue blocks were serially

sectioned onto unprepared glass slides. Xenografted tumors were stained with hematoxylin and eosin (H&E) for tumor verification. The slides were immunostained with MMP2 antibody 3158 (Abcam Inc, Cambridge, MA) and detected as previously described in Chapter 2.

Statistical Analysis

The standardized cT values were fit with a one-way ANOVA model with the experimental variable of interest (Ror2, etc) as the fixed effect. To verify the one-way ANOVA model, a nonparametric statistical procedure, Kruskal-Wallis test, was performed to compare the standardized cT among the cell lines. Error bars are shown as standard error of the mean.

Ror2 expression was evaluated across RCC tumor histologies using a Student's T test to assign the p-value as shown. Because clear cell tumors are more common than other histologies, the difference in sample size is reflected in the standard deviations using an adjustment to account for unequal variances. A non-parametric Wilcoxon Rank Sum procedure was performed to confirm the significant result found using the parametric two sample t-test.

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CHAPTER 4

IDENTIFICATION OF ROR2 AS A HYPOXIA INDUCIBLE FACTOR (HIF) TARGET IN VON HIPPEL-LINDAU (*VHL)* ASSOCIATED RENAL CELL CARCINOMA

This work is modified from: Wright and Rathmell (2010), Journal of Biological Chemistry (1)

ABSTRACT

Ror2 is an orphan receptor tyrosine kinase with expression normally restricted to early stages of development. However, emerging evidence has placed aberrantly expressed Ror2, leading to an invasive phenotype, in several cancers including renal cell carinoma (RCC). Though Ror2 is currently identified as upregulated in an assortment of cancers, neither the regulatory role or mechanism of action have been delineated. We sought to place Ror2 in RCC's most commonly mutated pathway, the loss of the tumor suppressor von Hippel-Lindau, VHL, which causes hypoxia induced factor (HIF)-1α and 2α stabilization and the transcriptional activation of a broad repertoire of response genes. We found that Ror2 was indeed associated with pVHL loss in RCC as well as with VHL somatic mutations tightly coordinated with the induction of RCC. Additionally, knockdown and rescue analysis of HIF expression suggests that Ror2 is dependent on the pathologic stabilization of either HIF-1α or HIF-2α. Subsequent evaluation of the Ror2 promoter suggests that HIF-2 and its dimerization partner, aryl hydrocarbon nuclear transferase, ARNT, localize to the Ror2 promoter via a cryptic transcriptional element. This data substantiates a unique regulation pattern for Ror2 in the VHL-HIF axis that has the potential to be applied to other cancer etiologies.

INTRODUCTION

The tyrosine kinase Ror2 was initially identified as a homologue of the Trk neurotrophin receptors (2) and later as a member of the receptor tyrosine kinase superfamily (3). Ror2 is an orphan receptor with expression in the developing embryo identified in the embryonic limb buds, heart, primitive genitalia, developing somites, and mesenchymal cells in the developing lung, kidney, and cephalic regions (4-6). In the adult organism, Ror2 expression is present as a part of osteoblast differentiation, highly induced in the preosteoblast stage (7), and is suppressed as these cells terminally differentiate as osteocytes. This pattern of expression is inversely related to that of secreted frizzled related protein 1 (sFRP1), and can be transcriptionally suppressed by ectopic expression of sFRP1 in this cell type, but further insights into the major elements of Ror2 regulation are not known.

Aside from developmental programs regulating bone morphogenesis and primitive organ development, Ror2 has only recently been recognized to play a role in the adult organism. We have identified Ror2 expression as a characteristic of many renal cell carcinoma (RCC) cell lines and human tumors (8), where its expression is associated with increased cell migration and anchorage independent growth. Ror2 also plays a prominent role in osteosarcoma (9) and has recently been identified in squamous cell carcinoma of the head and neck, where expression parallels the development of invasive features of these tumors (10). Further, in a tumor genomic analysis of invasive gastric cancers, Ror2 was identified as a frequent target of mutagenesis (11). These findings place Ror2 as a frequently upregulated feature of human cancers, and in each case, is associated with invasive

tumor characteristics. Though Ror2 has been identified as a frequently overexpressed protein in a variety of tumor types, what regulatory or mechanistic events contributing to increased Ror2 expression in these tumors have yet to be deduced.

Ror2 was initially identified as a renal tumor antigen in cell lines derived from clear cell renal cell carcinomas which were known to have inactivating mutations of the von Hippel-Lindau (*VHL*) tumor suppressor. The majority of clear cell RCC tumors are characterized by mutation, methylation, or loss of the *VHL* gene (12-15). The most well-documented function of the pVHL protein is to act as the substrate recognition component of an E3 ubiquitin ligase complex which includes Elongin C, Elongin B, Cullin 2, and ring box protein 1 (Rbx1 or Roc1) (16-19). The substrates of pVHL E3 ligase activity most tightly associated with RCC are the hypoxia inducible factor (HIF)-α subunits (HIF-1α and HIF-2α), a family of transcription factors which coordinate much of the physiologic response to restricted oxygen availability (20-23). Under normal oxygen conditions, the prolyl hydroxylases (PHDs) hydroxylate the HIF subunits which are subsequently recruited by pVHL to an E3 ubiquitin ligase complex for ubiquitylation, leading to proteasomal degradation (24-26). Under low oxygen conditions or as a result of *VHL* inactivation, one or both of these HIF factors are stabilized leading to the formation of a transcriptional complex with aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIF-1□), inducing the transcription of a large panel of hypoxia responsive genes including vascular endothelial growth factor (VEGF), glucose transporter 1 (Glut1), prolyl hydroxylase family member Egln3 (also known as PHD3), among many others

(27, 28). This transcriptional response leads to the highly vascular phenotype of RCC and the transformed phenotype of the cells (29).

VHL mutation is an important event in the development of RCC, and activation of components of the HIF pathway can be detected early in pre-malignant cysts that precede the development of cancer in patients harboring a germline mutation in *VHL* (30). However, within the spectrum of *VHL* mutation, tumors can demonstrate either stabilization of one or both these HIF factors, and may promote the transcriptional activation of certain subsets of the repertoire of hypoxia response induced genes (28). Additionally, the *VHL* mutational subtype may itself mediate HIF expression patterns based on studies *in vitro* (31), which parallel tumor growth *in vivo* (32). In particular, examination of human RCC tumors has demonstrated that the molecular profile of tumors is highly dependent on the expression of HIF1 and HIF2 in comparison to those tumors solely expressing HIF2, with distinctions which correlate with divergent tumor activity (33).

Though *VHL* mutation and HIF dysregulation have been identified as major contributors to the RCC tumor phenotype, the specific molecular mechanisms associated with this pathway that contribute to RCC features of cell growth, invasion or metastasis remain an active area of investigation. Thus, we sought to examine the potential that Ror2 regulation was occurring as a part of the *VHL*/HIF axis in RCC. We found that Ror2 expression was definitively associated with the loss of pVHL as well as with *VHL* mutations most tightly correlated with HIF-2α dysregulation. Knockdown analysis and rescue experiments suggest that Ror2 is dependent on the pathologic stabilization of either HIF-2α or HIF-1α expression,

although it is not expressed as a component of the physiologic response to hypoxia. This finding prompted an examination of the Ror2 promoter, which suggests that HIF-2α and ARNT are localized to the Ror2 promoter, potentially utilizing a cryptic element for its interaction. Coupled with observations that Ror2 has been shown to be involved in the invasive tumor phenotype of RCC and other malignancies, this study places Ror2 in the *VHL*–associated HIF transcriptional pathway associated with *VHL* mutation-mediated stabilization of HIF factors providing support for a model where the spectrum of HIF target genes activated as a result of *VHL* mutation may contribute substantially to the phenotype of an individual tumor.

RESULTS

Ror2 expression in clear cell RCC is dependent on VHL status

Our previous work has shown that Ror2 is expressed in RCC tumors and RCC cell lines. However, the tumorigenic mechanism that contributes to this aberrant regulation of Ror2 in RCC remained uncertain. Since pVHL expression is tightly associated with clear cell RCC, and the RCC cell lines in which Ror2 was initially identified lack functional pVHL expression, we sought to evaluate the expression of Ror2 in RCC cell lines in relation to pVHL status. Whole cell extracts were examined by immunoblot for Ror2 expression in two independent sets of RCC cell lines. Ror2 was first examined in the *VHL* mutant cell line 786-0, stably transfected with empty expression vector (786-0 RC3), confirming Ror2 protein expression demonstrated by two independent antibodies targeting the Ror2 extracellular domain. However, when pVHL expression was restored by the addition of a HA-tagged *VHL* cDNA (786-0 WT8), Ror2 expression was undetectable using either antibody (Figure 4.1A). To explore the nature of this regulation as a transcriptional event or as a consequence of protein stabilization, the *VHL*-dependent expression of Ror2 in RCC was assayed at the mRNA level using qRT-PCR. The *VHL* (-) 786-0 RC3 vector control cell line had significantly enhanced Ror2 expression of transcript compared to the *VHL* (+) wildtype 786-0 WT8 derivative cell line, for which no transcript was detectable (Figure 4.1B), paralleling the protein levels.

To verify that the *VHL* dependent regulation of Ror2 observed was not cell line specific, we utilized the RCC4 paired cell line for further analysis. Our results

showed that *VHL*-null RCC4 cells stably transfected with vector (RCC4 2-1) expressed Ror2, whereas RCC4 cells stably expressing an HA-tagged wild type *VHL* cDNA (RCC4 3-14) displayed dramatically reduced levels of Ror2 protein as detected by immunoblot (Figure 4.1C). This *VHL* dependency was also confirmed at the transcript level as Ror2 mRNA was suppressed to a significant degree in the *VHL* (+) cell line RCC4 3-14 compared to the *VHL* (-) counterpart RCC4 2-1 (Figure 4.1D). These findings confirm that Ror2 expression is *VHL*-dependent at both the protein and mRNA levels suggesting a transcriptionally regulated mechanism of Ror2 expression is involved in multiple RCC cell lines.

