Effects of Dicer Inactivation in the Developing Mouse Adrenal Cortex and Micro-RNAs in Adrenocortical Carcinoma

by

Kenneth Thomas Krill

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Doctoral Committee:

Professor Gary D. Hammer, Chair
Professor Andrzej A. Dlugosz
Professor Eric R. Fearon
Professor Deborah L. Gumucio
Assistant Professor John Kim

DEDICATION

To my parents.

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Abstract

Adrenocortical carcinoma (ACC) is a rare yet highly aggressive form of cancer with limited treatment options and poor prognosis. Insulin-like growth factor 2 (IGF2*)* is one of the most highly expressed genes in sporadic ACC, and is an adrenal mitogen. In addition, a microRNA (miRNA), miR-483-3p, is located in an intronic region of *IGF2*, suggesting co-expression of IGF2 and miR-483-3p in ACC. miRNAs are small, endogenous, non-protein coding RNAs that are an important means of post-transcriptional gene regulation, and have been implicated in numerous physiologic and disease processes. This thesis describes the correlation between IGF2 and miR-483-3p in primary human ACC samples, providing rationale for the development of molecular tools designed to aid in the study of the role of IGF2 and miR-483-3p in ACC. Additionally, we describe the results of genetically deleting Dicer, a key miRNA processing enzyme, in the developing adrenal cortex. Adrenal Dicer knockout (KO) mice did not survive beyond 24-48 hours post-parturition, and were characterized by rapid failure of the adrenal cortex during late gestation at embryonic day 18.5 (E18.5). Specifically, Dicer KO adrenal cortical cells underwent apoptosis and were completely depleted by E18.5. The adrenal medulla, however, remained in E18.5 Dicer KO adrenals, suggesting that initial adrenal cortex formation was unperturbed by Dicer inactivation. To further characterize Dicer KO embryonic

adrenals, we subjected purified RNA isolated from control and KO adrenals at both E15.5 and E16.5 to mRNA and miRNA microarray analyses. Intriguingly, Dicer KO adrenals demonstrated significant up-regulation of transcripts belonging to the genes *Nr6a1 and Acvr1c,* whose functions in adrenocortical development and physiology are currently poorly understood. Finally, several down-regulated miRNAs in Dicer KO adrenals were consistently predicted to target mRNA transcripts from these genes. The increased expression of Nr6a1 and Acvr1c gene transcripts in Dicer KO adrenals may suggest a role for miRNA mediated regulation of these genes, which may in turn be important in normal adrenal development.

CHAPTER 1

Introduction

Four hundred and fifty years ago, an Italian anatomist by the name of Bartolomeo Eustachi (Eustachius) described the presence of the suprarenal bodies, '*Glandulae renis incumbentes*', in his work *Opuscula Anatomica*. He did not, however, offer any explanation on their function. At the time, Eustachius stated that no one had previously described the glands now known as the adrenals in any medical work, although there is some debate as to whether the early Roman physician Galen may have described them as early as the second century AD [1]. While Eustachius remains a well known establishment of the medical community in the twenty-first century due in part to the tube in the inner ear that bears his name, he is often overlooked concerning the history of the adrenal gland. It was not until nearly 300 years later, in 1855, that the adrenal glands finally came to the forefront of the burgeoning field of endocrinology. It was in this year that a renowned English physician, Thomas Addison, published his book, *On the Constitutional and Local Effects of Disease of the Suprarenal Capsules,* in which he described 10 cases of patients suffering with what would eventually come to be identified as Addison's Disease [2]. With the renewed interest in the 'suprarenal capsules' and their association with a profile of disease symptoms, the floodgates of research into the adrenal glands were opened. The

following year, Brown-Séquard proved in animal experiments that the adrenal glands were essential for life. Then, towards the beginning of the twentieth century, Sir William Osler demonstrated the temporary effective treatment of Addison's Disease by the administration of a crude, oral extract prepared from adrenal glands, proving that their still unidentified secretions were important for health. Through the early twentieth century into the 1930s, further experiments in animal models supported Osler's initial observation, and the involvement of the pituitary gland in what is now known as the hypothalamic-pituitary-adrenal (HPA) axis was first described. As advances in biochemistry were made through World War II and beyond, the steroid hormones of the adrenal cortex were isolated, and their structures determined and synthesized. In 1942 adrenocorticotropic hormone (ACTH), the pituitary hormone responsible for stimulating steroid production in the adrenal cortex, was isolated by Li and Sayers; shortly afterwards the anti-inflammatory effects of cortisol were discovered by Hench in 1948. By the end of the 1950s, aldosterone had been isolated and characterized. Jerome W. Conn, a University of Michigan Medical School alumnus and endocrinologist, was the first to describe the disease of primary aldosteronism which now bears his name [3].

In the 157 years since Addison rekindled scientific interest in the adrenal glands, the biochemical, anatomical, and physiologic aspects of the glands and their functions have been elucidated in great detail. Their role in maintaining electrolyte homeostasis, mediating physiologic responses to stress and external stimuli, and the regulatory feedback pathways involved in adrenal function are

now standard fare for any first year medical student around the world. The advent of the ‘molecular age‘ of research as the twenty-first century gets underway opens the door for new directions of adrenal research. The molecular ontogeny of the adrenal gland is one of these avenues. The molecular and cellular signaling processes necessary for adrenal formation are still poorly defined, and the molecular mechanisms of maintenance and homeostasis of the adrenal cortex are still in the relatively early stages of discovery. The molecular and genetic factors that keep the balance between adrenal physiology, pathophysiology, and neoplasia, and the events that upset this balance still remain elusive as well; they are also a subject of great interest for the current and next generation of physicians and researchers. The information gathered from genetic and molecular studies of the adrenal gland will provide insights and answers to questions regarding the factors that contribute to adrenal development, function, and disease. From the simple yet foundational advancements made in adrenal biology from the nineteenth century onwards, the knowledge gleaned from present day adrenal research will serve to propel our understanding of adrenal pathology well into the twenty-first century and beyond, ushering in a new era of treatments for adrenal and endocrine related disease.

Adrenal Structure and Function

The adrenal glands are bilateral endocrine organs situated superior to the kidneys. In mice, they are grossly spherical or ellipsoid in shape, with a fairly uniform radial organization and appearance when viewed in cross section. In

humans, the glands appear grossly pyramidal in shape, and often have invaginations that make them irregular in cross section, with the internal organization seemingly flattened or folded. The human adrenal is divided into the head, body, and tail, from the inferomedial to superior aspects, highlighting its irregular shape in comparison with the mouse. The adrenals in humans and mice are surrounded by a thin capsule that surrounds the cortical tissue. Current research suggests that this capsule plays an important function in the maintenance of the adrenal cortex [4]. The adrenal gland is divided into two grossly defined zones, the outer cortex and the inner medulla (Figure 1.1). The cortex is further subdivided into three concentric functional zones, which are defined by their biochemical and steroid producing profiles. Beginning from the outermost zone, these are the zona glomerulosa (zG), zona fasiculata (zF), and zona reticularis (zR). In rodents and other animals, the functional distinction between the zF and zR is often less distinct when compared with the human adrenal [5]. Vascular supply is provided via branches from the aorta, renal, and inferior phrenic arteries, which form a capsular plexus that flows into a capillary network in the outer margin of the zG. Sinusoids in the zF, zR, and medulla eventually drain into the adrenal central vein, which then flows back into the venous return system. Innervation of the adrenal gland is well established in regards to the direct regulation of catecholamine synthesis by the adrenal medulla. Sympathetic preganglionic and cholinergic fibers derived from the splanchnic nerves synapse directly on the cells of the medulla. Recent studies, however, have demonstrated that the cortical cells may also receive direct

neuronal synapses [6,7]. In addition, evidence exists supporting the notion that the cortex may also contain sensory innervation, with afferent nerve fibers providing feedback to the central nervous system in response to adrenal specific stimuli. Finally, the presence of numerous neuropeptides and transmitters in the adrenal cortex and medulla also suggest a rich network of innervation in the gland [8-10].

As stated previously, the adrenal gland is divided grossly into the medulla and cortex. The adrenal medulla is comprised of chromaffin cells, so named by the Prague histologist Alfred Kohn at the turn of the nineteenth century because of their apparent affinity to chromium salts that resulted in a yellow-brown staining [11]. Indeed, it had been observed earlier in that same century by the French physician, Edmé Félix Alfred Vulpian, that an unknown substance in the adrenal medulla reacted with perchloride of iron to produce an emerald green stain not seen elsewhere in the human body [3]. It is now known that the unknown substances that caused the colored stains in Kohn and Vulpian's experiments were catecholamines synthesized by the adrenal medullary cells. This discovery was made shortly after Kohn coined the term "chromaffin", when adrenaline (epinephrin) was isolated and synthesized. Epinephrine, norepinephrine, and dopamine are the endogenous secretions of the adrenal medulla, and belong to a class of compounds known as catecholamines. They are characterized by an amine group containing a 3,4-dihydroxyphenyl (catechol) nucleus, and are synthesized from the amino acid tyrosine involving a series of biochemical steps mediated by enzymes produced by the medullary cells. The

majority of medullary cells synthesize, store, and secrete epinephrine. A small minority lack the enzyme phenylethanolamine N-methyl transferase (PMNT), which is necessary to catalyze the final step from norepinephrine to epinephrine; these cells can only secrete norepinephrine. Interestingly, PMNT expression is induced by glucocorticoids, raising the possibility of cortical-medullary signaling in the maintenance of epinephrine secreting medullary cells [12]. The biological functions of the catecholamines are mediated through adrenergic receptors found on the cell membranes of target cells. This class of receptors is broadly divided between α and β receptors, with each class further subdivided into subtypes α_1, α_2 and β_1, β_2. Catecholamines produces a variety of physiologic effects, although their effects on blood pressure and glucose metabolism are among the most widely known. Among the hemodynamic effects of catecholamines are increased myocardial contraction (β_1), vasoconstriction (α), and vasodilation (β_2). The metabolic effects of catecholamine stimulation include a hyperglycemic effect that is facilitated in part by increased hepatic glucose production [10]. These effects together, are in part responsible for the so-called “fight or flight” stress response that the medullary catecholamines are known to facilitate.

Despite the importance of the adrenal medulla in synthesizing and storing catecholamines, the adrenal cortex plays an equal, if not more critical role, in maintaining physiologic homeostasis by secreting functionally distinct hormones from a population of cells thought to have common origins. The adrenal cortex produces three main classes of steroid hormones: mineralocorticoids,

glucocorticoids, and sex steroids. The separately controlled secretion of these steroids is critical, and is accomplished in part by cellular differentiation and zonation of the adrenal cortex (Figure 1.2). Mineralocorticoids (primarily aldosterone) are produced by the zG, primarily under the influence of the renin-angiotensin system. Renin is produced by the kidney in response to decreased perfusion, decreased sodium balance, and sympathetic stimulation. Renin acts upon its zymogen substrate, angiotensinogen, which is produced by the liver, to generate angiotensin I. Angiotensin converting enzyme converts angiotensin I into active angiotensin II, which acts on the adrenal zG to stimulate aldosterone secretion. Aldosterone exerts its primary effects on the distal convoluted tubule of the kidney nephron to promote the resorption of sodium from the urine. In doing so, aldosterone facilitates the maintenance of fluid and electrolyte balance in the organism. The secretion of glucocorticoids is the task of the zF, and is controlled primarily by the HPA axis through the effects of ACTH, a peptide hormone secreted by the anterior pituitary. ACTH is a product of a larger precursor peptide, pro-opiomelanocortin (POMC) that is secreted by the anterior pituitary. POMC undergoes a series of endoproteolytic cleavage steps to produce ACTH, in addition to several other melanocortin-related peptides. While ACTH secretion is episodic and correlates with the circadian rhythm of the organism, stress, both emotional and physical, is also a significant stimulator of ACTH release into the bloodstream. The physiologic effects of glucocorticoids are too numerous and substantial to describe fully in this work, but briefly, glucocorticoids affect metabolic functions by promoting protein catabolism,

hepatic gluconeogenesis, and inhibiting glucose uptake and usage in peripheral tissues. Additionally, prolonged exposure to glucocorticoids leads to a re-distribution of fat mass, resulting in characteristic “moon facies”, truncal obesity, and other features of Cushing’s syndrome. Glucocorticoids also negatively regulate growth and wound healing, and repress the inflammatory response of the immune system, making them highly effective anti-inflammatory drugs. Finally, the last class of steroid hormones produced by the adrenal cortex are androgens, with dehydroepiandrosterone sulfate (DHEA-S) being one of the primary adrenal androgens. Like the secretion of glucocorticoids from the zF, androgen secretion from the zR is stimulated primarily by ACTH. While the testes produce more androgens than the zR, adrenal androgens are converted to more potent androgens in the periphery. In rodents and humans, both the zF and zR are capable of synthesizing androgens, although the sulfation of steroids appears to be specific to the zR [9,10].

This complex organization of the adrenal gland is a result of its equally complex developmental program. In the next section, the developmental steps of the adrenal gland will be outlined, with an emphasis on the underlying molecular mechanisms and the relatively new field of adrenal stem cells and maintenance.

Adrenal Development

Adrenal development is characterized by a series of discrete histological events under the control of specific molecular signaling mechanisms (Figure 1.3). The adrenal cortex originates from a cellular condensation of the coelomic

epithelium known as the urogenital ridge, which additionally gives rise to both the kidney and gonads. In the mouse, the adrenogonadal primodrium (AGP), which eventually gives rise to both the adrenal cortex and the gonads, becomes apparent at approximately embryonic day 9.5 (E9.5). An analogous initial phase also occurs in the human fetus at around the 4th week of gestation. The orphan nuclear receptor, steroidogenic factor 1 (Sf1) is a useful molecular marker to distinguish this group of cells. After this point, the adrenal and gonadal lineages become distinct. By E12.5, neural crest cells infiltrate the developing adrenal primordium, eventually becoming the adrenal medulla. The same occurs in the human at approximately the 9th week of gestation. In both humans and mice, the developing adrenal cortex is initially comprised of what is known as the fetal adrenal cortex. This fetal cortex is eventually replaced by the definitive adult cortex, and regresses in humans during the weeks following birth. In mice, the fetal cortex (sometimes referred to as the X-zone) enlarges until 3 weeks of age, then in males degenerates following puberty whereas in female mice the X-zone persists until the first pregnancy [13-16].

The expression of Sf1 is necessary for adrenal and gonadal development, as Sf1 deficient mice die shortly after birth and lack both adrenals and gonads [17]. Sf1 expression is first detectable in the mouse at E9.5, when it initiates the transcriptional signaling cascades that lead to adrenocortical development, differentiation, and maintenance. However there are numerous other regulatory factors and signaling pathways that have been implicated in adrenal development and maintenance. *Wt1,* or *Wilms Tumor 1*, is also required for

adrenal formation, and *Wt1* null mice demonstrate an adrenal aplasia phenotype [18]. Additionally, the CBP/p300-interacting transactivator with ED-rich tail 2 (*Cited2)* and the pre-B cell leukemia homeobox 1 (*Pbx1)* transcription factor have been reported to be necessary for adrenal development. In the case of *Cited2,* null mice demonstrate complete adrenal agenesis and other developmental defects [19]. It has been since shown that Cited2 interacts with Wt1 to modulate Sf1 dosage and maintain the proper threshold of gene expression necessary for adrenal development [20]. The *Pbx1* gene product has also been shown to be necessary for normal adrenal development, as *Pbx1* mutant mice have greatly reduced Sf1 expression and the complete lack of adrenal glands [21]. It has recently been shown that Pbx1 interacts with Hox and Prep1 to form a transcriptional complex that is capable of regulating the fetal expression of Sf1 unique to the fetal X-zone cells. [22]. Finally, the same report by Zubair et. al. demonstrates that Sf1 itself is involved in an auto-regulatory loop that is initiated following fetal expression of Sf1 under the regulation of the Pbx1-Hox-Prep1 complex, together defining the fetal enhancer (FAdE) of Sf1. At approximately E14.5 in the mouse adrenal, Sf1 expression is believed to shift from being driven by FAdE in the fetal cortex to being dependent upon a definitive, adult cortical enhancer (DAdE) that has yet to be defined. Lineage tracing experiments prove that adult cortical cells expressing Sf1 under the control of DAdE are derived from cells once expressing Sf1 via the FAdE enhancer [23]. These data have led to the current hypothesis that the adrenal cortex is maintained throughout life by

a population of putative stem or progenitor cells in the adrenal capsule that are derived from the early fetal cortex.

The concept of adrenal turnover and regeneration is not novel. In 1883 Gottschau proposed a theory of cellular migration in the adrenal cortex that has been experimentally supported over the years. Proliferation has been primarily observed in the outer periphery of the cortex, as enucleation (removal of the inner cell mass of the adrenal while leaving the outer capsule intact) results in the gradual regrowth of the adrenal cortex from the remaining capsule and peripheral cortical tissue, complete with functional zonation [24-26]. In addition to enucleation experiments, transplantation studies of the adrenal cortex also support the concept of continued cortical turnover and replenishment.

Until recently, however, the evidence for a population of adrenal stem or progenitor cells, and the molecular mechanisms that maintain this population has been sparse. Several reports published within the last five years have generated evidence that the Sonic hedgehog (Shh) signaling pathway may play a role in these putative adrenal stem or progenitor cells. The sonic hedgehog signaling pathway is involved in the development of numerous vertebrate organ systems [27]. Recent studies have shown that Shh ligand is expressed in the cortical subcapsular region. Ablation of Shh in the adrenal cortex results in decreased cortical size and proliferation, suggesting that Shh is necessary for the maintenance of the cortex [28-30]. Insight into the role of these Shh expressing cells comes from the elucidation of cells that respond to Shh, and hence express the downstream effector of the sonic pathway, Gli1. Interestingly, Gli1 expression

is restricted to the cells of the outer capsule of the adrenal gland, and is not expressed in the underlying cortex. Furthermore, these Gli+ cells can be lineage traced to Sf1 expressing cortical cells and indeed give rise to fully differentiated cells of the cortex. These specific studies by Ching & Vilain, King et. al., and Huang et. al. provide evidence that Gli1+/Shh-/Sf1- cells in the capsule give rise to an undifferentiated Shh+/Gli- subcapsular cell before becoming fully differentiated Sf1+/Shh-/Gli- definitive cortical cells [28-30]. Taken together, these data suggest both a signaling and cell lineage relationship between Shh expressing subcapsular cells and Gli1 expressing capsular cells that underlies the homeostatic maintenance of the adrenal cortex.

