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Dysregulation Of Meox2 Following Wt1 Mutation In Kidney Development And Wilms Tumorigenesis

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A

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by

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Dedication

To my mother, Sylvia Ann Dozier-Rayne, for the love and support she provided me throughout my entire life. She quoted, “Knowledge is power”, and to always do my best in what I do in my life. She inspired me to pursue the highest degree I can obtain and live life to the fullest. Furthermore, I dedicate my thesis to my father, Boualone Nosavanh, my grandmother, Christine Taylor, and to my siblings, Barry, Jessica, Joseph, Margie and Michael. They have provided me with the utmost love and encouragement. My younger siblings tell me that they look up to me as their role model, which makes me feel as if I have accomplished more than I ever imagined. To my friends, I am so fortunate to have you as a part of my life and express great appreciation for your support, encouragement, and positive energy throughout my graduate career.

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DYSREGULATION OF *MEOX2* FOLLOWING *WT1* MUTATION IN KIDNEY DEVELOPMENT AND WILMS TUMORIGENESIS

Publication No._____

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Wilms tumor (WT) is a childhood tumor of the kidney and a productive model for understanding the role of genetic alteration and interactions in tumorigenesis. The Wilms tumor gene 1 (*WT1*) is a transcriptional factor and one of the few genes known to have genetic alterations in WT and has been shown be inactivated in 20% of WTs. However, the mechanisms of how *WT1* mutations lead to Wilms tumorigenesis and its influence on downstream genes are unknown. Since it has been established that *WT1* is a transcriptional regulator, it has been hypothesized that the loss of *WT1* leads to the dysregulation of downstream genes, in turn result in the formation of WTs. To identify the dysregulated downstream genes following *WT1* mutations, an Affymetrix GeneChip Human Genome Array was previously conducted to assess the differentially expressed genes in the *WT1*-wildtype human and *WT1*-mutant human WTs. Approximately 700 genes were identified as being significantly dysregulated. These genes were further prioritized based on their statistical significance, fold change, chromosomal region, spatial pattern of gene expression and known or putative cellular functions. Mesenchyme homeobox 2 (*MEOX2*) was one of the most significantly upregulated genes in *WT1*-mutant WT. MEOX2 is known to play a role in cell proliferation, apoptosis, and differentiation. In addition to its biological roles, it is expressed during early kidney development in the

condensed mesenchyme similar to *WT1*. Furthermore, the use of the Match® web-based tool from the BIOBASE Biological Data base identified a significant predicted WT1 binding site within the first intron of *MEOX2.* The similarity in spatial gene expression in the developing kidney and the significant predicted WT1 binding site found in the first intron of *MEOX2* lead to the development of my hypothesis that *MEOX2* is upregulated via a *WT1*-dependent manner.

Here as a part of my master's work, I have validated the Affymetrix GeneChip Human Genome Array data using an independent set of Wilms tumors. *MEOX2* remained upregulated in the mutant *WT1* Wilms tumor by 41-fold. *Wt1* and *Meox2* gene expression were assessed in murine newborn kidney; both *Wt1* and *Meox2* were expressed in the condensed, undifferentiated metanephric mesenchyme. I have shown that the *in vivo* ablation of *Wt1* during embryonic development at embryonic day (E) 13.5 resulted in the slight increase of *Meox2* gene expression by two fold. In order to functionally demonstrate the effect of the loss of *Wt1* on *Meox2* gene expression in undifferentiated metanephric mesenchyme, I have generated a kidney mesenchymal cell line to genetically ablate *Wt1 in vitro* by adenoviral infection*.* The ablation of *Wt1* in the kidney mesenchymal cell line resulted in the upregulation of *Meox2* by 61-fold. Moreover, the upregulation of *Meox2* resulted in the significant induction of *p21* and *Itgb5.* In addition to the dysregulation of these genes the ablation of *Wt1* in the kidney mesenchymal cells resulted in decrease in cell growth and loss of cellular adherence. However, it is uncertain whether the upregulation of *Meox2* caused this particular cellular phenotype. Overall, I have demonstrated that the upregulation of *Meox2* is *Wt1*-dependent during early kidney development.

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Abbreviations

Δ, Ablated

ΔCt, Delta Ct

4-OH-Tamoxifen, 4-hydroxyl-Tamoxifen

A, Adenine

A1, Allele 1

ABC, Avidin-biotin-peroxidase

BLAST, Basic local alignment search tool

BSA, Bovine serum albumin

C, Carboxyl

Cy, Cytosine

CGH, Comparative genomic hybridization

ChIP, Chromatin immunoprecipitation

Chr., Chromosome

Cited1, Cbp/p300-interacting transactivator 1

CKO, Conditional knockout

CMV, cytomegalovirus

CXXC4, CXXC-type zinc finger protein 4

DDR1, Discoidin domain receptor family, member 1

DDS, Denys-Drash syndrome

Del, Deletion

dH_2O, distilled water

DIC, Differential interference contrast

DMEM, Dulbeccos's modified Eagle's medium

E, Embryonic day

eGFP, enhanced green fluorescent protein

Ex, Exon

FBS, Fetal bovine serum

FDR, False Discovery Rate

FS, Frame Shift

FST, Follistatin

FWT1/FWT2, Familial predisposition genes of WT on chromosome regions 17q12-q21 and 19q13.4

G, Guanine

GAPDH, Glyceraldehyde phosphate dehydrogenase

GFP-FITC, GFP fluorescein isothiocyanate

H19, Imprinted maternally expressed transcript (non-protein coding) H19 allele

HIPK2, Homeodomain-interacting protein kinase 2

IF, Immunofluorescence

IFN-γ, Interferon gamma

IGF2, Insulin-like growth factor

IHC, Immunohistochemistry

Ins, Insertion

Itgb5, Integrin Beta 5

IRES, Internal ribosome entry site

KTS, Lysine, threonine, and serine

LOH, Loss of heterozygosity

LOI, Loss of imprinting

MEOX2, Mesenchyme homeobox gene 2

MET, Mesenchymal-to-epithelial transition

MLLT3, Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3

MMC, Metanephric mesenchymal cells

MOI, Multiplicity of infection

N, Amino

NF-κ-B, Nuclear Factor κ-B

NLS, Nuclear localization signaling

NTC, Non-treated cells

P1, Post natal day 1

PAX6, Paired box gene 6

PBS, phosphate buffered saline

PCR, Polymerase chain reaction

PRRX1, Paired related homeobox 1

qPCR, Quantitative PCR

rpm, Revolutions per minute

SD, Standard deviation

T, Thymine

TAg, T-Antigen

Term, Termination of transcription

TGF-β, Transforming growth factor β

VSMCs, Vascular smooth muscle cells

WAGR, Wilms tumor, aniridia, genitourinary malformation, and mental retardation

Wilms tumor, WT

WT1, Wilms tumor suppressor gene 1

WTX, Wilms tumor on the X located at Xq11.1

X, Fold change

Chapter 1: Introduction

Wilms Tumor

Wilms tumor (WT) is an embryonal malignancy of the kidney that represents approximately 95% of all pediatric kidney tumors and accounts for 6% of all pediatric cancers (1-4). The overall annual incidence of Wilms tumor in North America is 1 in 10,000 children, resulting in 600-700 new cases each year (3, 4). Approximately 75% of these WTs occur in children under the age of 5 years, with a peak occurrence between 3-4 years of age (5). WTs can be classified into three clinical categories based on its occurrence: 1) the sporadic category, which describes children with no unusual physical features or family history of WT; 2) the familial category, which describes children with family history of WT; and 3) the syndromic category, which describes children with congenital anomalies, such as Wilms tumor, aniridia, genitourinary malformation, and mental retardation (WAGR) syndrome, Denys-Drash syndrome (DDS), and Beckwith–Wiedemann syndrome (BWS). Between 98-99% WT cases are sporadic, while only 1-2% are familial (1, 3, 6). The majority of WT cases (90-95%) are unilateral (occurring in one kidney). However, bilateral cases (occurring in both kidneys) are also reported, particularly in patients with congenital anomalies as well as a family history of WTs (1, 7). Because WT is thought to occur during early kidney development, it serves as an excellent model to study the relationship of cancer to development by understanding normal kidney development and how a disruption during this process results in Wilms tumorigenesis.

Kidney Development

The mammalian kidney arises during embryogenesis from the interaction between the differentiated epithelial cells of the ureteric bud and undifferentiated mesenchymal cells (8, 9). In mice, the ureteric bud epithelium invades the uncondensed, undifferentiated metanephric mesenchyme, which causes the metanephric mesenchymal cells to condense around the ureteric bud (**Figure 1.1 A-C**). Reciprocal interactions occur between the ureteric bud and the metanephric mesenchyme. The ureteric bud induces the metanephric mesenchyme to simultaneously give rise to stromal mesenchyme, which is rich in growth factors and corresponding receptors for the adjacent epithelial ureteric bud. Concurrently, the metanephric mesenchyme further condenses around the ureteric bud to undergo a mesenchymal-to-epithelial transition (MET) (8-11). Simultaneously, the induced mesenchyme forms into the comma and S-shaped bodies as the ureteric bud continues to branch out and proliferate (**Figure 1.1 D-E**) (8-10). The S- and comma-shaped bodies of the mesenchyme completely differentiates into epithelial cells to make up the majority of the cells of the nephron, such as the tubules and glomeruli (**Figure 1.1 F-G**) (8-10). The extensive branching of the ureteric bud results in the generation of the collecting ducts of the mature kidney, and the kidney continues to develop into the mature kidney after birth in mice (8-10). The stages of kidney development in humans occur similar to that in mice.

WT is thought to arise from undifferentiated metanephric mesenchyme. The classic histology of WTs, in both humans and mice, is triphasic consisting of mesenchymal, stromal, and epithelial cells that suggest disruption(s) during the cellular differentiation in the cells of the maturing nephron (**Figure 1.2**). Some

individual WTs consist of two of the three cell types; one cell type can be predominant over the other cell types present in the tumor. Because of the classic triphasic histology of WT, it is thought that there was a disruption during early kidney development, particularly in the cellular differentiation. This disruption may inhibit the cells to differentiate into the components of the nephron and cause them to overproliferate, in turn resulting in WT.

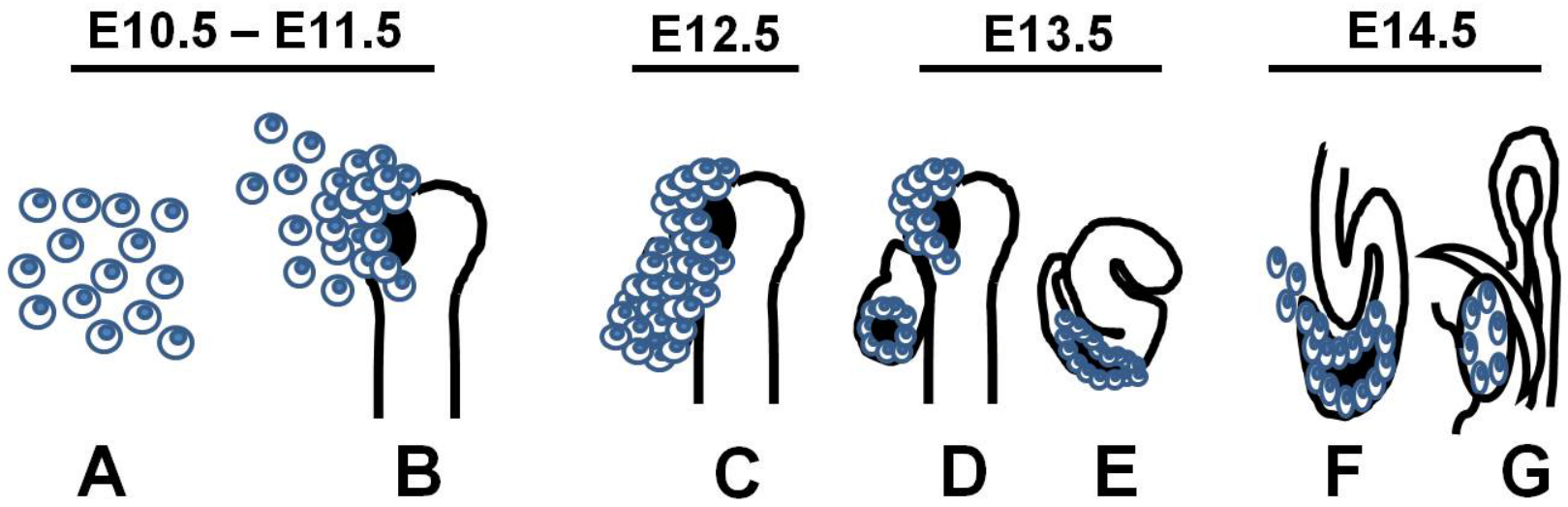


Figure 1.1. Kidney Development

Kidney development in mice starts at embryonic day (E) E10.5-11.5. In the beginning, the uncondensed mesenchyme **(A)** is invaded by the ureteric bud **(B)**. At E12.5, the mesenchyme becomes condensed **(C)**. At E13.5, comma-shaped bodies and S-shaped bodies **(D and E)** start to appear during kidney development. At E14.5, the tubular component of the nephron continues to develop into the functionally distinct cells of the proximal convoluted tubule, descending and ascending tubule **(F)** and the mesenchymal cells transition to become epithelized and differentiate into specialized cells of the glomerulus and tubules of the mature nephron **(G)** In humans, kidney development occurs similar to that in mice (10, 12). (Adapted from (12))

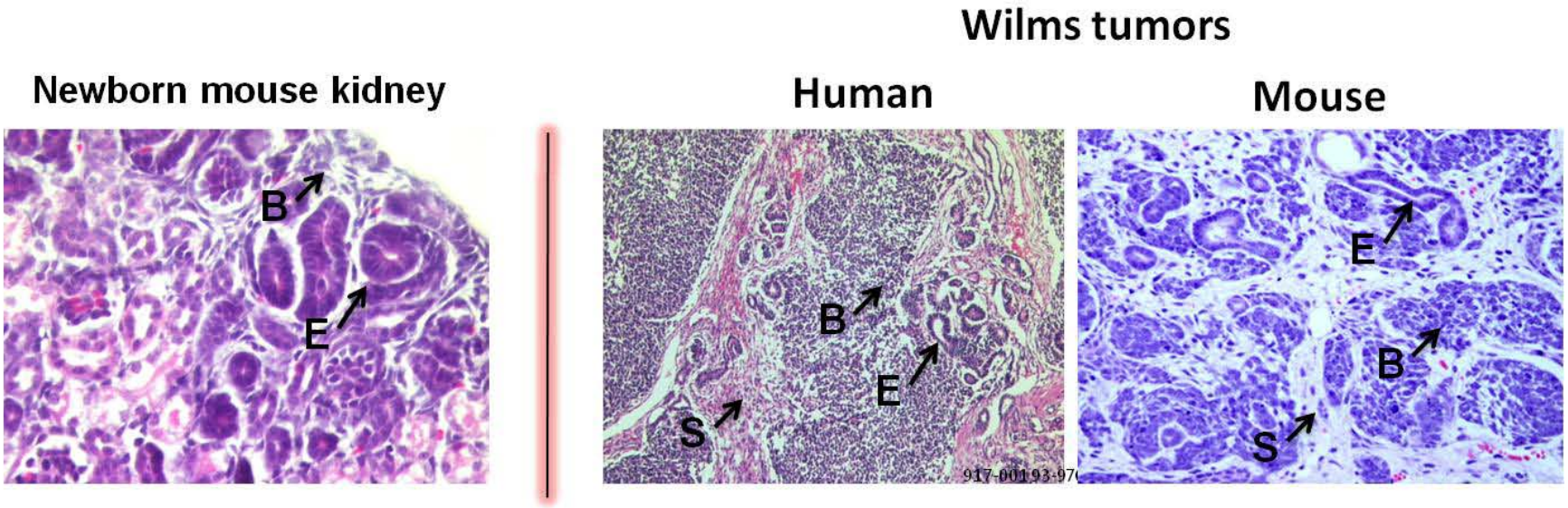


Figure 1.2. Hematoxylin and Eosin Staining of Newborn Mouse Kidney and WTs

The triphasic histology of WTs in both human and mouse illustrates the early stages of kidney development (B- Blastemal, S- Stromal, and E- Epithelial cells). (Provided by Gao, F. and Hu, Q.)

Genetic Alterations in Wilms Tumor

WT was the second tumor to meet the criteria of the Knudson's two hit model of cancer, in which there are two rate-limiting events, or 'hits', in turn resulting in cancer formation (13). The two rate-limiting events are a first 'hit', (which can be either a germline or somatic mutation), which may be followed by a second 'hit', always a somatic mutation (13). Besides the two rate-limiting events of the classic Knudson's two hit model, it has been suggested that there could be multiple genetic alterations and/or multiple mutated genes involved in an individual WT. Cytogenetic, genetic linkage, loss of heterozygosity (LOH), loss of imprinting (LOI), and DNA sequencing studies provided data that identified alterations that occur during Wilms tumorigenesis.

Cytogenetic studies, such as G-Banding, fluorescent in situ hybridization (FISH) and comparative genomic hybridization (CGH), revealed chromosomal alterations, including the gain and loss of chromosomes and chromosomal regions in WTs. The chromosomal gains in WTs were observed in chromosomes 6, 7, 12, 13, 17, 18, and 20 and the chromosomal losses noted in chromosomal regions 1p, 2q, 3, 7p, 11p, 11q, 14q, 16q, 17p, and 22q (14-17). These chromosomal regions may harbor genes that contribute to Wilms tumorigenesis.

As of now, genetic linkage studies mapped chromosomal region 17q12-q21 as familial Wilms Tumor gene 1 (*FWT1*) and chromosomal region 19q13.4 as familial Wilms tumor gene 2 (*FWT2*) as familial predisposition regions in large WT families. These regions may contain genes that predispose to WT (18, 19). However, no genes have yet been identified within these regions that contribute to the familial

predisposition of WT. The mapping of additional chromosomal regions in WT families is still an ongoing process.

Additional to cytogenetic analyses, LOH studies identified the loss of chromosomal region 11p in tumors versus constitutional DNA from the same individual. The demonstration of LOH at loci 11p in ~40% of WTs suggested that the inactivation of the gene within this particular region is essential to Wilms tumorigenesis (20-22). In addition to the LOH of 11p chromosomal region, WAGR patients carried constitutional deletions encompassing band 11p13 (17). Kaneko *et al.* reported that subsequent observation of 11p13 deletions in tumor DNA from patients who did not carry germline 11p13 deletions indicated somatic mutations were also essential during tumorigenesis (23). Collectively, these analyses of 11p13 deletions led investigators to localize and clone the first WT gene, Wilms tumor gene 1 (*WT1*) (24). This particular gene is mutated in ~20% of WTs (1).

DNA sequencing analysis data identified additional genetic alterations in identifiable genes in WTs. The tumor suppressor protein p53 (*TP53*), located at chromosome region 17p13, is mutated in 5% of WTs (25, 26). The tumors that carry *p53* mutations contain abnormal cells that are poorly developed with enlarged distorted nuclei (25, 26). Germline and somatic mutations of *TP53* include either missense mutations that occur in the exons 5, 6, and 10, and lead to the inactivation of the protein (25). Catenin (cadherin-associated protein), beta 1 (*CTNNB1*), located at chromosomal region 3p21, is mutated in 15% of sporadic WTs (27, 28). Somatic mutations of *CTNNB1* include protein-stabilizing mutations in exon 3 (27, 28). Additional mutations occur in exons 7 and 8 of *CTNNB1* (29, 30). Recently, Wilms tumor gene located at Xq11.1 (*WTX*) was discovered to be mutated in ~20% of WTs

(31-34). Somatic mutations of *WTX* include either whole or partial deletion of *WTX* or nonsense mutations resulting in termination codons (31-34). The discovery of all the genetic alterations and identifiable mutated genes involved during Wilms tumorigenesis is still in progress. *WT1* is one of the genes that is commonly mutated in WTs and essential during kidney development as later described. It is important to further study the loss of *WT1* in order to determine and establish its fundamental role during Wilms tumorigenesis.

Wilms Tumor Suppressor Gene 1 (*WT1*)

Located at 11p13 in humans, the *WT1* gene consists of ten exons and encodes a zinc finger transcriptional factor which contains a proline glutamine-rich protein-interaction domain and four carboxy-terminal zinc finger domains (24, 35) **(Figure 1.3A**). Four isoforms of the WT1 protein result from two different alternative splice sites. Alternative splice I inserts the entire exon 5, which is made up of 51 nucleotides and encodes 17 amino acids **(Figure 1.3B**) (36). Alternative splice II inserts 9 nucleotides in exon 9 that encodes an additional three amino acids, lysine [K], threonine [T], and serine [S] **(Figure 1.3B**) (36).

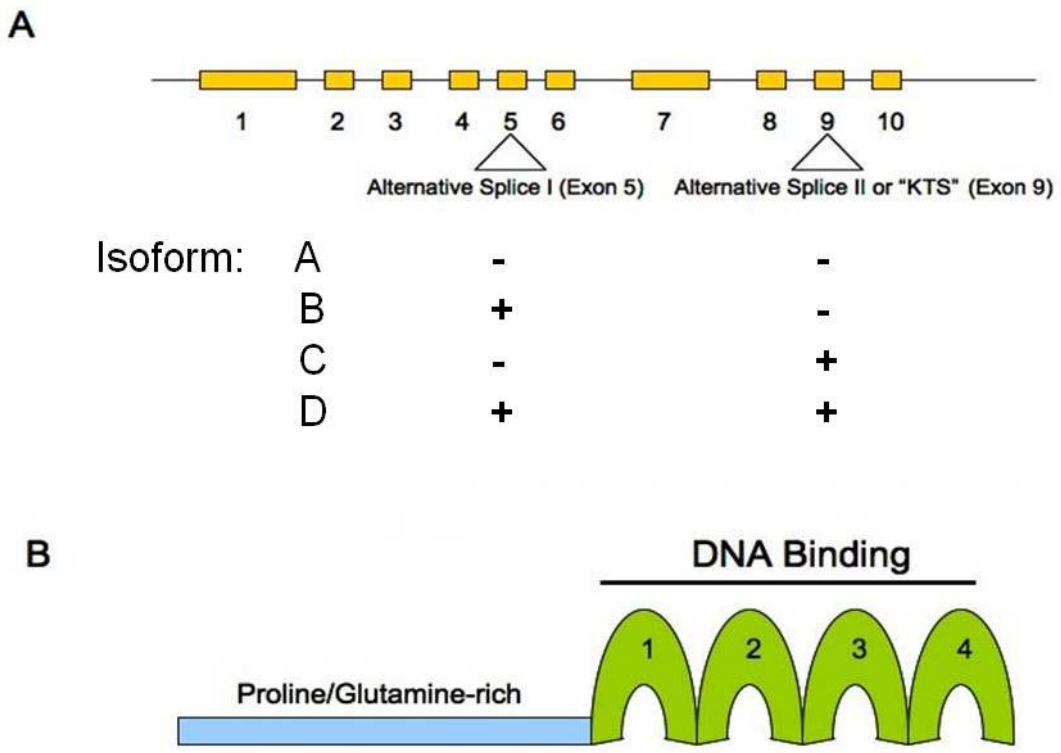


Figure 1.3. WT1 Gene and Protein

1.3A. Exons are indicated by yellow and the alternative splice sites are located within exons 5 and 9 that result in four different isoforms of the WT1 protein (36).