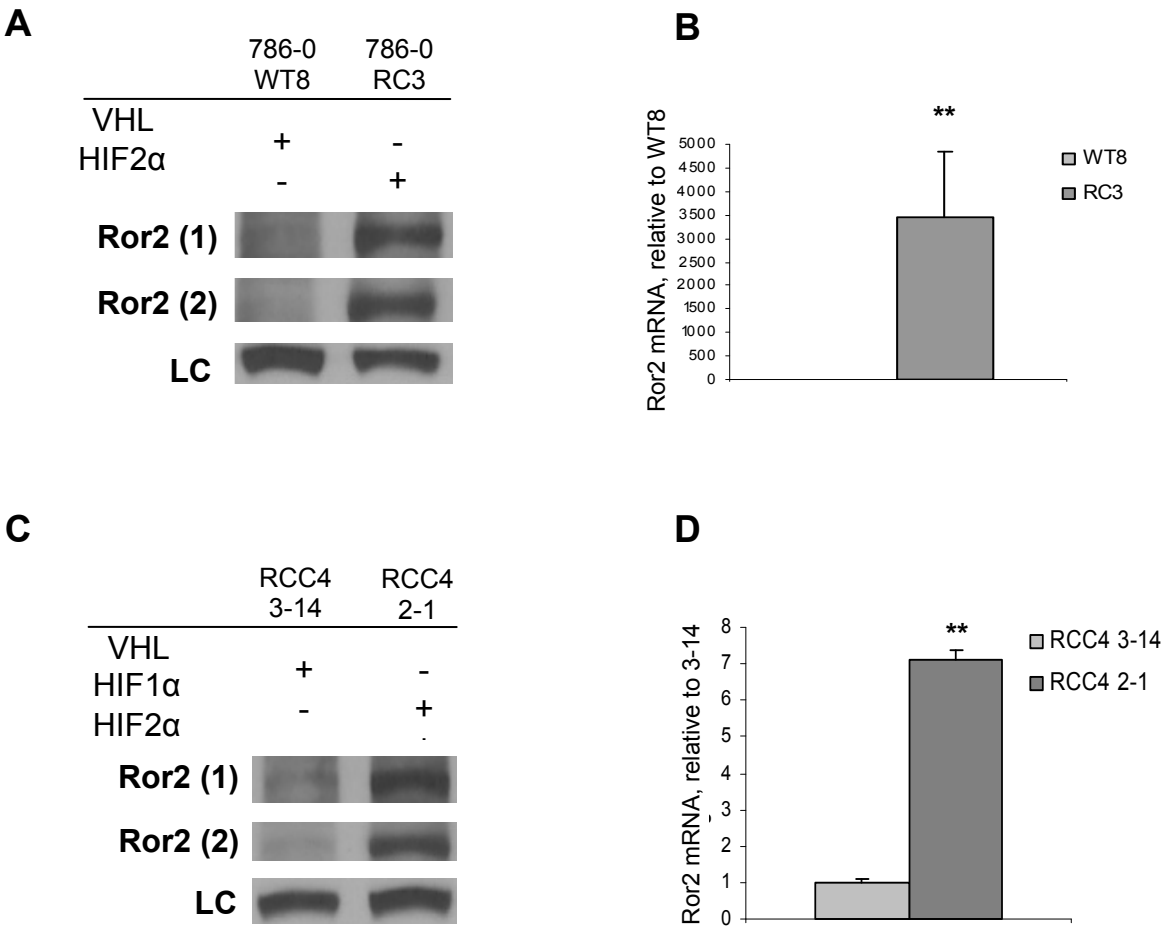


Figure 4.1: Ror2 is regulated by *VHL* status.
A. Ror2 is expressed when VHL is mutated in the 786-0 paired cell line. Whole cell extracts from 786-0 cells expressing (786-0 WT8) or lacking expression (786-0 RC3) of pVHL were immunoblotted with monoclonal Ror2 antibody - Ror2 (1), polyclonal Ror2 antibody - Ror2 (2) and Ku80 antibody as a loading control (LC).
B. Ror2 expression is regulated at the transcriptional level in the paired 786-0 cell lines. qRT-PCR analysis data is normalized to β-actin as an RNA internal standard and displayed as expression levels relative to the pVHL expressing member of the cell line pair. Significant differences were observed as Ror2 mRNA levels were suppressed in the VHL (+) cell line (light gray), relative to the paired vector transfected VHL (-) control (dark gray), **p<0.0001.
C. Ror2 is expressed when VHL is mutated in the RCC4 paired cell line. Whole cell extracts from RCC4 cells expressing (RCC4 3-14) or lacking expression (RCC4 2-1) of pVHL were immunoblotted with monoclonal Ror2 antibody - Ror2 (1), polyclonal Ror2 antibody - Ror2 (2) and Ku80 antibody as a loading control (LC).
D. Ror2 expression is regulated at the transcriptional level in the paired RCC4 cell lines. qRT-PCR analysis data is normalized to β-actin as an RNA internal standard and displayed as expression levels relative to the pVHL expressing member of the cell line pair. Significant differences were observed as Ror2 mRNA levels were suppressed in the VHL (+) cell line (light gray), relative to the paired vector transfected VHL (-) control (dark gray), **p=0.0001.

As regulation of Ror2 by wild type pVHL protein was evident at both the protein and mRNA levels, we also sought to evaluate the expression of Ror2 using the tight genotype/phenotype correlation seen with representative *VHL* mutations associated with the various *VHL* disease subtypes. We examined a panel of *VHL* somatic point mutants expressed in 786-0 cells, representative of the mutant *VHL* transgenes seen in the major subtypes of *VHL* disease associated with cancer predisposition. *VHL* Type 2B mutations are associated with the most diverse presentation of *VHL* disease and are thought to be more highly penetrant for the development of RCC. In these stable clonal cell lines expressing representative pVHL proteins, overexpression of Ror2 was detected by immunoblot only in the absence of pVHL or in the presence of a representative mutant pVHL of *VHL* disease, subtype Type 2B (Figure 4.2). This mutation, arginine 167 to glutamine (R167Q), has previously been associated with HIF-2α stabilization similar in extent to the *VHL* (-) cells (31). Further, representative mutations of *VHL* disease subtypes 2A, which displays minimal deregulation of HIF factors and low risk for renal cell carcinoma and Type 2C, which retains complete regulation of HIF factors and produces no risk for renal cell carcinoma, were completely able to reverse the aberrent Ror2 expression. This data ties the expression of Ror2 *VHL* mutation, and to correlative genotype/phenotype *VHL* regulation and its expression in RCC.

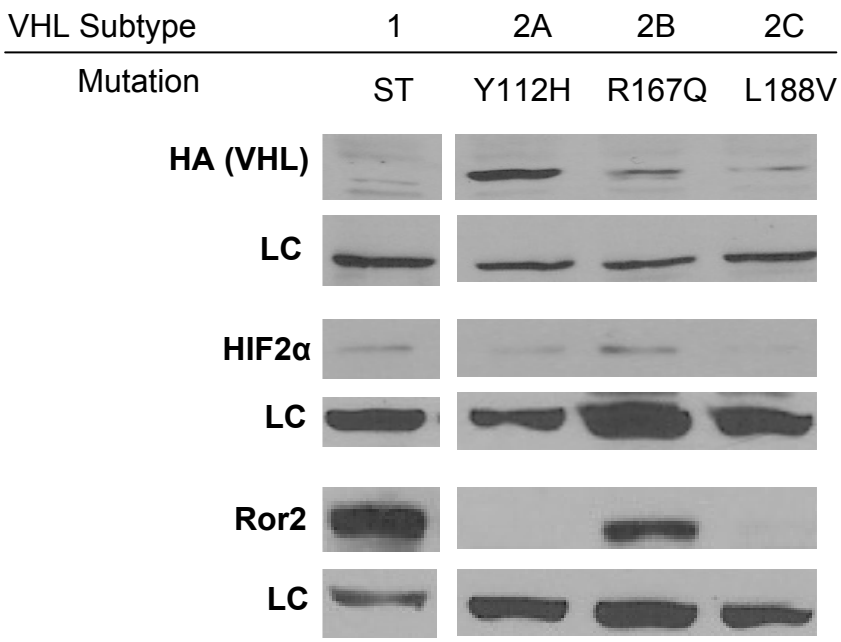
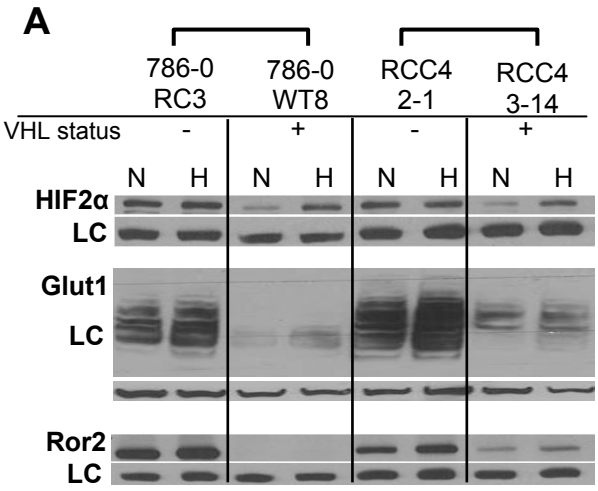


Figure 4.2: Expression of Ror2 in *VHL* somatic mutation subtypes.
A panel of *VHL* point mutant transgenes expressed in 786-0 cells, which recapitulate the mutant *VHL* genes representative of the major subtypes of *VHL* disease, was subjected to immunoblot analysis. There is graded HIF-2α expression associated with the *VHL* mutations, with the greatest HIF-2α levels present in the cells expressing the *VHL* type 2B mutant and the vector transfected cells which lack pVHL expression. Expression of Ror2 is permitted only in the most penetrant subtype, *VHL* Type 2B.

Minimal induction of Ror2 under hypoxic conditions

The findings that Ror2 expression was associated with *VHL* loss or mutations associated with HIF deregulation suggested that Ror2 expression was correlated with HIF-2α stabilization. Thus, we asked if Ror2 might be regulated by the cellular hypoxic response. Multiple strategies were evaluated as stimulants of Ror2 expression, beginning with prolonged exposure to 1% hypoxia. 786-0 and RCC4 derived cells were exposed to 1% oxygen over a 16 hour period of induction. HIF levels were elevated at baseline in the vector transfected control *VHL* mutant cell lines, and further induction with hypoxia was not observed. As expected, physiologic stabilization of HIF-2α in response to hypoxia was observed by immunoblot in each of the pair of cell lines expressing functional pVHL. At the protein level, no discernable change in Ror2 was observed, in contrast to induction of the established hypoxia target Glut1 (glucose transporter-1) - Figure 4.3A. We further examined Ror2 as a target of the hypoxic response by evaluating Ror2 protein levels in the paired 786-0 RCC cells after 48 hours of stimulation with cobalt chloride ($CoCl_2$), a potent hypoxia mimetic. Again, in contrast to the effect on HIF-2α levels, which were induced as predicted in those cells expressing wild type pVHL, Ror2 protein levels were at most only minimally induced in the cell lines with functional *VHL* in contrast to the vivid induction seen with the established hypoxia target Egln3. $CoCl_2$ treatment did not further induce expression levels in the cell line lacking functional *VHL* (Figure 4.3B). Additional studies extending the period of hypoxic induction out to 72 or 96 hours failed to induce Ror2 levels farther (data not shown).