In addition to the Shh signaling pathway, other molecular mechanisms have been implicated in adrenal maintenance. The Wnt/β-catenin pathway is involved in the adrenal gland during development, and is critical for the maintenance of the adult cortex. Loss of β-catenin in the adrenal cortex during development results in complete adrenal aplasia, and continuous impairment of the Wnt/β-catenin signaling pathway in adult mice results in the gradual failure of adrenocortical replenishment and maintenance [31]. Similarly, the dosage-sensitive sex reversal, adrenal hypoplasia congenita (AHC) critical region on the X chro- mosome, gene 1 (*Dax1*) gene is an orphan nuclear receptor that functions primarily to repress Sf1-mediated transcription in peripheral cortical progenitor cells to maintain the adrenocortical stem/progenitor population. Humans with mutations in DAX1 manifest clinically with adrenocortical failure due to adrenal hypoplasia or aplasia, and mice lacking the gene exhibit a gradual

decrease in adrenal proliferation and steroidogenesis [32]. Interestingly, Wnt signaling has been shown to induce Dax1 transcription, supporting the notion that adrenocortical maintenance and regeneration is under the control of a complex regulatory network of numerous signaling and transcription factors [33].

Adrenal Neoplasms

The relationship between physiologic tissue regeneration and pathologic tumorigenesis has long been recognized, and the adrenal cortex is no different in this regard. Adrenal masses are relatively common in the general population, with an incidental detection rate of up to 10% [34]. There are numerous types of adrenal neoplasms that include metastases from other tumors and those originating from non-adrenal cell types such as myelolipomas or lymphomas. However, the three types tumor types arising from adrenal restricted cell populations are adrenocortical adenomas (ACA), adrenocortical carcinomas (ACC), and pheochromocytomas. ACAs, by virtue of their nomenclature, are generally benign, and account for up to 80% of adrenal neoplasms [34]. Pheochromocytomas are neoplasms of the adrenal medulla, and are often characterized by a clinical presentation consistent with excessive catecholamine production and can be detected by elevated serum and urinary catecholamine metabolites (metanephrins). For both ACAs and pheochromocytomas, surgical resection is often curative, and patients generally have good prognoses. In sharp contrast, ACCs represent an extremely small fraction of all adrenal

neoplasms on an annual basis, yet the clinical outcome for patients diagnosed with ACC is usually poor.

Adrenocortical carcinomas have a reported incidence ranging from 0.5-2 cases per million per year. This represents less than 1% of all annually reported cancers. ACC is an aggressive cancer, and most cases are relatively advanced when diagnosed, contributing to a 5 year survival rate of less then 10% [35]. Treatment options for ACC are limited, and ACCs are often unresponsive to standard chemotherapeutic agents [36]. Surgical resection is typically the treatment of choice for localized disease, and adjuvant therapy with mitotane (o,p'-DDD; an isomer of the insecticide DDD which is derived from DDT) is often utilized to prevent recurrence in patients. Unfortunately, the effectiveness of mitotane is limited by its toxicity [37], and while additional strategies including the addition of streptozocin to adjuvant therapy, or the use of an etoposide, doxorubicin, and cisplatin (EDP) based therapy for non-operable disease have helped increased survival, the need for more targeted ACC treatments still remain [38,39].

The genetic mechanisms that drive the development of ACC have been the subject of recent study, and may hold the key to developing novel treatment strategies for this disease. There are several genetic causes of ACC that have been described over the years, and are often associated with disease syndromes. Mutations that render the *TP53* tumor suppressor gene inactive, which define the Li-Fraumeni syndrome, can predispose affected individuals to a higher risk of developing ACC and other types of cancer [40]. The multiple

endocrine neoplasia type I syndrome (MEN1) involves genetic abnormalities in the *MEN1* gene, a putative tumor suppressor. Patients diagnosed with this autosomal dominant syndrome variably develop parathyroid pituitary, pancreatic, and occasionally, adrenal tumors [35,41]. The Wnt/β-catenin signaling pathway is also implicated in adrenal development and tumorigenesis [31,42]. Mutations in the *APC* gene, which negatively regulates β-catenin (CTNNB1*)*, are known to predispose affected patients to familial adrenal neoplams, although these often tend to be non-malignant adenomas [43]. In contrast, mutations in CTNNB1 itself that render it insensitive to regulatory factors are described primarily in cases of sporadic adrenal tumors which are also typically benign, suggesting mutations in APC or CTNNB1 alone may not be sufficient to drive the formation of ACC [44]. However, cases of ACC that demonstrate APC or CTNNB1 mutations are correlated with poorer outcome and shorter disease free survival [45]. The most common genetic abnormalities seen in sporadic ACCs, however, are those in the locus that encodes the insulin-like growth factor 2 (*IGF2)* gene. Beckwith-Wiedemann syndrome (BWS) is characterized by a loss of imprinting defect at the 11p15.5 locus containing the *IGF2, p57^{KIP2} (CDKN1C),* and *H19* genes*,* and results in excessive IGF2 expression and decreased CDKN1C and H19 expression. CDKN1C is a cyclin-dependent kinase inhibitor, and negatively regulates cell cycle progression. H19 encodes a 2.3 kb non-protein coding transcript whose function remains unknown. It is thought to potentially act as a tumor suppressor, and is able to decrease IGF2 transcript levels in vitro [46]. BWS is characterized by macrosomnia, macroglossia, organomegaly, ear and

renal abnormalities, and an increased incidence of childhood tumors including ACC [35,47]. Normally IGF2 is expressed from the paternal allele, while CDKN1C and H19 are expressed from the maternal allele. In BWS, IGF2 can be expressed from both alleles, or CDKN1C and H19 expression may be silenced entirely due to epigenetic or genetic defects at the 11p15.5 locus that alter the relative contributions of each allele [47]. Similarly, sporadic ACCs are often associated with genetic defects at the 11p15.5 locus, with increased IGF2 expression and concomitant down-regulation of CDKN1C and H19 [48]. In more recent studies utilizing microarray analysis on human ACC samples, IGF2 is confirmed as being consistently among the most highly up-regulated transcripts in ACC versus normal adrenals or even adrenal adenomas [49-52]. Furthermore, increased IGF2 expression is also correlated with increased malignancy of adrenal tumors [53].

Although there is overwhelming evidence implicating IGF2 expression in the development of ACC, the exact role of IGF2 in ACC is still not fully understood. Both IGF1 and IGF2 are known to play important roles in the development and differentiation of the normal adrenal gland. IGF receptors, IGF binding proteins, and the IGF1/IGF2 peptides are synthesized in the adrenal glands of a variety of species, and have mitogenic effects on adrenocortical cells in vitro and in vivo. These effects are mediated through the activity of the IGF1 receptor (IGF1R), which is expressed in both the normal adrenal and in ACC cell lines such as NCI-H295R [46,51]. However, while postnatal mice engineered to over-express Igf2 exhibit increased adrenal weight, they do not develop ACC

[54]. Similarly, other mouse models engineered to increase Igf2 expression and decrease the function of either H19 or Cdkn1c fail to develop ACC, despite the fact that they more closely recapitulate clinical features of BWS [55,56]. These reports suggest that neither increased Igf2 expression nor dysregulation of the 11p15.5 locus alone are sufficient to cause ACC. Therefore, other genetic defects must also be present. Recent work from our lab provides evidence of a synergistic role for defects in both the Wnt/β-catenin signaling pathway and *Igf2* locus, supporting a genetic "multi-hit" progression leading to the development of ACC. Research into the genetic causes of ACC has led to the investigation of new therapeutic targets in this rare yet aggressive cancer. Recent work on IGF2 signaling in ACC has led to the initiation of clinical trials for drugs targeting this pathway, which show significant promise [51,57]. Additional research may yet provide ACC patients with new, effective treatments for this disease.

MicroRNAs

The first microRNA (miRNA) was described by Lee et. al. in 1993, when the heterochronic *C. elegans* gene *lin-4* was found to encode small RNAs with complementary sequences to the 3' untranslated region (UTR) of the *lin-14* gene transcript [58,59]. At the time, this observation remained largely a novelty of post transcriptional gene silencing in the nematode, and these small RNAs were initially referred to as small temporal RNAs (stRNAs) due to their temporally regulated expression and their role in regulating developmental timing [60]. It quickly became evident that these newly described stRNAs functioned in a post-

transcriptional gene silencing (PTGS) role, and had some aspects in common with PTGS phenomena that had been previously observed in plants.

PTGS had been described in plants, when it was observed that transgenes introduced into petunias with the intent of over-expressing an enzyme necessary for flower pigmentation had the opposite effect [61]. Although what Napoli had reported was eventually recognized as a specific type of PTGS known as RNA interference (RNAi), it was not until later in the decade that Fire and Mello described this phenomenon in greater detail [62]. In the years following the discovery of *lin-4* in nematodes, similarities between these new stRNAs (now known as miRNAs) and the RNAi pathway were noted. For example, the ~22 nucleotide length of mature stRNAs was recognized to be nearly identical to that of RNAs involved in RNAi [63,64], and the downstream genes and mechanisms necessary for proper processing of RNAs destined for RNAi mediated gene repression appeared to be shared by stRNAs. For example, both RNAi and stRNAs were soon demonstrated to require the activity of Dicer, an RNase III enzyme, and both also require a class of proteins known as Argonautes, which catalyze their PTGS activity [65]. However, researchers also observed several key differences that suggested that despite their similarities, RNAi and stRNAs were two distinct pathways. First, RNAi was described as primarily the result of exogenously supplied double stranded RNA molecules, while stRNAs were derived from the organism's genome [62]. Second, RNAi was shown to induce degradation of target mRNA transcripts [63,66], while stRNAs were initially described as inducing translational repression

[67]. Finally, *lin-4* does not form perfect duplexes with its cognate target, *lin-14* [68], unlike RNAs involved in the RNAi pathway which typically rely on perfect sequence complementarity with their targets [69].

Seven years following the discovery of the *lin-4/lin-14* relationship in nematodes, several breakthroughs were reported in the field of both RNAi and miRNAs. The RNA directed effector protein complex (RNA Induced Silencing Complex [RISC]) and RNase III enzyme (Dicer) required for RNAi were described, which allowed researchers to better characterize the mechanistic similarities between RNAi and miRNA mediated gene silencing described above [64,70-72]. Additionally, a second miRNA was discovered. In 2000, the heterochronic regulatory RNA, *let-7,* was discovered and described in detail, including its conservation across the animal kingdom [60,73,74]. This confirmed that miRNAs were not simply a curiosity in nematodes, but belonged to a post transcriptional gene silencing pathway that is conserved through metazoan evolution. Within one year, miRNAs were recognized as a distinct subclass of non-coding, phylogenetically conserved RNAs within the genome [75], and reports describing additional miRNAs began to accumulate [76-78]. It was collectively agreed by leading researchers of the emerging miRNA field to refer to this new subclass of RNAs as miRNAs, displacing the previously used terminology of heterochronic and small temporal RNAs. By the end of the decade, the field of miRNAs had expanded exponentially, and today, our understanding of their functions in development, physiology, and disease continues to grow. Current research has shed significant light on the

evolutionary and genomic origins of miRNAs, their cellular biogenesis, and the mechanisms by which they repress the expression of target genes. As discussed in the subsequent sections of this introduction, it is now known that miRNAs are expressed from endogenous loci within the genome of an organism, and function by inducing the degradation of target mRNA transcripts to repress gene expression. Furthermore, miRNAs can affect multiple target genes, and are therefore closely integrated in gene regulatory networks as a result of this pleiotropism. miRNA biogenesis has also been demonstrated to be necessary for the embryologic development in mammals, and specific miRNAs are capable of regulating physiologic processes such as cell renewal and differentiation. Finally, miRNAs have been implicated in the regulation of disease processes, particularly the development of human cancers. As discussed in this introduction, miRNAs can act as both tumor suppressors and oncogenes by regulating gene transcripts that are responsible for mediating tumorigenesis, and recently, have shown promise in their use as prognostic markers in human cancer.

MicroRNA Biogenesis

Micro-RNAs are derived from endogenous loci within the genome of an organism and undergo progressive processing steps to form a mature miRNAs (Figure 1.4). The majority of mammalian miRNAs can be found in clusters of functional transcriptional units. Approximately 80% are transcribed from intronic regions of either protein or non-protein coding transcriptional units, while a

minority may be found in exons [79-82]. Additionally, many mammalian miRNAs have multiple paralogues of each other, presumably due to gene duplications that occurred during the phylogeny of the organism [82]. These paralogues often have identical sequences in the “seed” region (positions 2-7 relative to the 5’ end of the miRNA) and are therefore thought to be redundant, although differential expression in vivo may suggest distinct roles for these various isoforms [83]. Biogenesis of miRNAs requires multiple processing steps of the initial miRNA transcript, and is under multiple layers of regulatory control [84,85].

miRNAs are primarily transcribed by RNA polymerase II (Pol II) and many primary transcripts are capped and polyadenylated, characteristic of Pol II transcription [86-89]. However, a small number of miRNAs can be transcribed by RNA polymerase III [90]. The use of Pol II allows for the control of miRNA transcription by the range of Pol II associated transcription factors. For example, p53 and the Tcf/Lef transcriptions factors associated with Wnt/B-catenin signaling are able to directly regulate transcription of miR-34 and miR-483, respectively [91,92]. As a result, miRNAs transcribed by Pol II can be specifically regulated under a number of conditions and cell types.

The primary miRNA transcripts (pri-miRs) generated by RNA polymerases can vary in length from several hundred bases to several hundred kilobases and carry local stem loop structures [86,93]. The first step in the canonical miRNA processing sequence involves endonucleolytic cleavage at the stem of the hairpin structure, which releases a small hairpin known as a precursor miRNA (pre-miR). This process is mediated by the Microprocessor complex, a putative

650kD multimeric protein complex which includes the RNAse III enzyme Drosha [94,95] and the dsRNA binding protein Dgcr8 [96,97]. The Microprocessor recognizes potential pri-miRs for processing by the single stranded RNA sequences that flank the ~33bp stem of the hairpin structure. Dgcr8 interacts with this portion of the pri-miR, and in conjunction with Drosha, cleaves 11 base pairs away from the single strand/double strand junction at the hairpin base [98]. The Microprocessor complex itself is under complex regulation, including an auto-regulatory loop whereby Drosha cleaves its own co-factor, Dgcr8 [99]. Additional regulation at this point in the miRNA pathway has been attributed to transforming growth factor beta (TGF-β) and bone morphogenic protein (BMP) signaling. It has been demonstrated that following signaling by BMPs or TGF-β, activated downstream Smad proteins can increase the recruitment of pri-miRs to the Microprocessor complex, enabling more efficient cleavage by Drosha. This mechanism is believed to occur in part by the ability of Smad1, 3, and 5 to bind a nucleotide sequence in the stem structure of specific pri-miRs that resembles the consensus sequence recognized by the Smad DNA binding domain. Additionally, activated Smads can also directly bind to the RNA helicase p68, a subunit of the Microprocessor complex; this interaction is required for Smad mediated pri-miR processing. Although miR-21 and miR-199a were among the first miRNAs described to be subject to this regulatory mechanism, subsequent studies have demonstrated numerous other miRNAs that can respond to BMP/TGF-β signaling in this manner [100,101].

Following Drosha mediated cleavage, the pre-miRNA is then transported out of the nucleus by Ran-GTP and the Exportin-5 nuclear transport protein [102]. The pre-miR sequence is inconsequential for recognition by Exportin-5, as the length of the hairpin and 3' overhangs appear to be sufficient [103]. Once in the cytoplasm, another RNAse III enzyme, Dicer, in complex with the transactivator RNA (tar) binding protein (TRBP) [104], cleaves the hairpin loop from the stems. The resulting ~22 nucleotide Dicer-Trbp bound RNA duplex is then joined by an Argonaute (Ago) protein, and a glycine tryptophan repeat containing 182kD protein (GW182) to generate the miRISC effector complex. One strand of the ~22 nucleotide duplex is retained as the guide strand or mature miRNA. The other strand is referred to as the passenger strand or miRNA* (miRNA "star" strand) and is degraded. Generally, selection of which strand becomes the miRNA* is dependent on the relative thermodynamic stability of the two ends of the duplex. The strand with the less stable 5' end is retained, while the other becomes the miRNA* strand [105]. Once incorporated into the Ago-RISC complex, a mature miRNA is then able to exert its post-transcriptional regulatory effects on target mRNAs. Due to the partially complementary binding between miRNAs and target sequences in the 3' UTR of mRNA transcripts, it is possible for a given miRNA to potentially target and bind to multiple mRNAs [106,107]. This phenomenon has the effect of allowing a single miRNA to have potentially pleiotropic downstream effects, and complicates the identification of mRNA targets. In metazoans, miRNA binding sequences almost invariably reside in the 3' UTR of target transcripts, and are often present in multiple copies.