1.3B. Proline and glutamine rich domain (blue) and four zinc fingers that serve as the DNA binding domain (green) of the WT1 protein (36). (Adapted from (7))

Wt1 in Kidney Development

At E9.5 in mice, the first expression of Wt1 is in the intermediate mesenchyme lateral to the coelomic cavity (37). Wt1 is later expressed in the metanephric mesenchyme (uncondensed and condensed) (**Figure 1.4**) and its maximum expression is in the S- and comma-shaped bodies (9, 38-42). In the adult kidney, it is restricted to the podocytes of the glomerulus (**Figure 1.4**) (10, 42). The essential role of Wt1 during kidney development was not recognized until a mouse model was generated for the purpose of investigating the role of Wt1 in development, homeostasis, and WT development.

Kreidberg *et. al.* (1993) first generated a *Wt1* mouse model (43). Heterozygous mutant *Wt1* mice appeared to be normal; however homozygous mutant *Wt1* mice were typically embryonic lethal between E13 and E15 (43). Homozygous mutant *Wt1* mice failed to complete gestation due to systemic edema and pericardial bleeding (43). Besides embryonic lethality, the major consequence of *Wt1* mutation was renal agenesis (43). Hu *et. al.* (2011) also demonstrated that the *in vivo* ablation of *Wt1* in a conditional inducible knock out mouse model resulted in a complete block in nephron development, in which no glomeruli were present, and there was no differentiation of condensed mesenchyme past the comma-shaped body stage (44). All together, Wt1 has an essential role, direct or indirect, in the survival and differentiation of the metanephric mesenchymal cells during kidney development.

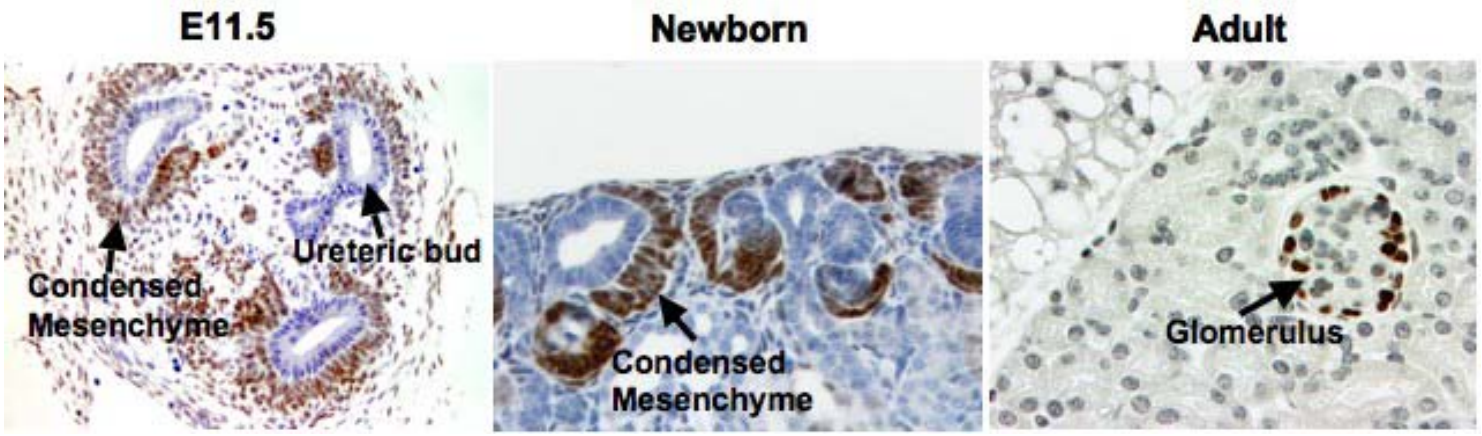


Figure 1.4. Wt1 Protein Expression during Murine Kidney Development

Wt1 is expressed in the uncondensed and condensed mesenchyme (**left**) and expression remains high throughout kidney development, especially in the S- and comma-shaped bodies (**middle**). Its expression is restricted to the podocytes of the glomerulus (**right**) (45). (Provided by Gao, F. and Hu, Q.).

WT1 in Wilms Tumor

Germline and sporadic mutations of *WT1* in WTs consist of alterations such as deletions or insertions leading to frameshift and/or truncation, missense and nonsense mutation, or splice site mutations that result in the inactivation of the WT1 protein (**Figure 1.5**) (1, 32, 46). These mutations lead to the inactivation of the protein (1, 32). *WT1* germline mutations occur in children with congenital anomaly syndromes, such as WAGR and DDS, and have a high risk of developing WTs (1, 32). In regards to *WT1* mutations in sporadic WTs, numerous of studies have gathered data providing different frequencies of *WT1* mutations ranging from 10-20% (47-50). Furthermore, patients with homozygous mutations within *WT1* provided evidence that mutations in both alleles at the *WT1* locus are critical in WT development and define the functional role of *WT1* in WT as a tumor suppressor gene (1, 47). Even though genetic studies identified germline and somatic mutations that result in the inactivation of *WT1* in WTs, the effect of the loss of *WT1* during Wilms tumorigenesis remains unknown. Since WT1 is a transcriptional factor that regulates the activation or repression of genes, the driving hypothesis is that the inactivation of *WT1* results in the dysregulation of downstream genes that are involved in key cellular pathways during embryogenesis that contribute to Wilms tumorigenesis.

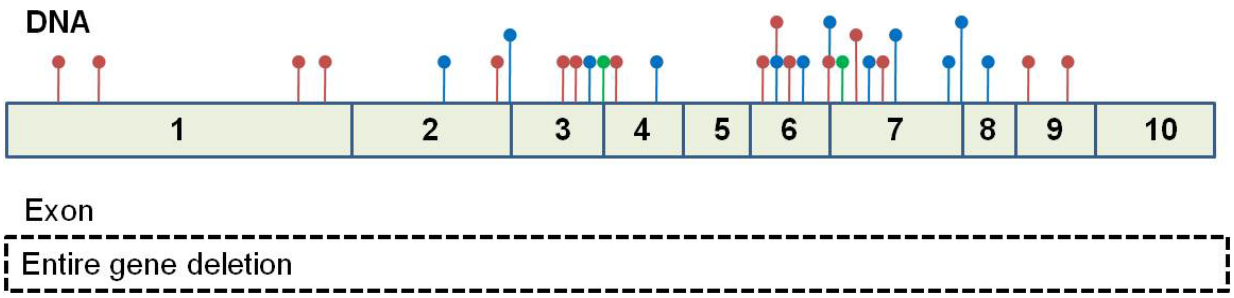


Figure 1.5. *WT1* mutations detected in WTs

Germline mutations, dispersed throughout the entire *WT1* gene, detected in patients with Wilms' tumor-associated phenotypes. Similar mutations were detected in somatically mutated WTs. Location of gene mutations are designated by red (insertion or deletions and frameshift mutations), blue (nonsense mutations), and green (splice site mutations). (Adapted from (51)).

Genes Regulated by WT1

Genes regulated by WT1 have been identified and are involved in pathways that are essential in cellular proliferation, apoptosis, cell survival, and cellular differentiation. The identification of the majority of genes regulated by WT1 was done by promoter assays, electrophoretic mobility shift assay (EMSA), co-immunoprecipitation (co-IP), microarray analyses, and co-transfection assays (52-124). These *in vitro* assays were conducted with human cell lines derived from normal and cancerous tissues and/or murine cell lines (52-124). A collection of published genes thought to be regulated by WT1 is displayed in **Appendix Table 1**. However, it is unclear whether the inactivation of *WT1* has an influence on their gene expression during Wilms tumorigenesis. To determine the effect of the loss of *WT1* on the downstream genes, a previous Affymetrix GeneChip Human Genome Array was used as an approach to identify genes that are differentially expressed in human *WT1*-mutant WTs when compared to *WT1*-wildtype WTs. Approximately 700 genes were significantly differently expressed from this array (Ruteshouser, E. C.). These genes were prioritized by their False Discovery Rate (FDR, 0.05), statistical significance (p-Value <0.05), fold change (>2X), chromosomal region, and gene expression pattern. Moreover, extensive literature searches on their known or putative cellular functions narrowed the number of genes for further investigation to 223 genes. The effect of the loss of *WT1* resulted in the differentially expression of a variety of genes; however, it remains unknown whether the effect of the inactivation of *WT1* dysregulates their gene expression during early kidney development, which could lead to the development of WTs.

Rationale

WT1 is known to be both an essential gene during kidney development and a tumor suppressor of WT. *WT1* is reported mutated in WTs, however the effect of the loss of *WT1* during Wilms tumorigenesis remains uncertain. Potentially, WT1 transcriptionally regulates genes involved in signaling pathways that control cellular processes, such as cellular proliferation, differentiation, apoptosis, and cell adhesion. The hypothesis of the inactivation of *WT1* in WT is that it results in the transcriptional dysregulation of downstream genes that lead to Wilms tumorigenesis. To test the hypothesis, a microarray was conducted and identified numerous genes that were either upregulated or downregulated in *WT1*-mutant WTs. The microarray data support the hypothesis that the inactivation of *WT1* transcriptionally dysregulates downstream genes. I have propose to investigate the loss of *Wt1* during murine kidney development to determine whether the gene is expressed during early kidney development, whether Wt1 regulates, directly or indirectly, the gene during kidney development, and whether their dysregulation in gene expression following a *Wt1* mutation lead to the formation of WT.

I have taken the first step to determine the effect of inactivation of *WT1* on the genes dysregulated in WTs by validating the microarray data by quantitative PCR (qPCR) using an independent set of WTs to rule out any false discoveries from the microarray data (**Chapter 2**). The qPCR validation revealed a stronger gene regulation relationship between *WT1* and *MEOX2.* Therefore, the goal of my project is to further assess the effect *MEOX2* gene expression following a *WT1* mutation during kidney development.

Because it is thought that a disruption in the cellular differentiation process during early kidney development leads to Wilms tumorigenesis, I generated a number of conditional *Wt1* immortalized undifferentiated metanephric mesenchymal cell lines (MMCs) (**Chapter 3**). I used this cell line to determine the expression level of *Meox2* before and after the ablation of *Wt1***.** To assess the dysregulation of *Meox2* following a *Wt1* mutation during early kidney development *in vivo,* I used the *Wt1* conditional knockout mouse model to ablate *Wt1* at E11.5 and determined the dysregulation of *Meox2* at E13.5 (Chapter 4). Results from both the *in vivo* and *in vivo* studies indicated that the inactivation of *Wt1* results in the upregulation of *Meox2* during early kidney development. Furthermore, the upregulation of *Meox2* from the *in vitro* analyses showed an effect on *Meox2* established downstream genes, *p21* and *Itgb5.* My studies of the effect of gene expression of *Meox2* following a *Wt1* mutation suggest that Wt1 regulates *Meox2* during both early kidney development and Wilms tumorigenesis.

Chapter 2: Upregulation of *MEOX2* in *WT1*-mutant tumors: Validation of WT1 Putative Transcriptional Targets by qPCR

WT1 is expressed during early kidney development and encodes a transcriptional factor and tumor suppressor that recognizes several DNA motifs such as the early growth response-1 (EGR-1) binding site, TCC repeats, and a high affinity WTE site (55, 71, 101, 125, 126). As displayed in **Appendix Table 1**, WT1 putatively activates or represses its downstream genes. Thus, the effect of the loss of *WT1* during Wilms tumorigenesis could be the increased or decreased expression of downstream genes during early kidney development. Our recent microarray data identified genes that were dysregulated due to the inactivation of *WT1* in WTs. Some of these dysregulated genes are known to be normally expressed in the kidney, while others were not.

Our previous Affymetrix GeneChip array data revealed numerous genes that were significantly dysregulated in *WT1*-mutant WTs. As previously mentioned in **Chapter 1**, stringent statistical analysis of the microarray data and literature searches further narrowed down the number of genes to 21 as the most significant dysregulated genes in *WT1*-mutant WTs. Seven out of the 21 genes were selected, based on their biological relevance(s) in kidney development and cancer, for further investigation (**Table 2.1**). Two of these genes, Discoidin domain receptor family, member 1 (*DDR1*) and Homeodomain-interacting protein kinase 2 (*HIPK2*), are involved in cellular proliferation and/or apoptosis (**Table 2.2**) (127). Three of these genes, CXXC-type zinc finger protein 4 (*CXXC4*), Follistatin (*FST*), and *HIPK2*, play roles in signaling pathways which promote cellular proliferation and differentiation (**Table 2.2**) (127). Another gene, Myeloid/lymphoid or mixed-lineage leukemia, translocated to 3

(*MLLT3*), is involved in transcriptional regulation (**Table 2.2**) (127). Additionally, two genes, Mesenchyme Homeobox gene 2 (*MEOX2),* and Paired related homeobox 1 (*PRRX1*), are located in chromosomes 1 and 7, which have been identified in WTs with genetic aberrations. The dysregulation of these genes following mutation *WT1* suggests that WT1 potentially regulates them during kidney development. Like WT1, these genes are expressed during embryogenesis, which also supports the hypothesis that WT1 transcriptionally regulates these genes. Before we can further study the effect of inactivation of *WT1* on the gene expression of *CXXC4*, *DDR1*, *FST*, *HIPK2, MEOX2*, *MLLT3*, and *PRRX1*, the problem of false discovery from the array data has to be resolved.

A method to assess any false discoveries from the microarray is by quantitative PCR (qPCR) that quantifies the relative or absolute gene expression level within a given sample. In order to further investigate the effect of inactivation of *WT1* on the gene expression of *CXXC4*, *DDR1*, *FST*, *HIPK2, MEOX2*, *MLLT3*, and *PRRX1*, I validated all these genes with RNA isolated from an independent set of WTs by qPCR. Here, it is reported that the gene expression of *MEOX2*, one of the seven selected genes, stood out from the other validated genes and was significantly upregulated in *WT1*-mutant tumors. This suggested that WT1 transcriptionally, directly or indirectly, regulates *MEOX2* during early kidney development and Wilms tumorigenesis.

Table 2.1. Selected Differentially Expressed Genes from the Affymetrix GeneChip Array

Gene ID	Protein	Location	Fold Change
CXXC4	CXXC-type zinc finger protein 4	4q22-q24	2.5-5X ↓
DDR1	Discoidin domain receptor family, member 1	6p21.3	2.5-3X ↓
FST	Follistatin	5q11.2	6-17X ↑
HIPK2	Homeodomain-interacting protein kinase 2	7q32-q34	2-5X ↓
MEOX2	Mesenchyme Homeobox gene 2	7p22-p21	19X ↑
MLLT3	Myeloid/lymphoid or mixed-lineage leukemia, translocated to 3	9p22	5-7X ↑
PRRX1	Paired related homeobox 1	1q24	4-7X ↑

Table 2.2. Biological and Cellular Description of the Selected Genes

Gene ID	Function*	Process*	Component*
Cxxc4	DNA binding	Wnt Signaling Pathway	Cytoplasm
	PDZ domain binding		Cytoplasmic membrane
	Metal ion binding		Cytoplasmic vesicle
	Zinc ion binding		
Ddr1	ATP binding	Cell adhesion	Basolateral plasma membrane
	Kinase activity	Ear development	Integral to membrane
	Nucleotide binding	Embryo implantation	Integral to plasma membrane
	Protein binding	Cell proliferation	Membrane
	Protein kinase activity	Mammary gland alveolus development	Plasma membrane
	Protein tyrosine kinase activity	Peptidyl-tyrosone phosphorylation	
	Receptor activity	Protein phosphorylation	
	Transferase activity	Transmembrane receptor protein tyrosine kinase signaling pathway	
	Transmembrane receptor protein tyrosine kinase activity		
Fst	Activin binding	BMP Signaling Pathway	Extracellular region
	Heparan sulfate proteoglycan binding	Female gonadal development	
		Gamete development	
		Hair follicle development	
		Hemopoietic progentitor cell differentiation	
		Keratinocyte proliferation	
		Cell differentiation	
		Pattern specification process	
Hipk2	ATP binding	DNA damage response	PML body
	SMAD binding	SMAD protein signal transduction	Centrosome
	Nucleotide binding	Anterior/posterior pattern formation	Cytoplasm
	Protein binding	Apoptosis	Nulear body
	Protein kinase activity	BMP Signaling Pathway	Nuclear membrane
	Transcritpion corepressor activity	Phosphorylation	Nucleus
	Transferase activity	DNA binding	
	Virion binding	JNK cascade	
		Cell Proliferation	
		Smoothen signaling pathway	
		TGF-beta receptor signaling pathway	
Meox2	DNA binding	Angiogenesis	Cytoplasm
	Sequence-specific DNA binding	Limb development	Nucleus
	Sequence-specific DNA binding transcription factor activity	Multicellular organismal development	
	Transcription regulator activity	Palate development	
		Regulation of transcription, DNA-dependent	
		Skeletal muscle tissue development	
		Somite specification	

Gene ID	Function*	Process*	Component*
Mllt3	Protein binding	Anterior/posterior pattern formation	Nucleus
		Regulation of transcription, DNA-dependent	Cytoplasm
		Segment specification	
Prrx1	DNA binding	Artery morphogenesis	Nucleus
	Sequence-specific DNA binding	Cartilage development	
	Sequence-specific DNA binding transcription factor activity	Embryonic cranial skeleton morphogenesis	
	Transcription regulator activity	Embryonic skeletal system morphogenesis	
	Transcription repressor activity	Inner ear morphogenesis	
		Middle ear morphogenesis	
		Multicellular organismal development	
		Palate development	
		Regulation of transcription, DNA-dependent	

Information provided by:

Mouse Genome Informatics (MGI)*

Materials and Methods

Wilms Tumor Samples

Twelve WT samples were used to validate the microarray data. Previously, WT samples, after informed consent, were collected from sporadic WT patients (50, 128). The DNA extracted from the tumors were analyzed for mutations by PCR amplification by the use of primers flanking each of the 10 exons of *WT1*, and followed by single-strand conformation polymorphism (SSCP) to confirm the abnormality (50, 129). The identification of *WT1* mutations was by the direct sequencing of aberrant PCR products (50)..

Isolation of RNA from Human Wilms Tumor Samples

The isolation of RNA was previously achieved by either acid guanidinium thiocyanate-phenol-chloroform extraction, described by Chomczynski, P. and N. Sacchi (130), RNAqueous®-4PCR Kit from Applied Biosystems (Carlsbad, California), or RNeasy Midi Kit from Qiagen (Valencia, CA). Depending on the method used to isolate the RNA, it was kept in solution in either diethyl pyrocarbonate (DEPC) treated water or in ethanol. Samples were stored at -80°C.

Quantitative PCR

qPCR is an efficient quantitative approach to validate the dysregulated genes from the microarray data. cDNAs were prepared by reverse transcription of 500 ng of total RNA from human *WT1*-wildtype and *WT1*-mutant Wilms tumors using TaqMan Reverse Transcription reagents with random hexamers from Applied Biosystems (Carlsbad, California). cDNAs were then amplified in duplicate in an ABI 7900HT Sequence Detection System thermal cycler using SYBR Green PCR Master Mix (Applied Biosystems). Primer Express v.3 software (Applied Biosystems) was used to design primer sets for the individual genes (**Table 2.3**). To determine the specificity of these primer sets, a dissociation curve of each primer set was assessed using human fetal kidney by qPCR analysis. Expression of glyceraldehyde phosphate dehydrogenase (*GAPDH*) was determined as an endogenous gene expression control. The standard deviation (SD) was determined for duplicate measurements of gene expression.

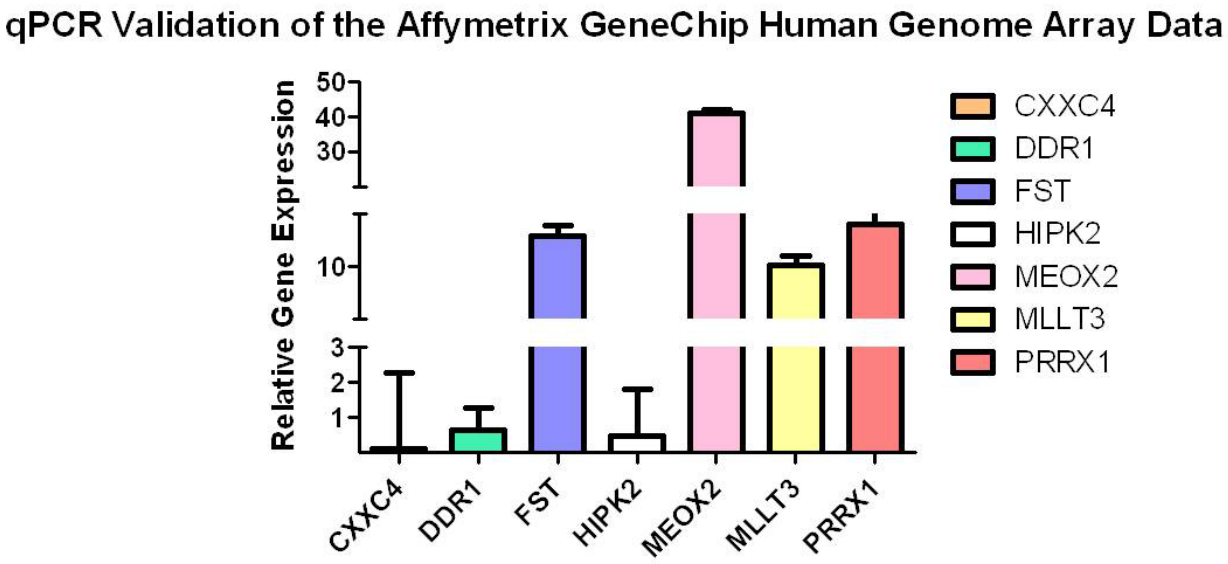
Table 2.3. Primer Sets designed by Primer Express v.3 Software for the qPCR Validation

Name	Sequence (5'-3')
CXXC4 F	AAAACCTGGCACTTCACTAGAGAGA
CXXC4 R	CTTTAAAAGAACCATCGGAATGCT
DDR1 F	CCCACCATCAGCTACCCAAT
DDR1 R	AAGTCTGCGATTTTGATGGTGAA
FST F	TGCTGGGCAGATCTATTGGAT
FST R	GATATCTTCACAGGACTTTGCTTTGA
HIPK2 F	CACCATGACACACTTACTCGATTTT
HIPK2 R	CACCCGACGCTTGCAGAT
MEOX2 F	CTGCGGAGGCGGAGAA
MEOX2 R	CTTGTAATTTCCTTCCTGGGAGTCT
MLLT3 F	GCAGCAGATCGTGAACCTTATAGA
MLLT3 R	CGAGCAAAGATCAAATCAAATGT
PRRX1 F	CAGCGTCTCCGTACAGATCCT
PRRX1 R	CGTTATGAAGCCCCTCGTGTAA

Results

Validation of the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array

CXXC4, DDR1, FST, HIPK2, MEOX2, MLLT3, and *PRRX1* were validated by qPCR with a total of 12 WTs samples, six *WT1*-wildtype WTs and six *WT1*-mutant WTs. *CXXC4, DDR1,* and *HIPK2* were downregulated and *FST, MEOX2, MLLT3,* and *PRRX1* were upregulated in *WT1*-mutant WTs by qPCR analysis (**Figure 2.1 and Table 2.4**). The upregulation of *MEOX2* stood out far from the rest of the dysregulated genes in the *WT1*-mutant WTs, therefore suggesting that WT1 would normally transcriptionally repress the gene expression of *MEOX2,* but the loss of *WT1* results in the overexpression of *MEOX2.*



	CXXC4	DDR1	FST	HIPK2	MEOX2	MLLT3	PRRX1
Relative Gene Expression	0.11	0.63	12.94	0.45	41.07	10.09	14.1
p-Value	0.06675	0.2017	0.00385	0.06469	0.00411	0.04114	0.06611

Figure 2.1. Validation of the Dysregulated Genes in the *WT1*-mutant WTs

In this graph, the y-axis represents the gene expression detected by the specific designed primer sets relative to the gene expression in the *WT1*-wildtype WTs. The individual color of the bars represents the gene found upregulated or downregulated in the *WT1*-mutant WTs.