As the primary event of hypoxia response signaling occurs at the transcriptional level, effects of chemical induction of the hypoxia response were examined using quantitative RT-PCR. We again examined the effect of $CoCl_2$ treatment on activation of Ror2 and the hypoxia response. The HIF target transcript Egln3 (a HIF dependent prolyl hydroxylase) served as an additional control to Glut1 to demonstrate the effect of these treatments on hypoxia response gene induction. In response to $CoCl_2$ treatment, we observed an appropriate and significant increase in Egln3 mRNA after 24 hours. However, no significant change was observed in the levels of Ror2 transcript in response to this treatment (Figure 4.3C, left). A second, more potent, chemical mimetic of hypoxia, the prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG), was tested to determine if maximal induction of this pathway may be sufficient to generate an induction in Ror2 levels after 24 hours of treatment. Both control transcripts (Glut1 and Egln3) were robustly induced in response to this treatment within the 786-0 WT8 cell line (with functional *VHL*) demonstrating a functional hypoxia pattern of transcriptional response. Again, the more potent hypoxia mimic, DMOG, also failed to show a statistically significant increase in total Ror2 transcript levels (Figure 4.3C, right). Therefore, although *VHL* inactivation is associated with the dysregulated expression of Ror2, activation of the hypoxia response by either potent chemical inducers or profound exposure to low oxygen levels were not sufficient to engage the transcript or protein expression of Ror2.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786-0 RC3
786-0 WT8
RCC4 2-1
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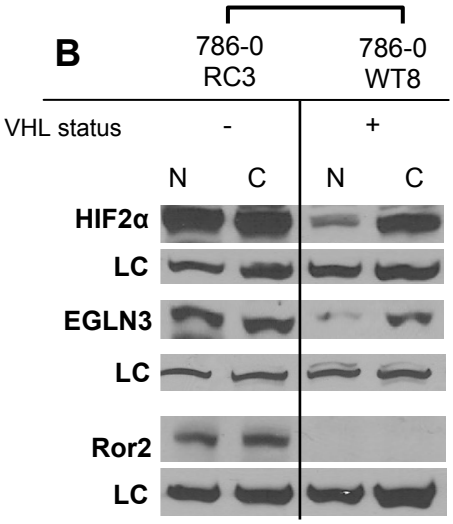
HIF2α
LC
Glut1
LC
Ror2
LC


B
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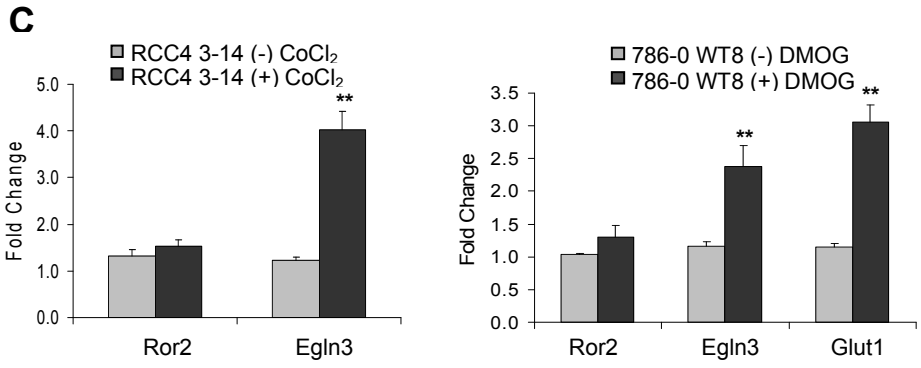
C
RCC4 3-14 (-) CoCl2
RCC4 3-14 (+) CoCl2
Fold Change
5.0
4.0
3.0
2.0
1.0
0.0
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Ror2
Egln3
786-0 WT8 (-) DMOG
786-0 WT8 (+) DMOG
Fold Change
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
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Ror2
Egln3
Glut1

Figure 4.3: Minimal hypoxia induction of Ror2 expression.
A. Ror2 expression is minimally induced under 1% hypoxic (H) conditions. Whole cell extracts from 16 hour normoxic (ambient oxygen, N), and hypoxic (1% oxygen, H), treated cell cultures were immunoblotted for HIF-2α, Ror2, Glut1, and Ku80 as a loading control (LC). HIF-2α and Glut1 induction is expected due to protein stabilization under conditions of hypoxia in cells expressing VHL and confirms the exposure of cultures to the hypoxic condition but minimal change in the Ror2 protein level is detected.
B. Ror2 expression is minimally induced after 48 hours of exposure to cobalt chloride (C). Normoxic (N) and cobalt chloride, $CoCl_2$, treated (C) protein samples were immunoblotted with HIF-2α antibody to show that HIF-2α was induced in the VHL (+) cell lines. However, Ror2 levels remained stable to this manipulation. Ku80 antibody used as a loading control (LC).
C. *Left* - Ror2 mRNA levels are minimally induced under hypoxic-like conditions in RCC4 cells. qRT-PCR analysis of the hypoxia mimetic cobalt chloride ($CoCl_2$) transcriptional induction after 24hr in the *VHL* expressing cell line RCC4 3-14 demonstrate induction of the HIF target gene Egln3 (**p=0.0015) in response to treatment with hypoxia mimetic, confirming HIF transcriptional activity. *Right* - Ror2 mRNA levels are minimally induced under hypoxic-like conditions in 786-0 WT8 cells. qRT-PCR analysis of the hypoxia mimetic DMOG transcriptional induction after 24hr in the *VHL* expressing cell line 786-0 WT8 demonstrate induction of the HIF target genes Egln3 (**p=0.0091) and Glut1 (**p=0.0015) in response to treatment with the hypoxia mimetic, confirming HIF transcriptional activity. Ror2 transcript levels show minimal enrichment upon treatment. Transcript values are shown as normalized to β-actin RNA internal standard and relative to the unstimulated cells of each set of paired cells.

Ror2 is regulated by HIF-2α expression

Since HIFα-mediated transcriptional induction is critical to the role of *VHL* mutation in RCC (29) and Ror2 expression was increased at the transcriptional level in a *VHL*-dependent manner, we sought to further evaluate whether Ror2 expression shared any association with HIFα dysregulation. As the *VHL*-deficient 786-0 cells express only HIF-2α, we used these cells to examine the linkage between HIF stabilization and HIF-dependent transcriptional regulation of Ror2 by suppressing HIF-2α in 786-0 cells with two independent shRNA constructs (shRNA constructs kindly provided by W. Kaelin, Dana Farber Cancer Institute (34)). Stable knockdown of HIF-2α protein was achieved with both shRNA constructs compared to a scrambled control shRNA. Ror2 protein levels correlated linearly with HIF-2α, being markedly reduced when HIF-2α levels were suppressed by shRNA (Figure 4.4A). We additionally evaluated the effects of HIF-2α knockdown on transcript levels. This experiment demonstrated a reduction in HIF-2α transcript by shRNA suppression to at or below physiologic levels, and a corresponding reduction in Ror2 mRNA with both shRNA vectors (Figure 4.4B), further implicating this pathway in the mechanism of Ror2 expression in RCC.

As we found Ror2 to be suppressed when HIF-2α was knocked down, we also asked if Ror2 expression can be rescued. Using a HIF-2α double proline to alanine (DPA) mutant cDNA plasmid, which is resistant to hydroxylation and *VHL*-mediated degradation (generously provided by B. Keith and M.C. Simon, University of Pennsylvania), we examined 786-0 cells stably expressing either of the HIF-2α shRNA constructs transiently transfected with this HIF-2α DPA mutant. We

observed transient rescue of high levels of HIF-2α expression in the 786-0 HIF-2α knockdowns and saw a delayed partial rescue of Ror2 correlated with the highest levels of HIF-2α rescue (Figure 4.4C) suggesting that Ror2 is expressed downstream of HIF-2α and its expression is directly tied to HIF-2α expression in the context of *VHL* inactivation.

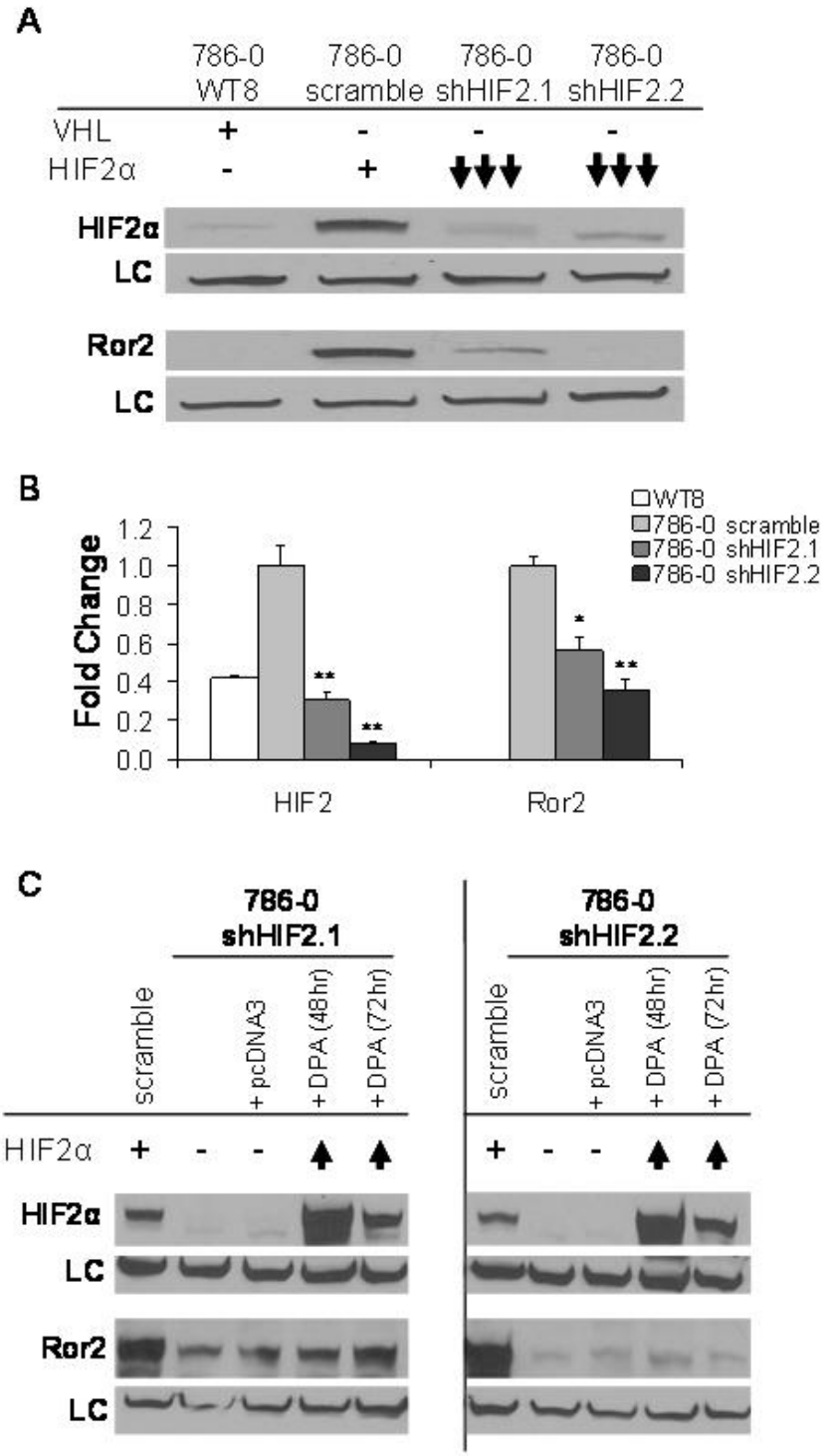
A
786-0 WT8
786-0 scramble
786-0 shHIF2.1
786-0 shHIF2.2
VHL
HIF2α
HIF2α
LC
Ror2
LC
B
WT8
786-0 scramble
786-0 shHIF2.1
786-0 shHIF2.2
Fold Change
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HIF2
Ror2
C
786-0 shHIF2.1
786-0 shHIF2.2
scramble
+ pcDNA3
+ DPA (48hr)
+ DPA (72hr)
HIF2α
HIF2α
LC
Ror2
LC

Figure 4.4: Ror2 is regulated by HIF-2α expression.
A. Ror2 expression is suppressed in 786-0 HIF-2α shRNA knockdown cells. Whole cell extracts from RCC cells expressing a *VHL* transgene (786-0 WT8) or lacking expression of *VHL* (786-0) were further transfected with a control scramble shRNA (scramble) or a HIF-2α short hairpin RNA retrovirus (shHIF2) and immunoblotted with HIF-2α, Ror2, and Ku80 (LC) antibodies.
B. Ror2 mRNA levels are suppressed in 786-0 HIF-2α shRNA knockdown cells. qRT-PCR analysis of the 786-0 HIF-2α shRNA knockdown cells verified effective HIF-2α knockdown in two independent knockdown cell lines (**p=0.0022, p=0.0001). Ror2 expression was concordantly suppressed with HIF-2α knockdown (*p=0.0172, **p=0.0023).
C. Ror2 expression can be rescued by HIF-2α overexpression. 786-0 cells stably expressing scramble and shHIF2 were rescued with empty vector (pcDNA) or proline hydroxylation resistant double proline to alanine HIF2 mutant (DPA) for 48 and 72 hrs or GFP control. Induction of Ror2 expression is seen when HIF-2α expression is rescued with the DPA HIF2 mutant in the two shRNA cell lines.