Recent reports suggest some miRNAs can be generated through several non-canonical pathways. For example, a number of intronic miRNAs known as "mirtrons" have been shown to mature without Drosha mediated cleavage if they are of the proper size to form a hairpin consistent with a pre-miR. In this case, the hairpin consists of the entire host intron and bypasses Drosha mediated cleavage. Instead, mRNA splicing is responsible for the first step of biogenesis, and the pre-miR is excised as an intronic lariat structure that is subsequently linearized, then exported directly to the cytoplasm for further downstream processing by Dicer. These mirtrons are characterized by flanking sequences that correspond to the 5' splice site if a 5' miRNA is formed, or the 3' splice site if a 3' miRNA is formed [108,109]. This is in contrast with canonical intronic miRNAs, which still require subsequent processing by Drosha following transcription to form a pre-miR that can be exported to the nucleus for Dicer processing. Mirtrons have subsequently been described in additional organisms including mammals, plants, and avians using deep sequencing techniques [110-112]. More recently, additional miRNA biogenesis pathways have been described in mammalian cell lines. Three independent reports were published in the summer of 2010 that describe the biogenesis of miR-451, an unusually highly conserved miRNA shown to be important for erythropoiesis [113]. Further investigation into miR-451 demonstrates that its maturation is dependent on the endonuclease activity of Ago2, and is refractory to Dicer inactivation [114-116]. The implications for this are significant, as miR-451 is perfectly conserved in vertebrates, suggesting that the retention of Ago2's unique catalytic activity

among the mammalian Argonaute proteins may be evolutionarily constrained to maintain this non-canonical miRNA biogenesis pathway. Additionally, Yang et. al. also demonstrate the ability of a reprogrammed miR-451 backbone to confer Dicer independent expression of other miRNAs, a feature which could show promise as a molecular tool to aid in the study of miRNAs [115]. Finally, a recent report by Havens et. al. claims to have identified yet another non-canonical miRNA biogenesis pathway, in which the biogenesis of the predicted mirtrons miR-1225 and miR-1228 is independent of most of the canonical miRNA biogenesis components, including Dgcr8, Dicer, Exportin-5, or Ago2 [117]. Instead, the authors report that Drosha appears to be the only component required for the maturation of these miRNAs, which they term splicing-independent mirtron-like miRNAs (simtrons). However, the authors were unable to identify the binding partners and additional components of this newly described simtron biogenesis pathway, and further research is necessary to elucidate this novel mechanism.

MicroRNA Mediated Silencing Mechanisms

The canonical miRNA mediated gene regulatory pathway requires the binding of mature miRNAs to their cognate mRNA targets through partially complementary sequences in the mRNA's 3' UTR. There are several experimentally and bioinformatically defined rules involving miRNA-mRNA interactions [118,119]. First, positions 2-7 (the seed region) must generally be continuous and perfectly matched, as mismatches in this sequence greatly affect

miRNA mediated repression; second, bulges or mismatches must be present in the central region of the miRNA to preclude endonucleolytic cleavage by Ago2; finally, there must also be partial complementarity in the 3‘ end of the miRNA to ensure stability [119-121]. Although these factors are not exhaustive in describing miRNA-mRNA interactions, they are considered to be among the most important. Recently, it has been demonstrated that bulged pairing at nucleotide positions 5-6 in the miRNA-mRNA pair can in some instances provide enough stability for the propagation of the seed interaction [122]. This observation, which was primarily seen in the mouse brain, has the potential to expand the number of predicted miRNA binding sites that would need to be considered when performing target prediction analyses.

Since their discovery in 1993, the mechanism by which miRNAs are thought to inhibit their mRNA targets has been a controversial subject. Evidence supports the possibility of both transcriptional degradation and/or translational repression, (Figure 1.5) and initially, miRNAs were thought to primarily function through the latter mechanism [123,124]. Indeed, this was recognized as one of the hallmark differences between canonical RNAi and miRNA mediated repression in plants, both of which are primarily characterized by degradation of the transcriptional target. There have been four distinct methods proposed to explain miRNA mediated translational inhibition in animals: inhibition of translational initiation, inhibition of elongation, co-translational protein degradation, and premature termination of translation [123,124].

Translation of mature mRNAs into protein broadly falls into 3 discrete stages: Initiation, elongation, and termination. The majority of miRNA mediated mRNA decay mechanisms involve perturbation of the initiation and elongation steps [118,125]. Additionally, miRNAs have been reported to facilitate the destabilization of target mRNAs through removal of the m^7G cap (decapping) [126,127] and the poly A tail (deadenylation) [128-130]. In the initiation stage of eukaryotic translation, the eIF4E subunit of the eukaryotic translation initiation factor (eIF4) binds to the m^7G cap at the 5' end of the mRNA. The other subunits in eIF4 recruit and stabilize the 40S ribosomal subunit. At the 3' end, polyadenylate binding protein 1 (PABP1) binds to the poly A tail of the mRNA, and in turn binds to the eIF4G subunit of eIF4. In doing so, the mRNA becomes circularized, and translation is initiated. Elongation can then begin when the 60S ribosomal subunit joins the protein complex at the AUG start codon [131].

Inhibition of translational initiation by miRNAs is thought to involve the Argonaute proteins that are bound with mature miRNAs as part of the RISC. It has been shown that human AGO2 has sequence similarities with the eIF4E translation initiation factor subunit that binds to the m^7G cap of mRNAs to begin the initiation process. In addition, AGO2 is able to bind a sepharose bound m^7G analog in vitro and suggests that binding of AGO2 to the m^7G cap can displace eIF4 factors and prevent initiation [132]. Both cell culture and cell free systems supported this notion, and also showed that non-cap dependent translation driven by an internal ribosomal entry site (IRES) was not silenced by miRNAs [126,127,133]. Subsequent studies in the fly and nematode also provided

evidence that the miRNA silencing machinery targets the mRNA cap structure or interferes with the cap binding complex [134,135]. In addition, Chendrimada et. al. reported that eIF6 and the 60S ribosomal subunit co-immunoprecipitate with AGO2 and other miRISC components [136]. By binding eIF6 and its associated 60S ribosomal subunit, AGO2 may be able to prevent the association of the small and large ribosomal subunits, inhibiting initiation. However, this is a controversial subject regarding miRNA mediated translational inhibition, as Eulalio et. al. demonstrated in a subsequent report that eIF6 does not appear to be required for miRNA mediated silencing [137].

Experimental evidence also supports the notion that miRNAs can repress target mRNAs at the post-initiation or elongation stages of translation. Early experiments in *C. elegans* showed that miRNA targets remained associated with polysomes despite a marked reduction in protein expression [67,138]. Other experiments in mammalian cells involving sucrose sedimentation experiments supported the theory that miRNAs may be blocking elongation at a later translational stage following initiation [139-141]. These studies showed that miRNAs appeared to associate with their mRNA targets undergoing translation, but without the protein product being detectable. To explain these results, Nottrott et. al. [140] proposed a model of co-translational degradation, where the nascent polypeptide is degraded as it exits the ribosome. In contrast, Petersen et. al. [141] suggested that miRNAs might induce the premature termination of translation, resulting in the drop-off of ribosomes from the target transcript, resulting in an incomplete and unstable polypeptide.

In yet another mechanism, miRNAs have been shown to induce the degradation of their mRNA targets, complementing their reported ability to induce translational repression. In plants, mRNA target degradation is believed to be the most common mechanism of miRNA mediated gene silencing, similar to the mechanism seen in RNAi. In contrast, animal miRNAs, until recently, were thought to repress targets mainly through translational inhibition, as outlined above. However, earlier studies reported the ability of animal miRNAs to degrade their cognate mRNA targets [142-144], raising the possibility that miRNA mediated mRNA degradation might be more common in animals than originally thought. In eukaryotes, mRNA degradation follows two primary pathways which are both initiated by a shortening of the poly A tail. Following this deadenylation step, mRNAs can be degraded 3‘ to 5‘ by the exosome, or degraded 5‘ to 3‘ following removal of the m^7G cap [145]. It has been shown that miRNA mediated mRNA degradation in animals is dependent on the 5’ to 3’ mRNA decay pathway in which they are first deadenylated [128,129,146,147]. This deadenylation step appears to be facilitated by the CAF1-CCR4-NOT deadenylase complex, which is required for miRNA mediated deadenylation as depletion of these components results in the up-regulation of miRNA targets [146,148,149]. Genome-wide studies with the goal of determining the contribution of both translational repression and transcriptional degradation to miRNA mediated gene silencing have shown that animal miRNAs have only a modest effect on the translation of mRNA transcripts [150] [151]. More recently, Guo et. al. demonstrated using ribosomal profiling techniques in mammalian cells

that ≥84% of miRNA mediated repression occurs due to mRNA transcript destabilization, while only a small fraction of repression is due to reduced translational efficiency [152]. These data together support the notion that mammalian miRNAs function primarily at the mRNA transcript level, and have minor effects on protein synthesis. Furthermore, in cases where translational inhibition does occur, it appears to do so at the translational initiation stage [152,153]. These observations are inconsistent with the previously proposed models of nascent polypeptide degradation or premature ribosomal drop-off.

Despite these advances made in delineating the roles of translational repression and mRNA degradation in miRNA mediated gene silencing, the temporal order of miRNA mediated silencing is still being elucidated. Recent studies now support a more parsimonious model that is able to unify the observed miRNA mediated gene silencing mechanisms of translational inhibition and mRNA degradation. These reports provide data that suggest miRNAs initially inhibit translation of their target mRNAs, but then subsequently induce degradation of the mRNA transcript [154-156]. Research into the GW182 protein, a core component of the miRISC, has demonstrated its ability to mediate both translational repression and deadenylation of target mRNAs [157]. Indeed, it was observed in earlier reports that GW182 is critical for miRNA silencing, as depleting this protein abrogates miRNA activity [137,158]. It is now believed that GW182, through its C-terminal “silencing domain” facilitates in part the silencing effects of miRNAs by interacting with poly-A binding proteins (PABP) and recruiting the CCR4-NOT deadenylase complex (reviewed in [159])

Emerging Role of MicroRNAs in Development & Cancer

Since their discovery in 1993, miRNAs have been implicated in developmental processes, as *lin-4* null nematodes exhibit developmental defects consistent with inappropriate *lin-14* expression [58,59]. Given the ubiquitous and highly conserved nature of miRNAs, it is therefore not surprising that miRNAs play critical roles in the developmental regulation of higher organisms. The first *Dicer* KO mouse was described by Bernstein et. al. in 2003 [160]. In this mouse model, the authors report that loss of *Dicer* in the developing mouse is embryonic lethal at approximately embryonic day 7.5 (E7.5), and is accompanied by loss of embryonic stem cells (ES cells) in mutant embryos. Indeed, subsequent studies have confirmed the role of *Dicer* in regulating pluripotency and differentiation of stem cells in various models [161-163]. Since then, numerous tissue specific knockout models in the mouse have confirmed the importance of *Dicer* in the development of various organ systems [164-170]. The necessity for *Dicer*, and presumably miRNA biogenesis, through the course of organismal development is irrefutable. However, the subsequent steps to further understand this observation involve uncovering the underlying mechanisms of specific miRNAs in the development of the organism.

Another rapidly advancing area of research involving miRNAs is the investigation into their role in disease processes, especially tumorigenesis. It has been established that genetic mutations that perturb the miRNA biogenesis pathway are implicated in tumorigenesis. Mutations in TRBP2 not only impair miRNA biogenesis, but have also been identified in sporadic and hereditary

carcinomas [171], as have inactivating mutations in Exportin-5, which result in the trapping of pre-miRNAs in the nucleus to inhibit miRNA biogenesis [172]. Also, several reports within the last year have emerged that describe heterozygous loss of function mutations of the human *DICER* gene in the context of several familial syndromes. The patient cohorts studied presented with a heterologous pattern of tumors that includes sertoli-leydig cell tumors of the ovary, embryonal rhabdomyosarcomas, and pleuropulmonary blastomas [173-175]. In addition, sporadic perturbations in DICER expression have been reported in numerous cancer types [176-182]. However, until recently, little data implicating total DICER loss in tumorigenesis was available. This was addressed in a recent report demonstrating that homozygous Dicer null murine sarcoma cells exhibit increased doubling time and higher apoptotic activity compared to heterozygous Dicer null cells [183]. When injected into mice, these Dicer null sarcoma cells are able to maintain their tumorigenicity, albeit with slight impairment compared with heterozygous Dicer null cells. In this case it would appear that complete loss of Dicer activity slightly inhibits tumorigenesis, raising the possibility that inhibiting Dicer activity in vivo may hold viable therapeutic use.

Individual miRNAs have also been implicated in the process of tumor formation and metastasis, with many of them acting as tumor suppressors or oncogenes. The ability of miRNAs to inhibit multiple genetic factors involved in tumorigenesis, and the observation that many of these same genetic factors can induce the expression of miRNAs has led to the understanding that miRNAs and their targets form complex regulatory networks. In many cases, these networks

consist of feed-back and feed-forward regulatory loops, demonstrating that miRNAs that integrate themselves into key oncogenic pathways can result in an intricate balancing effect between pro- and anti- oncogenic signals. As a result, miRNAs can act as tumor suppressors if they target transcripts that encode oncogenic proteins, and conversely, act as oncogenes if they target known tumor suppressors. For example, one of the most closely studied miRNA families, let-7, is able to repress several oncogenes including HMGA2, RAS, and MYC [184-186], thereby functioning as a tumor suppressor. Conversely, MYC is able to repress let-7 expression [187], and is able to repress the widespread expression of other miRNAs [188], including those with anti-tumorigenic and pro-apoptotic activity such as miR15a/16-1, miR-26, and miR-34 family members [187]. In addition, MYC can induce the expression of the miR-17-92 polycistronic cluster [189], which has been demonstrated to repress negative regulators of the PI3-kinase signaling pathway, and tumor suppressor proteins such as BIM, PTEN, and CDKN1A [190-192]. The miRNAs encoded by the miR-17-92 cluster are often amplified in lymphoma, small cell carcinoma of the lung [193]. They have also been shown to be expressed in developing mouse tissue [194], and deletion of the miR-17-92 cluster is embryonic lethal [83], illustrating its role in both tumorigenesis and development.

Similarly, the network of tumor suppressor proteins in the cell is also highly integrated with miRNA input and regulation. A canonical example is that of the regulatory network linking miRNAs and the tumor suppressor gene *P53*. The expression of miRNAs belonging to the miR-34 family have been demonstrated

to be directly induced by P53 [195]. These miRNAs have been shown to promote cell cycle arrest, senescence, and apoptosis by negatively regulating the expression of proteins that inhibit these processes, and include targets such as BCL2 and CDK4 [91]. More recent publications have reported other miRNAs that appear to be induced by P53, including miR-192, miR-194, miR-215, and miR-605 [196,197]. These miRNAs target MDM2, an important negative regulator of P53, suggesting a miRNA mediated feed forward regulatory loop initiated by P53 activation. miR-149* has also been shown to be up-regulated in human melanoma in response to P53. Intriguingly, miR-149* acts as an oncogene by inhibiting glycogen synthase kinase 3α and increasing the expression of Mcl1, an anti-apoptotic protein that inhibits PUMA, a pro-apoptotic protein that is also induced by P53 activation [198]. In contrast, miR-380-5p and miR-504 act to repress P53 and promote cellular survival and tumorigenesis [199,200]. Likewise, miR-372 and miR-373 have been demonstrated to inhibit P53 mediated apoptosis in testicular germ cell tumors [201]. Finally, in an even more complex interaction with miRNAs, P53 has been demonstrated to modulate miRNA processing through an association with P68 and Drosha [202] that promotes pri-miR processing. In this model, it would be possible for mutant P53 to interfere with this regulation, and in addition to the direct effects of P53 loss on downstream anti-tumor mechanisms, could result in decreased expression of cancer relevant miRNAs.

Lastly, miRNAs are an area of interest regarding their use as diagnostic and cancer therapy tools. For example, let-7 is often down-regulated in lung

cancer [203], miR-196a is up-regulated in pancreatic ductal carcinoma [204,205], and miR-483-3p, miR-483-5p, and miR-21 are up-regulated in adrenocortical carcinoma [206,207]. Because many miRNAs are uniquely and differentially expressed in certain tissues, their expression profiles in conjunction with clinical context could be used to determine various qualitative and quantitative characteristics of tumors such as tissue of origin, stage, grade, and overall outcome. Further empirical data regarding miRNA expression in various cancers is still required, however, and will likely be an ongoing area of study for the foreseeable future. The potential use miRNAs as therapeutic targets has yet even more progress that must be made before this concept becomes a practical clinical reality. Currently, molecular tools such as synthetic antisense oligonucleotides (antagomirs) designed to inhibit specific miRNAs [142], miRNA "sponges" [208], and miRNA mimics that can behave as endogenous miRNAs show considerable effect and practicality in many in vitro models, and in the case of antagomirs, some success in vivo [209]. However, many challenges remain, such as safe and efficient delivery of therapies designed to perturb miRNAs involved in a disease state. Target specificity is another concern, as off target effects of miRNA based therapy could have significant side effects in normal, healthy tissue given the relative ubiquity of miRNAs and their biogenesis pathway.

Despite the progress made over the past 20 years in understanding the biologic functions of miRNAs, it is clear that much work remains to be completed to elucidate mechanisms of miRNA mediated regulation in development and

disease. This work addresses the role of Dicer and miRNA biogenesis in the developing mouse adrenal gland, as well as investigating the correlation between miR-483-3p and IGF2 expression in human adrenocortical carcinoma. It is hoped that the work described herein will provide novel avenues for further research into adrenal miRNA expression and function.

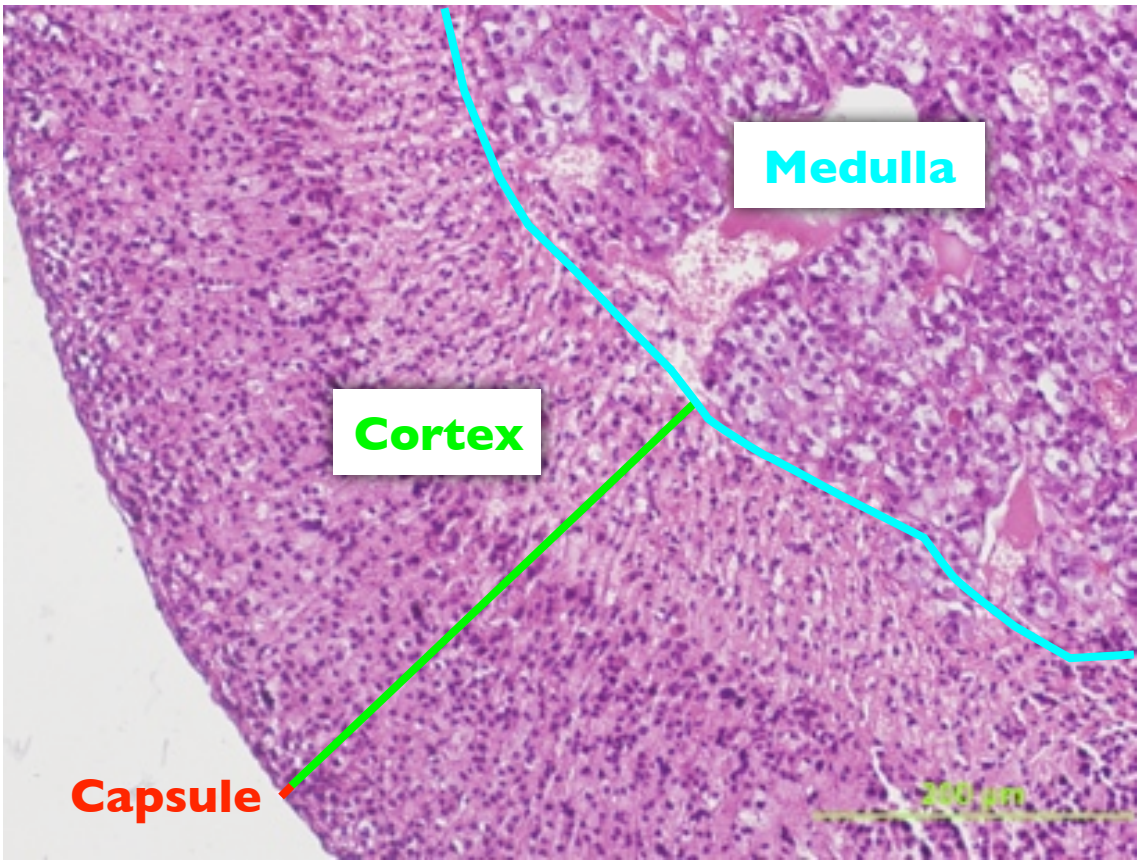

Figure 1.1 Histology of mouse adrenal.