Discussion

It is important to identify genes that play a role in regulating cellular proliferation and differentiation. Upstream genetic alterations, like mutant *WT1,* result in the dysregulation of genes which may contribute to tumorigenesis. Our recent array analysis revealed that there are potentially 223 genes that were dysregulated following a *WT1* mutation. Literature searches, assessment of gene expression pattern(s), and functional role(s)) in kidney development and/or cancer focused studies to 21 genes. Seven out of the 21 genes, *CXXC4*, *DDR1*, *FST*, *HIPK2, MEOX2*, *MLLT3*, and *PRRX1,* were validated by qPCR. The validation data confirmed that these genes were dysregulated in *WT1*-mutant WTs.

When I compared the qPCR data to the array data, the dysregulation of their gene expression remained consistent and validated the array data. However, some the genes, *CCCX4, DDR1, HIPK2,* and *PRRX1,* were no longer significantly dysregulated in the *WT1*-mutant tumors.

One reason why there was a change in their significance may have resulted from the overall technique. Both microarray and qPCR use a reverse transcriptase enzyme (RT) that generates a complementary cDNA to the mRNA. However, in microarrays, the fluorescent labeled cDNAs are hybridized to their synthetic complementary DNAs attached on the microarray slide. The probes used in microarrays vary in their length, ranging from 10 to 99 bps. Instead of using a probe to hybridize to the complementary cDNA, primers or probes are designed to amplify a specific region of the gene (100-200 bps) in qPCR. Depending on the length and location of the probes or primers, the assessment of the significance in their gene

expression by qPCR may be slightly and/or drastically different from the microarray data.

Another potential reason for the differences in the degree of gene expression change may be due to the cell variation within the WT sample. As mentioned in Chapter 1, Wilms tumor could be made up of a mixture of mesenchymal, stromal, and epithelial cells, in which the relative proportions of these cells vary broadly in each tumor. The RNA isolated from these tumor samples may have one cell type predominant over the other cell types residing in the tumor. There is a possibility the genes from **Table 2.1** are expressed in a specific cell type.

Regardless, *FST*, *MEOX2*, and *MLLT3* were significantly dysregulated in *WT1*-mutant WTs. However, the dysregulation of *MEOX2* gene expression stood out more compared to the other genes. *MEOX2* is a member of a novel subclass of the homeobox gene super family (131). In humans, *MEOX2* is located at chromosomal region 7p21, one of the chromosomal regions found altered in WTs (131, 132). Homeobox genes, such as *MEOX2,* regulate cellular growth and differentiation during embryonic development and disease progression (133, 134).

Additionally, *MEOX2* is expressed during early embryonic development. Between E9-E9.5, *Meox2* is expressed in all of the formed somites and in the sclerotome, a population of cells lying between the paraxial mesoderm and the limb buds (131, 132). At E14.5, *Meox2* is expressed in the condensed mesenchyme of the kidney. *Meox2* gene expression in the developing kidney decreases as the metanephric mesenchymal cells differentiate into epithelial structures of the developing kidney (42).

Because of *MEOX2* drastic and significant change in mutant-*WT1* WTs, biological functions, and embryonic gene expression, *MEOX2* is potentially a direct candidate downstream gene of WT1 during Wilms tumorigenesis. These suggest that there is a transcriptional relationship between WT1 and *MEOX2* expression in WT. However, more investigation is needed to completely understand the relationship between WT1 and *MEOX2,* such as when and where does the loss of *WT1* affect the gene expression of MEOX2 during kidney development.

Chapter 3: Generation of a Kidney Mesenchymal Cell Line

Mammalian kidney development results from reciprocal interactions between the ureteric bud and the undifferentiated metanephric mesenchyme. Because WT is thought to arise from undifferentiated metanephric mesenchyme, it is important to study Wilms tumorigenesis during early kidney development. In order to study Wilms tumorigenesis at the cellular and molecular level, we intended to use human embryonic kidney (HEK) 293 cells, which were transformed with sheared adenovirus 5 DNA to be cultured and passaged for a long period of time (135). However, experiments to determine the effect of the loss of *WT1* in the HEK293 cells were not feasible, because these cells did not expression high amounts of endogenous WT1 by IHC. Therefore, I generated an immortalized undifferentiated kidney mesenchymal cell line in which endogenous WT1 is robustly expressed and can be genetically ablated to efficiently study the effect of the inactivation of *Wt1* on putative downstream target genes.

Materials and Methods

Mouse Strains and Breeding

The *Wt1*$^{flox/flox}$ mouse generated and described by Gao et, al, (112) and the Immortomouse® (CBA;B10-Tg(H2Kb-tsA58)6Kio/Crl) purchased from Charles River (Wilmington, MA) were crossed together to obtain *Wt1*$^{flox/flox}$; *Immorto* mice. The purpose of this mating cross was to generate a mouse *Wt1*$^{flox/flox}$ strain that carried the Immorto allele for conditional cellular immortalization as described by Jat *et. al* (136). The mating cross consisted of *Wt1*$^{flox/flox}$; *Immorto* female mice and *Wt1*$^{+/-;}$*Cre-ER*^{TM} male mice was to generate the following genotypes:*Wt1*$^{-/flox}$; *Immorto*, *Wt1*$^{+/flox}$; *Immorto*, *Wt1*$^{-/flox}$; *Cre-ER*^{TM}, *Wt1*$^{+/flox}$; *Cre-ER*^{TM}, *Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM} and *Wt1*$^{+/flox}$; *Immorto*; *Cre-ER*^{TM}. The *Cre-ER*^{TM} inducible model will allow the Cre recombinase to recognize the loxP sites around exons 8 and 9 of *Wt1*, which, in turn, will result in a mutant, non-functional WT1 protein (**Figure 3.1**) in mice with the genotype of *Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM}. All of the embryos from these crosses will be genotype as described below.

Dissociation of the Kidney Mesenchymal Cells

The morning of the vaginal plug was defined as E.5. All of the embryos were harvested at E13.5 and the kidney rudiments were dissected out. Both of the kidney rudiments from each embryo were temporarily stored on ice in 50 μl of 1X trypsin from MediaTech (Manassas, VA). Once all the kidneys were dissected out from each embryo, the kidney rudiments in the 1X trypsin were placed in the water bath at 37°C for 5 minutes. The cells from kidney rudiments were then dissociated into single cell suspensions by the use of the 10 μL pipette.

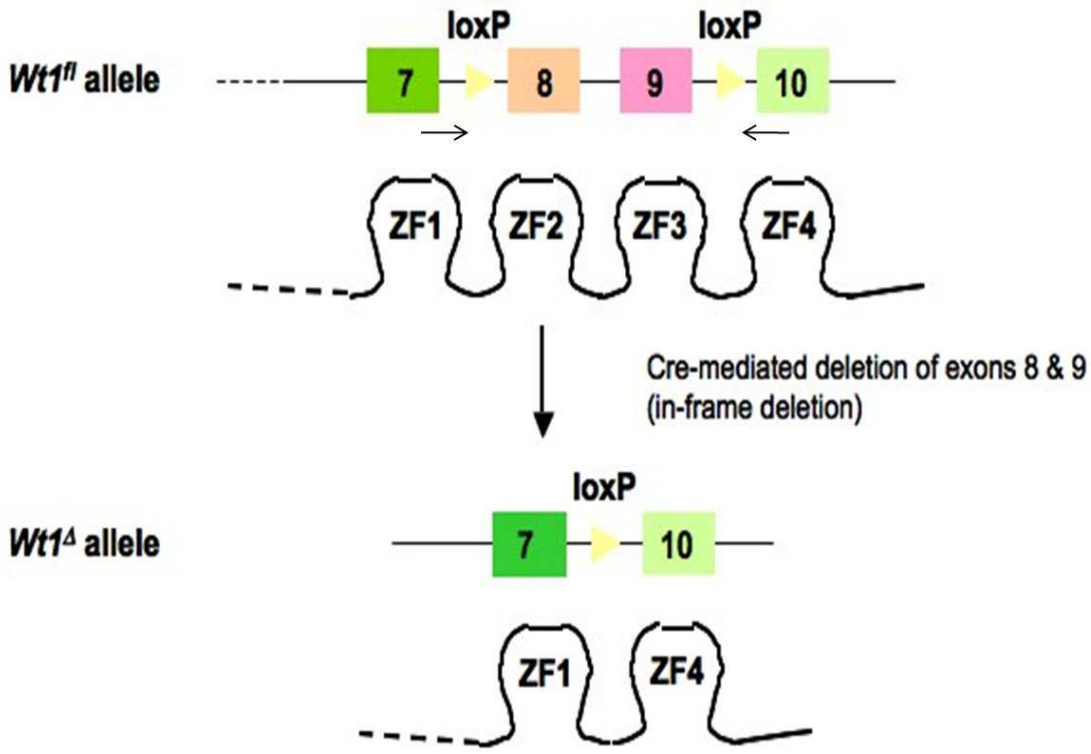


Figure 3.1. Schematic Diagram of the Cre-Lox System

The 1.55 and 1.75 primers (black arrows) are located around the loxP sites around exons 8 and 9 on the floxed allele. Once the Cre-recombinase recognizes these loxP sites, an in-frame deletion is created, resulting in the deletion of exons 8 and 9 to generate a mutant, non-functional WT1 protein.

Culturing of the Metanephric Mesenchymal Cells (MMCs)

Cells were seeded in a 24-well plate and cultured in permissive conditions: Dulbecco's modified Eagle's medium (DMEM) supplemented with 60 U/mL of interferon γ (IFN-γ) from Sigma-Aldrich (St. Louis, MO) and 100 U/mL of antibiotics (penicillin, streptomycin, and amphotericin B) (MediaTech), 10% Fetal Bovine Serum (FBS), and cultured at 33°C. At this temperature, the temperature sensitive large T-antigen (TA-g) was produced to ultimately result in the immortalization of the MMCs (136). The MMCs were subsequently passaged after enzymatic dissociation in 1X trypsin (MediaTech) in DMEM for 5 minutes at 37°C. Once the MMCs reached 40 passages, they were considered an immortalized cell line (136).

Molecular Characterization of the MMCs

A total of 20 clones (the *Wt1*$^{+/fl}$; *Immorto* MMCs) were subcloned by end point dilution. Colonies were selected based on their cell morphology that were consistence with the established mesenchymal cell morphological traits, such as being elongated, spindle-shaped, and fibroblastic looking (136). The isolated cells did not resemble epithelial cells, which appear to be cuboidal-shaped and well organized. The F11249 *Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM}, F11254 *Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM}, and F12055 *Wt1*$^{-/flox}$; *Immorto* cell lines were not yet subcloned. Immunocytochemistry analyses of all the cell lines indicated whether the cell lines were mesenchymal using two mesenchymal markers, Vimentin and Cited1, and an epithelial marker, Cytokeratin. The MMC cell lines were seeded onto round, glass cover slips (Fisher) and fixed with methanol for 10 minutes and washed off with 1X PBS three times for 10 minutes each. Cells were incubated with 10% goat serum in PBS for 1-2 hour at room temperature or

overnight at 4°C, depending on the antibody. The fixed cells were then incubated overnight at 4°C with either anti-WT1 monoclonal antibody (1:100) (DAKO), anti-MOX2 monoclonal (1:50) (Santa Cruz), anti-Vimentin monoclonal (1:100), anti-Cited1 (1:100) (Fisher) or anti-Cytokeratin monoclonal (1:100) from Sigma-Alrich. After being washed three times with PBS, the fixed cells were incubated with conjugated Alexa Fluor 594 anti-mouse secondary antibody (Invitrogen). The cells on the cover slips were mounted in VECTASHIELD mounting media (Vector Laboratories) and images were captured by a Leica Epifluorescence/Brightfield Microscope.

Adenovirus Vectors

The Cre recombinase and enhanced green fluorescent protein (eGFP) within an internal ribosome entry site (IRES) expression cassette (Ad-Cre-IRES-eGFP) packaged inside an Adenovirus vector (type 5, DE1/E3) was purchased from Vector Biolabs (Philadelphia, PA). The viral infection control was a recombinant human adenovirus type 5 expressing eGFP under the control of a cytomegalovirus (CMV) promoter (Ad-CMV-GFP) purchased from Vector Biolabs. The purpose of eGFP was to monitor the expression of the viral construct after the viral infection. The Adeno-Cre-eGFP and Adeno-eGFP stocks (titer of $1x10^{10}$ PFU/ml) were stored in DMEM with 2% Bovine Serum Albumin (BSA), and 2.5% Glycerol.

Conditional Ablation of *Wt1* in the MMCs

After 24hrs of seeding the F11249 *Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM} cells or the F11254 *Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM} cells into a 24-well plate, the cells were treated with 1µM of 4-hydroxy tamoxifen (4-OH tamoxifen) for 48hrs. The mutant form of the ligand binding domain of the estrogen receptor (ER^{TM}) prevents binding of its natural ligand, 17β-estradiol, at normal physiological concentrations, but allows the ER^{TM} domain responsive to 4-OH tamoxifen (137-139). The fusion of *Cre* with *ER*^{TM} results in the ER^{TM}-dependent cytoplasmic sequestration of Cre by Hsp90, which prevents *Cre*-mediated recombination (140, 141). However, binding of 4-OH tamoxifen disrupts the interaction Hsp90 that permits access of *Cre-ER*^{TM} to the nucleus and initiation of recombination. The Cre recombinase recognizes the loxP sites flanking exons 8 and 9 of *Wt1*. The enzymatic reaction by Cre recombinase results in a mutant, non-functional WT1 protein (**Figure 3.1**).

The ablation of *Wt1* in the F12055 *Wt1*$^{-/flox}$; *Immorto* MMC cell line was done by adenoviral infection that carried the Cre recombinase. The *Wt1*$^{-/fl}$; *Immorto* MMCs were infected with 10 multiplicity of infection (MOI) of Adeno Cre for 48 hours. The medium was aspirated and replaced with fresh DMEM supplemented with 60 U/mL of IFN-γ and 100 U/mL of antibiotics.

Genotyping

DNA isolated from the MMCs was used for genotyping by PCR amplification conditions (**Table 3.1**) by the primer sets in (**Table 3.2**). The detection of *Wt1*-null allele was accomplished as described by Kreidberg et, al (43) The presence of the *Wt1* flox allele was determined using the *1.75/1.55* primer set, in which primers are located around the loxP sites flanked around exons 8 and 9. The *Cre-ER*^{TM} transgene was detected by the *ZP3-se* and *ZP3-as* primer set (44). The *Immorto* allele was detected by the primer set *Immo I* and *Immo II*. The recombined *Wt1* flox allele (*Wt1*$^{\Delta}$) was detected by PCR amplification using the *ckodelF* and *1.55* (112).

Table 3.1. PCR Conditions for Animal Genotyping

	Initial Denature	Cycle Denature	Cycle Anneal	Cycle Extension	Cycles	Final Extension	Product Size
Primer Set							
Wt1-F/PGK	94°C - 3 min	94°C - 45 sec	58°C - 45 sec	72°C - 50 sec	35	72°C - 7 min	250 bps
1.55/1.75	94°C - 3 min	94°C - 45 sec	58°C - 45 sec	72°C - 50 sec	35	72°C - 7 min	150 and 200 bps
ckodelF /1.55	94°C - 3 min	94°C - 45 sec	58°C - 45 sec	72°C - 50 sec	35	72°C - 7 min	450 bps
ZP3-se/ZP3-as	94°C - 3 min	94°C - 45 sec	58°C - 45 sec	72°C - 50 sec	35	72°C - 7 min	600 bps
Immo I/Immo II	94°C - 4 min	94°C - 30 sec	58°C - 1 min	72°C - 1 min	35	72°C - 7 min	1 kb

Table 3.2. Primer Sequences for Animal Genotyping

Name	Sequence (5'-3')
Wt1-F	GTG ACC CCG CAG CTA GCC
PGK	CCA TTT GTC ACG TCC TGC
1.55	TGC CTA CCC AAT GCT CAT TG
1.75	GAA ACT GTT TGT AAC GAG AG
ckodelF	GCT AAC ATA TGG GAG ACA TT
ZP3-se	TCC AAT TTA CTG ACC GTA CAC CAA
ZP3-as	CCT GAT CCT GGC AAT TTC GGC TA
Immo I	AGC GCT TGT GTC GCC ATT GTA TTC
Immo II	GTC ACA CCA CAG AAG TAA GGT TCC

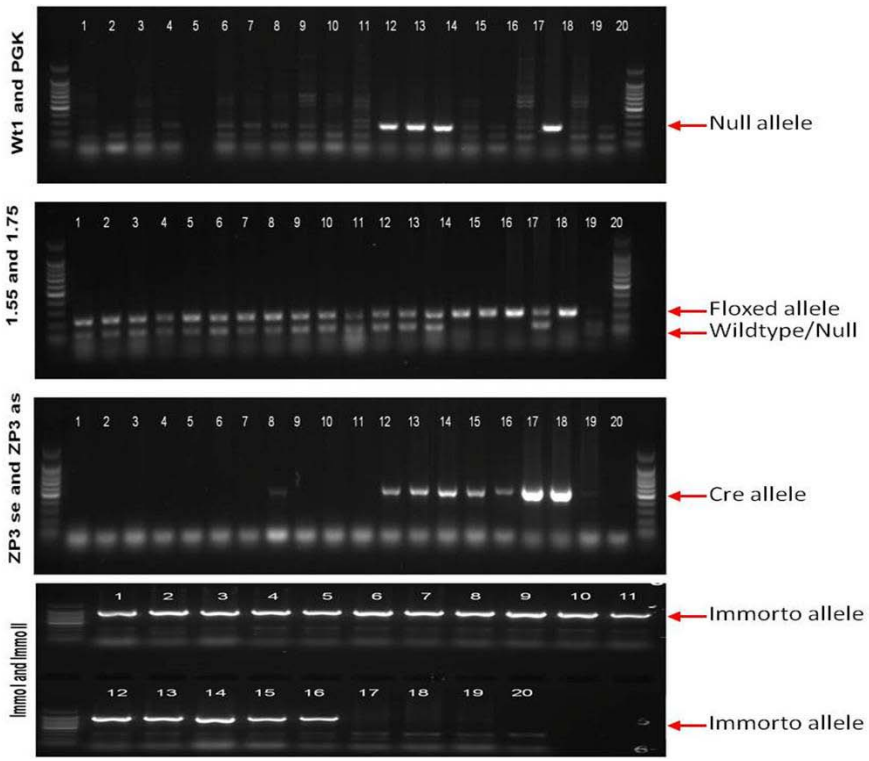
Results

Genotyping of the MMCs

Genotyping confirmed that clones # 1, 2, 3, 4, 7, 10, 11, 12, 15, 16, and 18 were *Wt1*$^{+/fl}$; *Immorto* MMCs and F11249 E3 and F11254 E2 cell lines were *Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM} MMCs (**Figure 3.2**). Cell line F11254 E3 was *Wt1*$^{fl/fl}$; *Immorto*; *Cre-ER*^{TM}, and F12055 was *Wt1*$^{-/fl}$; *Immorto* (**Figure 3.5**).

Two of the cell lines, F11249 *Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM} and F11254 *Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM} were tamoxifen-inducible cell lines, which *Wt1* can be genetically ablated by the Cre-recombinase. By PCR analysis, the ablation of *Wt1* occurred after 48 and 72 hrs as shown in **Figure 3.3**. However, the ablation of *Wt1* in these cells was incomplete, as indicated by the residual presence of the floxed allele.

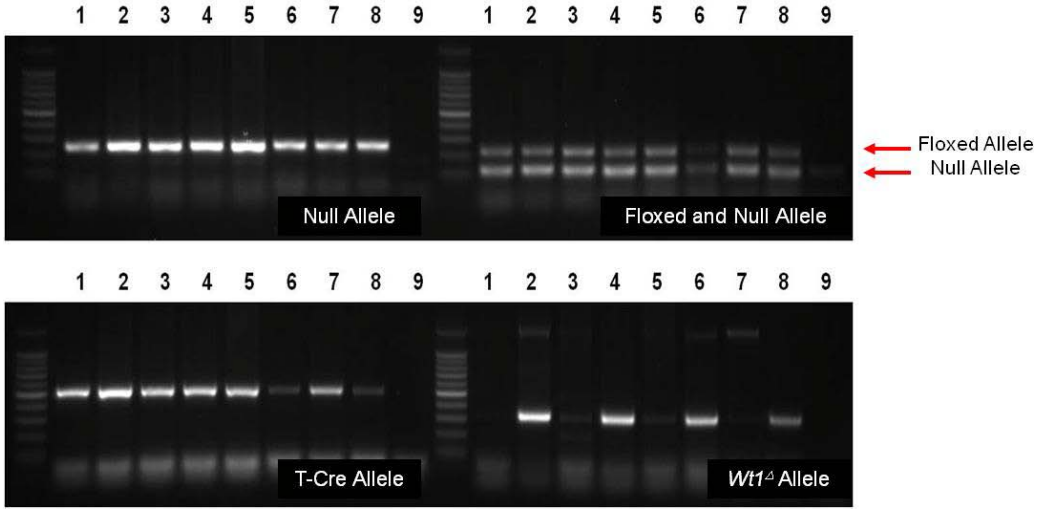
The ablation of *Wt1* in the F12055 *Wt1*$^{-/flox}$; *Immorto* MMCs cell line was achieved by adenoviral infection. As shown in **Figure 3.5**, GFP positive cells indicated that the cells were successfully infected by the adenovirus. PCR analysis confirmed the presence of the *Cre-ER*^{TM} transgene and demonstrated the recombination of the *Wt1*^{fl} allele 48 hrs after of the adenoviral infection (**Figure 3.5**). Most important, the ablation of *Wt1* after the adeno-viral infection of Cre-recombinase was complete; the floxed allele was no longer present (**Figure 3.5**).



Lane#	MMCs DNA Samples	Genotype
1	F736 E6 clone#1	*Wt1*$^{+/fl}$; *Immorto*
2	F736 E6 clone#2	*Wt1*$^{+/fl}$; *Immorto*
3	F736 E6 clone#3	*Wt1*$^{+/fl}$; *Immorto*
4	F736 E6 clone#4	*Wt1*$^{+/fl}$; *Immorto*
5	F736 E6 clone#7	*Wt1*$^{+/fl}$; *Immorto*
6	F736 E6 clone#10	*Wt1*$^{+/fl}$; *Immorto*
7	F736 E6 clone#11	*Wt1*$^{+/fl}$; *Immorto*
8	F736 E6 clone#12	*Wt1*$^{+/fl}$; *Immorto*
9	F736 E6 clone#15	*Wt1*$^{+/fl}$; *Immorto*
10	F736 E6 clone#16	*Wt1*$^{+/fl}$; *Immorto*
11	F736 E6 clone#18	*Wt1*$^{+/fl}$; *Immorto*
12	F11249 E3 #1 (15)	*Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM}
13	F11249 E3 #2 (14)	*Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM}
14	F11254 E2 #5 (15)	*Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM}
15	F11254 E3 #2 (16)	*Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM}
16	F11254 E3 #5 (17)	*Wt1*$^{-/flox}$; *Immorto*; *Cre-ER*^{TM}
17	#52 (Fl allele Control)	*Wt1* $^{fl/fl}$
18	#70 (Δ allele Control)	*Wt1* $^{-/\Delta}$
19	#20 (Cre(-) Control)	*Wt1* $^{+/+}$
20	dH_2O	-

Figure 3.2. PCR Analyses of the MMCs using *Wt1-F/PGK*, *1.55/1.75*, *ZP3 se/ZP3 as*, *and Immo I/Immo II* primer sets

Cells isolated from E13.5 embryos were genotyped using the established primer sets to detect the *Wt1* null allele, floxed allele, *CreER*^{TM} allele, and the *Immorto* allele (**Table 3.1**). Genotypes are shown to the right.