Ror2 is regulated by both HIF-1α and HIF-2α expression

Observing that Ror2 could be regulated by HIF-2α, we also explored the HIF-2α dependent regulation in the context of concurrent HIF-1α stabilization and examined the more ubiquitously expressed HIF-1α subunit as a potential regulator of Ror2 expression. Though many HIF-1α and HIF-2α targets overlap, some are dependent on only one or the other subunit, HIF-1α or HIF-2α, deriving the assumption that each of these subunits may have unique or context specific functions (28). Utilizing the RCC4 cell line, which expresses both the HIF-1α and HIF-2α subunits, we were able to examine this proposition by suppressing HIF-1α and HIF-2α independently using a lentiviral shRNA system which included an iRES GFP for stable selection (lentiviral constructs kindly provided by W. Kim, University of North Carolina). Transfecting cells with lentiviral shRNA viral particles with suppression titrated by increasing GFP expression, we observed that effective suppression of HIF-1α and HIF-2α could be achieved without non-specific suppression of the complementary HIF proteins. However, as has been observed previously, some positive compensation in levels of the alternate HIF factor was observed (32). In particular, when HIF-2α was suppressed, an increase in the basal levels of HIF-1α were detected, although the net cellular levels of HIF appeared to be reduced. In this system, which preserved the exclusive deregulation of one HIF factor, we observed that Ror2 protein levels were highly suppressed when HIF-2α levels were reduced (Figure 4.5A) and HIF-1α were sustained, or even enhanced, further implicating the HIF-2α pathway in the role of Ror2 regulation. However, when HIF-1α protein levels were suppressed, particularly at the upper end of HIF-1α

suppression, there was also a measurable reduction in Ror2 protein expression (Figure 4.5A), suggesting that the regulation of Ror2 can also be influenced by HIF-1α expression. Utilizing the most highly suppressed cell line for each knockdown (based on selection for the highest level of GFP expression), this phenomenom was recapitulated at the transcript level, demonstrating suppression of Ror2 transcript with shRNA knockdown of either HIF-1α or HIF-2α (Figure 4.5B). Additionally, simultaneous knockdown of both factors produced an additive effect on Ror2 transcript (data not shown). This observation that Ror2 transcription is clearly linked to HIF stabilization, but that the maintenance of one stabilized HIF factor fails to convey a functional redundancy, suggests that the overall amount of HIFα present in the cells influences the levels of Ror2 that is expressed. These results imply that Ror2 expression is deregulated in cancer as a result of *VHL* inactivation and is dependent on sustained or pathologically high level expression of either HIF subunit, HIF-1α or HIF-2α.

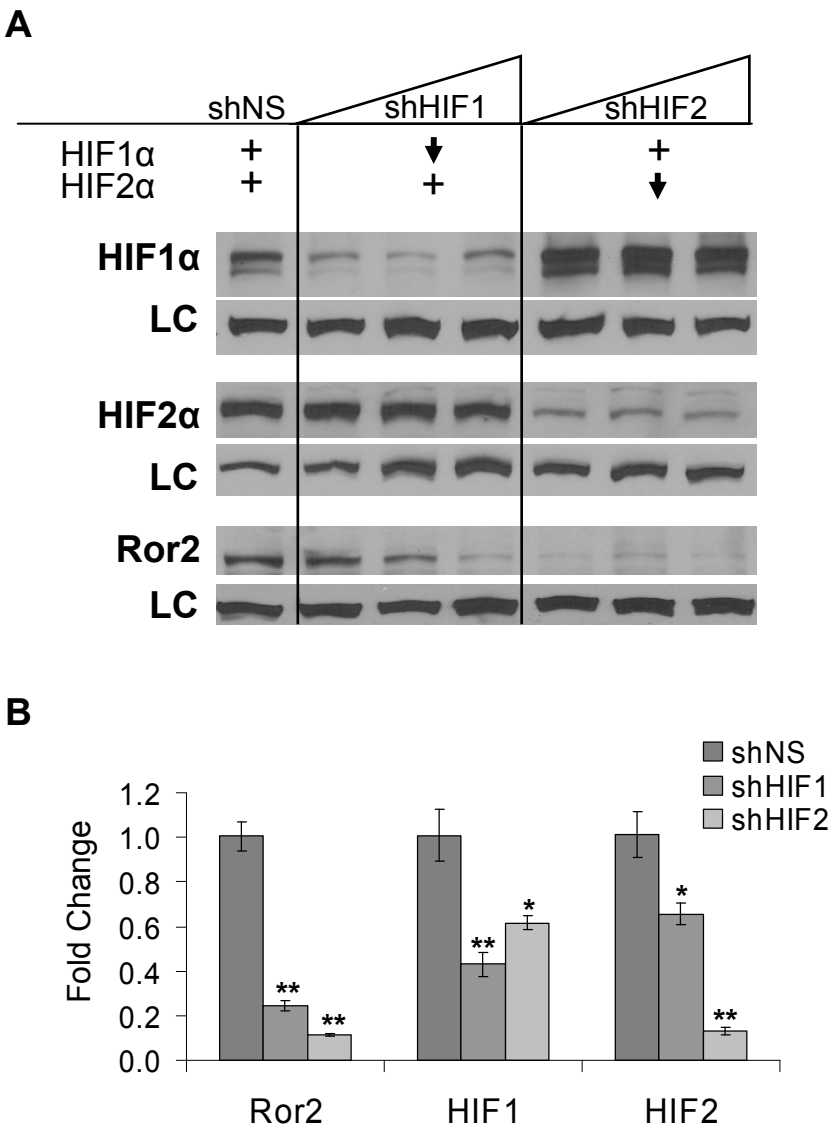

Figure 4.5: Ror2 is regulated by HIF-1α and HIF-2α expression.
A. Knockdown of either the HIF-1α or HIF-2α subunit suppresses Ror2 expression. Cells expressing both HIF-1α and HIF-2α were subjected to lentiviral shRNA of each subunit and sorted by GFP expression. Three populations sorted for increasing GFP expression were assayed for each knockdown and compared to a non-specific sequence (shNS). When HIF-2α levels were suppressed, HIF-1α levels remain constant, while Ror2 was downregulated. Similarly, when HIF-1α levels were highly suppressed and HIF-2α was stable, Ror2 was also downregulated.
B. Ror2 expression is reduced in a graded manner at the transcriptional level (**p=0.0002, p <0.0001) with highest level of suppression, with this effect observed most visibly for HIF-1α knockdown. Suppression of Ror2 was observed at all levels of knockdown of HIF-2α. qRT-PCR analysis of the HIF-1α (**p=0.0074, *p=0.0165) and HIF-2α (**p=0.0002, *p=0.0277) suppression at the highest level were used to assay for Ror2 and both HIF-1α and HIF-2α knockdown lead to a loss of Ror2 expression.

HIF complex localizes to the Ror2 promoter

Observing that Ror2 could be regulated by both HIF-1α and HIF-2α, we examined the Ror2 genomic sequence and untranslated regions for evidence of a hypoxia response element which could account for HIF-mediated transcriptional activation. None of the previously reported canonical hypoxia response element binding sites (5'-CACGTA-3', or the degenerate sequence 5'-BACGTSSK-3') were detected in the immediate proximity of the Ror2 gene. Although isolated matching sites were found throughout the sequence 10kb upstream and 2kb downstream, we decided to assess if HIF proteins could specifically interact with the immediate 5' region of the Ror2 promoter. The HIF-2α only expressing cell line 786-0 was utilized for our studies. Chromatin immunoprecipitation (ChIP), using HIF-1α, HIF-2α, and ARNT/HIF-1β antibodies, was performed to analyze HIF interactions with the Ror2 promoter, utilizing four primer sets covering a 1kb region overlapping the transcriptional start site. HIF-1α immunoprecipitation in this experiment was used as a control, as this factor is not expressed in 786-0 cells. As a positive control, known primers targeting the hypoxia response element (HRE) of Egln3 (also known as prolyl hydroxylase 3, PHD3) were utilized to confirm HIF complex/chromatin interactions (35) providing validation of the system.

We consistently observed positive interactions with HIF-2α and ARNT in both the Egln3 control, as well as with primer set B and less consistently with primer set C in the Ror2 promoter (Figure 4.6). A second set of primers encompassing the same general region as primer set B were designed, and also consistently demonstrated a positive interaction (data not shown). Over multiple evaluations, the A and D

primers did not demonstrate an interaction with HIF-2α or ARNT in these flanking segments of the Ror2 promoter (Figure 4.6). Upon further examination, the region covered by both sets of Primer Pair B was found to encompass an aryl hydrocarbon response element (Table 4.1) shown to be important for binding ARNT. These data suggest that we may have identified a cryptic site suitable of HIF complex binding in the immediate promoter region of Ror2.