Hematoxylin & eosin stain of an adult mouse adrenal gland illustrating the cellular zonation characteristic of the adrenal. The outer-most **capsule** surrounds the gland, and is derived from mesenchymal cells of the stroma around the developing gland. The **cortex** is further subdivided into the zona glomerulosa (zG), zona fasciculata (zF), and zona reticularis (zR). The zR is not present in rodents, but is the source of androgen precursors in primates. The **medulla** consists of neuroendocrine cells derived from the neural crest, and synthesizes catecholamines such as epinephrine and norepinephrine. Scale bar = 200 μm.

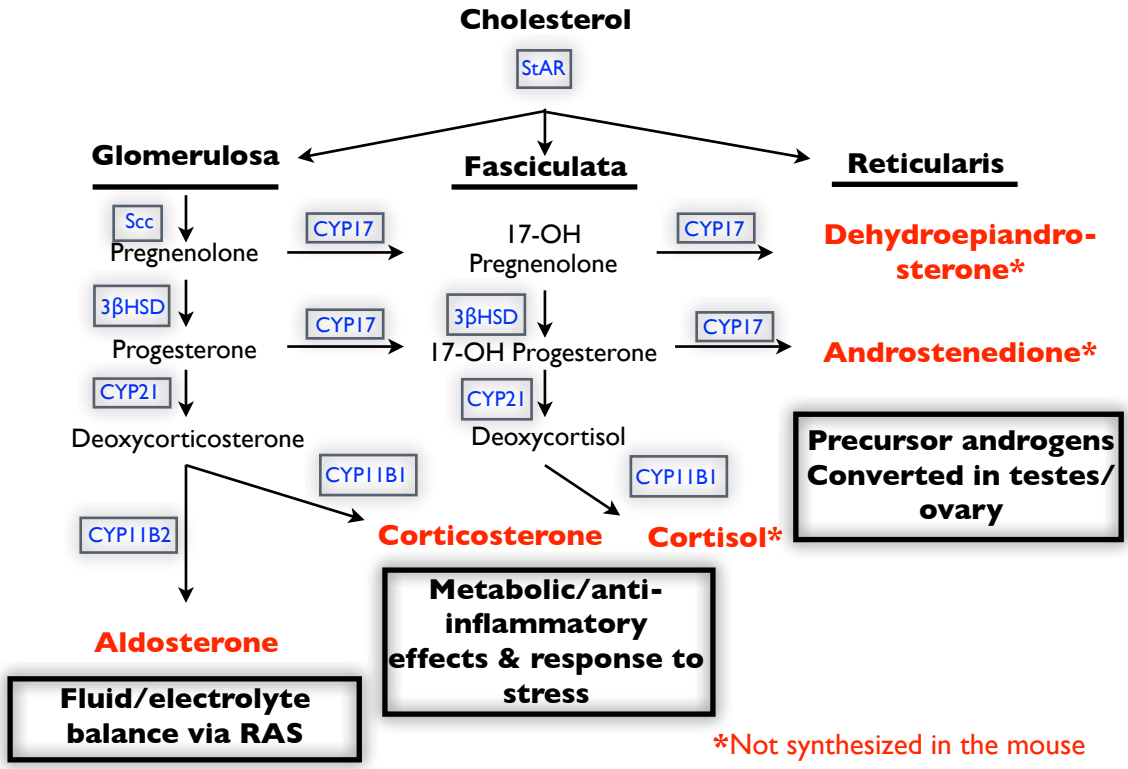

Figure 1.2 Steroidogenic pathways of the adrenal cortex

Flow chart illustrating the steroidogenic pathways in each of the cortical zones of the adrenal gland. Mice lack the Cyp17 enzyme, and therefore, cannot produce cortisol or precursor androgens. The predominant glucocorticoid produced by the mouse adrenal is corticosterone.

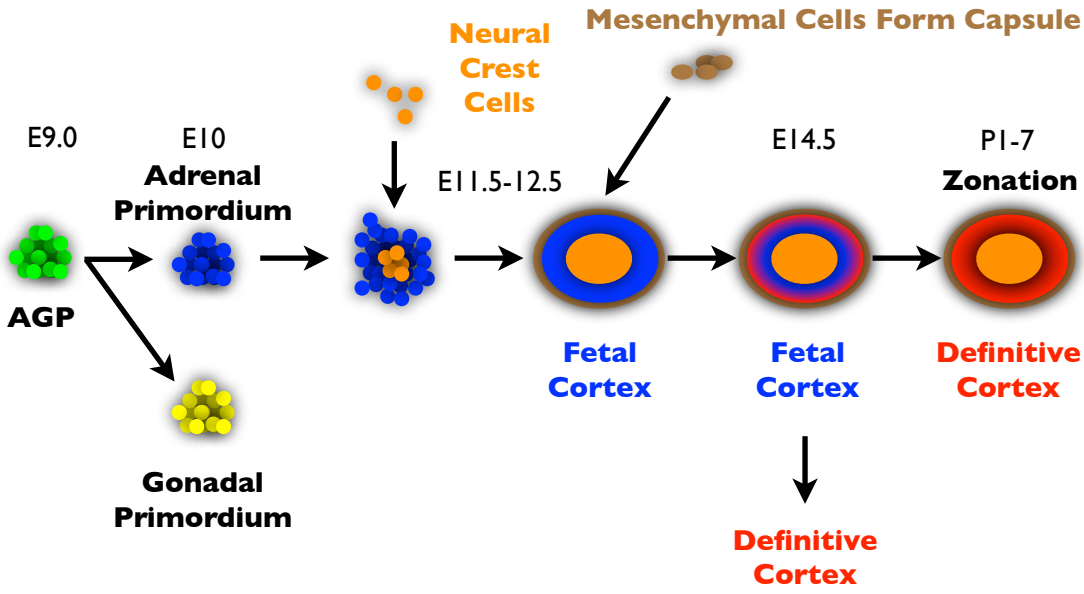

Figure 1.3 Adrenal development in the mouse

Adrenal development in the mouse begins at E9.0 with the coalescence of the adrenogonadal primordium (AGP), a bi-potential mass of cells that gives rise to both the adrenal cortex and gonads. The orphan nuclear receptor Steroidogenic Factor 1 (Sf1) is detectable in the AGP at this time, and acts as a master regulator for adrenal and gonadal development. Loss of Sf1 results in adrenal and gonadal agenesis. Following the separation of the adrenal and gonadal primordia, the fetal cortex begins to coalesce beginning at E10. Shortly thereafter, cells from the neural crest migrate into the developing fetal cortex to populate what will become the adrenal medulla. The adrenal capsule is derived from mesenchymal cells in the surrounding stroma, and is hypothesized to be be the location in which adrenal stem/progenitor cells reside. At approximately E14.5, the fetal cortex begins to be displaced by the adult or definitive cortex. Transcriptional control of Sf1 is believed to shift from the use of a fetal enhancer to a still undefined definitive enhancer. As development progresses, the fetal cortex is continuously displaced by the expanding definitive cortex. In rodents, the fetal cortex persists in females until the first pregnancy, whereas in males the fetal cortex fully disappears by 3 weeks of age.

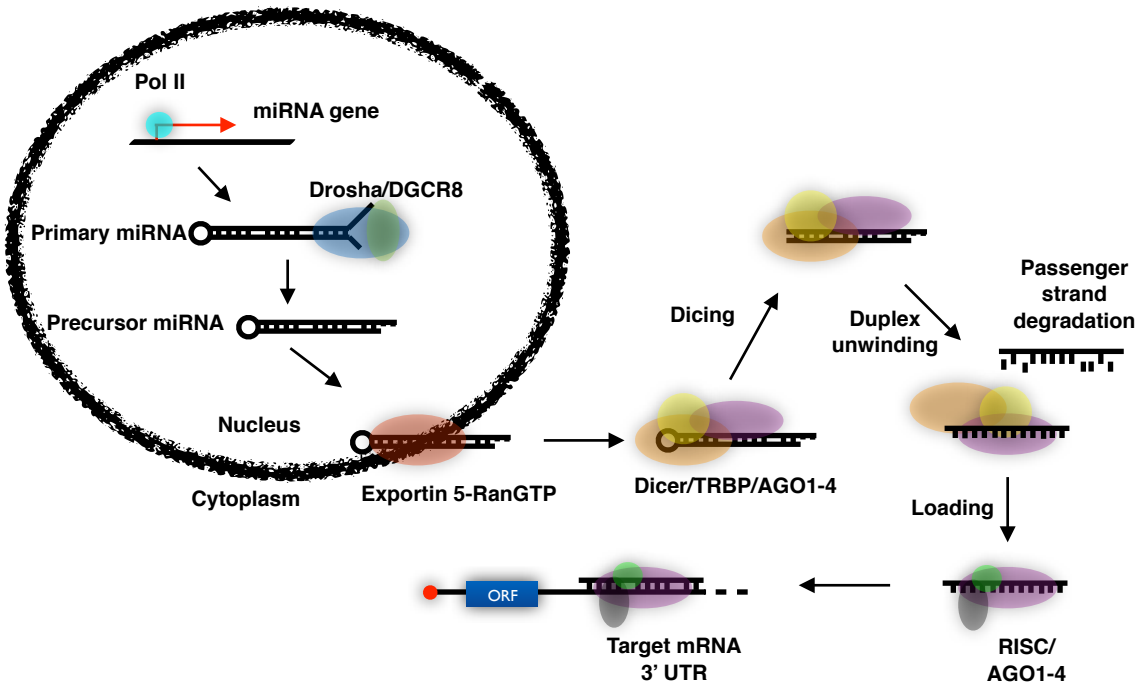

Figure 1.4 miRNA biogenesis

Canonical miRNA biogenesis in animals. miRNAs are found in discrete transcriptional units in the genome, often in clusters, and can also be found in coding or non-coding regions of protein expressing genes. Most are transcribed by RNA polymerase II, and the resulting primary miRNA is ~70 nucleotide hairpin structure that is recognized and cleaved by the Microprocessor complex consisting of the Drosha RNase III enzyme and its cofactor, DGCR8. The resulting product is known as a precursor miRNA (pre-miR), and is transported out of the nucleus into the cytoplasm via the Exportin-5 nuclear transport protein. In the cytoplasm, a protein complex consisting of Dicer, TRBP, and Argonaute (AGO) proteins removes the loop from the pre-miR, and helicases unwind the duplex. One strand of the duplex is retained (typically the strand with the less stable 5' pairing) and the other is degraded. The remaining strand is incorporated into the miRNA-induced silencing complex (miRISC), a protein complex that facilitates downstream mRNA repression mediated primarily Argonaute proteins and the GW182 protein. The miRISC bound miRNA binds to target mRNAs through partially complementary sequences located in the 3' untranslated regions of the RNA transcript.

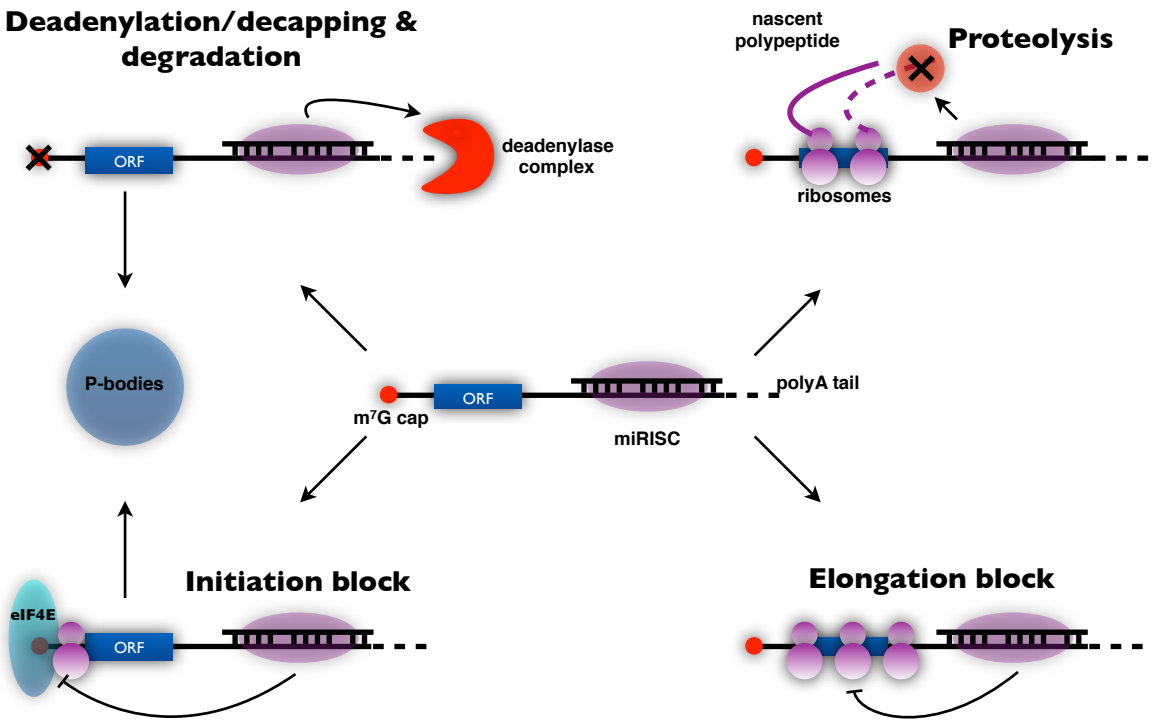

Figure 1.5 Mechanisms of miRNA mediated post transcriptional gene regulation

Evidence for multiple mechanisms of miRNA mediated gene regulation has been observed, and the main proposed methods are described here. miRNAs have been demonstrated to induce the destabilization of target mRNAs by the deadenylation and subsequent decapping of the target transcript. Current research suggests this is the predominant mechanism in miRNA mediated gene silencing. Additional proposed mechanisms involve translational repression, either by preventing the formation of the translational initiation complex, proteolysis of the nascent peptide as it exits the ribosome, and inhibition of translational elongation. Current theory holds that miRNAs induce translational repression at the initiation step, then subsequently facilitate the degradation of target mRNAs. This parsimonious model helps reconcile the conflicting data regarding miRNA mediated gene silencing mechanisms.

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CHAPTER 2

Correlation Between MicroRNA-483-3p and IGF2 in Human Adrenocortical Carcinoma

This chapter is comprised of in vitro work performed to establish the correlation between IGF2 and miR-483-3p expression in human ACCs. All work was performed independently under the mentorship of Gary Hammer, MD, PhD. I would also like to acknowledge Guido Bommer for his assistance in designing the miR-483 sponge, and Victoria Kelly for her assistance with luciferase assays.

Introduction

Adrenocortical carcinoma (ACC) is a rare yet highly aggressive endocrine malignancy, with an incidence ranging from 0.5-2 cases/year per million people, and accounts for less than 1% of all reported cancers [1]. Prognosis for patients with ACC is unfortunately poor, with a 5 year survival rate of 22% [2]. Unfortunately, most patients with ACC present with advanced metastatic disease, where the 5 year survival is less than 10%, and most cases of ACC present with this advanced form of the disease [1]. Treatment options for ACC have historically been extremely limited. Surgical resection is treatment of choice for localized disease. Additionally, radical ACC resection in conjunction with adjuvant mitotane therapy and radiotherapy (XRT) has been demonstrated to

prolong recurrence-free survival in patients presenting with advanced disease [3-6]. However, the benefits of mitotane are relatively modest, and use of the drug at therapeutically effective doses is limited by toxicity [5]. While small studies suggest some benefits of alternative adjuvant therapies [7], ACC has the distinction of being relatively unresponsive to most chemotherapuetic treatments [8].

There are only a few well characterized genetic causes of sporadic adrenocortical tumorigenesis that have been described over the years. Inactivating mutations in the *TP53* tumor suppressor gene, which is the cause of the Li-Fraumeni syndrome, are known to predispose affected individuals to the development of ACC, among other types of cancer. Additionally, mutations in the *APC* gene, which are characteristic of the adenomatous polyposis coli syndrome, can lead to the development of adrenal neoplasms, although these tend to often be non-malignant adenomas. The multiple endocrine neoplasia type 1 syndrome (MEN1), which is associated with a predisposition towards the development of endocrine tumors, is also associated with the formation of adrenal adenomas, and to lesser extent, carcinomas [1,8]. The most common genetic abnormalities seen in sporadic ACC however, involve those in the locus that encodes the insulin-like growth factor II (*IGF2*) gene. Nearly 90% of sporadic ACCs are shown to have an abnormal up-regulation of IGF2 expression [9], overwhelming evidence that supports the association between IGF2 over-expression and the development of ACC. Indeed, the Beckwith-Wiedemann syndrome, which is due to a loss of imprinting defect at the 11p15.5 locus that results in excessive IGF2

expression, is strongly associated with ACC [10,11]. IGF2 is known to be a mitogen for adrenocortical tissue [12], and signals through the ubiquitously expressed type 1 IGF receptor (IGF1R), whose expression is also up-regulated in many ACCs [13]. Based on this body of work, current therapeutic efforts targeting IGF2 signaling in ACC have shown significant promise in clinical trials [14,15].