Lane #	Description
1	48 hrs Untreated Set 1
2	48 hrs 1 µM 4-OH-Tamoxifen Set 1
3	48 hrs Untreated Set 2
4	48 hrs 1 µM 4-OH-Tamoxifen Set 2
5	72hrs Untreated Set 1
6	72 hrs 1 µM 4-OH-Tamoxifen Set 1
7	72 hrs Untreated Set 2
8	72 hrs 1 µM 4-OH-Tamoxifen Set 2
9	dH_2O

Figure 3.3. Ablation of *Wt1* by Tamoxifen Treatment

As shown in duplicates, F11249 E3 *Wt1*$^{-/fl}$; *Immorto; Cre*^{TM} cell line was treated with 1 µM of Tamoxifen for 48 hrs and at 72 hrs the recombined *Wt1*$^{\Delta}$ allele is detected. However, the floxed allele was still present, which indicated only partial ablation of *Wt1*.

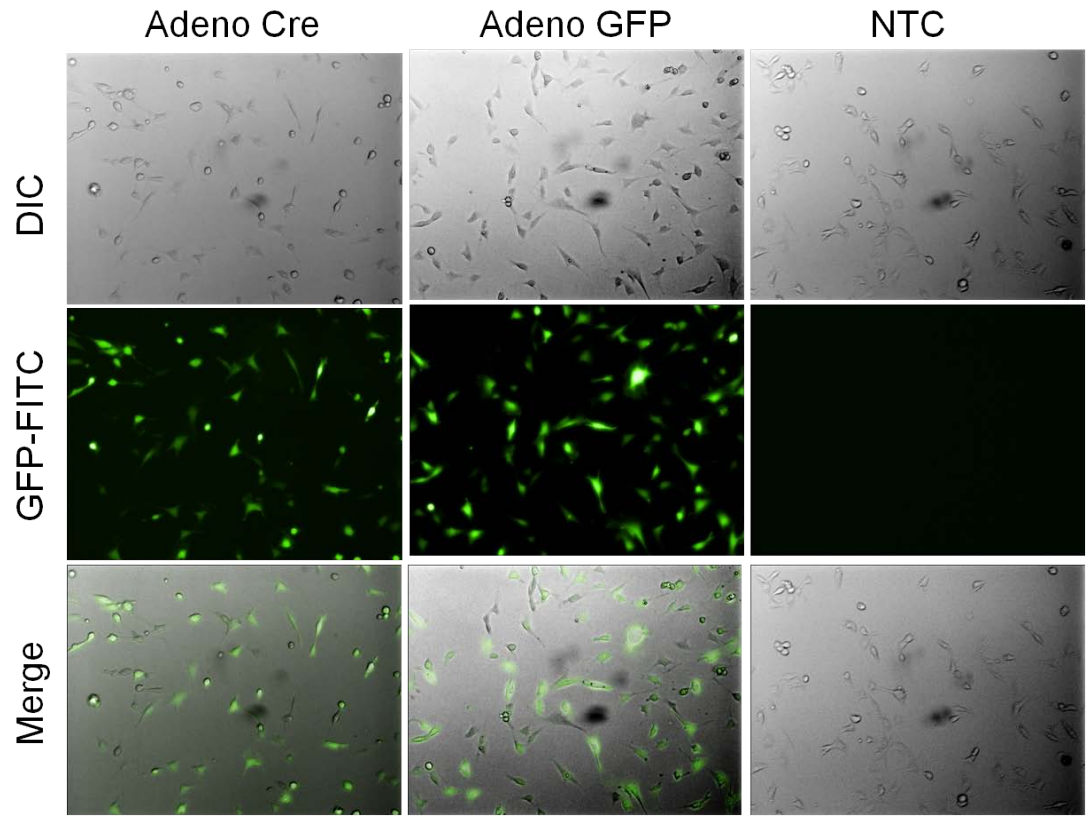


Figure 3.4. Viral Infection of Adeno-Cre-GFP in the F12055 *Wt1*$^{-/flox}$; *Immorto* MMC cell line

The top panel of images was taken by Differential Interference Contrast microscopy *(*DIC*)* and the middle panel of images was taken with a GFP-FITC (GFP-fluorescein isothiocyanate) filter to detect the excitation at 519 nanometers in the green region to indicate the cells that have been infected and expressed GFP. All of the images were taken at 20X magnification. The lower panel of images merged DIC and FITC images together to determine the number of cells that were infected by the adenovirus. NTC – Non Treated Cells

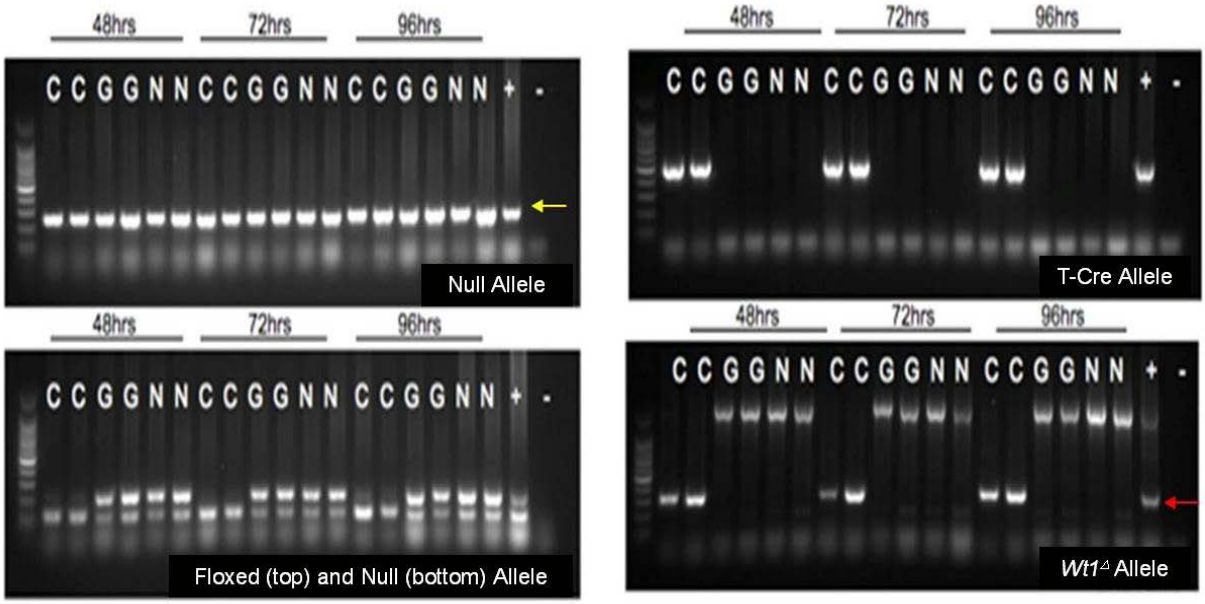


Figure 3.5. PCR Analyses of the Ablation of *Wt1* in the F12055 *Wt1*$^{-/flox}$; *Immorto* MMC cell line infected by Adeno-Cre-eGFP or Adeno-eGFP

The complete ablation of *Wt1* was detected after 48hrs. All the cells were confirmed to have the *Wt1* null allele (yellow arrow) and the recombined allele (red arrow) by using the primers in **Table 3.1**. (C=Cells infected with Cre and eGFP adenovirus, G= Cells infected with eGFP only, and N= Non-Treated cells)

Molecular Characterization of the MMCs

All of the cells displayed in **Table 3.3** reached passage 40 and were considered immortalized cell lines. The wild type *Wt1*$^{+/fl}$; *Immorto* MMCs were sub-cloned based on their cell morphology. Ten clones of *Wt1*$^{+/fl}$; *Immorto* MMCs were generated, and were vimentin positive and cytokeratin negative (**Appendix Figure 1**). Of the 10 clones, Clone# 10 expressed more of both Wt1 and Meox2 (**Figure 3.6 A**). *Wt1*$^{-/flox}$; *Immorto* cell line, F12055, expressed Wt1 and Meox2 similar to Clone #10 (**Figure 3.6 B)**. An established mesenchymal cell marker, Cbp/p300-interacting transactivator 1 (Cited1), was used to ensure that the MMCs were derived from the cap mesenchyme. *Cited1* is known to be specifically expressed in the condensed cap mensenchyme with no expression in the clefts between the ureteric bud or the surrounding stromal mesenchyme (142, 143). As shown in **Figure 3.7**, *Wt1*$^{+/flox}$; Immorto and F12055 *Wt1*$^{-/flox}$; *Immorto* MMCs were positive for Cited1 further indicating the mesenchymal status of the cell lines.

The *Wt1*$^{-/flox}$; *Immorto*; *CreER*^{TM} (F11249 E3 and F11254 E2) and the *Wt1*$^{-/flox}$; *Immorto* cell lines have not been sub-cloned based on their cell morphology. These cells are still a heterogeneous population of vimentin and cytokeratin positive cells. In addition, *Wt1*$^{-/flox}$; *Immorto*; *CreER*^{TM} (F11249 E3 and F11254 E2) did not express Wt1 (**Appendix Figure 2**).

Table 3.3 MMCs Cell Lines Derived from E13.5 Kidney Rudiments

Wt1, Meox2, Vimentin, Cited1, and Cytokeratin were the markers used to determine whether the cells were mesenchymal.

Cell Line	Genotype	Wt1	Meox2	Vimentin	Cited1	Cytokeratin
Clone # 1	*Wt1* $^{+/fl}$; *Immorto*	+	NT	+	NT	-
Clone # 2	*Wt1* $^{+/fl}$; *Immorto*	+ weak	NT	+	NT	-
Clone # 3	*Wt1* $^{+/fl}$; *Immorto*	+ weak	NT	+	NT	-
Clone # 4	*Wt1* $^{+/fl}$; *Immorto*	+	NT	+	NT	-
Clone # 5	*Wt1* $^{+/fl}$; *Immorto*	-	NT	+	NT	-
Clone #7	*Wt1* $^{+/fl}$; *Immorto*	+	NT	+	NT	-
Clone # 8	*Wt1* $^{+/fl}$; *Immorto*	+ weak	NT	+	NT	-
Clone # 10	*Wt1* $^{+/fl}$; *Immorto*	+	+	+	+	-
Clone # 11	*Wt1* $^{+/fl}$; *Immorto*	+ weak	NT	+	NT	-
Clone # 15	*Wt1* $^{+/fl}$; *Immorto*	+ weak	NT	+	NT	-
F11249 E3	*Wt1* $^{-/fl}$; *Immorto*; *CreER*^{TM}	-	NT	+	NT	+*
F11254 E2	*Wt1* $^{-/fl}$; *Immorto*; *CreER*^{TM}	-	NT	+	NT	+*
F12055	*Wt1* $^{-/fl}$; *Immorto*	+	+	+	+	+*

(+) - Expressed the marker

(-) - Did not express the marker

Weak – The intensity of the IF staining was weak compared to the control cell line (MCF-7 cells).

NT- Not Tested

***** - The cell line was not subcloned.

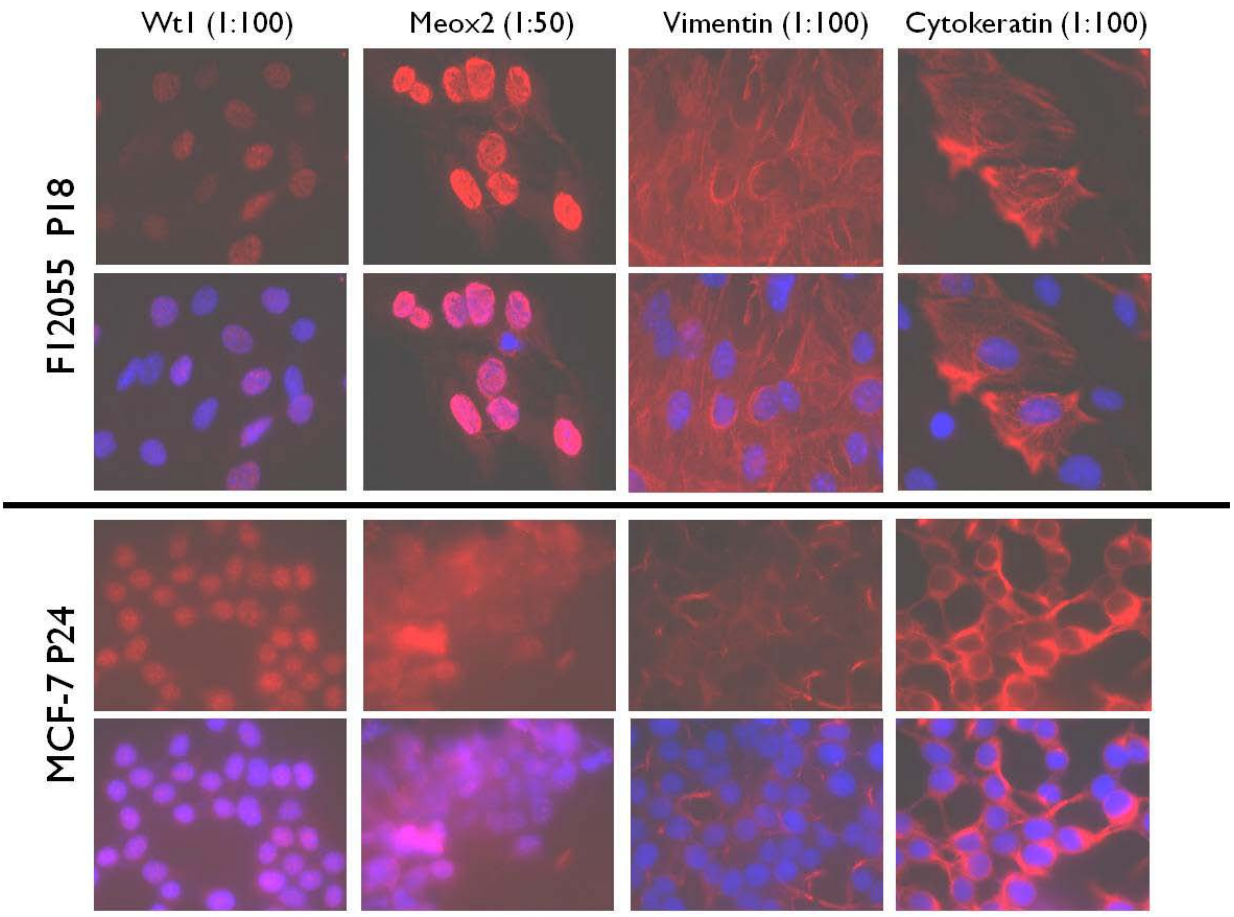


Figure 3.6. IF Images of the Generated MMCs from E13.5 Kidney Rudiments

A. The top panel of images is Clone #10 *Wt1*$^{+/flox}$; *Immorto* MMC cell line and the bottom panel of images is the control, MCF-7 cell line. As shown, Clone #10 *Wt1*$^{+/flox}$; *Immorto* MMCs were WT1, MEOX2, vimentin positive and cytokeratin negative. DAPI (blue) stains the nucleus.

B. The top panel of images is F12055 *Wt1*$^{-/flox}$; *Immorto* MMC cell line and the bottom panel of images is the control, MCF-7 cell line. F12055 *Wt1*$^{-/flox}$; *Immorto* MMC cell line expressed both WT1 and MEOX2. Because the F12055 *Wt1*$^{-/flox}$; *Immorto* MMC cell line were not sub-cloned, it is presently a heterogeneous population of vimentin and cytokeratin positive cells.

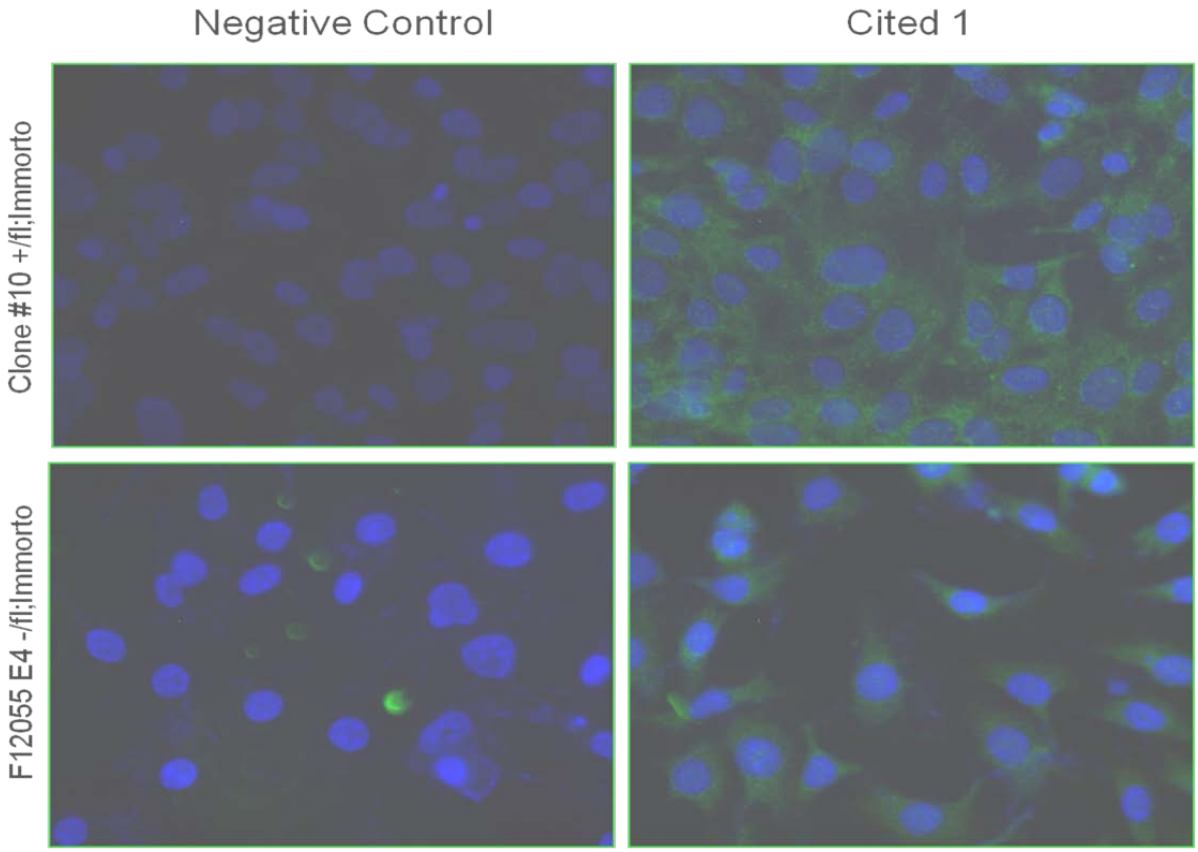


Figure 3.7. MMCs are Positive for Cited1: Biological Marker for Condensed Cap Mesenchyme

Clone #10 *Wt1*$^{+/fl}$; *Immorto* MMCs (top panel) and the F12055 E4 *Wt1*$^{-/fl}$; *Immorto* (bottom panel) were positive for CITED1, which confirmed that these cells were isolated from the condensed cap mesenchyme. DAPI (blue) stains the nucleus.

Discussion

The HEK 293 cell line was the first transformed human cell line. Since these cells were isolated from the kidney, I thought it would serve a good cellular model to study the effect of the loss of *Wt1* on the gene expression of *Meox2*. Hence, I proposed to manipulate the gene expression of Wt1 either by siRNA and/or shRNA to assess the effect on *Meox2* gene expression. However, these cells were more epithelialized cells and they did not express a robust amount of Wt1. Therefore, I generated several *Wt1* kidney mesenchymal cell lines: wildtype *Wt1* MMCs (*Wt1* $^{+/fl}$; *Immorto*) and the mutant *Wt1* MMCs F11249 E3 and F11254 E2 (*Wtl*$^{-/fl}$; *Immorto*; *Cre*^{TM}) and F12055 (*Wt1*$^{-/fl}$; *Immorto*).

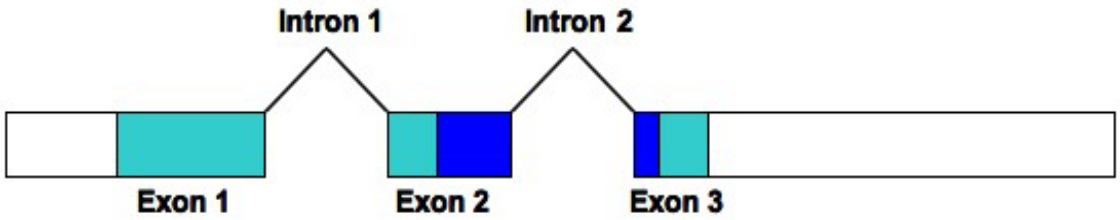
After the molecular characterization of all the cell lines (**Table 3.2**), I chose two cell lines, Clone #10 *Wt1*$^{+/flox}$; Immorto cell line and F12055 *Wt1*$^{-/flox}$; Immorto cell line for my experiments to study the effect of the inactivation of *Wt1* on the gene expression of *Meox2 in vitro*. Both of these cell lines displayed mesenchymal morphological traits of being elongated, spindle-shaped, and fibroblastic looking. Additionally, the total ablation of *Wt1* was achieved in the F12055 *Wt1*$^{-/fl}$; *Immorto* cell line after infection with Adeno-Cre-GFP. By using these molecular tools, we can understand how a *Wt1* mutation effects *Meox2* and other potential downstream genes in kidney mesenchymal cells that may contribute to Wilms tumorigenesis. We can assess *WT1*-ablated cells for tumorigenic phenotypes, such as increased cellular proliferation, inhibition of apoptosis, disruptions in cellular adhesion and migration. Furthermore, the cell lines generated were not just limited for the purpose of Wilms tumorigenesis, but can be used to experimentally study the loss of *Wt1* in other kidney cancers and during kidney development.

Chapter 4: Inactivation of *Wt1* effects the Gene Expression of *Meox2* during Kidney Development

Mesenchyme homeobox 2 (*MEOX2*), formally known as *GAX* and *MOX2,* was one of the seven genes from the prioritized microarray data and was significantly up regulated by 19-fold in the *WT1* mutant Wilms tumors. *MEOX2* is a member of a novel subclass of the homeobox gene super family which is located at chromosomal region 7p21 in humans and on chromosome 12 in mice (131, 132). *MEOX2* consists of three exons and two introns. It also contains a nuclear localization signaling (NLS) as shown in **Figure 4.1 A** (131). Homeobox genes like *MEOX2* encode homeodomains that correspond to a 60 amino acid helix-turn-helix DNA binding region, located near the C-terminus of the protein (**Figure 4.1 B**) (131, 133, 144). Homeobox proteins localize to the nucleus and are transcription factors which regulate the expression of lineage specific genes which, in turn, control cellular and organ differentiation (145).

MEOX2 is expressed during early embryonic development. Between E9-E9.5, *Meox2* is expressed in all of the formed somites and in the sclerotome, a population of cells lying between the paraxial mesoderm and the limb buds (131, 132). At E14.5, *Meox2* is expressed in the condensed mesenchyme of the kidney (**Figure 4.2**). *Meox2* gene expression in the developing kidney decreases as the metanephric mesenchymal cells differentiated into epithelial structures of the developing kidney (42). The embryonic gene expression of *Meox2* suggests that it has a specific role during development.

A.



B.