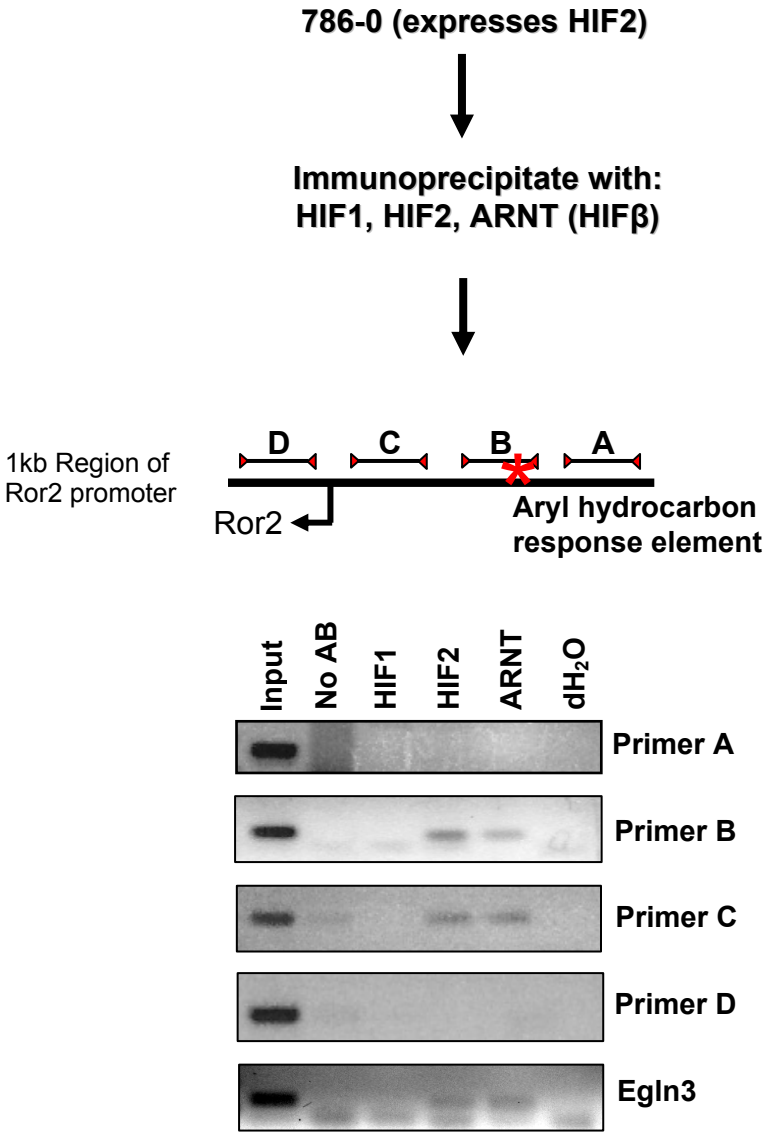

Figure 4.6: HIF-2α and ARNT interact with the Ror2 promoter. Cells lacking expression of *VHL* and overexpressing only HIF-2α (786-0) were subjected to chromatin immunoprecipitation with HIF-1α (used as a negative control), HIF-2α, and ARNT antibodies. Primers were designed targeting the 1kb region of the Ror2 promoter which overlaps the Ror2 start site. Using primers targeted against the promoter region containing a known hypoxic response element (HRE) of PHD3, also known as Egln3, we show that HIF-2α and its dimerization partner, ARNT, interact with the PHD3 promoter as expected. HIF-1α interaction is not detected, as this cell line does not express HIF-1α. When the Ror2 promoter was examined, HIF-2α and ARNT localized to the Ror2 promoter regions B and C similarly to the control promoter. The region covered by Primer B encompasses an aryl hydrocarbon response element (*CACGC) which recognizes the aryl hydrocarbon receptor nuclear translocator - also depicted in Table 4.1- Primer Pair B (1). However, interaction was not observed for flanking regions A and D.

	Predicted Primer Products
Primer B (1)	**AGGAGCTGGGGCTGCAGCTGGGGCCCCGGACCTGCCTCCTGCGCCCGCCCTC GGGCCTGCGCCTCGGAACGCTTGAACACAAAGGTGGCGCCTTGGGGGCGTCA GCCGGAGTTCGGCGGGTCTGCCCACGCAGGAGAGGCGCCCAGAGCG**
Primer B (2)	**GAACACAAAGGTGGCGCCTTGGGGGCGTCAGCCGGAGTTCGGCGGGTCTGCC CACGCAGGAGAGGCGCCCAGAGCGGGTGCCCCCGACTTTCGGGGGCGACAC ACCCGGGGATCTAGGTCACAAATC**

Table 4.1: Predicted product of the Ror2 promoter primers. Two independent sets of primers (highlighted in blue and green, respectively) targeting the 1kb region of the Ror2 promoter encompassing the aryl hydrocarbon response element (CACGC - highlighted in red) were designed to analyze HIF associated proteins and their ability to bind to the Ror2 promoter.

DISCUSSION

Ror2 is a developmentally regulated kinase normally expressed in mesenchymal cells of organs such as the brain, lung, heart, and kidney but is normally repressed in most adult tissues, including the kidney (8). However, this kinase is derepressed in a subset of human RCC tumors (8) as well as other cancers (9-11), where it is emerging as a major factor promoting tumor cell migration and tumor invasion phenotypes. We sought to identify the mechanism of regulation for Ror2, contributing specifically to its stable expression in RCC. The most commonly referenced mutation in RCC pathology is of the *VHL* gene, with over 70% of RCC tumors displaying inactivation of *VHL* leading to the subsequent stabilization of one or more HIF factors, notably HIF-1α and HIF-2α. The stabilization of these potent transcriptional activators leads to the expression of multiple hypoxia response genes responsible for such phenotypes of renal tumors as prolific angiogenesis, cell invasion, and metastasis (36).

We first observed that Ror2 was expressed in a *VHL* dependent manner in RCC. This was further corroborated by our analysis of the *VHL* somatic point mutations that recapitulate the *VHL* mutant transgenes which represent the major subtypes of *VHL* disease. This analysis demonstrated that Ror2 is expressed most highly in the subtype which maintains expression of the mutant *VHL*, but promotes the most significantly deregulated level of HIF-2α and is thought to lead to a highly penetrant form of RCC, Type 2B. Further, our work has previously shown that the *VHL* Type 2B mutation, which was unable to suppress the Ror2 expression of *VHL* mutant cells, is dysregulated for HIF-2α both *in vitro* (31) and *in vivo* (32). This ties

Ror2 to an important aspect of RCC tumorigenesis, as a potential contributor to invasive forms of this cancer. Further, this work linked Ror2 expression indirectly to dysregulated stabilization of HIF, as Ror2 expression was observed in cell culture *VHL* somatic mutation models which produce either stabilization of both HIF factors (*VHL* null), or the exclusive stabilization of HIF-2α (*VHL* 2B).

Though the HIF-α subunits share certain similarities, they also have divergent responsibilities (28). As Ror2 has been shown to be *VHL* dependent and our data suggested a role for a correlation with HIF stabilization, it provided a natural extension to analyze the independent effects of HIF-1α and HIF-2α subunits on Ror2 expression. Normally, transcriptional targets that are unique to one of the HIF factors are not affected by genetic repression of the uninvolved HIF factor. Additionally, transcriptional targets that are mutual to HIF-1α and HIF-2α , such as Glut1 or VEGF, typically require the depletion of both factors, due to transcription factor compensation, in order to impact expression. We wanted to tease out the difference in expression and analyze if exclusively one or both HIF-α subunits had an effect on Ror2 expression. Our investigations point toward a clear correlation between HIF-2α stabilization and Ror2 expression in 786-0 cells, in which genetic knockdown and rescue experiments confirm this linear association. In cells expressing both HIF-1α and HIF-2α, the depletion of either factor was sufficient to reduce Ror2 expression at the transcript and protein levels. This suggests that with graded suppression of either HIF-1α or HIF-2α, Ror2 is coordinately expressed in a similar manner, and that functional redundancy between the factors may be dose dependent. Such a pattern of regulation is not typical of most transcriptional

components of the hypoxia response pathway, although similar patterns of atypical regulation have been previously reported for HIF targets (28). In the case of Ror2, the depletion of either factor from the maximally stabilized state causes the loss of Ror2 expression, indicative of a dosage effect of HIF factors on the transcriptional activation of Ror2.

Prior hypoxia target investigations suggest that the Ror family ortholog *cam-1* is a HIF-dependent hypoxia target in *C. elegans* (37). However, though the HIF pathway has been studied extensively, Ror2 has yet to be identified as target of the hypoxia response pathway in mammalian cell screens. Activation of the hypoxia response pathway via stabilization of one or both of the HIF-α subunits is a commonly observed phenotype in RCC tumors, and physiologic upregulation of HIF factors as a result of strict decreases in oxygen availability is an important cellular process in a variety of disease states. We thus extended our analysis to address this issue. Despite the observation of clear linear connections between HIF stabilization and Ror2 expression, we observed minimal induction of Ror2 expression in these studies, even after prolonged exposure to hypoxia, or chemical poisoning of the prolyl hydroxyation activity with potent inhibitors. These data suggest that within the context of this tumor cell system, physiologically relevant exposure to hypoxia may not be sufficient to induce changes in Ror2 expression levels compared to the normoxic controls. This surprising result may shed further light on the mechanism of Ror2 regulation as a cancer associated kinase. Sustained oncogenic levels of HIF factors are not routinely attained in response to hypoxic insult. However, in renal cell carcinoma, as a result of *VHL* inactivation, or in other

tumors, notably squamous cell carcinoma of the head and neck, HIF is induced to high constitutive levels, which may account for the identification of a novel HIF target associated with cancer.

Whether it is the distinctly high levels of HIF factors associated with *VHL* loss or the maintenance of high levels for sustained periods of time which contributes to Ror2 expression is not certain. However, constitutive stabilization of HIF factors occurs broadly across many types of tumors, such that Ror2 as a tumor associated kinase may be encountered even more frequently. One potential reason hypoxia may fail to upregulate Ror2 expression is that Ror2 transcriptional regulation may be steps removed from HIF induction. Certainly several factors downstream of HIF activation could be considered as intermediate transcriptional regulators of the expression of Ror2, though which of these factors may contribute to regulation of Ror2 expression either developmentally or in the tumor physiology scenario remains to be determined. However, in our analysis of the Ror2 promoter, we found that HIF-2α and ARNT can be localized to a small region of the immediate Ror2 promoter region, which lacks any known hypoxia response element binding sequence. This interaction of the HIF complex at a cryptic Ror2 promoter site is suggestive of a direct mechanism of HIF regulation. However, this finding does not preclude the possibility that one or more additional transcriptional co-factors may be required to coordinate activation in a manner which is dependent on *VHL* loss or HIF stabilization, or that more distant conserved HRE sites may be utilized for HIF-dependent transcriptional regulation.

An additional possibility exists to link the dependence on *VHL* inactivation and HIF stabilization in clear cell renal cell carcinoma, and the failure of Ror2 to exhibit hypoxic induction. *VHL* mutation also mediates alternate effects on cell signaling external to the well studied effects on HIF regulation. For example, pVHL is known to affect the stabilization of a non-HIF associated protein, JADE-1 (38), and in a sophisticated genetic screen in *C. elegans*, at least four HIF-independent but *VHL*-dependent genes were identified (39). Thus, our results may reflect the activation of a gene that is HIF dependent only in the setting of a *VHL* mutation because of other context specific cues. Further, patterns of tumor cell invasion have been suggested to be dependent on aspects of *VHL* loss which are not clearly dependent on HIF stabilization. The extent to which Ror2 may play a role in promoting these invasive features of tumors will need to be further investigated.

RCC is a difficult to treat cancer that offers few effective options for treatment. We previously reported that Ror2 expression has a distinct role in RCC tumor cell biology (8) and has potential to be important for components of tumor invasion. We have since demonstrated a mechanism of Ror2 regulation that, with respect to clear cell RCC, can be linked to *VHL* loss and is a component of the HIF dependent transcriptional program in clear cell RCC cell lines. This sustained stabilization of HIF is a feature of many tumors, some of which are independent of *VHL* mutation, and is associated with advanced disease. Here we offer a unique mechanism of regulation that offers insight into how Ror2 is deregulated in RCC that has the potential for application in many other cancers.