While IGF2 itself has been implicated in ACC, a microRNA locus, miR-483, is located within the 2nd intron of the *IGF2* gene. MicroRNAs (miRNAs) are small, endogenous, non-protein coding RNAs that effect post transcriptional regulation by targeting partially complementary “seed” sequences in the 3’ UTR of target mRNAs. In conjunction with the miRISC complex, miRNAs are able to facilitate the destabilization and degradation of their target mRNA transcripts, and repress translation of target mRNAs [16,17]. Although initially overlooked, the importance of miRNAs in the regulation of oncogenic processes is now widely appreciated. Perturbations in DICER expression have been described in numerous cancer types, with conflicting data on the usefulness of over-expression versus under-expression of DICER as a predictor of prognostic outcome. For example, in chronic lymphocytic leukemia, lung, breast, and ovarian cancers, lower expression of DICER is associated with poor prognosis [18-21]. On the other hand, increased DICER expression is correlated with worse outcome in primary cutaneous T-cell lymphomas, colorectal carcinoma, prostate cancer, and triple negative breast cancer [22-25]. These data suggest the presence of more complicated mechanisms besides simple

gain or loss of miRNA biogenesis in tumorigenesis. Lastly, heterozygous germline mutations in DICER have been identified in several familial syndromes that are characterized by pleuropulmonary blastoma, multinodular goiter, ovarian cancer, and Sertoli-Leydig cell tumors [26-28].

In addition to perturbations in DICER expression, individual miRNAs have recently gained attention for their roles in the pathogenesis of tumor formation. Many of the miRNAs identified regulate proliferation, differentiation, or apoptotic pathways; these miRNAs have been shown to function as both oncogenes or tumor suppressors, and are referred to as 'oncomirs' [29-31]. Current research suggests that many of these oncomirs hold potential as therapeutic targets or prognostic biomarkers indicative of the severity of a tumor [32,33].

In this study, we aimed to determine whether IGF2 and miR-483 expression cold be correlated in human ACC samples and the human ACC cell line, H295R. As previously described, the IGF2 mRNA levels were significantly higher in ACC samples compared to normal adrenals [14]. miR-483 transcript levels, specifically the miR-483-3p strand, were similarly high in ACCs versus normal adrenal tissue samples. To further study miR-483-3p, we constructed a lentiviral over-expression vector capable of expressing mature miR-483 at levels similar to ACCs and the H295R cell line to aid in the investigation of potential miR-483-3p targets and physiologic effects. Additionally, we tested a luciferase sensor and miRNA "sponge" for miR-483 to also aid in pursuing these goals. We were successful in constructing these molecular tools, but were unable to elucidate the mechanistic aspects of miR-483-3p expression in vitro.

Materials and Methods

Human Tissue Samples

A subset of frozen tissue samples from a larger sample set consisting of human normal adrenals (n=3) and both low IGF2 expressing (IGF2-LOW; n=5) and high IGF2 expressing (IGF2-HIGH; n=6) ACCs were kindly donated by Dr. Thomas J. Giordano, Departments of Pathology and Internal Medicine, University of Michigan Medical School, Ann Arbor MI [9]. All patient data were kept strictly confidential in accordance with institutional IRB guidelines.

RNA Isolation

RNA isolation on human tissue samples was performed using a modified TRIzol Reagent protocol (Ambion, Life Technologies, Carlsbad, CA). Briefly, following the phase separation step in the manufacturer's protocol, the aqueous phase was removed and subject to an acid phenol chloroform separation step using an equal volume of 5:1 phenol:chloroform solution, pH 4.5 (Life Technologies, Carlsbad, CA). After spinning to phase separate the sample, the aqueous phase was removed and the RNA was precipitated with 0.5mL 100% isopropanol, 1µL linear acrylamide, and 10 µL 3M sodium acetate pH 4.5 (Life Technologies, Carlsbad, CA). Samples were mixed by inversion, then stored at -20°C overnight. The samples were then spun for 10 minutes at 4°C in a microcentrifuge to pellet the RNA, then washed with 1mL of 75% ethanol before being spun again for 5 minutes at 4°C, and finally resuspended in up to 100µL of

nuclease free water. RNA was quantified on a Beckman DU640 spectrophotometer.

Quantitative Real Time PCR for IGF2 and miR-483-3p

For RNA isolated from human tissue samples, up to 200ng of RNA was reverse transcribed using the iScript system (Bio-Rad Laboratories, Hercules, CA) to generate cDNA. The resulting cDNA was diluted 1:5 in nuclease free water, and 2μL was used for downstream amplification with appropriate primers using Power SYBR Green PCR Master Mix and an ABI 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA). Data analysis was performed using the $2^{-\Delta\Delta C(T)}$ method [34]. Primers for each amplified gene are as follows: β-Actin (*ACTB*) Fwd 5'-CCAACCGCGAGAAGATGA and Rev 5'-TCCATCACGATGCCAGTG; Igf1 (*IGF1)* Fwd 5'-TGTGGAGACAGGGGCTTTTA and Rev 5'-ATCCACGATGCCTGTCTGA; Igf2 (*IGF2)* Fwd 5'-GCTGGCAGAGGAGTGTCC and Rev 5'-GATTCCCATTGGTGTCTGGA; IGF1 receptor (*IGF1R)* Fwd 5-'AAAAACCTTCGCCTCATCC and Rev 5'-TGGTTGTCGAGGACGTAGAA. All transcripts were normalized to β-Actin. Taqman based miRNA quantitative real time PCR kits for miR-483-3p and U6 control RNA were purchased from Applied Biosystems by Life Technologies (Carlsbad, CA) and used according to the manufacturer's instructions to determine relative expression of miR-483-3p. Correlation between miR-483-3p and IGF2 transcript levels was done using linear regression analysis on

miR-483-3p and IGF2 ΔCt values for each sample with the Graphpad Prism software suite (La Jolla, CA).

miR-483 Over-expression Vector

To generate a vector capable of stably expressing miR-483-3p at high levels, a 550 base pair product that consisted of the pre-miR-483 hairpin structure and 305 and 170 base pairs of flanking sequence upstream and downstream, respectively, was amplified using a high fidelity Platinum Taq DNA Polymerase from H295R genomic DNA. The 550bp product was gel purified and cloned into a pCRII-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), then subcloned into the EcoRI restriction site in the MCS of the pMSCV-Puro pro-viral vector (Clontech, Mountain View, CA). The over-expression vector was confirmed by DNA sequencing (University of Michigan Sequencing Core), grown and isolated in Dh5α E. Coli cells, and transfected into the SW13 cell line. Transfected cells were grown in DMEM supplemented with 10% fetal bovine serum, antimicrobials, and 3μg/μL puromycin to select for cells containing the over-expression vector. Confirmation of mature miRNA expression was assayed with the TaqMan based miRNA assay kit (Applied Biosystems) and normalized to U6.

miR-483 Sponge

Biological “sponges” constructed by inserting predicted miRNA binding sites has been described previously [35]. Complementary sense and antisense

oligonucleotides bearing 3 miR-483-3p binding sites (5'-AAGACGGGACCTAGGAGTGA-3'; perfect matches in the seed region with a non-complementary bulge in the central region of the sequence) in tandem and separated by spacers of 4 nucleotides were synthesized and purchased from Invitrogen. The oligos were annealed in vitro to generate a double stranded DNA construct with EcoRI compatible sticky ends. The pLentiLox 3.7 (pLL3.7) proviral vector (kindly donated by Guido Bommer) was digested with EcoRI, mixed with the DNA insert (sponge) containing miR-483-3p binding sites, and annealed using standard molecular cloning procedures. Insertion and orientation of the DNA insert was confirmed with DNA sequencing to ensure that the binding sites were properly inserted in the 3‘ UTR of the GFP reporter in the proviral vector. Lentiviral packaging was performed by the University of Michigan Vector Core. H295A and H295R cells were grown in DMEM F12 supplemented with 10% fetal calf serum, antimicrobials, and L-glutamine. Cells were transduced with 1mL 1x viral supernatant and 8µg/mL polybrene. After 24 hours, the media was replaced with fresh DMEM F12, and the cells monitored for GFP expression. Proliferation assays were performed by plating GFP positive H295R cells at a starting density of 5x10^5 cells/well in 12 well plates. Cells were followed and harvested on days 1, 2, 3, and 4 (short term) or days 3, 4, 5, and 6 (long term), and counted on a hemocytometer. To assess the transcript levels of the predicted miR-483-3p targets IGF1 and EGR1, the SYBR based semi-quantitative real-time PCR method described in the above section was used. Primer sequence for IGF1 was as above, and primers for EGR1 were as follows:

Fwd 5'-AGCCCTACGAGCACCTGAC and Rev 5'-GGTTTGGCTGGGGTAACTG. Expression data from H295 cells transduced with the miR-483-3p sponge were expressed relative to H295 cells transduced with control plasmid.

miR-483-3p Sensor Luciferase Assays

To generate luciferase reporters sensitive to miR-483-3p mediated knockdown, a DNA construct consisting of 2 perfectly complementary sites (5'-AAGACGGGAGGAGAGGAGTGA-3') to mature miR-483-3p was inserted into the EcoRI and SpeI sites downstream of the luciferase reporter in a modified pGL3Control plasmid (Promega, Madison, WI). Insertion was confirmed by DNA sequencing. H295R, H295A, or SW13 cells expressing the miR-483-3p over-expression vector were plated in 24 well plates at 5x10^4 cells/well. 24 hours after plating, cells were transiently transfected and harvested 48 hours later. Renilla luciferase (pRL-TK, Promega, Madison, WI) was co-transfected into cells and utilized to measure transfection efficiency. Cell lysates were assayed for luciferase activity using the Dual Luciferase Assay kit (Promega, Madison, WI) on an auto-injector luminometer.

Results

miR-483-3p Expression Correlates Strongly with IGF2 in Primary Human ACCs and Human ACC Cell Lines

We first determined whether IGF2 and miR-483 might be co-expressed in ACC. Previous microarray data from our lab, in collaboration with Dr. Tom Giordano (Depts. of Internal Medicine and Pathology, University of Michigan Medical School) have shown that increased IGF2 expression in human ACC samples contributed to the distinct genetic profile that separated ACCs from ACAs and normal adrenals (Figure 2.1). Further investigation revealed the existence of an annotated miRNA, miR-483, located in the second intron of the *IGF2* gene. The miR-483 locus, like most miRNA loci, is capable of producing two mature sequences, designated by a -3p or -5p suffix, depending on which strand of the precursor duplex is selected for miRISC loading. Given the strong correlation between ACC and IGF2 over-expression, we considered whether miR-483-3p, the more abundant product of the miR-483 locus, would strongly correlate with IGF2 expression and therefore, be characteristic in many cases of ACC.

Normal human adrenal tissue, and human ACC samples consisting of both low and high IGF2 expressing tumors were provided by Dr. Tom Giordano as part of our collaboration. We performed quantitative real time PCR for IGF2, IGF1, IGF1R, and ACTB (β-Actin) as an internal normalization control. Additionally, numerous human cell lines were also tested for IGF2 expression, including the NCI-H295A and NCI-H295R human ACC cell lines. Figure 2.2A

displays the significantly higher expression of IGF2 in human ACC samples relative to normal adrenals; the H295A/H295R human ACC cell lines also displayed very high IGF2 transcript expression relative to the RL251 cell line, a human ACC derived cell line that does not express IGF2. Also, non-ACC human cell lines (HEK293, HeLa, SW13) did not show elevated IGF2 expression. Following confirmation of IGF2 over-expression in primary human ACCs and human ACC cell lines, mature, endogenous miR-483-3p expression was measured in both sets of samples. TaqMan based quantitative real time PCR showed that like IGF2, miR-483-3p expression was significantly up-regulated in primary human ACCs, but not normal human adrenals. Likewise, the high IGF2 expressing human ACC cell lines H295A and H295R exhibited very high levels of endogenous miR-483-3p, whereas the non-IGF2 expressing RL251 cell line did not (Figure 2.2B).

We confirmed the observed correlation between IGF2 and miR-483-3p expression in primary human ACCs and human ACC cell lines by performing linear regression analysis on the ΔCt values calculated for IGF2 and miR-483-3p (relative to internal normalization controls ACTB and U6) for each sample tested. Figure 2.3A shows the correlation between IGF2 and miR-483-3p expression among primary human adrenal samples, while 2.3B illustrates the same correlation in human cell lines. Both analyses indicate that the correlation between IGF2 and miR-483-3p expression is robust, with R^2 values of .8021 and .9683 for primary human ACCs and human cell lines, respectively. The human ACC cell lines H295A and H295R demonstrated similar expression

profiles for IGF2 and miR-483-3p, suggesting that these cell lines may serve as a convenient in vitro model of IGF2/miR-483-3p high human ACC.

miR-483-3p Over-Expression Vector

Several molecular tools were developed to facilitate further study of miR-483-3p and its potential function in the pathogenesis of ACC. A miR-483-3p over-expression vector was developed to allow for stable expression in cell lines. In conjunction with sensor reporter constructs and knock-down tools specific for miR-483-3p, this construct was designed to facilitate the identification of potential mRNA targets, and the isolation of pathophysiologic effects of perturbing the level of intracellular miR-483-3p.

Figure 2.4 illustrates the cloning strategy utilized to generate the miR-483-3p over-expression vector. This expression vector would be predicted to express both the -3p and -5p miRNA products; however, we focused our analysis on the -3p product due in part to technical limitations of the TaqMan qPCR reagents that were available at the time. In addition to providing robust expression of the gene of interest, this vector can be directly transfected into cells, or can be packaged into viral particles to transduce cell lines that may be difficult to transfect using chemical means. A similar strategy has been shown previously in the past to be effective for the artificial expression of miRNAs [36]. SW13 (a cell line with low IGF2 expression) cells were transfected with either empty pMSCVpuro (pMSCV-Control) or miR-483 expressing plasmid (pMSCV-

miR483), then grown and passaged in cell culture media containing puromycin to maintain selective pressure for transfected cells.

We first assessed SW13 cells transfected with pMSCV-miR483 for mature miR-483-3p using a TaqMan quantitative qPCR assay specific for mature miR-483-3p (Figure 2.5). SW13 cells transfected with the pMSCV-miR483 vector expressed significantly more mature miR-483-3p than control SW13 cells transfected with pMSCV-Control vector. The amount of miR-483-3p expression was comparable to what was observed in H295R cells used as positive controls. These results indicate that the miR-483-3p expression vector was capable of robustly producing mature miRNA transcripts in SW13 cell lines. Although quantitative real time PCR was able to detect mature miR-483-3p in SW13 cells transfected with the pMSCV-miR483 vector, it was unknown whether the transcripts being produced were functionally active and capable of silencing gene targets.

A Luciferase Sensor and GFP 'Sponge' for miR-483-3p

At the time of these experiments, mRNA targets of miR-483-3p were not known. Although the target prediction algorithms used by Targetscan (www.targetscan.org) offered significant possible targets with phylogenetically conserved miR-483-3p binding sites, the lack of an empirically confirmed target made functional studies of miR-483-3p difficult. This made constructing an inhibitor to miR-483-3p challenging as there was no known positive control (i.e. a validated, endogenous target 3' UTR containing miR-483-3p binding motifs)

against which we could compare the efficacy of our inhibitor. To attempt to circumvent this limitation, we developed an artificial luciferase sensor construct that contained perfect complementary sequences to mature miR-483-3p. Using this approach, it is possible to make miRNAs behave similarly to siRNAs in mammalian cells by slightly modifying the 3' UTR target sequence of a reporter construct to be perfectly complementary to the miRNA sequence. Indeed, miRNAs in plants generally exhibit perfect sequence matching between the miRNA sequence and target mRNAs. Additionally, this perfect base pair matching commonly leads to the degradation of target mRNAs, characteristic of mammalian siRNA and plant miRNA pathways, and may provide a potentially greater degree of target inhibition.

To generate a miR-483-3p specific luciferase sensor, complementary oligonucleotides bearing two repeats of a sequence that perfectly match the mature miR-483-3p sequence flanked by EcoRI sticky ends was synthesized (See Materials & Methods). These oligos were then annealed in-vitro and cloned into pGL3-Control downstream of the luciferase stop codon and upstream of the PolyA sequence. If both the pMSCV-miR483 expression vector and the pGL3-Sensor constructs were functioning as intended, decreased luciferase activity would be observed in SW13 cells that stably expressed the pMSCV-miR483 expression vector. Indeed, SW13 cells expressing miR-483-3p exhibited less luciferase activity when the miR-483-3p luciferase sensor was introduced. In contrast, SW13 cells stably expressing the pMSCV-miR483 expression vector did not show decreased luciferase activity when pGL3-Control plasmid lacking the 3'

miR-483-3p binding sites was introduced (Figure 2.7). Likewise, SW13 cells stably transfected with the pMSCVpuro-Control plasmid were unable to decrease luciferase activity from either pGL3-Control or pGL3-Sensor plasmids. Taken together, these data demonstrate that the miR-483-3p transcript expressed from the pMSCVpuro-miR483 vector was able to inhibit a luciferase sensor designed to specifically respond to miR-483-3p.

The pGL3-Sensor construct was then tested to determine if it would be able to respond to endogenous miR-483-3p, as opposed to the artificial expression construct utilized in SW13 cells. pGL3-Sensor or pGL3-Control were transfected into both H295A and H295R cells that express high levels of endogenous miR-483-3p. Endogenous miR-483-3p expressed by these cell lines was able to significantly decrease the relative measured luciferase activity (Figure 2.8). Interestingly, it appeared that the H295R (295R) cells were more effective at repressing the miR-483-3p specific luciferase sensor, as measured relative luciferase activity was reduced by up to 3-fold whereas luciferase repression in H295A (295A) cells was less than 2-fold.