MEHPLFGCLRSPHATAQGLHPFSQSSLALHGRSDHMSYPELSTSSSSCIIAGYPNEEGMF
ASQHHRGHHHHHHHHHHHHQQQQHQALQSNWHLPQMSSPPSAARHSLCLQPDSGGPPELG
SSPPVLCSNSSSLGSSTPTGAACAPGDYGRQALSPADVEKRSGSKRKSDSSDSQEGNYKS
EVNSKPRKERTAFTKEQIRELEAEFAHHNYLTRLRRYEIAVNLDLTERQVKVWFQNRRMK
WKRVKGGQQGAAAREKELVNVKKGTLLPSELSGIGAATLQQTGDSLANEDSRDSDHSSEH
AHL

Figure 4.1. Representation of Meox2 Gene and Protein Sequence

A. *Meox2* is a homeobox gene that contains 3 exons (light blue) and 2 large introns. The homeobox domain (dark blue) is encoded by exons 2 and 3.

B. Amino acid sequence of Meox2 gene product. The homeodomain (highlighted in yellow) starts in exon2 at position 187 and ends in exon 3 at position 245. The NLS sequence (underlined in red) is located within exon 3.

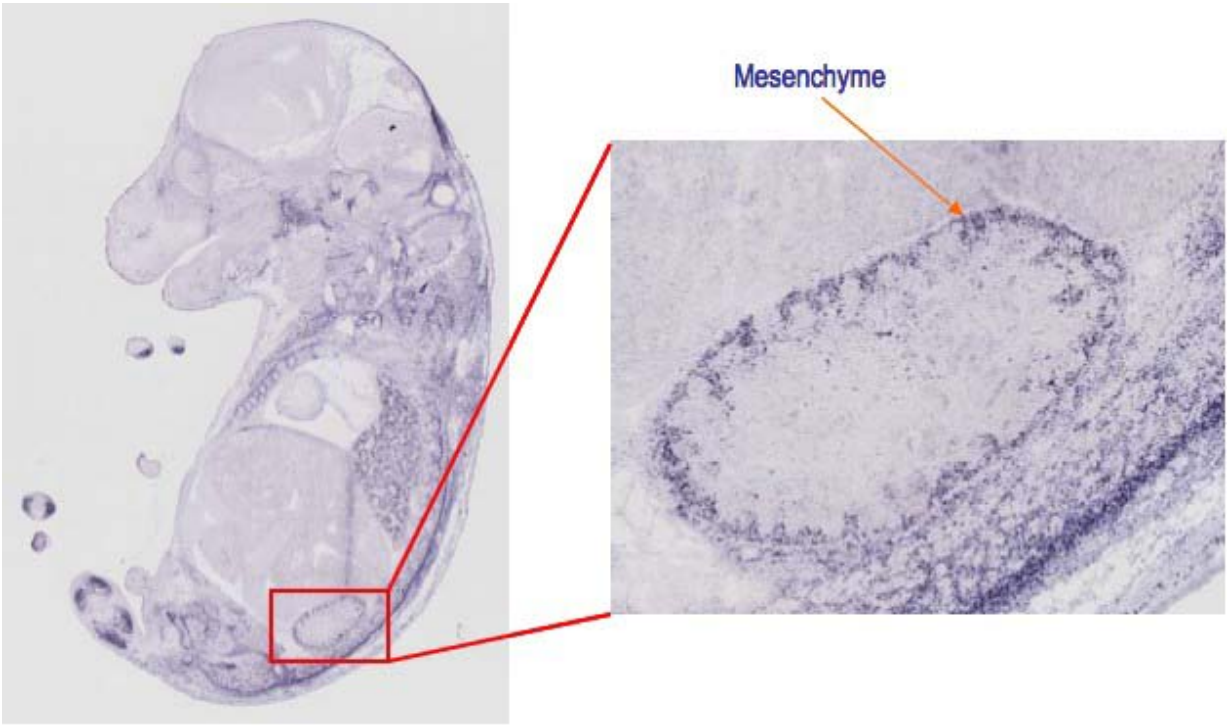


Figure 4.2. *Meox2* Gene Expression in E14.5 Mouse

At E14.5, *Meox2* is expressed in the condensed metanephric mesenchyme (Adapted from (146)).

In normal mouse mammary epithelial NMuMG cells, *Meox2* expression has been shown to be regulated by Tgf-β1 (147). After 16-24 hrs of Tgf-β1 stimulation, the gene expression of *Meox2* increased 2-8X in NMuMG cells. Dominant-negative mutants of *Smad3* and *Smad4* blocked the induction of *Meox2*, further indicating that the regulation of *Meox2* was dependent on the stimulation of Tgf-β1(147). Furthermore, the endogenous expression of *Meox2* by Tgf-β1 inhibits proliferation by inducing *p21* in the NMuMG cells (147). However, these data do not demonstrate that *Meox2* expression is dependent only by Tgf-β1 stimulation and whether the regulation of *Meox2* is cell specific. Other genes throughout development could regulate Meox2. *MEOX2* is upregulated in the *WT1* mutant WTs, and Meox2 and Wt1 are both expressed during kidney development in the condensed mesenchyme. These data suggest that Wt1 may regulate the gene expression of *Meox2* during developing kidney.

In order to assess whether there are any predicted WT1 binding sites in *MEOX2,* the Match™ web-based tool from the BIOBASE Biological Database was used to identify transcription factor binding sites in the *MEOX2* gene by a weight matrix search. The Match™ web-based tool was able to identify one predicted Wt1 binding site (cCCTCCccc) within the first intron of *MEOX2* suggesting that WT1 may regulate *MEOX2*. Having identified a putative WT1 binding site within the *MEOX2* gene, I sought to determine whether modulation of *Wt1* expression resulted in a corresponding change in *Meox2* expression *in vivo* in developing mouse kidney and also in the kidney mesenchymal cell lines. I further sought to determine the role, if any, of Tgf-β1 in regulating *Meox2* expression in these cells. Moreover, I investigated the phenotypic effect of *Wt1* ablation in this experimental system. Additionally, I

investigated whether the inactivation of *Wt1* affects the gene expression of the established regulator, Tgf-β1, of *Meox2.* The dysregulation of *p21* and *Itgb5* resulting from the upregulation of Meox2 was also assessed within this study.

Within this chapter, I have demonstrated that following the ablation of *Wt1* in E13.5 kidney rudiments and in the kidney mesenchymal cell lines, *Meox2* expression was upregulated. Data revealed that Tgf-β1 did not contribute to the dysregulation of *Meox2*. Moreover, the established downstream genes of Meox2 (*p21* and *Itgb5*) were dysregulated after the upregulated of *Meox2* in the kidney mesenchymal cells. A novel phenotype was observed in the presence of a *Wt1* mutation and the upregulation of *Meox2;* the cells decreased in cellular growth and loss the ability to adhere to the cell culture plates. Overall, these significant data further supported my hypothesis that the gene expression of *Meox2* is upregulated both *in vivo* and *in vitro,* following a *Wt1* mutation during early kidney development.

Materials and Methods

Mouse Strains

Refer to **Chapter 3** – Materials and Methods – Mouse Strains.

In Vivo Ablation of *Wt1*

The *Wt1*$^{-/flox}$ mouse model generated and described by Gao et, al, was used to genetically ablate *Wt1* exons eight and nine *in vivo* (112). Crosses were set up with the females' genotype *Wt1*$^{flox/flox}$ and the males' genotype *Wt1*$^{+/-}$; *Cre-ER*^{TM}. At E11.5, the females were injected with 3mg of Tamoxifen to ablate *Wt1*.

RNA Isolation from Embryonic Kidney Tissue

Kidneys were dissected from embryos at E13.5 and were placed into 1mL TRIzol purchased from Invitrogen (Carlsbad, CA). The tissues were completely homogenized in the TRIzol and precipitated by isopropanol. The RNA was rinsed off with 70% ethanol. The RNA pellet was resuspended in RNase-free distilled water. The RNA was treated with 1 μl of DNase 1 (Applied Biosystems) for 30 minutes to remove any traces of DNA contamination.

Genotyping of the Kidney Rudiments

Limbs of the dissected E13.5 embryos were used to extract DNA. PCR genotyping as previously described in Chapter 3. The PCR conditions are displayed in **Table 3.1**. The primer sets used for the genotyping are displayed in **Table 3.2**.

In Vitro Ablation of *Wt1* in the MMCs

Immediately after seeding into 60mm plates, F12055 MMCs were infected with 10 multiplicity of infection (MOI) of Adeno Cre for 48 hours. The medium was aspirated and replaced with fresh DMEM supplemented with 60 U/mL of IFN-γ and 100 U/mL of antibiotics. As a control, a second plate of cells was infected with Adeno GFP of 10 MOI. The recombined allele was detected by PCR as described in Chapter 3 in Table 3.1.

RNA Isolation from the Infected MMCs

After each time point (48, 72, and 96 hours), cells were harvested, centrifuged at 7500 revolutions per minute (rpm) for 5 minutes, and resuspended in TRIzol to isolate RNA.. A phenol-chloroform extraction was done to remove the residual salt in the RNA sample. The RNA was re-precipitated by 5M Ammonium Acetate, Linear Acrylamide, and 100% ethanol at -20C. The RNA pellet was resuspend in RNase-free distilled water. The NanoDrop spectrophotometer from Thermo Scientific (Wilmington, DE) was used to quantify the RNA. RNA samples were stored at -80°C.

qPCR

cDNAs were prepared from E13.5 kidney and MMCs RNA by reverse transcribing 1 μg of total RNA as described in **Chapter 2** Materials and Methods. The primer sets for *Wt1, Meox2, Tgf-β1, Smad3, p21,* and *intergrin β5* are displayed in **Table 4.1.**

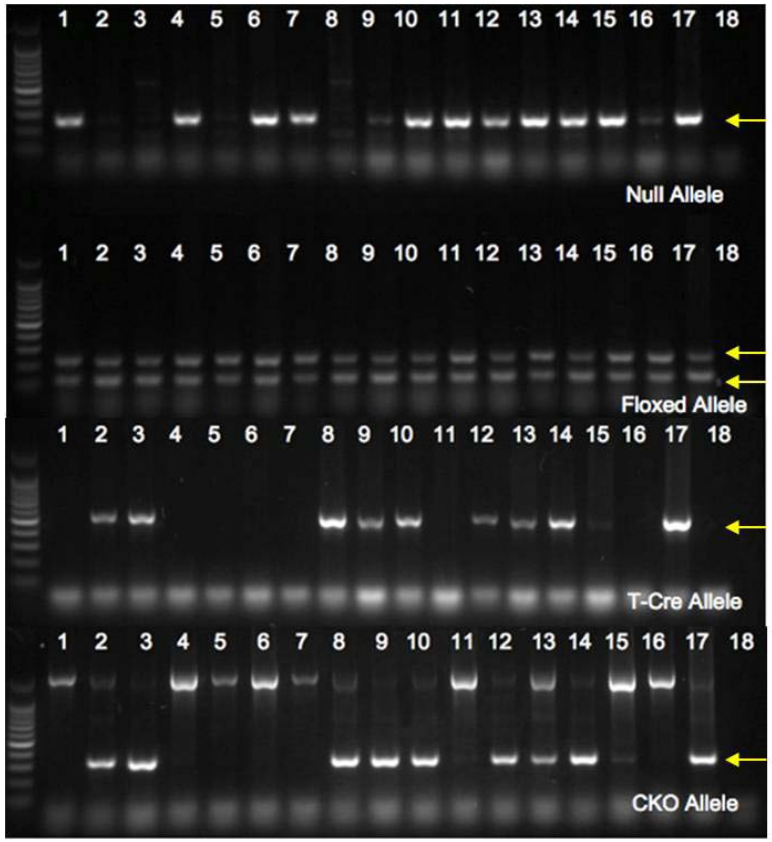
Table 4.1. Primer Sets designed by Primer Express v.3 Software for the qPCR analysis using cDNA from E13.5 kidney and MMCs

Name	Sequence (5'-3')
Wt1 F	CAAGGACTGCGAGAGAAGGTTT
Wt1 R	TGGTGTGGGTCTTCAGATGGT
Meox2 F	GCTGTCACCCGCAGACGTA
Meox2 R	AATCTGAGCTGTCGCTTTTCCT
Tgf-β1 F	AAACGGAAGCGCATCGAA
Tgf-β1 R	TGGCGAGCCTTAGTTTGG
Smad3 F	GGAATGCAGCCGTGGAAC
Smad 3 R	GACCTCCCCTCCGATGTAGTAGA
p21 F	ACGTGGCCTTGTCGCTGTC
p21 R	AATCTGCGCTTGGAGTGATAG
Itgb5 F	AGGCTGGGACGTCATTCAGA
Itgb5 R	GCGAACCTGTAGCTGAAGGT

Results

In Vivo Ablation of *Wt1* during Early Kidney Development

The Cre-mediated ablation of *Wt1* did occur, which resulted in the recombined allele produced by the CKO primer set as shown in **Figure 4.3**. However, the ablation of *Wt1* did not occur in all the cells, because the floxed allele was still present after the kidney rudiments were genotyped by PCR as described in Chapter 3 (**Figure 4.3**). However, *Wt1* was genetically knocked out in ~70% (.0004) of cells at E13.5 and resulted in the increase of Meox2 relative gene expression by 2X (.018) as shown in **Figure 4.4**.



Lane #	Genotype	Lane #	Genotype
1	*Wt1*$^{-/fl}$	10	*Wt1*$^{-/fl}$;*Cre*^{TM}
2	*Wt1*$^{+/fl}$;*Cre*^{TM}	11	*Wt1*$^{-/fl}$
3	*Wt1*$^{+/fl}$;*Cre*^{TM}	12	*Wt1*$^{-/fl}$;*Cre*^{TM}
4	*Wt1*$^{-/fl}$	13	*Wt1*$^{-/fl}$;*Cre*^{TM}
5	*Wt1*$^{+/fl}$	14	*Wt1*$^{-/fl}$;*Cre*^{TM}
6	*Wt1*$^{-/fl}$	15	*Wt1*$^{-/fl}$
7	*Wt1*$^{-/fl}$	16	*Wt1*$^{+/fl}$
8	*Wt1*$^{+/fl}$;*Cre*^{TM}	17	(+) *Wt1*$^{-/fl}$;*Cre*^{TM}
9	*Wt1*$^{+/fl}$;*Cre*^{TM}	18	(-) ddH_2O

Figure 4.3. PCR Analyses of the E13.5 Kidney Rudiments after the *in vivo* Ablation of *Wt1*

The E13.5 embryos were genotyped after 48 hrs of tamoxifen treatment to ablate *Wt1*. Genotypes of these embryos are to the right.

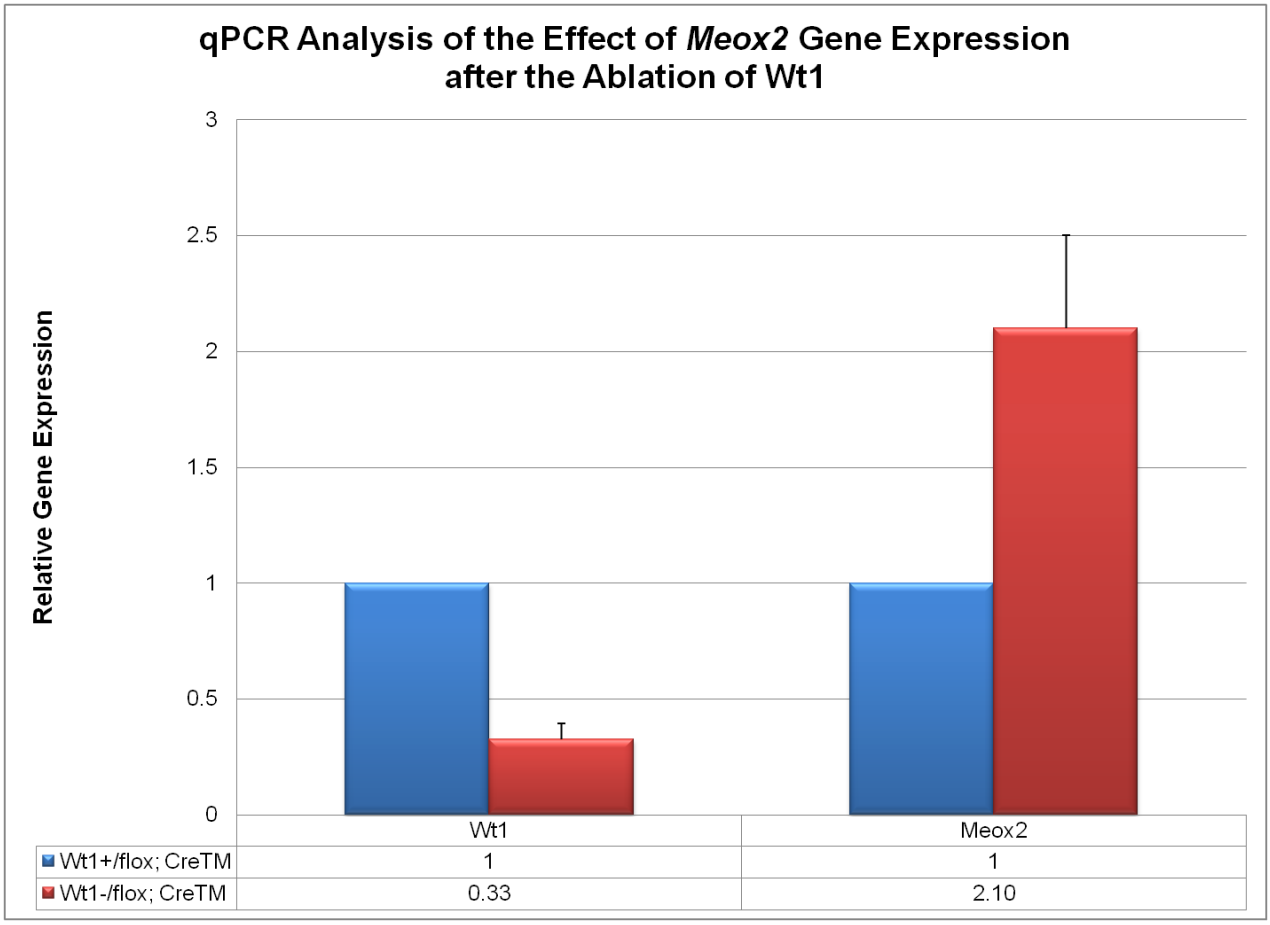


Figure 4.4. *Meox2* Gene Expression after the *in vivo* Ablation of *Wt1*

In this graph, the y-axis indicates the gene expression *Wt1* and *Meox2* in the $Wt1^{-/\Delta}$ kidney rudiments relative to the $Wt1^{+/\Delta}$ kidney rudiments. The blue bars represent the control samples $Wt1^{+/fl}$; Cre^{TM} (n=4) and the red bars represents the mutant samples $Wt1^{-/fl}$; Cre^{TM} (n=4). After the ablation, *Wt1* is knocked down by 70% (.0004) and Meox2 gene expression increased by 2X (.018).

Downstream Effects Due to the *In Vitro* Ablation of *Wt1*

In order to assess the loss of *Wt1* on the gene expression of *Meox2,* other potential downstream genes, and tumorigenic phenotypes, such as over cellular proliferation, inhibition of apoptosis, disruptions in cellular adhesion and migration, Wt1 was genetically knocked down in the generated F12055 $Wt1^{-/fl}$; *Immorto* kidney mesenchymal cells by adenoviral infection as described in the **Materials and Methods**. As shown in **Figure 4.6**, *Wt1* was knocked down by ~90% after 48 hrs of infection by qPCR analysis. After 48hrs and 72hrs of infection, there was no significant dysregulation of *Meox2*. After 96 hrs of infection, the gene expression of *Meox2* significantly increased by 61X (0.001) as shown in **Figure 4.5**.

It has been reported that the Tgf-β/Smad pathway mediates the activation of *Meox2* expression by Tgf-β1 (147). Additionally, a dominant negative mutant *Smad3* had an effect on the on blocking the endogenous Tgf-β/Smad signaling, which resulted in the late induction of *Meox2* mRNA levels (147). Thus, *Tgf-β1* and *Smad3* are known to be involved induction of *Meox2* expression. After 48 hrs of the knockdown of *Wt1*, *Tgf-β1* had a relative gene expression of ~2X (0.039) (**Figure 4.6**). However after 72 hrs and 96 hrs of the knockdown of *Wt1*, *Tgf-β1* expression was knocked down by approximately 40% as shown in **Figure 4.6**. For *Smad3*, its expression was at 1.58X (0.243) after 48 hrs of the knockdown of *Wt1. Smad3* expression fluctuated between 72 hrs and 96 hrs. After 72 hrs, *Smad3* expression was knocked down approximately 50%. After 96 hrs after the knockdown of *Wt1, Smad3* expression increased to 1.23 (0.799). Overall, this data indicated that the loss of *Wt1* has an effect on *Tgf-β1* expression, but not on *Smad3* expression. Moreover, it also suggest that the upregulation of *Meox2* expression was due to the loss of *Wt1.*

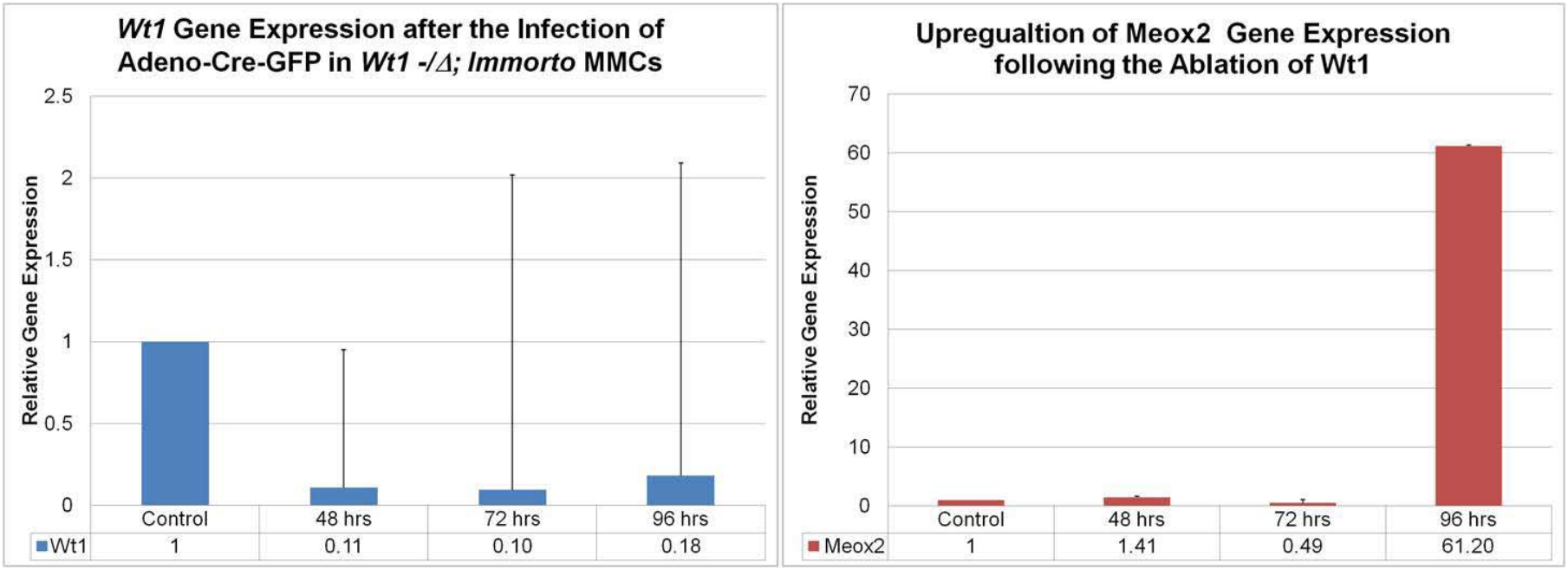
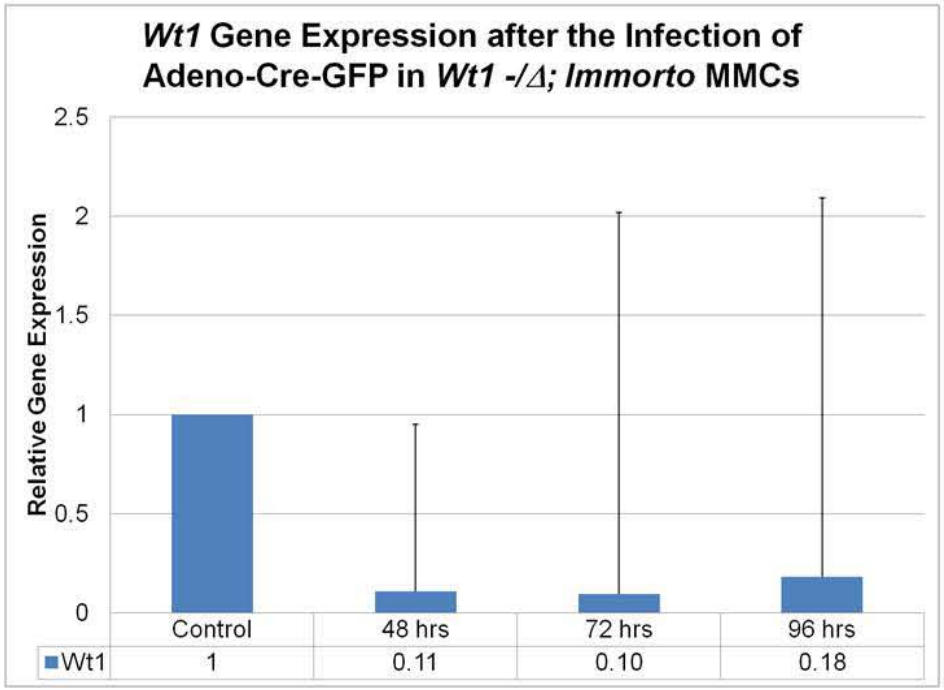


Figure 4.5. *Meox2* is Upregulated following the *in vitro* Ablation of *Wt1*

In the figure above, the y-axis indicates the gene expression *Wt1* (blue) and *Meox2* (red) in the $Wt1^{-/\Delta}$; *Immorto* MMCs relative to the $Wt1^{-/fl}$; *Immorto* MMCs. The x-axis is a table that provides the values of how much *Wt1* and *Meox2* were relatively expressed. The ablation of *Wt1* in MMCs resulted in the upregulation of *Meox2* expression after 96 hrs.