MATERIALS AND METHODS

Antibodies

Both monoclonal and polyclonal antibodies against Ror2 were obtained from RND Systems (Minneapolis MN). Antibodies against HIF-2α and Ku80 (loading control - LC) were obtained from Genetex (San Antonio TX). The HIF-1α antibody was obtained from BD Transductions (Franklin Lakes NJ) and the Glut1 and the Egln3 antibodies were obtained from Novus Biologicals (Littleton CO). For figures 4.3B and 4.5, a HIF-2α antibody obtained from RND Systems (Minneapolis MN) was used for analysis.

Chromatin Immunoprecitation (ChIP) Analysis

Cells were grown to 80% confluency before being crosslinked with 11% formaldehyde. The crosslinked DNA was then sonicated. Chromatin immunoprecipation (ChIP) analysis was performed with HIF-1α (BD Transduction, Franklin Lakes NJ), HIF-2α (RND Systems, Minneapolis MN) and ARNT1 (Novus Biologicals, Littleton CO). The resulting ChIP DNA was analyzed by PCR using primers targeting the known HRE region of Egln3/PHD3 (Reverse Primer: 5' TAGGCACAGTAAACAGGCCC 3'; Forward Primer: 5' AGCGTCCGTTCCCAGCTCAG 3') as previously described (35) as positive controls to validate the system. Additionally, primers were designed to target the 1kb region of the Ror2 promoter – Primer A (Reverse Primer: 5' ACGCGCTTGTCCCCACCGAC 3'; Forward Primer: 5' CTGCACTGCGCACCGGGACA 3'), Primer B (Reverse Primer: 5'

CGCTCTGGGCGCCTCTCCT 3'; Forward Primer: 5' AGGAGCTGGGGCTGCAGCTGGG 3'), Primer C (Reverse Primer: 5' AGCCCGCGCCAAGGAACCTC 3'; Forward Primer 5' ACGATGCGTCCGCTCCTCCT 3') and Primer D (Reverse Primer: 5' TCTGGCGTTCCGGCTTGTGC 3'; Forward Primer: 5' CGTCGGGCGAGATGCGAATGG 3').

Cell Culture and Cell Treatments

The 786-0 cell line and their paired derivatives, WT8 & RC3 (kindly provided by Dr. W. Kaelin, Boston MA), the 786-0 *VHL* point mutants knock-in cell lines and the RCC4 paired cell lines, 3-14 and 2-1 (kindly provided by Dr. M.C. Simon and Dr. B. Keith, Philadelphia, PA) were maintained in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, L-glutamine and non essential amino acids. For treated cells, the following conditions were utilized: cobalt chloride, $CoCl_2$ (Thermo Sci Acros Organics, Geel Belgium) was used at 100μM for times specified, dimethyloxalylglycine, DMOG (Cayman Chemical Company, Ann Arbor MI) at 1mM for 24 hours, or cells were incubated under conditions of 1% hypoxia for times specified.

Immunoblot Analysis

Proteins were extracted from cultured cells using cell lysis buffer consisting of 20mM Tris, 100mM NaCl, 1 mM EDTA, 1% NP-40 and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis IN). Samples were quantitated using Bradford reagent to

measure absorbance. 40-50ug of protein samples were then separated on 8% SDS-polyacrylamide gels, transferred to nitrocellulose membrane (GE, Pittsburg PA) and the proper horse radish conjugated secondary antibodies were used. Proteins were detected using the Amersham ECL-Plus System (Pittsburg PA) and visualized on autoradiography film (ISC BioExpress, Kaysville UT).

qRT-PCR

cDNA was made by reverse transcription from 0.5ug of total RNA (5PRIME Perfect Pure RNA Cultured Cell Kit, Gaithersburg MD) using oligo dT primers and the Superscript II RT-PCR reagents (Invitrogen, Carlsbad CA). Commercially available proprietary FAM labeled primers (Ror2, Egln3, Glut1, HIF1, HIF2 and β-actin) were used for amplification and the samples analyzed on the 7900H Fast Real-Time PCR System using recommended cycle conditions (Applied Biosystems, Foster City CA).

Transfections and shRNA

shRNA knockdown cells were generated by infecting cells with pRetroSuper (pRS) packaged shRNA for HIF-2α (kindly provided by Dr. W. Kaelin, Dana Farber Cancer Institute (34)). Cells were supplemented with puromycin in the media to maintain the shRNA for stable suppression. Rescue of HIF-2α shRNA cell lines were generated transiently by using a HIF-2α (mutated double proline to alanine, DPA) expressing plasmid in a pcDNA3 backbone (kindly provided by Drs. B. Keith and M.C. Simon, University of Pennsylvania). RCC4 lentiviral knockdown cells were generated by infecting the cells with pLL 5.0 packaged shRNA (with a GFP-expressing tag) for

HIF-1α and HIF-2α (generously provided by Dr. W. Kim, University of North Carolina) and GFP-positive cells were selectively sorted via GFP expression levels by the UNC Flow Cytometry Core Facility.

Statistical Analysis

A one-way ANOVA was used to analyze the standardized cT values with the specified qRT-PCR target (Ror2, Egln3, Glut1, HIF1, HIF2) as the fixed constant. Verification of the one-way ANOVA model was performed using Kruskal-Wallis test, a nonparametric statistical procedure, to compare the standardized cT values. Error bars represent standard error of means (SEM). P-values referenced as are followed: *$p<0.05$, **$p<0.01$.

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CHAPTER 5

SUMMARY, DISCUSSION AND CONCLUSIONS

SUMMARY

In the search to uncover potential biomarkers or cell intrinsic kinases for the difficult to treat epithelial cancer, RCC, we have discovered a novel putative cancer cell specific kinase target, Ror2. Biomarkers and cancer cell specific kinases are severely lacking within the renal carcinoma cancer field as well as other cancer etiologies. Depending on the cancer field being studied, few biomarkers have been identified and characterized with the specific intent of finding an amenable target. My goal was to identify and characterize cancer cell specific kinase(s) expressed on RCC with the ultimate goal of using the specific kinase(s) for future therapeutic development.

For our analysis, we utilized a phospho-RTK array using pVHL mutant cell lysates from cell lines to try to identify potential kinases. Within this array, we had several RCC specific positive and negative controls as well as internal controls provided by the company. EGFR is normally expressed abundantly on RCC and there was robust expression of EGFR on the RTK array as would be expected. However, VEGFR and PDGFR, which are not normally expressed on RCC, were also not expressed on the RTK array as expected. Though several receptor tyrosine kinases were identified from this screen that had the potential to be useful as RCC cancer therapeutics, we chose to look more closely at Ror2 as it was the most novel at the time of discovery and had not been previously associated with any cancer. In fact, Ror2 is a developmentally regulated kinase usually expressed in the developing kidney, heart and lungs. However, expression of Ror2 is not normally seen in adult tissues.

Two bone disorders that result in shortened or misshapen bones arise if hRor2 is mutated. Robinow disease results from missense, nonsense and frameshift mutations in the CRD, kringle and kinase domains whereas autosomal dominant Brachydactyly B arises from truncations of the entire kinase domain or just below the tyrosine kinase domain. Genes important for skeletal development have also been shown to have a significant impact on cancer. In fact, many labs are currently in the process of characterizing genes important for skeletal development including multiple Wnt and Hox genes that also impact cancer growth. It is intriguing that Ror2 has also been identified as having a key role in RCC's cancer etiology as it also plays a key role in skeletal development. Ror2 has previously been identified as having a potential role in the Wnt signaling pathway in several model systems where it is shown to bind different Wnts depending on context, but again what role Ror2 itself may play in RCC still remains to be elucidated.

As Ror2 was initially identified from a phospho-RTK array, we verified by immunoprecipitation that it was phosphorylated in RCC cell lines. We show that expression was not specific to one cell line as Ror2 was also found to be expressed in multiple RCC cell lines tested. Most notably, expression of Ror2 was not detected in our SV40 transformed control proximal tubule cell line, suggesting this was specific to RCC as it was not expressed in the "normal" control kidney cell line. Seeing expression in RCC cells lines, we also wanted to know if Ror2 could be detected in primary human tumors. Indeed, we show that Ror2 is expressed in a majority of RCC tumors analyzed both at the protein and mRNA level.

Additionally, by microarray analysis, we show human RCC tumors coordinately overexpresses with MMPs which demonstrates an ECM tumor phenotype for Ror2. This is fascinating as Ror2 is shown to be related to skeletal development and is now related with MMP2, which has also been shown to be important for skeletal and bone development. Additionally, MMP2 is a key component in the degradation of the cell basement membrane, or the extracellular matrix. The connection to MMP2 was further corroborated in RCC cell lines - when Ror2 was suppressed, MMP2 expression was also suppressed suggesting that Ror2 may play a role in directing MMP2 expression. Functional analysis assays suggest that Ror2 supports cellular migration, without affecting doubling time or cell viability. Further, Ror2 suppression has an impact on anchorage independent growth, a key component of tumor growth potential, leading to a dramatic decrease in overall colony size. Utilizing a xenograft model, we also show that Ror2 suppression influences tumor growth *in vivo*. Together, this data strongly suggests that Ror2 plays a considerable role in RCC tumorigenesis (1).

A model of regulation had yet to be determined for Ror2. Therefore, we also sought to place Ror2 in the most common deregulated pathway in RCC, the VHL pathway. We show that Ror2 is dependent on the inactivation of pVHL. Additionally, Ror2 regulation by VHL is defined by specific VHL subtypes that are highly predisposed to RCC. Further, the only known substrates of VHL inactivation, the HIFα subunits, were also assessed. We show that Ror2 is dependent on the stabilization of both HIF1α and HIF2α. This Ror2 dependency could be rescued with constitutively active HIF subunits. However, Ror2 could not be induced under

physiological hypoxic conditions suggesting that it may only require a component of the VHL/HIF pathway or it may not be a linear pathway. Seeing this interesting finding, we assessed the Ror2 promoter and found that HIF associated proteins (HIF2 and its binding partner ARNT) can and do bind and/or localize to the immediate Ror2 promoter. These findings suggest a novel mechanism of regulation for Ror2 not previously uncovered (2).

The identification of Ror2 opens up the cancer field to an avenue of Wnt signaling, not previously identified before now, that is relevant to RCC and also provides a tangible biological avenue to extracellular matrix remodeling. Additionally, the identification of a novel pathogenic HIF activation pathway not commonly seen in other cancers could be exploited in other pathogeneses. Not only has the identification of Ror2 introduced a potential druggable target, but also identifies a potential biomarker for RCC treatment and for other cancers.

DISCUSSION

VHL regulation of Ror2 is predictive of a more aggressive tumor type

Within the spectrum of *VHL* disease, there is a tight genotype/phenotype correlation where representative *VHL* mutations are associated with specific *VHL* disease subtypes. Our data shows that Ror2 is regulated by both *VHL* and HIF expression in RCC (2). Based on this finding, we also closely assessed the *VHL* disease subtypes and analyzed Ror2 expression. *VHL* disease patients fall into distinct genotypic categories that can predict the range of RCC presentation (3, 4) and have correlated HIF2 dysregulation. *VHL* Type 1 and Type 2B are highly dysregulated for HIF2α and both VHL subtypes are at high risk for developing ccRCC. Further, *VHL* Type 2B mutations are associated with the most diverse presentation of *VHL* disease and are thought to be the most highly penetrant for developing RCC. Indeed, in previous studies it is postulated that type 2B tumors may be more aggressive than their other Type 2 counterparts.