In the final part of this series of experiments, a biological sponge was designed to “soak” the activity of endogenous miR-483-3p by providing multiple miR-483-3p binding sites in a GFP reporter gene. The rationale behind this technique has been described previously, but briefly, the sponges are intended to be competitive inhibitors of endogenous miRNAs that are expressed from strong promoters and driven by RNA polymerase II [35]. A construct was cloned bearing multiple bulged miR-483-3p binding sites to into the 3’ UTR of the GFP gene in

the pLentilox 3.7 lentiviral vector. The plasmid was packaged into viral particles to overcome the inherent difficulty of transfecting the H295 cell line and a viral transduction approach was used to attain strong levels of GFP expression. While the cloning and transduction of the miR-483-3p sponge was successful, we were unable to conclusively determine if the sponge was functional. This was due in part to the lack of data regarding validated miR-483-3p targets, against which we could compare the effects of the sponge. Instead, we chose to assess proliferation because IGF2 is a known mitogen in ACC. We hypothesized that the co-expression of miR-483-3p may have a similar function, and therefore a functional miR-483-3p sponge might have a negative effect on proliferation of cells expressing this miRNA. However, pilot experiments that analyzed the proliferation of NCI-H295 cells transduced with the miR-483-3p sponge construct were inconclusive. Growth curves of H295 cells transduced with the miR-483-3p sponge relative to control plasmid were erratic and inconsistent over the time course of both a short term (4 days) and long term (6 days) experiment (Figure 2.9). The total number of viable cells at each experimental endpoint, however, were consistently lower in H295 cells transduced with the miR-483-3p sponge. The results from these pilot experiments did not definitively support the hypothesized effects on proliferation stemming from miR-483-3p inhibition, indicating the sponge may not have functioned effectively and may require further optimization, or that miR-483-3p does not play a significant role in promoting cellular proliferation as hypothesized.

We also determined a list of predicted miR-483-3p mRNA targets that, according to the array data cited previously, were down-regulated in IGF2/ miR-483-3p expressing primary human ACC samples. This list consisted of BMPR2, EGR1, H3F3B, IGF1, MLLT6, QKI, TMOD1, USP46, and VAMP2. We focused on IGF1 and EGR1 (Early growth response protein 1), as IGF1 is a known adrenal mitogen, and EGR1 has been demonstrated to possess tumor suppressor properties. Quantitative real-time PCR for IGF1 and EGR1 transcripts in H295 cells transduced with the miR-483-3p sponge demonstrated a slight increase of EGR1 expression relative to H295 cells transduced with control plasmid (Figure 2.10). In contrast, H295 cells transduced with the miR-483-3p sponge responded inconsistently with regard to IGF1 expression. Half of the replicates analyzed demonstrated a further decrease in IGF1 transcript, contrary to the expected result, and a only single replicate showed a marked increase in IGF1 expression. Together these data suggest the miR-483-3p sponge had a modest effect on de-repressing EGR1 transcript levels in transduced H295 cells. However, the data on IGF1 transcript levels in cells transduced with the miR-483-3p sponge were inconclusive, and would require additional investigation.

Discussion

This chapter describes the efforts to investigate the correlation between IGF2 expression in primary human ACCs and the expression of miR-483-3p, a miRNA that is located within the second intron *IGF2*. We also attempted to generate potential molecular tools that could aid in the study of miR-483-3p and its functional role in the human ACC cell line H295R. Our data, while preliminary in nature, supported the correlation between IGF2 and the co-expression of miR-483-3p with its host gene. We also performed several experiments to analyze potential physiological effects of miR-483-3p inhibition in vitro by testing a miRNA "sponge" designed to specifically respond to miR-483-3p.

The experiments designed to assess proliferation in H295 cells transduced with the sponge yielded inconclusive results, which may be due to several factors. One likely explanation may be due to poor efficacy of the sponge designed in this study. We designed the sponge with three potential miR-483-3p binding sequences that consisted a perfect seed region match and non-complementary bulge in the central region of the miRNA-target sequence. It is possible that three binding sites were not sufficient to effectively inhibit endogenous miR-483-3p activity in H295 cells, which express very high levels of this miRNA. Additionally, the binding sequences in the sponge were separated by only four nucleotides, which may have resulted in steric hindrance of miRISC bound miR-483-3p, preventing access to all three binding sites. Another explanation is that miR-483-3p does not significantly affect proliferation, and that its host gene, IGF2, primarily mediates the proliferative capacity of H295 cells.

Indeed, IGF2 has been specifically implicated as an autocrine regulator of proliferation in the NCI-H295R human ACC cell line [37]. Finally, there may be additional uncharacterized molecular pathways independent of miR-483-3p or IGF2 that promote proliferation in the H295 cell line.

We also compared a list of predicted miR-483-3p targets using the TargetScan algorithm (www.targetscan.org) with down-regulated gene transcripts found in primary human ACCs that express high IGF2 and miR-483-3p. Of the nine predicted targets to be down-regulated in human ACCs, we were interested to discover that IGF1 and EGR1 were among those target genes. IGF1 is a known adrenal mitogen, and in humans, is co-expressed in the adult adrenal in conjunction with IGF2. In ACC, IGF2 is up-regulated, and IGF1 expression often decreases, recapitulating the embryonic adrenal in which IGF2 is the predominant growth factor [12]. The prediction of IGF1 as a target of miR-483-3p raises the possibility of a regulatory mechanism in which IGF1 expression is repressed indirectly by IGF2. This could be a potential mechanism that maintains the balance between IGF1 and IGF2 expression in the adult adrenal.

Finally, EGR1 is an interesting predicted miR-483-3p target because of its tumor suppressor function that has been reported in the literature [38,39]. EGR1 is a zinc-finger transcription factor that has been demonstrated to induce the expression of p53, PTEN, and c-Jun, and is down-regulated in a variety of cancers including glioma, lymphoma, and carcinoma of the breast [40-44]. In contrast, EGR1 is often up-regulated in prostate cancer [45,46], and intriguingly, has been demonstrated to induce the expression of IGF1R [47], the key IGF2

receptor in ACC. However, given that EGR1 is down-regulated in the primary human ACCs analyzed in our array, this suggests that EGR1 may act in the role as a tumor suppressor in the context of ACC. In this case it would be reasonable to hypothesize that as a predicted target of miR-483-3p, EGR1 expression is inhibited in ACCs that express high levels of IGF2, contributing to the tumorigenesis of ACC.

More recent studies into the potential targets of miR-483-3p in the context of cancers have reported that miR-483-3p does indeed have a pro-oncogenic function in human neoplasms including Wilm's Tumor, colon, breast, and liver tumors. These same reports have also identified a potential role for miR-483-3p as an oncogene that inhibits apoptosis and promotes proliferation in the human ACC cell line H295R by repressing the pro-apoptotic protein, p53 up-regulated modulator of apoptosis (PUMA) [48,49].

Our data appeared to show robust correlation between IGF2 and miR-483-3p expression, suggesting the expression of the latter is dependent on the former. In addition, it has been recently demonstrated that expression of miR-483 can be induced by the Wnt/β-catenin signaling independent of IGF2, adding an additional layer of complexity to the regulation of miR-483 expression [50]. Recent studies have shown that other intronic miRNAs, most notably miR-21, can be regulated independently of their host genes in a similar manner. In the case of miR-21, it has been shown that TGF-β signaling can promote the processing and expression of miR-21 through a positive interaction between Smads, the downstream effector proteins of TGF-β signaling, and Drosha, the

RNase III enzyme responsible for processing primary miRNA transcripts into precursor transcripts ready for nuclear export [51,52]. Considering that both the Wnt/β-catenin and TGF-β signaling pathways are implicated in the process of tumor formation, the discovery that these signaling pathways can directly promote the expression of miRNAs involved in oncogenesis such as miR-483-3p and miR-21 means consideration must be given to identifying the targets of these miRNAs implicated in cancer.

The potential involvement of β-catenin in regulating the miR-483 locus is intriguing, as abnormal nuclear localization of β-catenin, indicative of active signaling, is characteristically associated with poorer prognosis and outcome in human ACC cases [53,54]. It has further been reported that Wnt/β-catenin targets are over-expressed in cases of human ACC [55]. Although IGF2 or β-catenin dysregulation is most often associated with poor outcome and prognosis in ACC, there are few studies that evaluate the simultaneous effects of both IGF2 and β-catenin dysregulation on ACC development. Igf2 over-expression alone in mouse models fails to induce ACC, although these mice exhibit non-malignant adrenal defects consistent with the Beckwith-Wiedemann syndrome seen in humans [56,57]. Mice engineered to constitutively express β-catenin have been reported to develop adrenal hyperplasia, dysplasia, increased adrenal vascularization, and ultimately neoplasia in aging mice [58]. This model of progressive adrenal pathology is similar to the multi-hit progression seen in colorectal cancer, suggesting that single genetic defects alone may not be enough to promote the development of ACC, and that other genetic hits are

necessary for carcinogenesis. A long term study conducted in our lab to investigate the potential synergistic effects of simultaneous dysregulation of Igf2 active β-catenin in the mouse adrenal gland is currently in press [59] and provides evidence supporting the hypothesis that multiple genetic hits may be required for the tumorigenesis of ACC.

We did not assess the specific human ACC samples used in this study for dysregulated β-catenin expression; nor did we assess ACC samples with high β-catenin expression for miR-483-3p. As such, we cannot comment as to whether β-catenin could be in part responsible for the high levels of miR-483-3p that are seen these samples. Given that miR-483-3p has oncogenic properties by repressing PUMA, leading to decreased apoptosis, it is possible that the IGF2 and β-catenin dysregulation seen in human ACCs converge to a common pathway that involves miR-483-3p and its repression of PUMA and other genes. Further studies would need to be performed to investigate this possibility in both ACCs and other cancers characterized by increased β-catenin expression.

The field of miRNAs and the understanding of their involvement in development and disease has expanded tremendously since we initially began these studies. Although we were successful in generating several molecular tools that might be useful in studying the effects of miR-483-3p in vitro, the abundance, quality, and cost effectiveness of commercially available tools that have rapidly come on the market has made working with miRNAs significantly faster. With the increased availability of commercially produced molecular tools designed to study the function of individual miRNAs, it has become much easier

for researchers to perturb miRNAs of interest in cell culture systems, or even whole animal models. To illustrate this, while we were investigating the relationship between IGF2 and miR-483-3p in the NCI-H295 cell lines, the previously cited reports correlating miR-483-3p with poorer disease prognosis and establishing PUMA as a target were published by an independent laboratory [48]. As a result, we therefore elected to expand our studies by addressing the role of miRNAs in adrenal development by utilizing a genetic knockout approach that will be described in Chapter 3. However, there is still much work that can be pursued regarding the interplay between miR-483-3p, IGF2, and ACC. Future studies involving IGF2 and miR-483-3p would benefit greatly from the recent advances in techniques and molecular tools aimed at facilitating miRNA research.

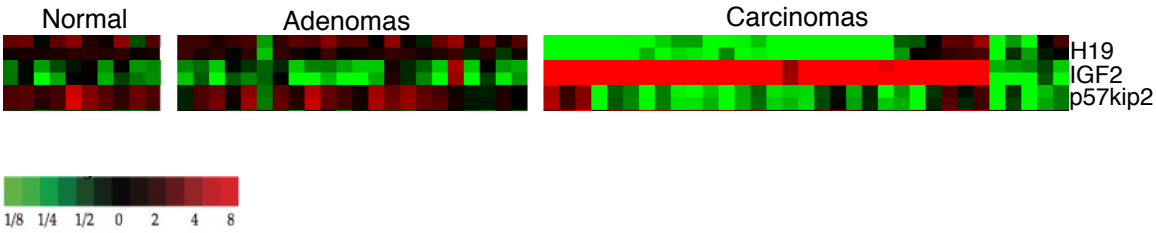


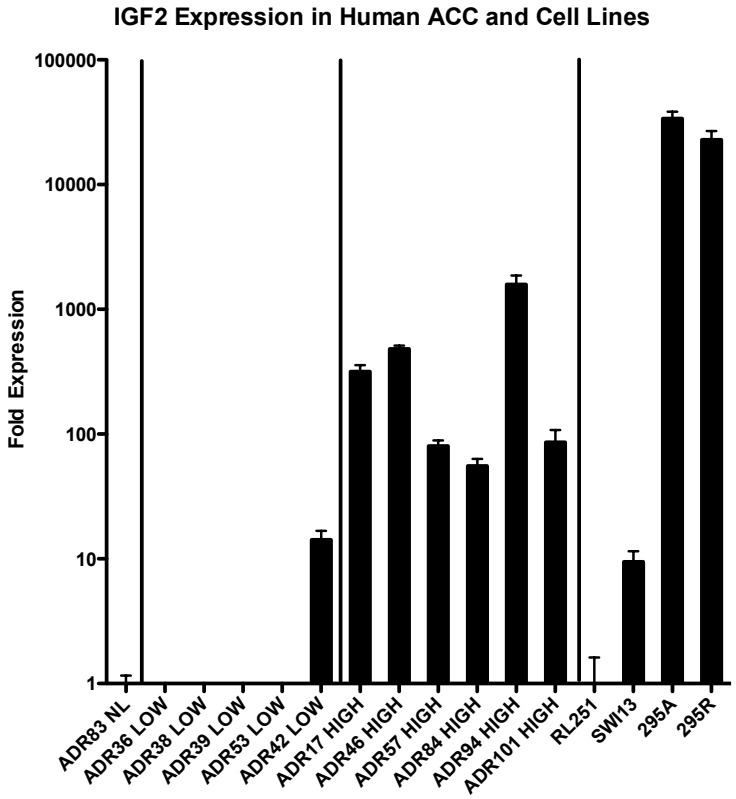
Figure 2.1 Tissue Classification & IGF2 Locus Heatmap of Human Adrenal Samples.

A heat map showing the histological classification and expression levels of the *IGF2* locus genes at 11p15.5 in normal human adrenals, adrenal adenomas, and adrenal carcinomas.

Figure 2.2 Relative fold expression of IGF2 and miR-483-3p in human adrenal tissue samples and cell lines.

Quantitative real-time PCR for both IGF2 (A) and miR-483-3p (B) transcript expression in human adrenal (normal, high IGF2, low IGF2) samples and human cell lines. Fold expression is relative to normal human adrenals (for ACC samples) and the RL251 cell line (for human cell lines).

A


IGF2 Expression in Human ACC and Cell Lines
Fold Expression
100000
10000
1000
100
10
1
ADR83 NL
ADR36 LOW
ADR38 LOW
ADR39 LOW
ADR53 LOW
ADR42 LOW
ADR17 HIGH
ADR46 HIGH
ADR57 HIGH
ADR84 HIGH
ADR94 HIGH
ADR101 HIGH
RL251
SW13
295A
295R

B

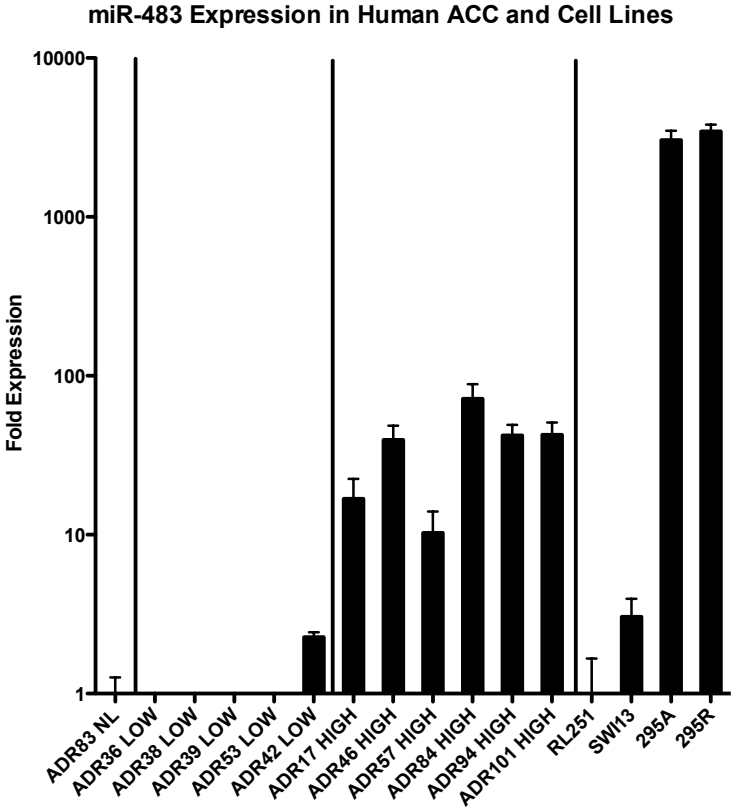
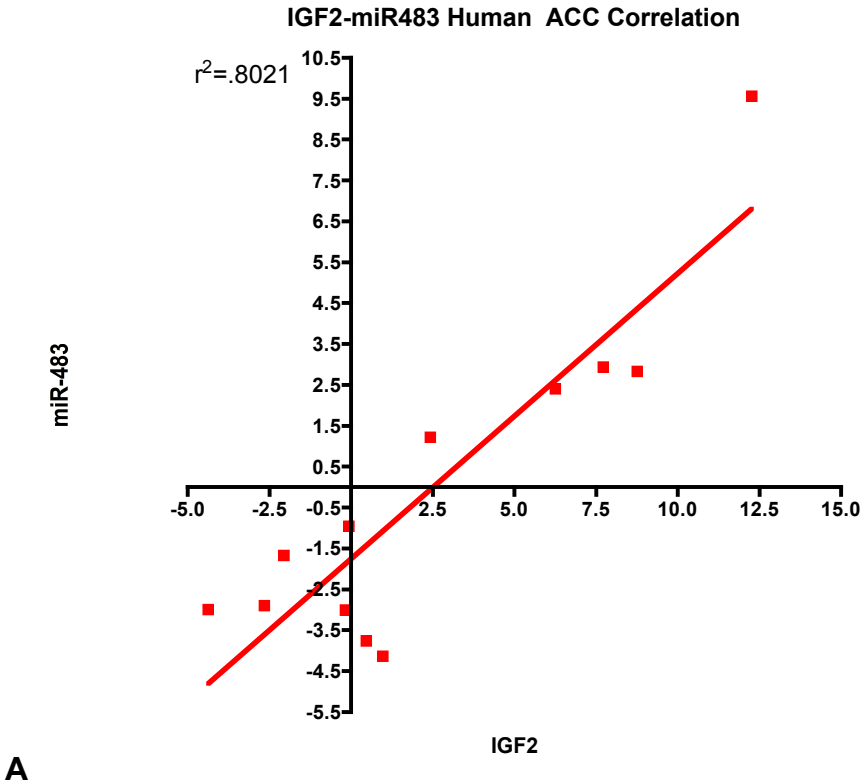
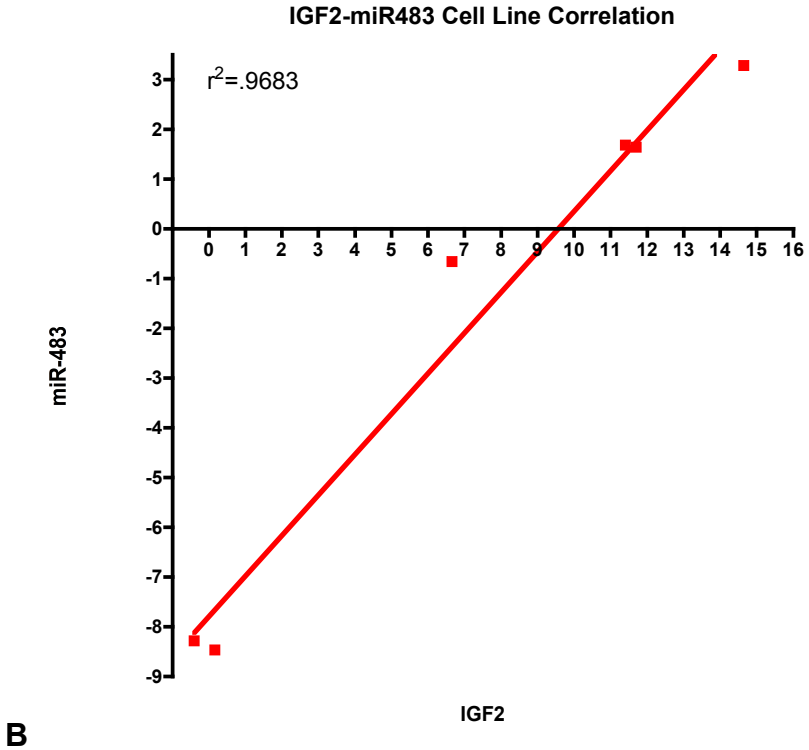

miR-483 Expression in Human ACC and Cell Lines
Fold Expression
10000
1000
100
10
1
ADR83 NL
ADR36 LOW
ADR38 LOW
ADR39 LOW
ADR53 LOW
ADR42 LOW
ADR17 HIGH
ADR46 HIGH
ADR57 HIGH
ADR84 HIGH
ADR94 HIGH
ADR101 HIGH
RL251
SW13
295A
295R

Figure 2.3 Correlation between IGF2 and miR-483-3p expression in human adrenal tissue and cell lines.