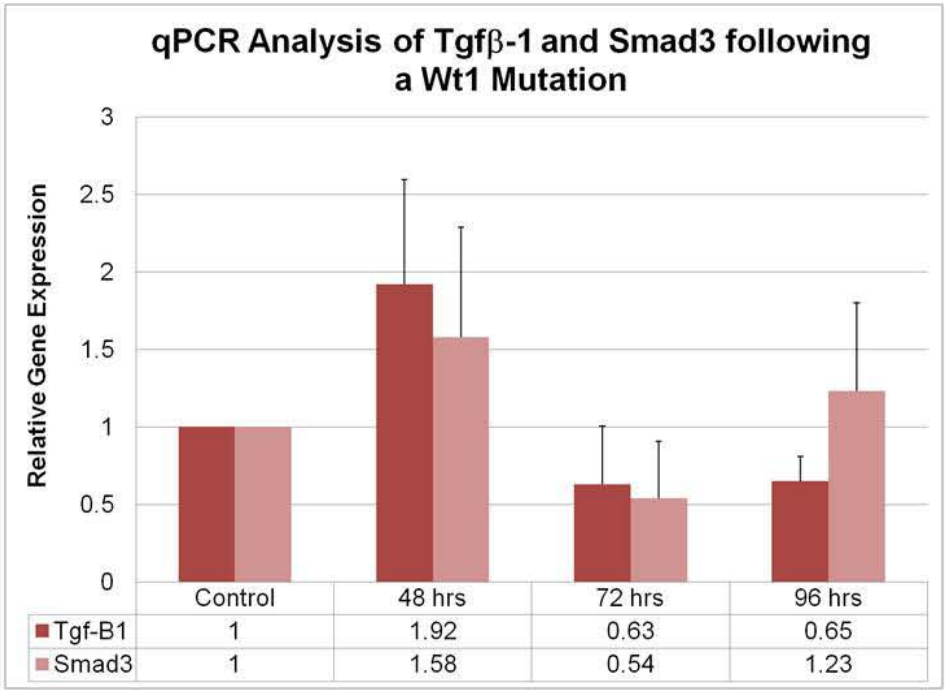


Figure 4.6. The Effect of Loss of *Wt1* on T*gf-β1* and *Smad3* Gene Expression

In the figure above, the y-axis indicates the gene expression *Wt1* (blue), *Tgfβ-1* (red), and *Smad3* (pink) in the $Wt1^{-/\Delta}$; *Immorto* MMCs relative to the $Wt1^{-/fl}$; *Immorto* MMCs. The x-axis is a table that provides the values of how much *Wt1, Tgf-β1,* and *Smad3 were* relatively expressed after the ablation of *Wt1* in the MMCs.

Dysregulation of Meox2 Targeted Genes and Cellular Phenotype

I have demonstrated that *MEOX2* is upregulated following the ablation of *WT1* in WTs, during early kidney development, and in kidney mesenchymal cells. It is known that the upregulation of *Meox2* results in the induction of *p21* and the repression of *Itgb5* (145, 148-150) (151). By qPCR analysis, after 96 hrs of infection, the upregulation of *Meox2* in MMC cells resulted in the significant induction of *p21* (0.0002) and decreased the expression of *Itgb5* (0.01) (**Figure 4.7**). In addition to this, an abnormal cellular phenotype was observed. As shown in **Figure 4.8**, the number of the *Wt1*$^{-/\Delta}$; *Immorto* MMCs cells on the plate appeared to be less after 72 hrs of infection compared to the control cells. After 96 hrs of infection, there was a significant decrease in the number of adherent cells and an increase of floating cells.

To determine whether these cells were apoptotic, the floating cells were stained with Trypan blue reagent which stains dead cells. Surprisingly, the majority of the floating cells were viable. In **Figure 4.9**, the number of *Wt1*$^{-/\Delta}$; *Immorto* MMCs was compared to the number of the *Wt1*$^{-/fl}$; *Immorto* MMCs by counting the number of cells after they were stained with the Trypan blue reagent. The total number of *Wt1*$^{-/\Delta}$; *Immorto* MMCs started to decrease after 72hrs (0.080) (**Figure 4.9**). After 96 hrs of infection, there was a significant decrease in the total number of *Wt1*$^{-/\Delta}$; *Immorto* MMCs compared to the control (0.044), which ~40% of the *Wt1*$^{-/\Delta}$; *Immorto* MMCs total number of cells were viable and in suspension (**Figure 4.9**).

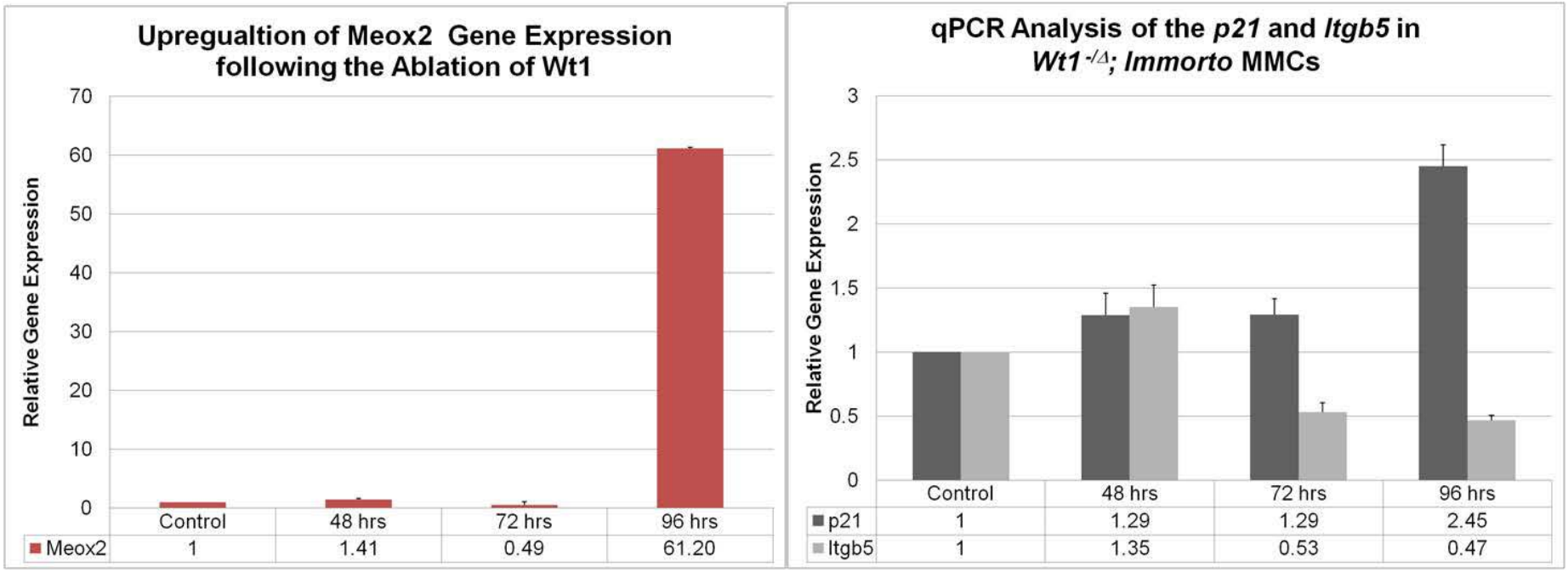


Figure 4.7. Induction of *p21* and the Repressiom of *Itgb5*

In the figure above, the y-axis indicates the gene expression *Meox2* (red), *p21* (black), and *Itgb5* (gray) in the *Wt1*$^{-/\Delta}$; *Immorto* MMCs relative to the *Wt1*$^{-/fl}$; *Immorto* MMCs. The x-axis is a table that provides the values of how much *Meox2, p21,* and *Itgb5 were* relatively expressed. After 96hrs of *Wt1* ablation, the overexpression of *Meox2* resulted in the significant induction of *p21* (0.0002) and the knockdown of *Itgb5* (0.01).

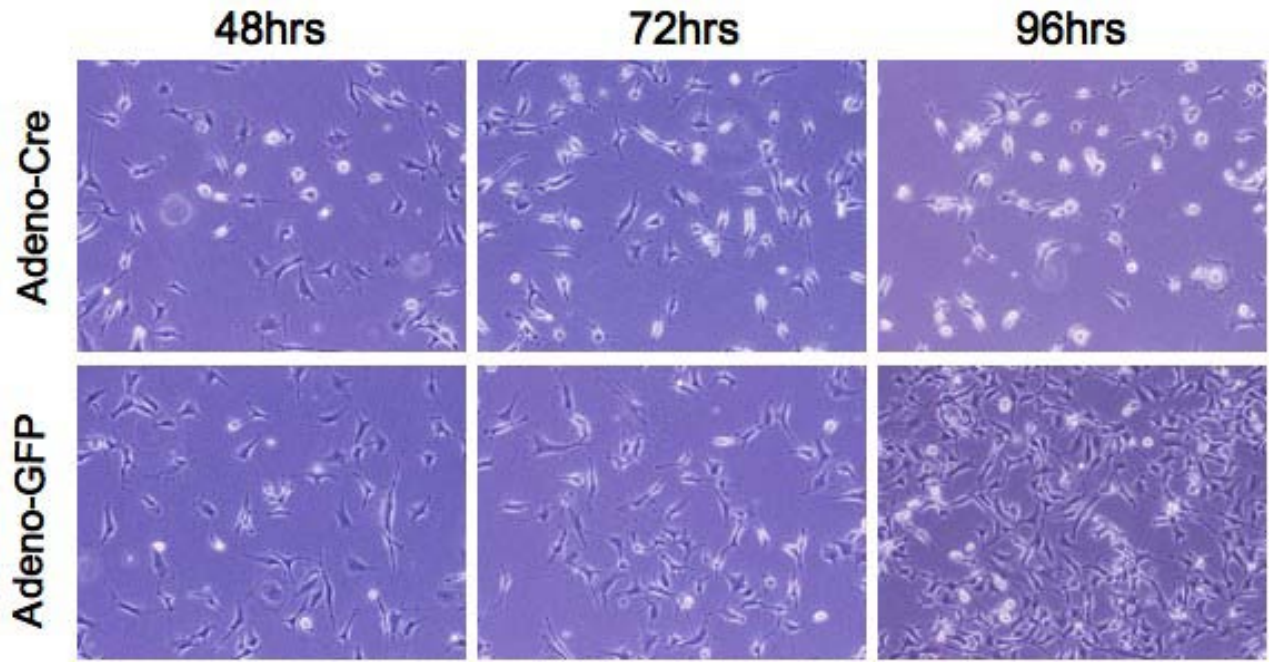
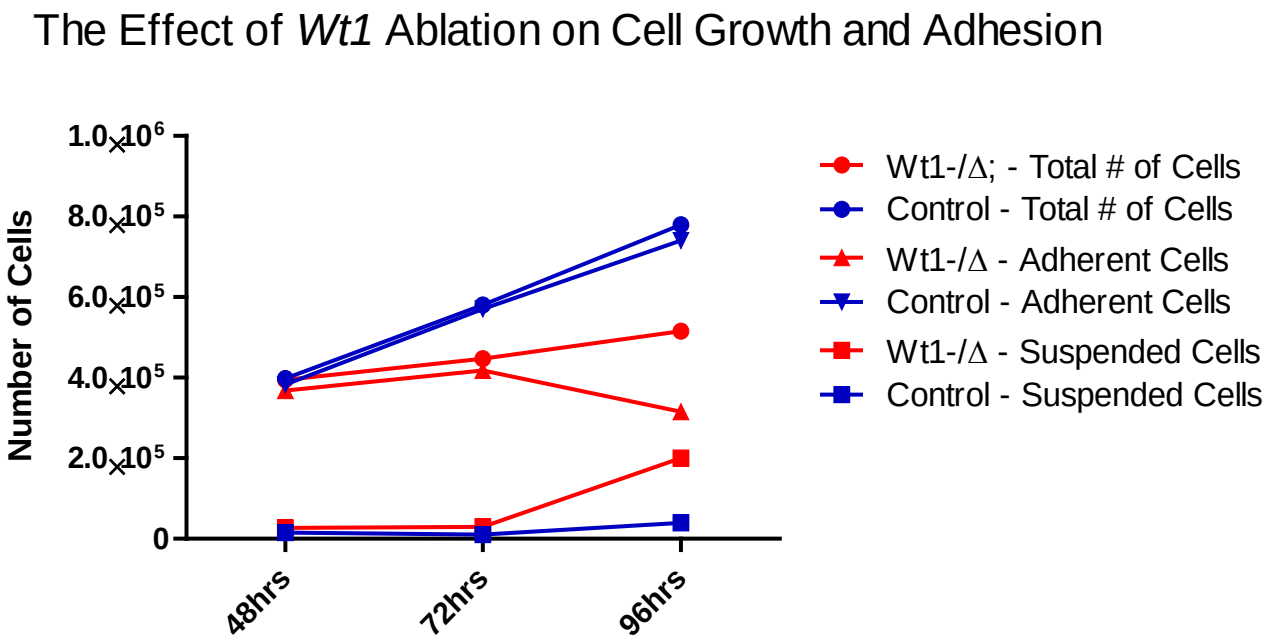


Figure 4.8. The Effect of *Wt1* Ablation in *Wt1*$^{-/flox}$; *Immorto* MMCs: Visual Observation in Decrease Cell Number and Loss of Cell Adhesion

As shown above, the top panel of images is infected cells with Adeno Cre (*Wt1*$^{-/\Delta}$; *Immorto* MMCs) and the bottom panel of images is infected cells with Adeno GFP (*Wt1*$^{-/fl}$; *Immorto* MMCs) at different time points.



p-Values

	Total Number of Cells	Adherent Cells	Suspended Cells
48hrs	0.905	0.653	0.102
72hrs	0.080	0.038	0.136
96hrs	0.044	0.019	0.011

Figure 4.9. The effect of *Wt1* Ablation in *Wt1*$^{-/flox}$; *Immorto* MMCs: Quantification of Cell Proliferation and Adhesion

In this figure, the red lines indicate the mutant, *Wt1*$^{-/\Delta}$; *Immorto* MMCs and the blue lines indicate the *Wt1*$^{-/fl}$; *Immorto* MMCs (control). Lines with circle indicate the total number of cells, lines with triangles indicate the number of adherent cells, and lines with squares indicate the number of suspended cells at 48 hrs, 72 hrs, and 96 hrs.

Discussion

The functional role of Meox2 has been studied in vascular smooth muscle cells (VSMCs), endothelial cells, and cardiomyocytes. Within these cell types, Meox2 induces the cyclin-dependent kinase inhibitor, *p21* (145, 148-150). Moreover, *Meox2* has shown to induce apoptosis in VSMCs (150). Meox2 specifically repressed β_3 and β_5 integrins in VSMC and may lead to the disruption of integrin-regulated signaling pathways (151). In endothelial cells, Meox2 inhibits cellular proliferation and endothelial cell tube formation (152). All together, Meox2 regulates genes that are involved in cellular proliferation and cell adhesion. However, the role of Meox2 has not been extensively studied during kidney development, in particular, kidney mesenchymal cells.

According to the literature, the mediated-regulation of *Meox2* results from the stimulation of Tgf-β1 (147). It is known that TGF-β inhibits cellular proliferation of specific cells types and mutated in various human cancers; TGF-β has the functional role of being a tumor suppressors (153-155). *TGF-β2* and *TGF-β3* were up-regulated in mutant WTs in the microarray data (156). Could it be that during kidney development, *TGF-β2* and *TGF-β3* were transcriptionally repressed by WT1? During Wilms tumorigenesis, could the loss of *WT1* upregulate the gene expression of *TGF-β2* and *TGF-β3* which resulted in the upregulation of *MEOX2?* TGF-β1 was the only TGF-β gene assessed on the array. Therefore, the other TGF-β genes need to be assessed following *WT1* mutation as discussed **in Chapter 5**.

Because *Meox2* was expressed at high levels in WTs and during kidney development following a *Wt1* mutation, it may play a role as an oncogene, which

would cause cells to survive and overproliferate. The overexpression of *Meox2* following a *Wt1* mutation resulted in the induction of p21 and the downregulation of *Itgb5*. In addition, there was a decrease in cell number and an increase in the number of floating cells which goes against the concept of an oncogenic phenotype. However, the floating cells were viable and potentially still growing. If these cells were considered tumorigenic and remain viable and proliferative, they could metastasize throughout the body, which would define *Meox2* as an oncogene. However, we do not know whether this cellular phenotype was due to the overexpression of *Meox2* or from the loss of *Wt1*.

Overall, my data supports my hypothesis that the upregulation of *Meox2* is *Wt1*-dependent during early kidney development (*in vivo studies*), especially in the condensed mesenchyme (*in vitro studies***).** The dysregulation of downstream targeted genes of Meox2 followed the upregulation of *Meox2* in the MMCs***.*** A novel cellular phenotype was discovered, in which the cells decreased in number and lose their ability to adhere after the ablation of *Wt1* and/or the upregulation of *Meox2*. Of course there needs to be more analyses done to further investigate the effect of the loss of *Wt1* on *Meox2* gene expression and the role of over expressed Meox2 in the kidney mesenchymal cells as discussed in **Chapter 5**.

Chapter 5: Conclusions and Future Directions

Conclusions

Wilms tumor is a childhood cancer of the kidney that represents approximately 95% of all pediatric kidney tumors and accounts for 6% of all pediatric cancers (1-4). The classic histology of WTs in both humans and mice is triphasic, which consist of mesenchymal, stromal, and epithelial cells. The data from WT histology suggest that there was a disruption during cellular differentiation of the mesenchymal cells during early kidney development. Most of our understanding of the association between WTs and kidney development derived from the investigation of *WT1* and the histology of WTs. However, the role of WT1 during Wilms tumorigenesis is unclear. WT1 is a transcriptional factor that is known to activate or repress genes involved in cellular proliferation, differentiation, apoptosis, cellular migration, and cellular adhesion. Therefore, it is hypothesized that the inactivation of *WT1* has a reverse effect on downstream genes that are involved in cellular proliferation, differentiation, apoptosis, cellular migration, and cellular adhesion that potentially lead to Wilms tumorigenesis.

Recently, an Affymetrix GeneChip Human Genome Array was conducted with human with *WT1*-wildtype and *WT1*-mutant WTs. Numerous genes were dysregulated in *WT1*-mutant WTs, which *MEOX2* was one of the significantly dysregulated genes upregulated by 19X in *WT1*-mutant tumors. *MEOX2* was validated by qPCR and remained significantly upregulated in *WT1*-mutant tumor by 41X.

In order to test the hypothesis that *Meox2* is upregulated in a *Wt1*-dependent manner, *Wt1* was genetically ablated. A qPCR analysis was conducted comparing the control kidneys to the *Wt1* ablated kidneys, which resulted in the upregulation of *Meox2*. Since Wt1 and Meox2 were both expressed in the mesenchyme, a kidney mesenchymal cells line was generated in which *Wt1* could be genetically ablated with Cre recombinase to determine the effect of *Wt1* mutation on *Meox2* gene expression. Once again, the effect of the ablation of *Wt1* resulted in the significant upregulation of *Meox2*. The significant upregulation of *Meox2* following a *Wt1* mutation, specifically in the mesenchyme, further supported the hypothesis that the upregulation of *Meox2* is *Wt1*-dependent. The inactivation of *Wt1* and/or the upregulation of *Meox2* induced *p21,* repressed *Itgb5*and revealed a unique cellular phenotype in which the cells displayed a loss of cellular adhesion and a decrease in cellular growth. It is not certain whether the induction of *p21* and the repression of *Itgb5* are the cause of the cellular phenotype. However, there is more to investigate how the loss of Wt1 leads to the formation of WT by first further investigating the actual functional role of Meox2 during kidney development as later discussed. By further studying the dysregulated genes in WTs, such as *MEOX2*, we can develop therapeutic strategies to prevent and improve treating Wilms tumors. Additionally, we can understand how the remaining 80% of WTs were formed independent of *WT1* mutation.

Future Directions

Even though the upregulation of *MEOX2* expression following a *WT1* mutation was quantitatively assessed in WTs during developing kidney at E13.5 and in the mesenchymal cell line, there are still gaps remaining in understanding the mechanistic relationship between WT1 protein and *MEOX2* gene. In order to determine how the Wt1 protein and *Meox2* gene interact, directly or indirectly, a Chromatin Immunnoprecipitation (ChIP) sequence would provide evidence that Wt1 is directly regulating the gene expression of *Meox2.*

At E13.5, *Meox2* was significantly upregulated in the kidney rudiments following *Wt1* ablation. To determine whether the loss of *Wt1* affected *Meox2* gene expression throughout kidney development, more developmental time points are required. These studies will provide data to specifically show when and where the upregulation of *Meox2* occur during kidney development after the loss of *Wt1*.