Previous work from our lab shows that *VHL* type 2B mutation is dysregulated for HIF2 expression much so as the VHL Type 1 mutation (5). Further, these studies suggest a tight connection between the ratio of HIF expressed and its stability. When we assessed Ror2 expression in the representative somatic mutational VHL subtypes, we saw high expression of Ror2 only in the VHL Type 1 and Type 2B. Both of these subtypes are highly predisposed to ccRCC and have a high level of HIF2α dysregulation. It is interesting to see that Ror2 is associated with *VHL* subtypes that can lead to the most aggressive forms of ccRCC. More notably, HIF2α itself is considered to be the more tumorigenic of the two HIFα subunits as

downregulation of HIF2α is necessary for tumor suppression by pVHL (6-8). These findings not only imply a possible role for Ror2 in the *VHL* disease subtypes, but also suggest that the potential RCC target kinase Ror2 is associated with the more tumorigenic of the HIF subunits leading to a more aggressive form of RCC.

Ror2 expression defines an ECM tumor phenotype in RCC

Our analysis of Ror2 shows that it is linked to the loss of VHL expression. ECM remodeling itself has also been tied directly to VHL expression as restoring pVHL to pVHL-deficient cells partially restored the ECM organization phenotype previously lost because of pVHL inhibition (9, 10). Additionally, previous data shows that VHL directly interacts with and has a role in the assembly of the extracellular matrix (9, 10). Further, cells with mutated VHL have increased expression of MMPs including MMP2 (11). Ror2 is dependent on the loss of pVHL expression and can direct MMP2 expression, immediately suggesting a potential role for Ror2 at the ECM.

Seeing that Ror2 was expressed in a large majority of the human RCC tumors, we wanted to assess if Ror2 also plays a role in RCC tumorigenesis. RCC is a lethal epithelial cancer that is the most common kidney malignancy (12) with over half of those diagnosed presenting with metastasis. For epithelial cells to metastasize, they must first acquire mesenchymal characteristics that permit proliferation outside of their normal periphery. Ror2 is primarily expressed in rising mesenchymal cells in the developing kidney immediately suggesting a potential role for Ror2 in RCC tumor development. Indeed, genes important for ECM remodeling

including MMP2 and Twist1 were found to be tightly correlated with Ror2 overexpression and both have been linked to RCC. MMPs are widely known to be key factors in tumor expansion and promote metastasis by degrading the structural basement membrane. In recent years, a tight link has been established connecting MMPs to RCC. In fact, previous studies show that increased MMP2 expression is correlated with patients that have poor prognostic variables such as survival, aggressiveness and grade in RCC (13, 14). Seeing that Ror2 has a direct role in MMP2 expression is exciting as it tightens the link between Ror2 and RCC tumor biology. Thus, Ror2 overexpression and its link to MMP2 may represent an important transitional event in the development of invasive renal cell carcinoma.

HIF uniquely regulates Ror2 expression

In an effort to uncover the mechanism of Ror2 regulation in RCC, we chose to analyze the VHL/HIF pathway - as VHL is the most commonly mutated gene leading to HIF dysregulation - to identify pathways in which Ror2 had not been previously implicated. Our analysis uncovered a novel and uncommon method of HIF regulation for RCC that may have broader applications. We found that Ror2 is not only regulated by pVHL expression but is also dependent on the stabilization of either HIF1 or HIF2, even though Ror2 expression is minimally induced under physiological hypoxic conditions.

The most well-documented function of pVHL is to act as the substrate recognition component of an E3 ubiquitin ligase complex (15-18). The most identifiable substrates of pVHL are the hypoxia inducible factor (HIF) subunits. The

HIF subunits are oxygen sensing transcription factors that play vital roles in the cell. When the HIF1α and HIF2α were suppressed individually, Ror2 expression was reduced in both the HIF1α only and HIF2α only RCC4 cell lines. This level of regulation can be seen at both the protein and mRNA levels via immunoblot and qRT-PCR analysis. Normally, transcriptional targets that are unique to one of the HIF factors are not affected by genetic repression of the uninvolved HIF factor. Additionally, transcriptional targets that are mutual to HIF-1α and HIF-2α, such as Glut1 or VEGF, typically require both factors to be depleted to have an influence on expression due to transcription factor compensation. However, in our studies, we found that when either HIF-1α and HIF-2α is suppressed individually, we see a graded level of Ror2 expression with increasing HIF suppression.

This graded regulation of Ror2 by the HIF subunits is uncommon for HIF regulated genes as regulation by HIF1α and/or HIF2α require both to be present or completely depleted to fully function in its regulatory role. However, both HIF factors do not need to be depleted to have an impact on Ror2 expression. Within the literature, few other genes have been identified that share this atypical regulatory property. Within the pseudohypoxic RCC tumors themselves, there is a difference of expression between HIF1 and HIF2 expressing tumors and HIF1 only tumors. A recent study performed by Gordan et al. analyzing human RCC tumors demonstrates that the molecular profile of tumors is highly dependent on the expression of HIF1 and HIF2 in comparison to those tumors solely expressing HIF2, with distinctions that correlate with divergent tumor activity (19). The distinctive

graded HIF regulation seen with Ror2 could represent a new category of genes indicative of a unique class of regulation.

The HIF factors play important roles in many cellular contexts in addition to *VHL* loss and renal cell carcinoma including angiogenesis and invasion and metastasis (20). Both HIF subunits (HIF1 and HIF2) dimerize with HIF-1β/ARNT and bind conserved hypoxia response elements (HREs), oxygen dependent promoters/enhancers of target genes, leading to the induction of hypoxia inducible target genes including Glut1 and prolyl hydroxylase family member Egln3 (also known as PHD3). Seeing that Ror2 has a distinctive HIF regulation and to explore the possible contribution of this factor to the global cellular response to hypoxia, the hypoxic induction of Ror2 was analyzed. It was surprisingly difficult to stimulate Ror2 expression levels under low oxygen conditions. Using a variety of low oxygen induction methods, including 1% hypoxia and hypoxia mimetics such as cobalt chloride, Ror2 levels were minimally induced at best. To further delineate the hypoxic response pathway, we utilized a more potent hypoxia mimetic, DMOG. After DMOG stimulation for 24 hrs, minimal induction of Ror2 was seen again at the transcriptional level utilizing qRT-PCR. However the controls Egln3 and Glut1, direct targets of the hypoxia response pathway were sufficiently induced under hypoxia-like conditions as expected since 786-0 WT8 cells express a wild-type *VHL* gene. Several possibilities arise from this interesting finding. We may need to induce hypoxia for an extensive time period – as the Huang lab at the University of Utah provided data suggesting hypoxia can be induced long-term (as far as two weeks) (21). Conversely, Ror2 may be an indirect target of the HIF activation pathway so

may require even more HIF activation than the cells can withstand in a hypoxic environment or Ror2 may complex with an unidentified cofactor that may inhibit hypoxia with pVHL inactivation. This intriguing finding prompted examination of the Ror2 promoter to analyze if Ror2 specifically interacted with HIF proteins.

HIF proteins bind or localize to the Ror2 promoter

Seeing that Ror2 is minimally induced under physiological hypoxic conditions but is dependent on the stabilization of the HIF subunits, we wanted to closely analyze the Ror2 promoter region to determine if HIF associated proteins also bind or localize to the Ror2 promoter. We analyzed the 1kb region of the Ror2 promoter that contains the transcriptional start site as it encompasses an aryl hydrocarbon response element which recognizes the aryl hydrocarbon receptor nuclear translocator. To begin, we first utilized primers against a known HIF target, a prolyl hydroxylase family member, PHD3 (also known as Egln3) as a control (22) to test the interactions of this genes' promoter with HIF2 and ARNT in the HIF2 only expressing cell line, 786-0. Specifically, we chose published PHD3/Egln3 primers (22) targeted against the hypoxic response element (HRE) of Egln3, where HREs are oxygen dependent promoters/enhancers of target genes. Using the Egln3 primers, we show that HIF2 and its dimerization partner, ARNT, interacts with the Egln3 promoter as expected but not with HIF1 as this cell line does not express HIF1. For our Ror2 promoter analysis, we designed primers that emcompassed the transcriptional start site of Ror2 and asked if HIF localizes to the promoter. Indeed, when the Ror2 promoter was assayed, we show that the HIF associated proteins,

HIF2 and ARNT, bind or localize to the Ror2 promoter similarly to the control promoter, Egln3. Not only have we shown that Ror2 expression is dependent on the physiological stablization of the HIF subunits but we now show that HIF associated genes bind and/or localize to the Ror2 promoter. This finding is especially intriguing as our data suggests that Ror2 is HIF dependent but minimally induced under hypoxia.

This could be because Ror2 may be part of an epigenetic component that has a different and possibly unknown posttranslational modification, though this is only speculative. Indeed, a novel regulation may be established as the Ror2 promoter encodes an aryl hydrocarbon response element (AHRE) that is perhaps responsible for its interaction with HIF but not necessarily for its induction under physiological hypoxic conditions. Several possibilities exist that may explain the Ror2 results we see. ARNT may be preferentially sequestering the HIF-2 subunits away from putative HREs leading to minimal hypoxic induction of Ror2 when placed under cellular stress. Furthermore, the AHRE may represent a novel binding site of interaction between HIF/ARNT such that Ror2 transcriptional expression is not induced under physiological hypoxic exposure, though this novel interaction is only speculative with respect to Ror2.

The HIFα subunits contain a conserved asparagine residue that under normal oxygen conditions, factor inhibiting HIFα (FIH) hydroxylates preventing interaction with known transcriptional co-factors, CBP/p300, as well as with many other cofactors. It is possible that the FIH domain in the HIFα subunit may play a prominent role by recruiting an unidentified cofactor which may interact with Ror2 in

a unique way. Additionally, these results are based on our analysis of the VHL/HIF pathway and it is possible that other HIF activated pathways may play a contributory role. In conclusion, our efforts to analyze the mechanism of Ror2 regulation in RCC has uncovered a novel and uncommon method of HIF regulation where Ror2 is HIF dependent but not upregulated by physiologically induced hypoxia. This is intriguing as this novel method of regulation may have broader applications to other cancer etiologies.

Ror2 is a new kinase target in RCC

Growth promoting pathways are currently not examined closely in tumor oncology. Observing Ror2 and other developmentally regulated genes is of increasing value as a largely untapped area of solid tumor oncology is the explicit investigation of growth promoting pathways. These promoting pathways are distinctive and critical to early developmental patterning. Classifying the signaling molecules that play a physiologic role in early developmental processes, with potential to promote tumor growth or other pathologic characteristics in the adult, provides oncologists and the general cancer community an opportunity to define biological cause for various tumor attributes (23). Further, identifying molecules such as Ror2 that have restricted tissue expression in the adult organism provides a valuable chance for diagnostic or therapeutic implementation.