Linear regression analysis performed on real time PCR ΔCt values for IGF2 and miR-483-3p. Each square represents one sample. (A) Correlation between IGF2 and miR-483-3p for human adrenal tissue samples ($p<.05$; $R^2=.8021$). (B) Correlation between IGF2 and miR-483-3p for various human cell lines ($p<.05$; $R^2=.9683$).


IGF2-miR483 Human ACC Correlation
r2=.8021
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
-0.5
-1.5
-2.5
-3.5
-4.5
-5.5
-5.0
-2.5
2.5
5.0
7.5
10.0
12.5
15.0
miR-483
IGF2

A


IGF2-miR483 Cell Line Correlation
r2=.9683
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
miR-483
IGF2

B

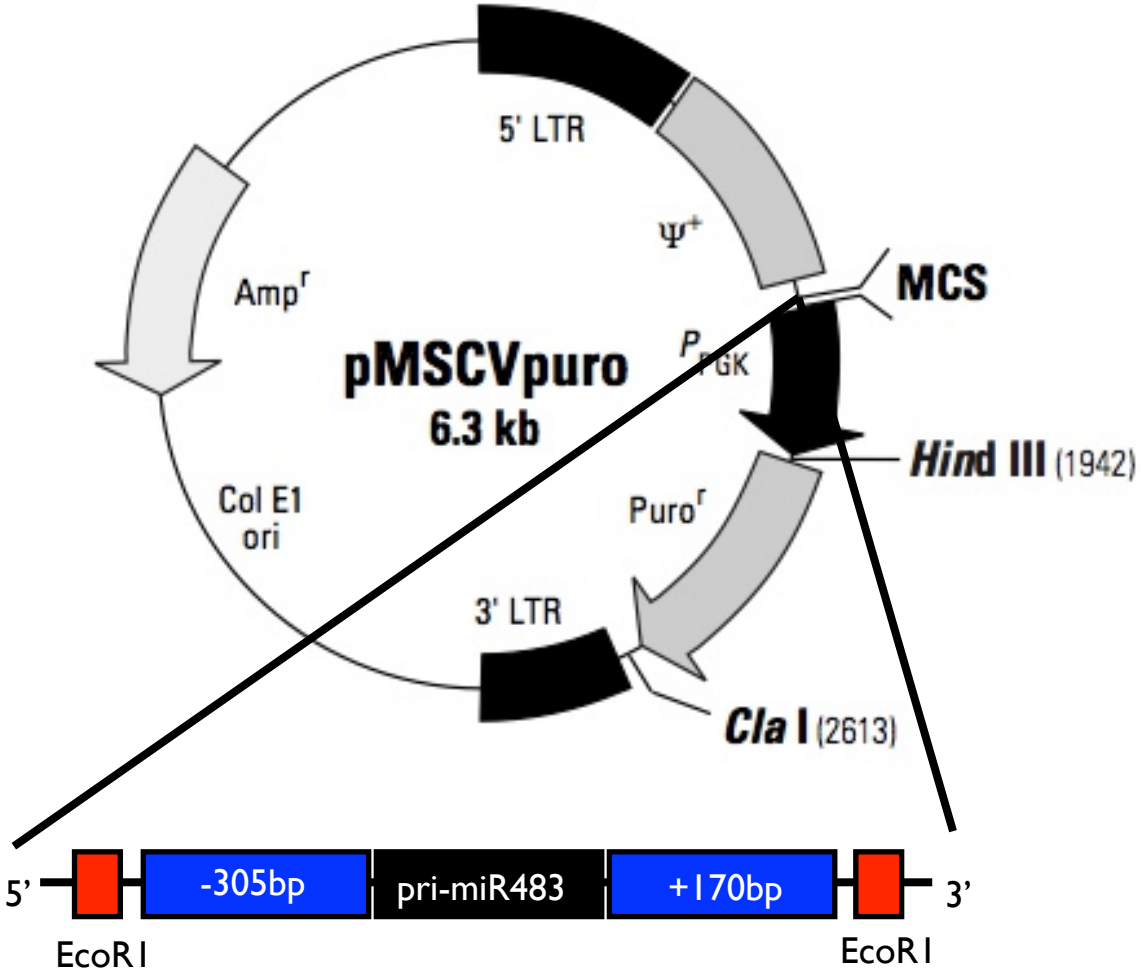


Figure 2.4 miR-483-3p over-expression vector construction.

Schematic showing the strategy used to develop the miR-483 expression vector.

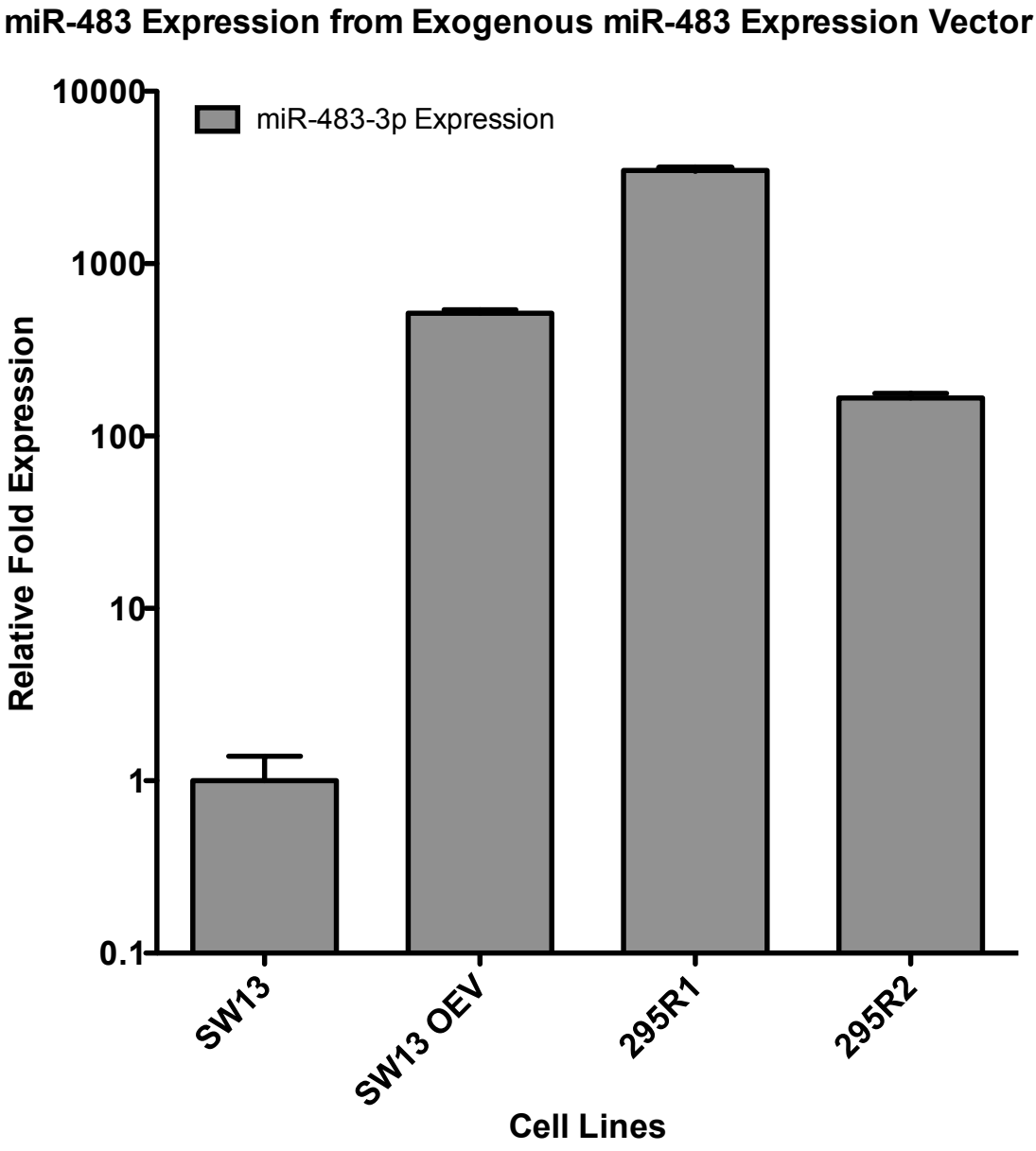


Figure 2.5 Quantitative real time PCR on cell lines transfected with miR-483-3p expression vector.

miR-483-3p expression in SW13 cells that do not express endogenous miR-483-3p. Fold changes relative to SW13 cells transfected with control pMSCVpuro vector (SW13 OEV). H295R cells known to express endogenous miR-483-3p used as positive controls (295R1 & 295R2).

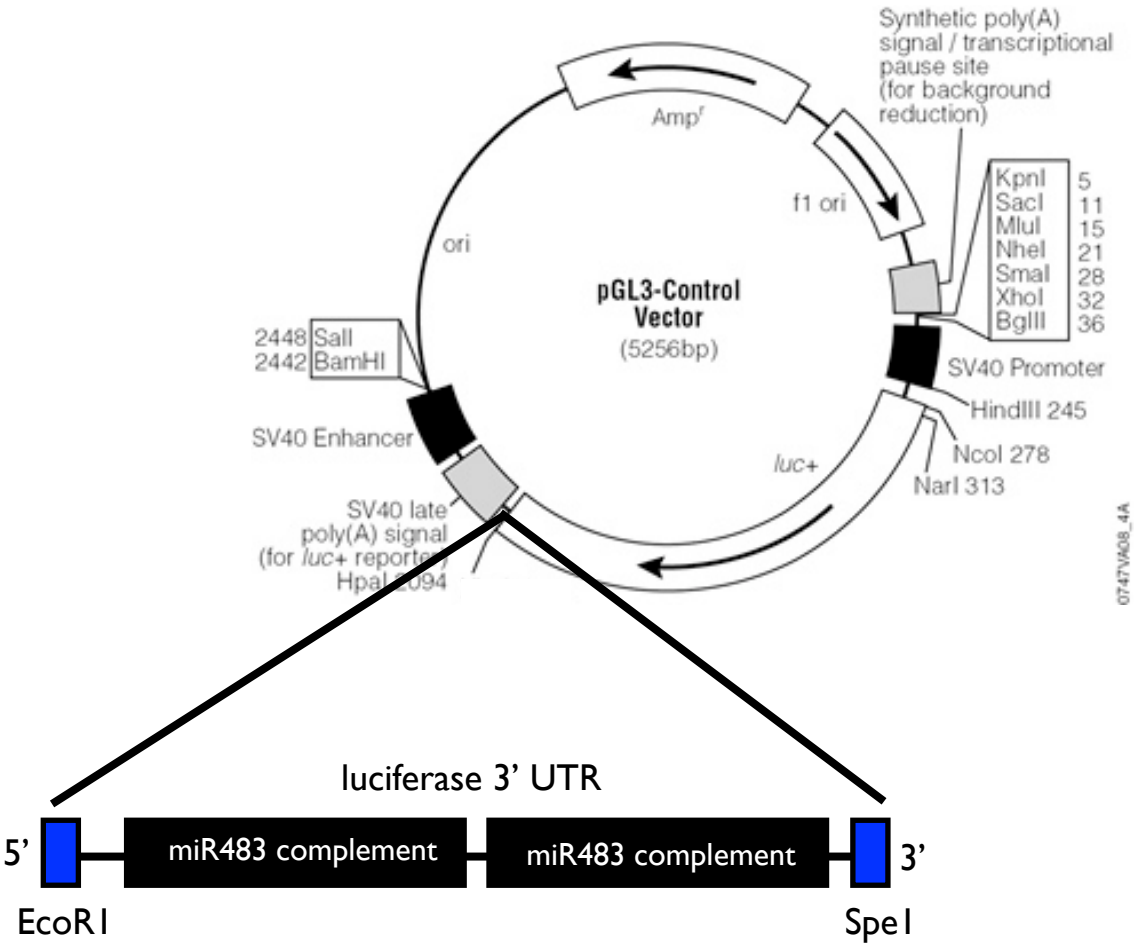


Figure 2.6 miR-483-3p luciferase reporter construction.

Schematic illustrating the strategy used to generate miR-483-3p luciferase sensor using the pGL3-Control plasmid.

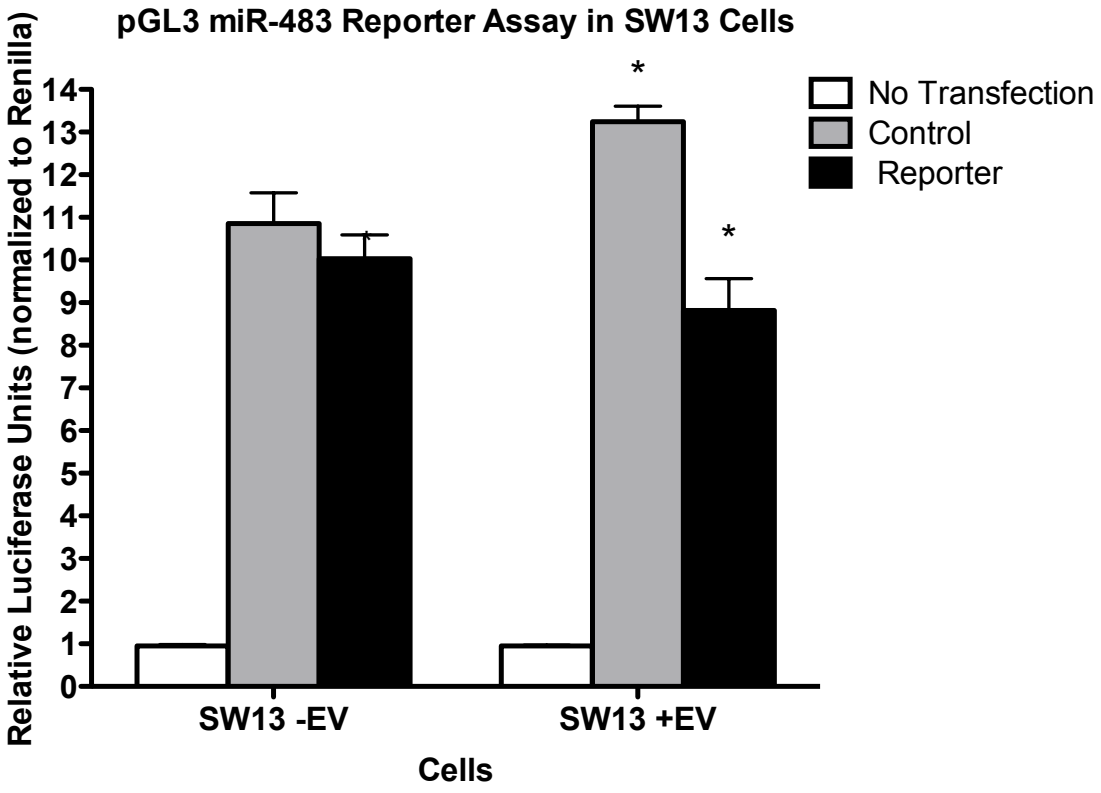


Figure 2.7 Luciferase activity in SW13 cells transfected with miR-483-3p expression vector.