It has been reported that the TGF-β/Smad pathway positively mediates the gene expression of *Meox2.* I sought to determine whether Wt1 regulates *Meox2* expression by altering the TGF-β/Smad pathway. According to my data, the gene expression of *Tgf-β1* was downregulated when *Wt1* was ablated in the mesenchymal cells. According to our microarray data, TGF-β2 and TGF-β3 were both upregulated in the *Wt1*-mutant WTs. Thus, TGF-β2 and TGF-β3 could also regulate the gene expression of *MEOX2.* For future experiments, a qPCR will be conducted to analyze whether Wt1 dysregulates Tgf-β2 or Tgf-β3 in kidney rudiments and in the MMC cell lines that could lead to upregulation of *Meox2.* If either of these Tgf-β proteins are upregulated after the ablation of *Wt1*, it would suggest two possibilities: 1) that Wt1

represses the expression Tgf-β2 and Tgf-β3 and/or 2) that the regulation of Meox2 is dependent on the isoforms of Tgf-β proteins. The combination of the two possibilities would suggest that Wt1 is upstream and transcriptionally regulates the TGF-β/Smad pathway and *Meox2* expression.

The ablation of *Wt1* is known to slightly induce apoptosis during kidney development (157). In the MMCs, the ablation of *Wt1* after 96hrs resulted in the significant increase of *Meox2,* decrease of adhesive cells, and the decrease in cell growth. The floating cells were viable, however, they could be undergoing early induction of apoptosis. In the future, this experiment needs to be repeated and extended to determine whether these cells will eventually become apoptotic by Fluorescence Activated Cell Sorting (FACS) or transferase dUTP nick end labeling (TUNEL) assay. In addition, testing different cellular adhesion coatings, such as gelatin, collagen, fibronectin, poly-D-lysine, or poly-L-lysine, with *Wt1* ablated cells may reduce the loss of cellular adhesion.

In order to assess whether the decrease in cell growth and loss of cellular adhesion was solely achieved by the upregulation of Meox2, future experiments would also include overexpressing *Meox2* independently of Wt1. A construct with a pCMV promoter that overexpresses full-length *Meox2* has been generated in the lab. The pCMV-Meox2 construct could be transiently transfected into a wildtype *Wt1*$^{+/fl}$; *Immorto* MMCs cell line to determine whether these phenotypes resulted solely from the overexpression of *Meox2*.

These future directions will set the stage to study the relationship between Wt1 and Meox2 during kidney development, to understand the role of Meox2 during kidney

development, and to develop strategies to reverse the phenotypes, such as the dysregulation of downstream genes of Meox2, decrease in cell growth and loss of cellular adhesion. Once the relationship between Wt1 and Meox2 are fully understood, we will have an insight on the functional role(s) of WT1 and MEOX2 during Wilms tumorigenesis and have the advantage of being able to develop better therapeutics for WTs.

Appendix

Appendix Table 1. Putative Targets of WT1

A, Activate and **R**, Repress

Gene ID	Protein	Location	Regulation	Reference
ABCB1	ATP-bndg cassette sub-fam B mbr 1 (MDR-1)	7q21.12	R	McCoy, 1999
AMHR2	anti-Mullerian hormone receptor 2	12q13.13	A	Klattig, 2007
AR	androgen receptor	Xq12	R	Shimamura, 1997
AREG	amphiregulin	4q13.3	A	Lee, 1999
BAG3	BCL2-associated athanogene 3	10q26.11	A	Cesaro, 2010
BAK1	BCL2 antagonist/killer 1	6p21.31	A	Morrison, 2005
BCL2	B-cell lymphoma prot 2 beta isoform	18q21.33	A	Mayo, 1999
BCL2A1	BCL2-related protein A1	15q25.1	A	Simpson, 2006
CCNE1	cyclin E1	19q12	R	Loeb, 2002
CCNG1	cyclin G1	5q34	A	Wagner, 2001
CDH1	cadherin 1, type 1; E-cadherin	16q22.1	A	Hosono, 2000
CDH5	cadherin 5, vascular endothelial cadherin	16q21	A	Kirschner, 2010
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21)	6p21.31	A	Englert, 1997
COL4A1	alpha 1 type IV collagen	13q34	A	Hosono, 1999
COL4A2	alpha 2 type IV collagen	13q34	A	Hosono, 1999
CSF1	colony stimulating factor 1	1p13.3	R	Harrington, 1993
CTGF	connective tissue growth factor	6q23.2	R	Stanhope-Baker, 2000
CXXC5 (WID)	CXXC-type zinc finger protein 5	5q31.3	A	Kim, 2010
CX3CL1	chemokine (C-X3-C) ligand1	16q13	A	Kim, 2007
DMTF1	cyclin-dependent myb-like protein 1, WT1-induced inhibitor of DSH	7q21.12	R	Tschan, 2008
EGFR	epidermal growth factor receptor	7p11.2	R	Englert, 1995
EGR1	early growth response 1	5q31.2	R	Madden, 1991
EPO	erythropoeitin	7q22.1	A	Dame, 2006
EPOR	erythropoeitin receptor	19p13.2	A	Kirschner, 2008
EREG	epiregulin	4q13.3	A	Kim, 2007
ESR	estrogen receptor	6q25.1	R	Han, 2008
GNAI2	G-protein alpha i-2	3p21.31	R	Kinane, 1996
HBEGF	heparin-binding EGF-like growth factor	5q31.3	A	Kim, 2007
HOXA10	homeobox gene	7p15.2	R	Andikyan, 2009
HSPA4	heat shock 70kDa protein 4 isoform a	5q31.1	A	Maheswaran, 1998

HSPG2	heparan sulfate proteoglycan 2 (perlecan)	1p36.12	A	Hosono, 1999
IFI16	interferon-inducible protein 16	1q23.1	A	Kim, 2008
IGF1R	insulin-like growth factor 1 receptor	15q26.3	R	Werner, 1993
IGF2	insulin-like growth factor 2	11p15.5	R	Drummond, 1992
IGFBP4	insulin-like growth factor binding prot 4	17q21.2	R	Wagner, 2001
IL10	interleukin-10	1q32.1	A	Sciesielski, 2010
INHA	inhibin alpha	2q35	R	Hsu, 1995
IRF8	interferon regulatory factor 8	16q24.1	R	Vidovic, 2010
ITGA4	integrin alpha 4	2q31.3	A	Kirschner, 2006
ITGA8	integrin alpha 8	10p13	R	Hosono, 1999
JUNB	Proto-oncogene JunB	19p13.13	A	Kim, 2007
MDK	midkine (neurite growth-promoting factor 2)	11p11.2	R	Adachi, 1996
MIS	Mullerian inhibiting substance	19p13.3	A	Nachtigal, 1998
MKP3	mitogen-activ prot kinase phosph 3 (DUSP6)	12q21.33	A	Morrison, 2008
MMP9	matrix metalloproteinase-9	20q13.12	R	Marcet-Palacios, 2007
MYB	c-myb myeloblastosis viral onc hom	6q23.3	R	McCann, 1995
MYC	c-myc oncogene	8q24.21	A	Han, 2004
MYCN	n-myc myelocytomatosis viral related onc	2p24.3	R	Zhang, 1999
NDRG2	N-myc downstream regulated gene 2	14q11.2	A	Svensson, 2007
NES	nestin	1q23.1	A	Wagner, 2006
NOV	novH, nephroblastoma overexpressed gene	8q24.12	R	Martinerie, 1996
NPHS1	nephrin	19q13.12	A	Wagner, 2004
NR0B1	nucl recep subfamily 0, grp B, mbr 1 (Dax-1)	Xp21.2	A	Kim, 1999
NR5A1/SF1	nuclear receptor subfamily 5, group A (SF-1)	9q33.3	A	Wilhelm, 2002
NTRK2	neurotrophin receptor TrkB	9q21.33	A	Wagner, 2005
ODC	ornithine decarboxylase 1	2p25.1	R	Moshier, 1996
PAX2	paired box protein 2	10q24.31	R	Ryan, 1995
PDGFA	platelet-derived growth factor alpha	7p22	R	Wang, 1992
PODXL	podocalyxin-like (podocalyxin)	7q32.3	A	Palmer, 2001
POU4F2	POU domain, class 4, transc factor 2	4q31.22	A	Wagner, 2003
RARA	retinoic acid receptor, alpha	17q21.2	R	Goodyer, 1995
RBBP7	Rb binding protein 7 (RbAp46)	Xp22.2	A	Guan, 1998
SALL2	Sal-like 2 (HSAL2)	14q11.2	R	Ma, 2001
SCRIB	Scribble	8q24.3	A	Wells, 2010
SDC1	syndecan 1	2p24.1	A	Cook, 1996
SLC6A6	solute carrier fam 6 (neurotrans transp, taurine, TauT)	3p25.1	A	Han, 2003
SNAI1	Snail	20q13.13	A	Martinez-Estrada, 2010

SNAI2	Slug	8q11.21	A	Kim, 2007
SOD1	superoxide dismutase 1	21q22.11	A	Minc, 1999
SOX9	transcription factor SOX9	17q24.3	A	Gao, 2006
SPRY1	Sprouty homolog 1	4q28.1	A	Gross, 2003
SREBP2	sterol reg element binding transc factor	17p11.2	A	Rae, 2004
SRY	sex determining region Y	Yp11.31	A	Matsuzawa-Watanabe, 2003
STAT3	signal transducer and activator of transcription 3	17q21.2	A	Inoue, 1998
STIM1	stromal interaction molecule1	11p15.5	R	Ritchie, 2010
TBXA2R	thromboxane A2 receptor	19p13.3	R	Gannon, 2008
TERT	telomerase reverse transcriptase	5p15.33	R	Oh, 1999
TGFB	transforming growth factor, beta 1	19q13.2	R	Dey, 1994
THBS1	thrombospondin 1	15q14	R	Dejong, 1999
VDR	vit D (1,25- dihydroxyvitamin D3) receptor	12q13.11	A	Wagner, 2001
VEGF	vascular endothelial growth factor	6p21.1	A	Hanson, 2007
WNT4	wingless-type MMTV integ site fam, member 4	1p36.12	A	Sim, 2002
WT1	Wilms tumor 1	11p13	R	Rupprecht, 1994

A, Activate and **R**, Repress

Appendix Table 2. Calculating the Relative Gene Expression of Validated Genes (n=12)

CXXC4:

Average	STDEV	Average	STDEV	WT-MT	Relative Gene Expression
7.009	7.009	10.856	10.856	-3.223	0.107
6.850	6.850	10.914	10.914		**-9.337**
7.176	7.176	9.937	9.937		
7.241	7.241	11.008	11.008		
10.029	10.029	12.556	12.556		
9.908	9.908	12.653	12.653		
6.287	6.287	8.368	8.368		
6.102	6.102	8.498	8.498		
6.511	6.511	12.169	12.169		
6.616	6.616	15.053	15.053		
8.126	8.126	8.111	8.111		
7.987	7.987	8.396	8.396		
7.487	**1.309**	**10.710**	**2.162**		

DDR1:

Average	STDEV	Average	STDEV	WT-MT	Relative Gene Expression
5.978	5.978	5.242	5.242	-0.661	0.633
6.041	6.041	5.241	5.241		-1.581
6.047	6.047	7.374	7.374		
5.976	5.976	7.400	7.400		
5.898	5.898	4.349	4.349		
6.040	6.040	4.279	4.279		
6.771	6.771	5.243	5.243		
6.807	6.807	5.217	5.217		
4.870	4.870	4.008	4.008		
5.000	5.000	4.040	4.040		
5.382	5.382	4.781	4.781		
5.337	5.337	5.046	5.046		
5.846	**0.609**	**5.185**	**1.132**		

FST:

Average	STDEV	Average	STDEV	WT-MT	Relative Gene Expression
9.966	9.966	6.678	6.678	3.694	12.938
10.074	10.074	6.810	6.810		
12.819	12.819	8.787	8.787		
12.951	12.951	7.781	7.781		
12.666	12.666	7.597	7.597		
13.033	13.033	8.333	8.333		
10.870	10.870	8.305	8.305		
15.658	15.658	9.423	9.423		
14.482	14.482	9.360	9.360		
9.735	9.735	9.119	9.119		
9.804	9.804	9.236	9.236		
12.005	**2.042**	**8.312**	**0.989**		

HIPK2:

Average	STDEV	Average	STDEV	WT-MT	Relative Gene Expression
3.610	3.610	4.184	4.184	-1.176	0.443
3.629	3.629	4.285	4.285		-2.260
1.780	1.780	4.038	4.038		
1.749	1.749	4.253	4.253		
4.315	4.315	3.109	3.109		
4.473	4.473	3.207	3.207		
2.546	2.546	5.863	5.863		
2.514	2.514	5.953	5.953		
1.880	1.880	2.244	2.244		
1.938	1.938	2.341	2.341		
2.843	**1.070**	4.341	4.341		
		4.413	4.413		
		4.019	**1.168**		

MEOX2:

Average	STDEV	Average	STDEV	WT-MT	Relative Gene Expression
13.369	13.369	5.934	5.934	5.360	41.069
13.473	13.473	6.361	6.361		
12.099	12.099	4.404	4.404		
12.305	12.305	5.087	5.087		
11.029	11.029	5.227	5.227		
10.810	10.810	5.996	5.996		
9.914	9.914	4.608	4.608		
9.179	9.179	5.214	5.214		
13.068	13.068	6.477	6.477		
13.152	13.152	6.621	6.621		
7.082	7.082	6.370	6.370		
7.104	7.104	5.962	5.962		
11.049	**2.308**	**5.689**	**0.754**		

MLLT3:

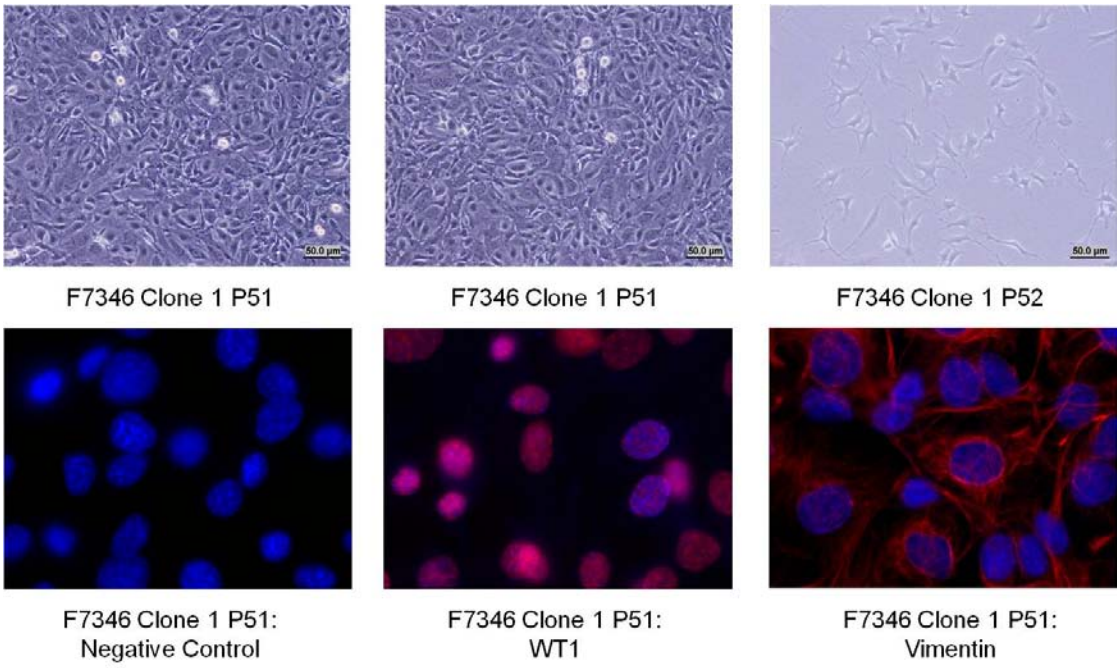
Average	STDEV	Average	STDEV	WT-MT	Relative Gene Expression
8.550	8.550	7.819	7.819	3.335	10.091
8.570	8.570	8.714	8.714		
14.635	14.635	7.666	7.666		
15.724	15.724	8.366	8.366		
7.384	7.384	7.832	7.832		
7.705	7.705	7.737	7.737		
12.549	12.549	8.303	8.303		
14.656	14.656	8.488	8.488		
15.321	15.321	10.149	10.149		
12.439	12.439	10.531	10.531		
13.323	13.323	8.562	8.562		
11.896	**3.232**	8.567	8.567		
		8.561	**0.909**		

PRRX1:

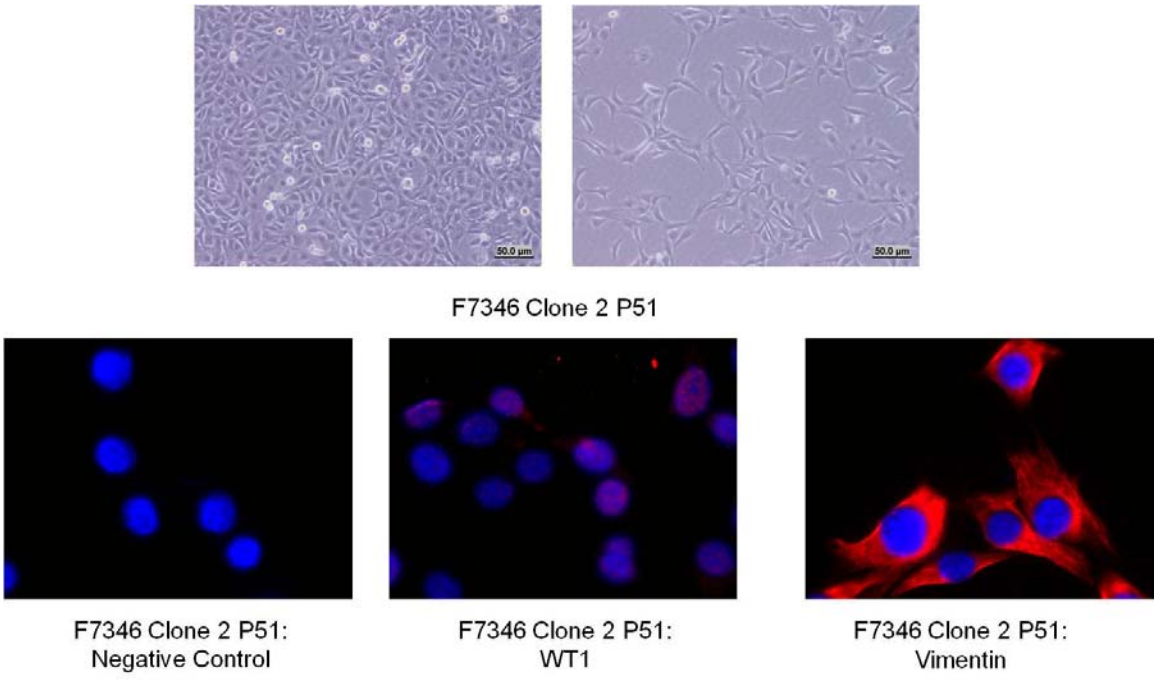
Average	STDEV	Average	STDEV	WT-MT	Relative Gene Expression
11.536	11.536	12.771	12.771	3.818	14.099
11.396	11.396	13.306	13.306		
11.826	11.826	6.368	6.368		
15.108	15.108	6.314	6.314		
15.303	15.303	7.699	7.699		
20.143	20.143	8.038	8.038		
11.795	11.795	7.693	7.693		
12.610	12.610	8.246	8.246		
12.896	12.896	9.331	9.331		
9.411	9.411	10.200	10.200		
9.648	9.648	9.129	9.129		
12.879	**3.040**	9.643	9.643		
		9.062	**2.210**		

Appendix Figure 1. IF Images of the Subcloned Kidney MMCs

Clone #1:



Clone #2:



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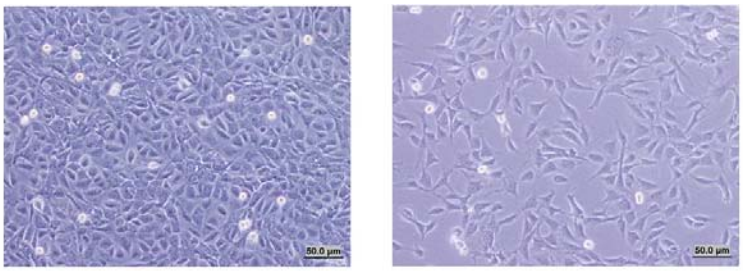

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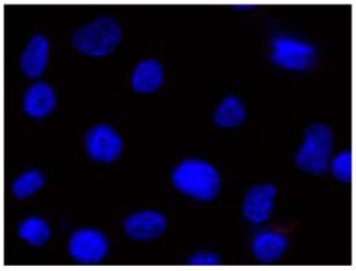

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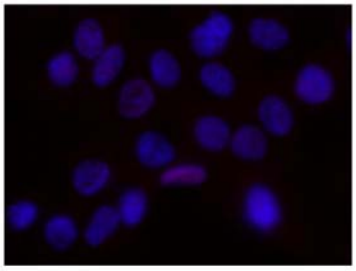

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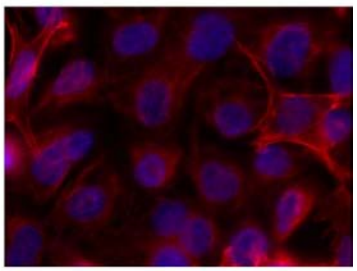

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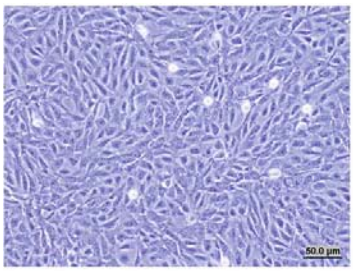

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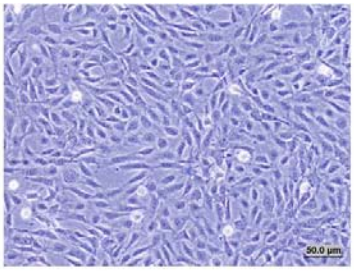

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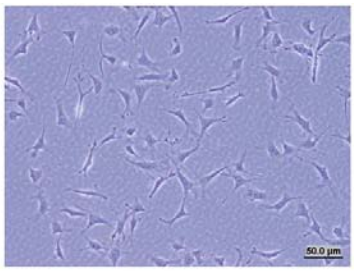

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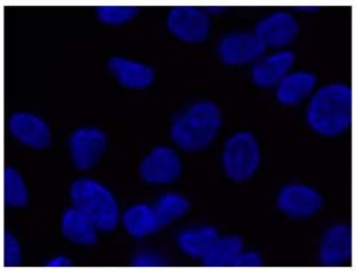

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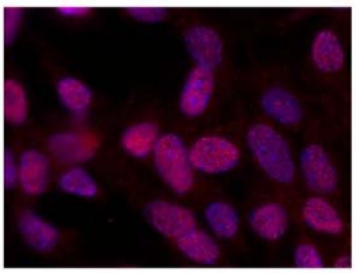

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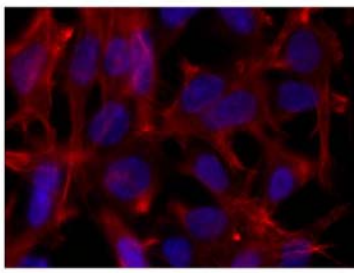

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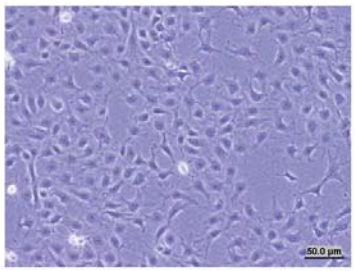

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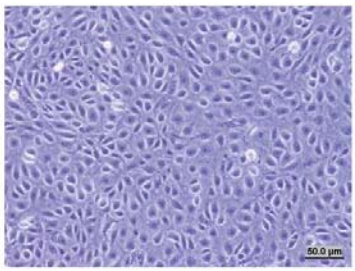