Within the renal carcinoma field as well as other cancer fields, few specific cell intrinsic kinase targets have been identified as amenable targets. Though targeted therapies exist for RCC, drug target optimization for clinical relevance and

further patient benefit still needs to be addressed. A prime example of this is sunitinib – though it has been shown to have efficacy in RCC treatment, it targets multiple kinase receptors, some of which are not expressed on RCC including VEGFR and PDGFR. However, within the field of RCC, the paradigm is shifting to wanting to specifically select patients for treatment based on specific molecular markers. As such, we undertook the task of searching for cell intrinsic kinases that may have the potential to be used as the basis for targeted therapy. In the hunt to identify novel cancer cell specific kinases, we identified several putative kinases. One identified kinase, Ror2, was chosen for characterization as it had not been previously associated with any cancer, but is developmentally regulated, and we show that it has a dramatic impact on RCC tumorigenesis and its regulation is VHL and HIF dependent. This finding of a potential kinase target is of importance to not only the RCC field but other cancer fields as well because Ror2 is normally expressed during development but is now de-repressed and is highly associated with cancer – having a significant role in RCC's tumorigenesis and regulated by HIF in a unique manner. Together, having an impact on RCC tumorigenesis and being tightly associated with the VHL/HIF pathway is of significant value as current targeted therapies against RCC are heavily focused on this pathway. Thus the discovery of a newly identified cancer cell intrinsic kinase in RCC, and now in other cancer etiologies, could have a drastic impact on the RTK inhibitor field.

WHAT THE FUTURE MAY HOLD

Ror2 is associated with an invasive phenotype in multiple cancers

Since the identification of Ror2 in RCC, Ror2 has since been identified in several cancers including metastatic melanoma, invasive gastric cancer, malignant squamous cell carcinoma and osteosarcoma (Table 1.2). Within all these tumors, Ror2 has been shown to be associated with an invasive phenotype (24-29). Specifically, in metastatic melanoma, Ror2 has a dramatic impact on cell motility, cellular invasion and metastasis (24) while in invasive gastric cancer, Ror2 has a role in cell invasion (25, 26). Within malignant squamous cell carcinoma, Ror2 has been associated with cell motility and cell polarity (27). In osteosarcoma, an adolescent bone cancer, Ror2 has been associated with cell proliferation, cell migration, invadopodia formation and ECM degradation (28, 29). All this data links Ror2 once again to an invasive phenotype but also ties it to skeletal development as it plays a prominent role in both bone cancer and at the ECM. Finding Ror2 in multiple cancers and being associated with an invasive phenotype suggests the potential to target Ror2, in not only RCC, but also opens up the door to target Ror2 in other cancer etiologies as well.

More pointedly, with respect to RCC, Ror2 is associated with the VHL disease subtype 2B, which is thought to be more invasive than its associate subtypes and is also highly coordinated with and possibly directs MMP2, a key component in the degradation of the extracellular matrix. Interestingly, MMP2 is also associated with bone development and is thought to play a key role at the ECM. Ror2 itself makes an amenable target as it has similar functions in different cancers that have been

independently assessed by multiple groups. At the time of discovery of Ror2 in RCC, Ror2 was shown to be associated with the Wnt signaling pathway and embryonic mutations in Ror2 leading to bone disorders. Intriguingly, Ror2 is now tied to both skeletal development and is associated with an invasive cancer phenotype in multiple cancers. This suggests that Ror2 certainly warrants closer focus in the clinics and possibly when thinking of future therapeutics.

Ror2's role in the EMT process

To analyze the signaling role of Ror2 in RCC, we had performed microarray analysis on RCC human tumors and analyzed gene expression. In our initial studies we found that Ror2 correlated with genes important at the ECM, but we also identified genes important for the epithelial to mesenchymal transition (EMT). When genes that most significantly associated with overexpressed Ror2 were examined, we saw genes important for the epithelial to mesenchymal transition including Twist1, Twist2, Snail2 and MMP2. Interestingly, epithelial to mesenchymal transition (EMT) also plays a critical role in tumor development and metastasis (30, 31). Within EMT, certain hallmarks define EMT transformation including the expression of the transcription factors Snail and Twist, which mediates the morphological changes seen in tumors (32-34). Specifically within renal cells, HIF also play a role in directing the expression of these transcription factors (35-37). Moreover, HIF1 has been shown to directly mediate Twist and Snail expression as well as the onset of EMT seen with hypoxic injury (35-37). Seeing these factors expressed when Ror2 is coordinately expressed suggests further association and more feasibility for analysis

of this phenomenon. However, the direct role Ror2 may play in the EMT process has yet to be elucidated.

To analyze this further, we plan to study the role of Ror2 in the EMT process specifically within RCC. Within the context of assessing Ror2's role in EMT, defined criteria need to be addressed. EMT is defined by changes in cell motility and cell to cell adhesion changes. As such, we plan to assay for increased cell motility, loss of cellular adhesion, and repression of E-cadherin and/or integrins. All three criteria will be addressed using functional assays (live cell migration, tight or adherence junction analysis). The experiments outlined above will aid in identifying the role of Ror2 in the EMT process as EMT plays critical roles in tumor development and metastasis. Based on previous data, Ror2 may play a direct role in this process as well.

Ror2 association with a more aggressive form of ccRCC

Our lab has also undertaken the task of trying to identify potential molecular subtypes within ccRCC. Looking closely at the clinical presentation and analyzing previous gene expression data suggested that more than one molecular subtype of clear cell RCC existed. As such, a recent microarray study was performed by Brannon et al. on 48 clear cell RCC tumors to address this issue (38). An unsupervised consensus clustering – where similar tumors are grouped - was performed on these primary tumors without any previous knowledge or clinical information such as grade and survival. From this analysis, two distinct ccRCC molecular subtypes were recognized. Seeing this distinction between the ccRCC

tumor subtypes prompted validation which was performed in 177 tumors and again the same two distinct molecular subtypes were present.

To further assess potential reasons and a rationale of why two distinct molecular subtypes were recognized, survival analysis was performed on the 177 tumors and there was a significant difference in survival. The two subtypes were designated as ccA and ccB, with ccA conferring a significantly better survival rate over ccB. The analysis was taken farther utilizing SAM (Significance Analysis of Microarrays) and pathway analysis. ccA tumors overexpressed RCC-like genes such as those associated with hypoxia and angiogenesis while ccB overexpressed genes associated with more aggressive pathways including genes that regulate wound healing, the cell cycle and EMT (38). Interestingly, Ror2 was also overexpressed in ccB molecular subtype, which confers to a more aggressive and immature phenotype. This again places Ror2 in a more aggressive subtype of RCC and suggests that Ror2 may play a key role in the poor survival seen in ccBs. This analysis not only distinguishes two molecular phenotypes that confer to a significant survival difference but once again distinctly associates Ror2 with a more aggressive subtype of RCC.

In the future, we would like to look more closely at a cohort of primary paraffin embedded ccB tumors that also have corresponding clinical information. Ror2 will be tested as a potential tumor biomarker of EMT events in the tumor. We would like to analyze these tumors by IHC for Ror2 expression to assay its potential to be a biomarker. Additionally, since Ror2 is regulated by both HIF factors, we will also assay for both HIF1 and HIF2 expression as well as markers of EMT including E-

cadherin. A Ror2 protein profile will be generated using these primary human tumors. This will be correlated with clinical information such as gender, metastatic potential and survival. Our previous data indicates that Ror2 is in a majority of ccRCC tumors (over 55%) suggesting the feasibility of accomplishing this goal. It will be interesting to see the role Ror2 plays in the ccRCC and its potential clinical benefit once these technologies are up and running and have the possibility to go from bench to benchside.

Ror2 as a potential biomarker and basis for therapeutic development

The above results suggest that Ror2 has the potential to be used as a possible predictive biomarker or even as a target for targeted therapy. Collaborations using Ror2 as the basis for drug discovery are currently in the process. One collaboration initiated with the UNC Center for Integrative Chemical Biology and Drug Discovery (CICBDD) will use Ror2's kinase domains as the basis to screen for potential kinase inhibitors. Utilizing the autophosphorylation properties of Ror2, we will use this as the substrate basis for a small molecule inhibitor screens. Potential kinase target hits will be analyzed using Ror2-expressing and Ror2-suppressed cells. Briefly, Ror2-expressing and Ror2-suppressed cells will be treated with potential inhibitors and an MTT assay will be used as a functional assay to assess cell viability. To move forward with an inhibitor, we will assess those inhibitors that abrogate Ror2-expressing cell viability while Ror2-suppressed cells are viable. To validate potential compounds, immunoprecipitation and autophosphorylation assays will be used to analyze Ror2 phosphorylation status

changes in response to drug treatment, while xenograft models will be used to analyze if the inhibitor causes stabilization or reduction of RCC tumors.

Within the RCC field, few if any validated tumor specific prognostic indicators of death or recurrence of metastatic RCC have been recognized. Additionally, predictive biomarkers are needed in the field as clinical decisions about the use of adjunct therapies are based on pathological factors such as tumor grade and stage specific factors. Though this information is good for identifying tumors less or more likely to recur, it is not ideal for patients who are in intermediate stages for RCC. As Ror2 is associated with the more aggressive form of RCC, it has the potential to be used as a biomarker to identify patients more (or less) likely to develop metastatic disease.

IN CONCLUSION

In the course of this study, we have succeeded in our goal of identifying and characterizing a potential kinase target for RCC. We show that Ror2 plays an important role in RCC tumorigenesis, is associated with the more aggressive form of ccRCC and is also tied to the most commonly mutated gene, pVHL, in RCC. This is the first time that Ror2 has been associated with RCC tumor biology and also associated with the VHL/HIF pathway. The discovery of Ror2 in RCC heralds a previously untapped field within the RCC world and has also linked a developmentally regulated gene found in RCC to other cancer fields.

The identification of Ror2 links RCC to a new potential biological avenue tightening the bond between RCC and the ECM. We show that Ror2 plays an important role at the ECM and our data suggests that Ror2 can also be linked to the epithelial to mesenchymal transition (EMT), an important feature of cancer metastasis. The discovery of Ror2 also opens up the RCC field to an avenue of Wnt signaling relevant to RCC that has not been previously uncovered. Moreover, Ror2 has a novel pathogenic HIF activation pathway not previously uncovered in other pathogenesis. Though this regulation by HIF is atypical, there has been evidence within the literature showing HIF dependent genes that were not drastically induced under physiological hypoxia. An example of this is endosialin/TEM1, which was suggested to be regulated by the Ets-1 transcription factor in cooperation with HIF-2α (39). Thus, the results we have discovered may help to further explain previously unresolved mechanisms for certain identified genes.

Additionally, Ror2 has been recognized as having a key role in several cancer etiologies, where it is shown to be associated with an invasive phenotype. The discovery of Ror2 in RCC and other cancer types has opened up a new and exciting field of study in the cancer world that warrants further exploration. Not only has the identification of Ror2 introduced the potential of a specific target for targeted drug therapy, but we have also identified a potential predictive biomarker of cancer that has the possibility to be applied to several cancer etiologies.

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