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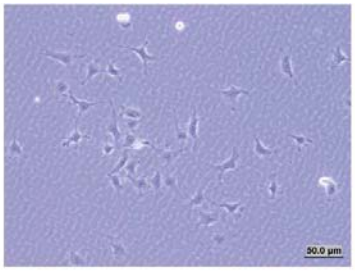

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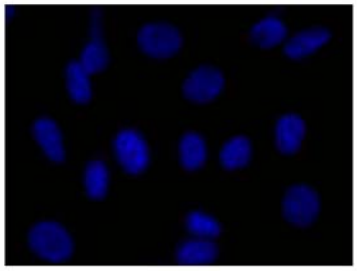

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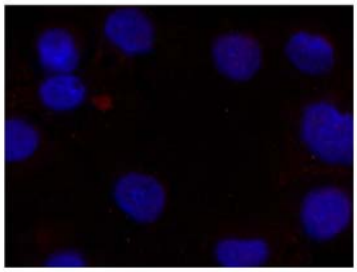

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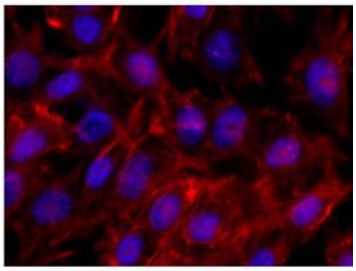

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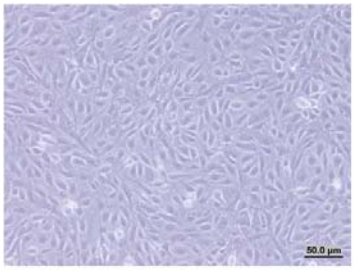
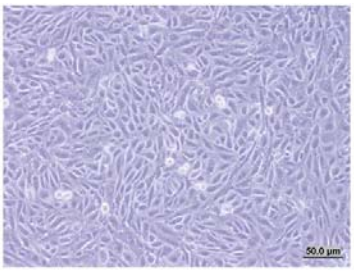


F7346 Clone 7 P51



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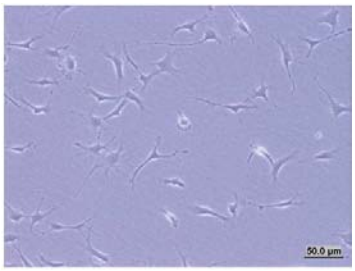

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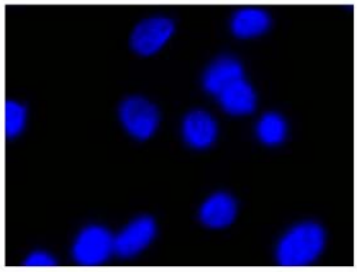

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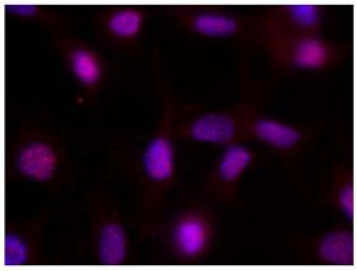

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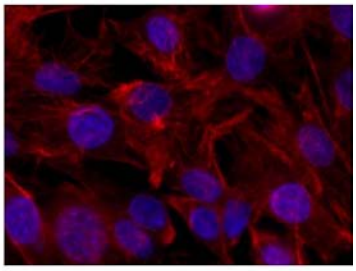

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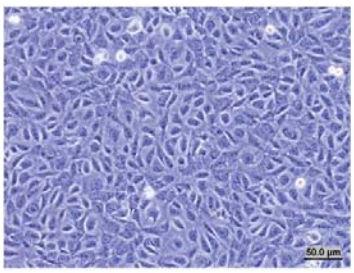
Vimentin

Clone #8:



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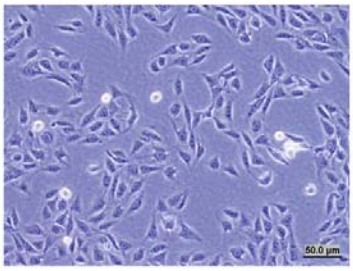

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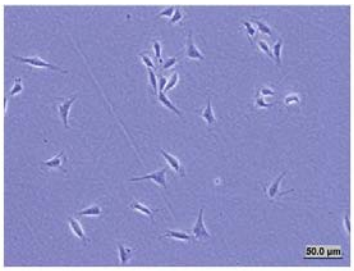

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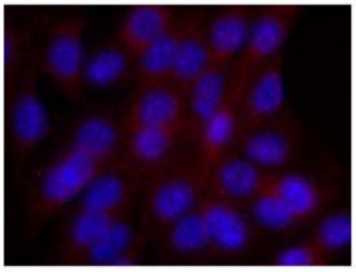

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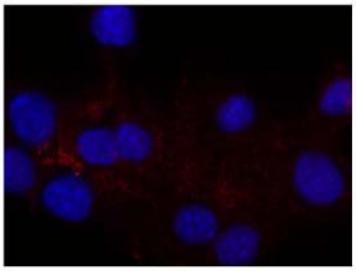

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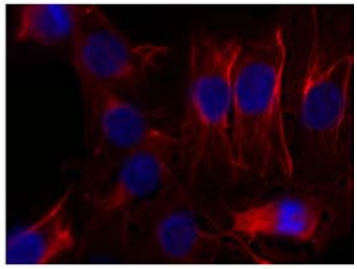

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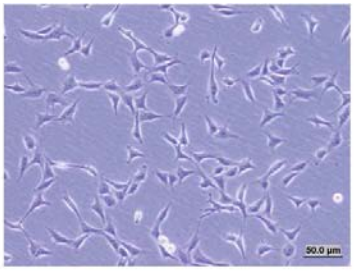

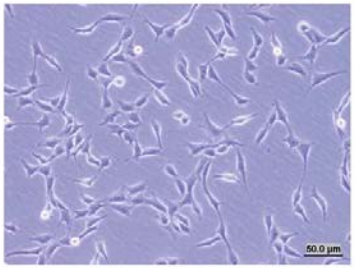

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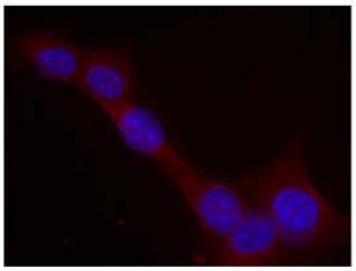

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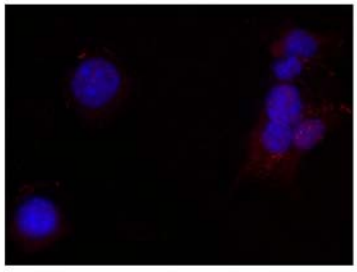

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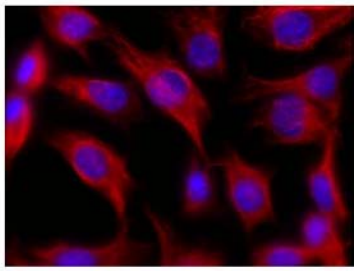

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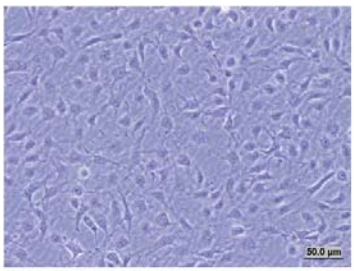

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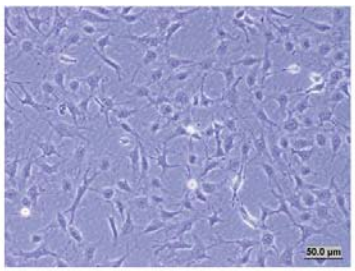

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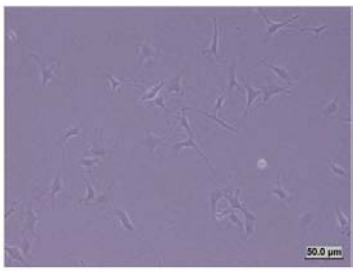

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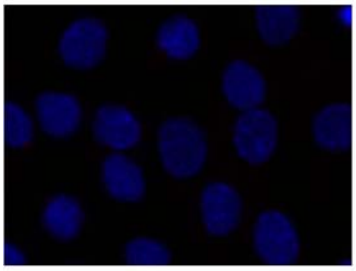

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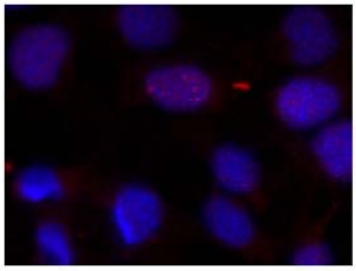

F7346 Clone 12 P51:
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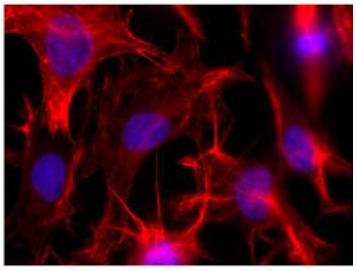

F7346 Clone 12 P51:
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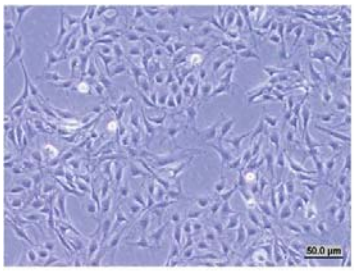

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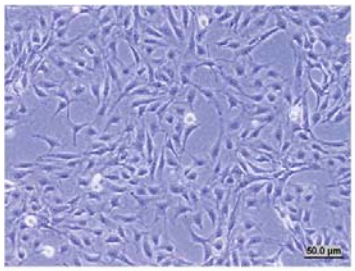

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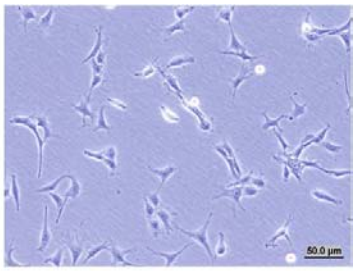

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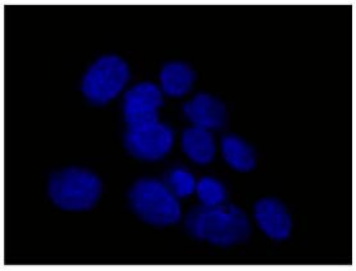

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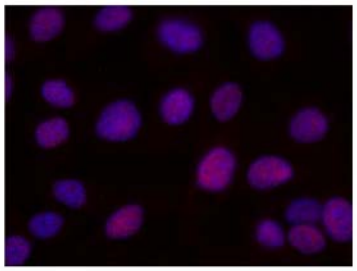

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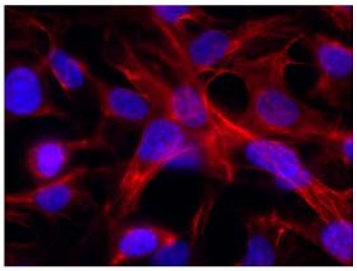
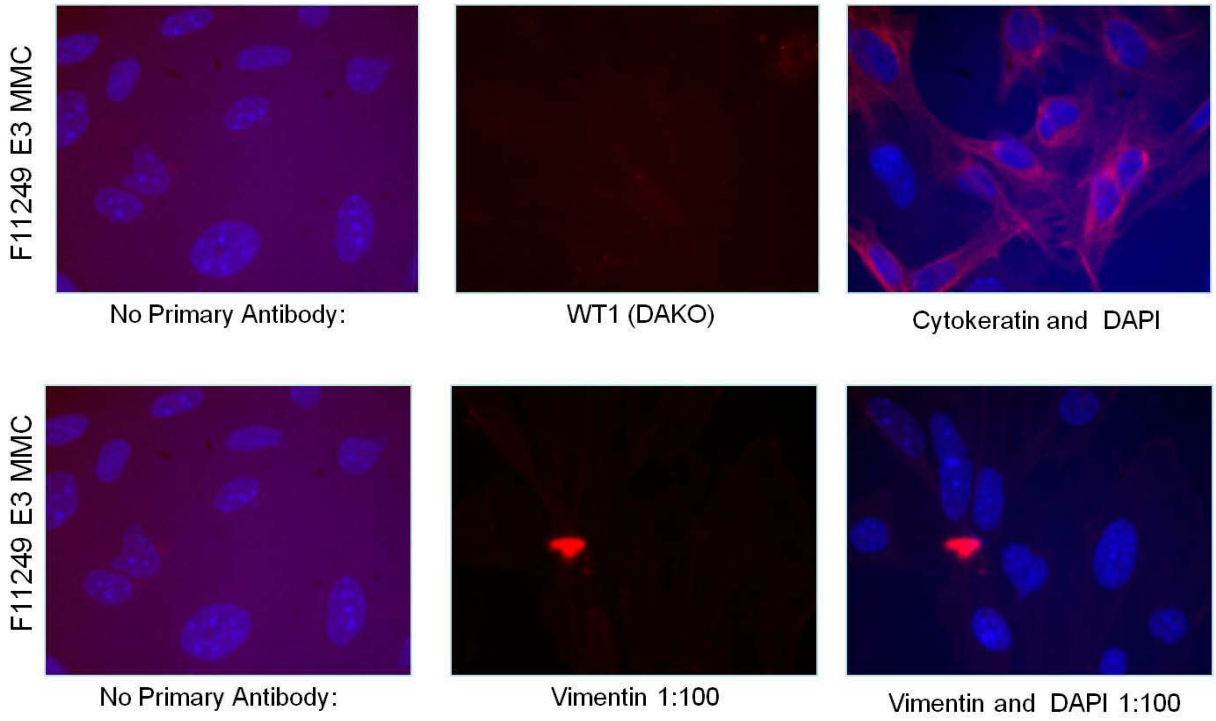
WT1



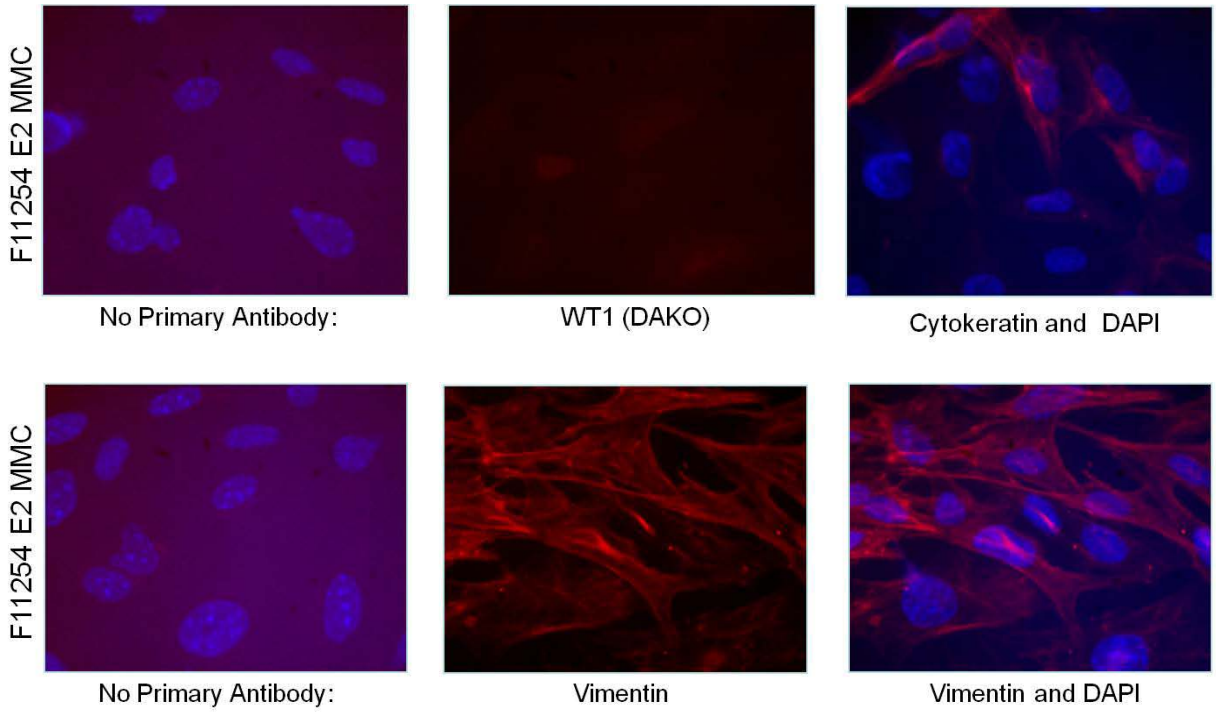
F7346 Clone 15 P51:
Vimentin

Appendix Figure 2: IF images of the MMCs with the *CreER^{TM}* Transgene

F11249 E3 *Wt1$^{-/fl}$*; *Immorto; CreER^{TM}*



F11254 E2 *Wt1$^{-/fl}$*; *Immorto; CreER^{TM}*



Appendix Table 3. Calculating the relative gene expression of *the Wt1*, *Tgf-β1, Smad3, Meox2, p21* and *Itgb5*after adenoviral infection

Wt1

48 Cre		**48 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
8.474	8.474	4.705	4.705	-3.224	0.107
7.634	7.634	4.291	4.291		
6.790	6.790	4.231	4.231		
7.633	0.842	4.409	0.258		
72 Cre		**72 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
7.059	7.059	5.519	5.519	-3.390	0.095
7.250	7.250	4.647	4.647		
10.483	10.483	4.458	4.458		
8.264	1.924	4.874	0.566		
96 Cre		**96 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
4.475	4.475	4.280	4.280	-2.474	0.180
6.599	6.599	3.757	3.757		
8.293	8.293	3.908	3.908		
6.456	1.913	3.982	0.269		

Tgf-β1

48 Cre		**48 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
7.185	7.185	8.021	8.021	0.942	1.921
6.078	6.078	6.605	6.605		
5.966	5.966	7.428	7.428		
6.410	0.674	7.351	0.711		
72 Cre		**72 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
6.123	6.123	6.646	6.646	-0.666	0.630
5.858	5.858	4.120	4.120		-1.587
6.600	6.600	5.818	5.818		
6.194	0.376	5.528	1.288		
96 Cre		**96 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
6.488	6.488	5.875	5.875	-0.616	0.652
6.186	6.186	5.920	5.920		-1.533
6.421	6.421	5.450	5.450		
6.365	0.159	5.749	0.259		

Smad3

48 Cre		**48 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
8.144	8.144	7.037	7.037	-1.584	0.334
9.148	9.148	7.086	7.086		-2.998
8.646	0.710	7.062	0.034		
72 Cre		**72 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
9.384	9.384	8.236	8.236	-0.541	0.687
8.685	8.685	8.808	8.808		-1.455
8.844	8.844	8.246	8.246		
8.971	0.366	8.430	0.328		
96 Cre		**96 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
9.549	9.549	7.396	7.396	-1.227	0.427
9.143	9.143	7.643	7.643		-2.341
8.425	8.425	8.397	8.397		
9.039	0.570	7.812	0.522		

Meox2

48 Cre		**48 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
11.250	11.250	11.392	11.392	0.494	1.409
11.435	11.435	12.061	12.061		
11.014	11.014	11.730	11.730		
11.233	0.211	11.728	0.335		
72 Cre		**72 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
8.186	8.186	6.156	6.156	-1.028	0.490
7.137	7.137	7.120	7.120		-2.039
7.370	7.370	6.333	6.333		
7.564	0.551	6.536	0.513		
96 Cre		**96 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
7.070	7.070	12.489	12.489	5.936	61.204
7.338	7.338	13.589	13.589		
6.953	6.953	13.088	13.088		
7.120	0.197	13.056	0.551		

p21

48 Cre		**48 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
5.319	5.319	5.705	5.705	0.366	1.289
5.192	5.192	5.478	5.478		
4.984	4.984	5.410	5.410		
5.165	0.169	5.531	0.155		
72 Cre		**72 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
4.848	4.848	5.507	5.507	0.367	1.290
4.824	4.824	5.331	5.331		
5.056	5.056	4.992	4.992		
4.909	0.127	5.277	0.262		
96 Cre		**96 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
4.945	4.945	6.272	6.272	1.294	2.452
5.278	5.278	6.575	6.575		
5.141	5.141	6.399	6.399		
5.121	0.167	6.415	0.152		

Itgb5

48 Cre		**48 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
7.532	7.532	8.897	8.897	0.436	1.353
7.782	7.782	7.623	7.623		
7.861	7.861	7.961	7.961		
7.725	0.172	8.160	0.660		
72 Cre		**72 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
8.833	8.833	7.327	7.327	-0.911	0.532
8.909	8.909	8.036	8.036		-1.880
8.765	8.765	8.411	8.411		
8.836	0.072	7.925	0.551		
96 Cre		**96 GFP**			
Average	**STDEV**	**Average**	**STDEV**	**Control-Infected**	**Relative Gene Expr**
8.485	8.485	7.234	7.234	-1.100	0.467
8.559	8.559	7.674	7.674		-2.143
8.507	8.507	7.345	7.345		
8.517	0.038	7.417	0.229		

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Vitae

LaGina Merie Nosavanh was born on May 15, 1985 in Salisbury, Maryland and raised in Frankford, Delaware. She attended Delaware State University located in Dover, Delaware beginning in 2003. LaGina received a NIH MARC (Minority Access to Research Careers) undergraduate training grant, and participated in the summer research programs at the following institutions: University of Texas – Health Science Center at Houston (2004), Stanford University (2005), and University of Michigan – Ann Arbor (2006). LaGina gave a poster presentation at the ABRMS (Annual Biomedical Conference for Minority Students). She conducted research in Dr. Fatma Helmy's laboratory at Delaware State University from Fall 2005 - Spring 2007. She presented her data at the Experimental Biology Conference. LaGina graduated Magna Cum Laude with a B.S. in Biology in 2007 and continued her education in Genetics at the University of Texas – Graduate School for Biomedical Sciences. In 2008, she joined Dr. Vicki Huff's laboratory, and gained research experience in development biology, pediatric cancer biology/genetics and molecular genetics to address the important questions in kidney development and Wilms tumorigenesis. LaGina attended the AACR (American Association for Cancer Research) National meeting to gain more knowledge in cancer research in 2009 at Denver Colorado. She presented a poster at the Genes and Development Spring Retreat in 2009 at Port Aransas, Texas. She also presented a poster at the 2009 Molecular Carcinogenesis and Genes and Development Joint Retreat at M.D. Anderson South Campus. In 2009, she received the Gigli Family Endowed Scholarship for 2009-2010. LaGina was granted a Masters in Genes and Development in December of 2011.

Her abstracts include:

Nosavanh, L. M., Ruteshouser, C. and Huff, V. Genes Dysregulated Following *WT1* Mutation and their Role in Wilms Tumorigenesis and Nephrogenesis. Abstract for poster presentation, Genes and Development Annual Retreat, 2009

Nosavanh, L. M., and Huff, V. Genes Dysregulated Following *WT1* Mutation and their Role in Wilms Tumorigenesis and Nephrogenesis. Abstract for poster presentation, Genes and Development and Molecular Carcinogenesis Joint Retreat, 2009

Helmy, F., **Nosavanh, L.,** Haynes, H. and Juracka, A. Phospholipid Profile of rat Testis, its Unique High level of Monolysocardiolipin and its Lipolytic Capabilities *in Vitro.* A Chromatographic and Densitometric Analysis. Abstract for poster presentation, The Experimental Biology Conference at Washington D.C. on May 3, 2007

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Her publications include:

F. Helmy, **L. Nosavanh**, H. Haynes and A. Juracka. 2008. Phospholipid profile of rat testis, its unique high level of monolysocardiolipin and its lipolytic capabilities in vitro. A chromatographic analysis. Cell Biochem. Funct. Vol. 26 (4). 434-42.

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