SW13 cells were transfected with either empty control pMSCVpuro (SW13 -EV) or pMSCVpuro-miR483 expression vector (SW13 +EV). The miR-483-3p luciferase reporter plasmid (Reporter), or control pGL3-Control plasmid (Control) were subsequently transfected into each cell type, and relative luciferase activity was measured.
(*p>.05)

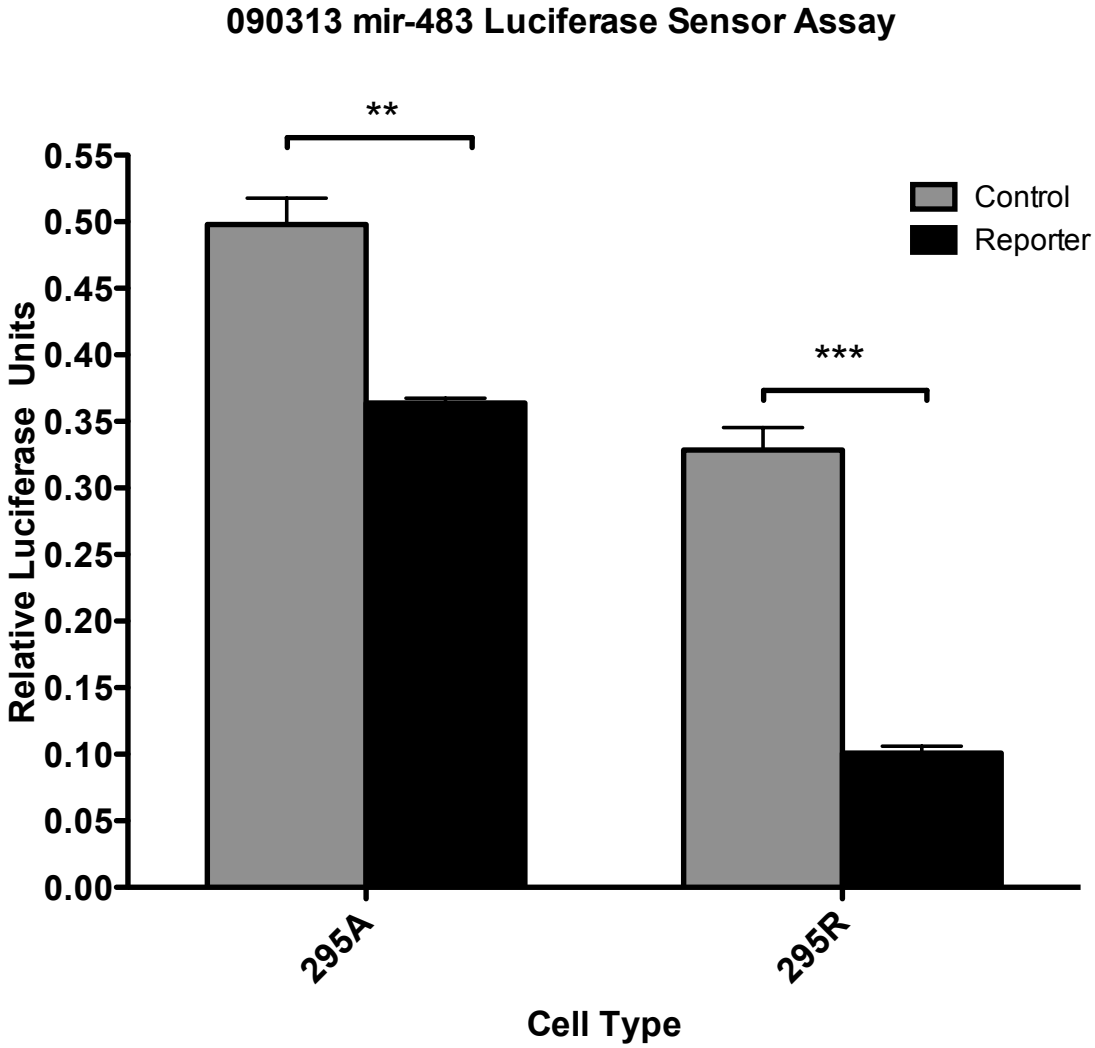


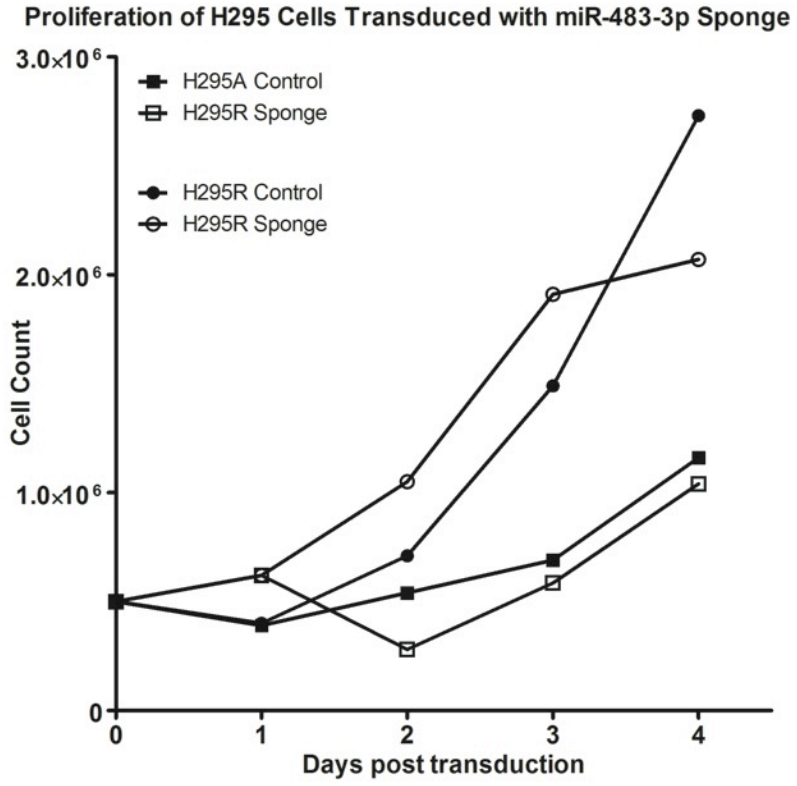
Figure 2.8 Luciferase assays on H295R cells transfected with the miR-483-3p sensor.

Effect of endogenous miR-483-3p expression on the luciferase reporter construct in H295A and H295R cells. Cells transfected with the miR-483-3p luciferase reporter (Reporter) show significantly less (*p<.05) luciferase activity compared to cells transfected with control pGL3-Control luciferase plasmid (Control).

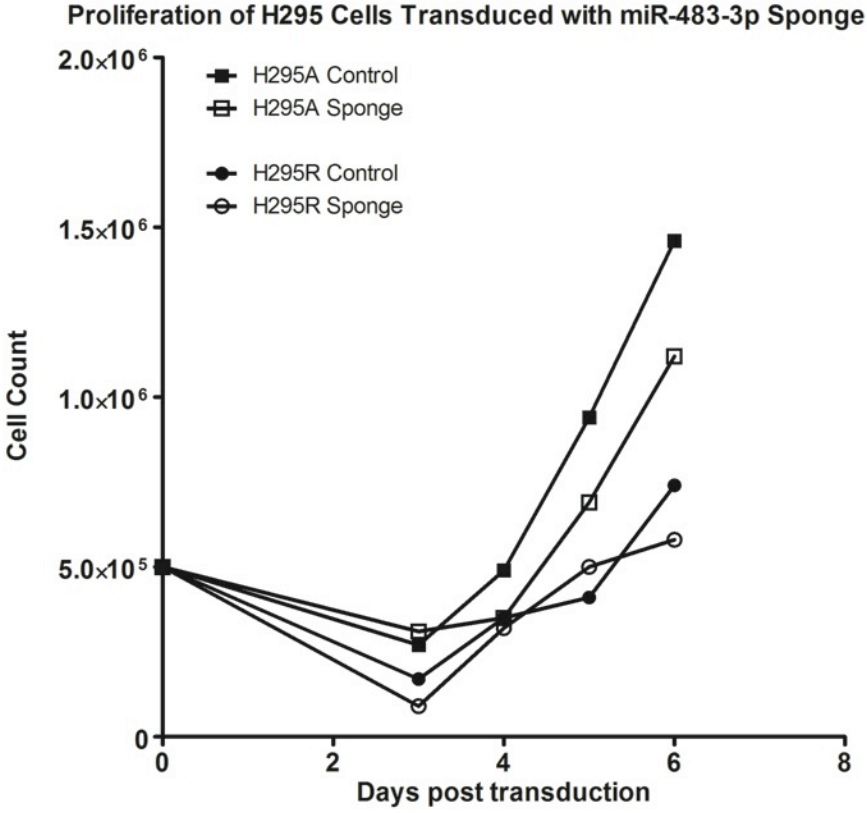
Figure 2.9 Growth curves of H295A and H295R cells transduced with the miR-483-3p sponge.

Growth curves of H295A and H295R cells transduced with either control plasmid or miR-483-3p sponge. Cells were plated at a density of 5 x 10^5 cells per well and followed for up to 4 days (A) or 6 days (B). Cells were harvested on days denoted by data points on the line graph and counted.

A


Proliferation of H295 Cells Transduced with miR-483-3p Sponge
3.0x10^6
2.0x10^6
1.0x10^6
0
Cell Count
H295A Control
H295R Sponge
H295R Control
H295R Sponge
0
1
2
3
4
Days post transduction

B


Proliferation of H295 Cells Transduced with miR-483-3p Sponge
2.0x10^6
1.5x10^6
1.0x10^6
5.0x10^5
0
Cell Count
H295A Control
H295A Sponge
H295R Control
H295R Sponge
0
2
4
6
8
Days post transduction

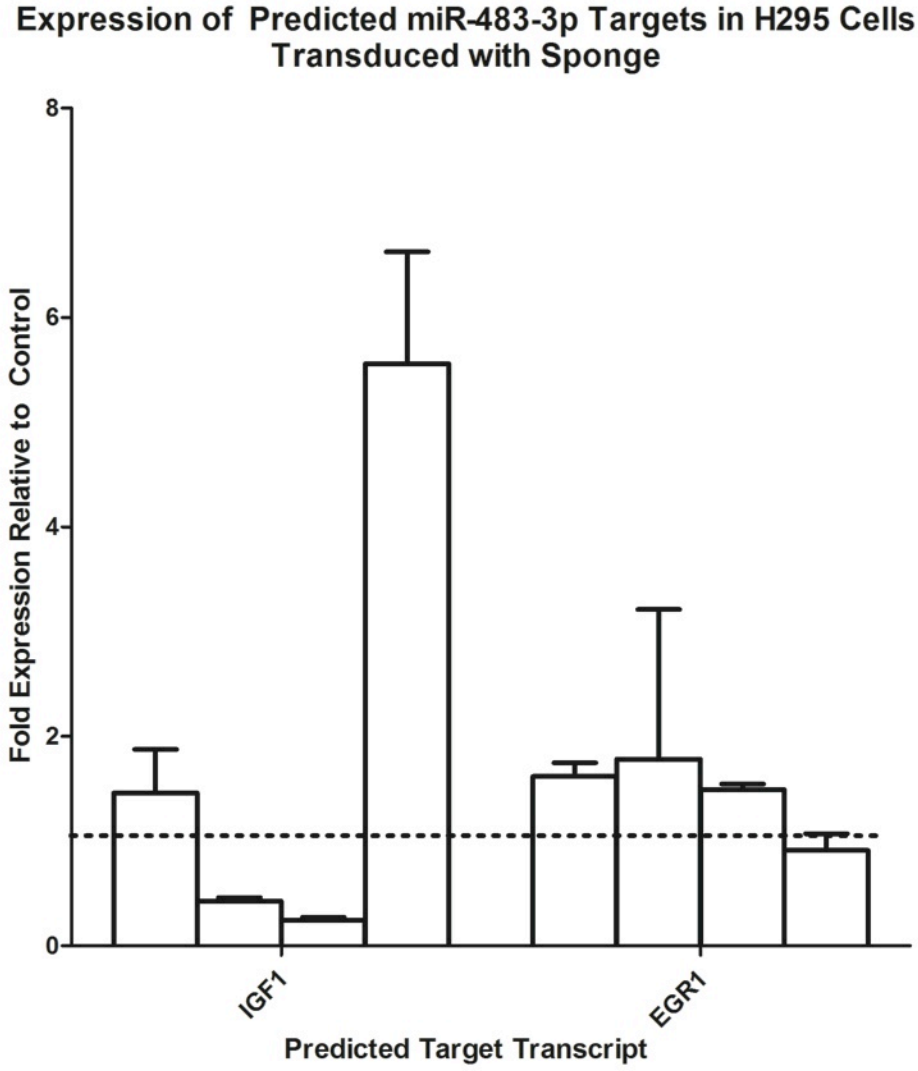


Figure 2.10 IGF1 and EGR1 transcript levels in H295 cells transduced with the miR-483-3p sponge.

Expression of IGF1 and EGR1 in H295 cells transduced with the miR-483-3p. Each column represents an independent replicate, and shows expression relative to a corresponding control (H295 cells transduced with control plasmid). The dotted horizontal line at 1 denotes the baseline expression in control samples. miR-483-3p sponge induced a modest increase in EGR1 transcript levels in 3/4 replicates analyzed. IGF1 response to the miR-483-3p sponge was inconclusive.

References

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CHAPTER 3

Differentially Expressed Genes and MicroRNAs in Embryonic Dicer Deficient Mouse Adrenals

The work described in this chapter was performed under the mentorship of Gary Hammer, MD, PhD. I would like to thank Katherine Gurdziel, MS, and Ann Grosse, PhD, for their invaluable insight and assistance in staging and harvesting embryonic adrenals, and in performing bioinformatic analysis on the microarray data. Katherine was heavily involved in the data and statistical analysis of Figures 3.6, and 3.8, as well as Tables 3.1 and 3.2, and had a significant role in the generation of these figures and tables. Katherine will also be a first co-author on a manuscript to be submitted for publication based on this work.

Introduction

The adrenal glands are bilateral structures located superior to the kidneys. They are critical components of the hypothalamic-pituitary-adrenal (HPA) axis, and have important functions in maintaining electrolyte and metabolic homeostasis, as well as regulating the stress response. The adrenal gland is comprised of two embryologically and functionally distinct cell types: The adrenal cortex, which is derived from the coelomic epithelia and intermediate mesoderm

known as the urogenital ridge; and the adrenal medulla, which is comprised of neuroendocrine cells derived from the neural crest [1,2].

The adrenal cortex initially forms as a coalescence of cells known as the adrenogonadal priomordium (AGP) at approximately embryonic day 9 (E9.0) [3]. This bi-potential mass of cells then further develops into two distinct cell populations that eventually comprise the steroid secreting cells of the gonad and adrenal cortex. It is at this time that steroidogenic factor 1 (Sf1), a key regulator of steroidogenic enzymes in the adrenal cortex and steroid secreting cells of the gonads, begins to be expressed in the AGP [4]. By E12.0, a distinct adrenal primordium consisting of fetal adrenocortical cells forms. At approximately the same time, medullary precursor cells from the neural crest begin migrating into and populating the fetal adrenal cortex [1]. Shortly thereafter, mesenchymal cells from the surrounding stroma coalesce to form the adrenal capsule, which is where a population of adrenocortical stem/progenitor cells is believed to reside [5]. As encapsulation progresses, the fetal adrenal cortex is replaced by the adult or definitive cortex, which has been shown to have regenerative properties that presumably facilitate the continued turnover and replenishment of adrenocortical cells throughout the life of the organism [6].

The necessity of Sf1 in adrenal development is absolute for the development and maintenance of the adrenal gland. Sf1-null mice die shortly after birth likely due to adrenocortical insufficiency, and also completely lack both adrenal glands and gonads [7]. However, there are numerous additional regulatory factors and signaling pathways that have also been implicated in the

specification, development and maintenance of the adrenal cortex. These include dosage-sensitive sex reversal, adrenal hypoplasia congenita critical region on the X chromosome, gene1 (Dax1); pre B-cell leukemia homeobox 1 (Pbx1); Wnt/β-catenin signaling; sonic hedghog signaling (Shh); Cdkn1c ($p57^{kip2}$) which is implicated in the intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenita, and genital anomaly syndrome (IMAGe); and most recently, Pod1 (Tcf21) [8-14]. The interplay between these regulatory and signaling pathways that occurs in the developing adrenal gland is highly complex and intricate, and the exact roles of each of them has yet to be fully understood.

Recently, the role of post-transcriptional regulation in the form of miRNAs has been investigated in development and physiology, including in the developing adrenal. miRNAs are short, endogenous, non-coding RNA transcripts first described in *C. elegans* [15]. Initially thought to be an idiosyncrasy found in nematodes, they are present in most eukaryotic cells, consistent with a critical evolutionary role for these transient and previously dismissed RNA transcripts. The canonical function of miRNAs is that of post-transcriptional regulation of gene expression, and this is accomplished by binding to target gene mRNAs through partially complementary sequences in the 3' untranslated region (UTR). In conjunction with a protein complex known as the miRNA Induced Silencing Complex (miRISC), miRNAs bind to target mRNA transcripts to inhibit translation by destabilizing the target transcript and facilitating degradation, or inhibiting the translational machinery [16,17]. These mechanisms have the effect of

subsequently inhibiting the protein expression of specific genes within a cell, fine tuning the gene expression within a cell to maintain homeostasis.

Since the groundbreaking observation in 1993 by Lee et. al., the field of miRNAs has rapidly expanded, and along with it our understanding of their functions in development, physiology, and disease processes. miRNAs appear to be crucial in development, as mice deficient in *Dicer*, the RNAse III enzyme required for miRNA maturation, do not survive beyond embryonic day 8.5 (E8.5), due to arrested embryonic development beginning at E7.5 [18]. Subsequent studies involving tissue-specific Dicer knockout mouse models show that Dicer is required for normal organogenesis and tissue maintenance in a variety of organs including the heart, lung, skin, muscle, and gonads [19-25]. Additionally, Dicer has shown to be required for the maintenance of embryonic and tissue stem cells [26,27], suggesting a role for Dicer and miRNA expression in regulating cellular differentiation.

In this study we utilized a genetic approach to ablate Dicer in the steroidogenic cells of the adrenal cortex. The resulting adrenocortical Dicer KO mice underwent normal adrenal development through embryonic day E14.5. However, the adrenal cortex underwent rapid degeneration beginning at E16.5. By E18.5, the adrenal cortex had completely failed to the point of there being a near complete absence of cortical tissue. MicroRNA and mRNA array analysis showed that Dicer KO adrenals had distinct expression profiles relative to wild type controls, including the up-regulation of Nr6a1, Igdcc3, and Acvr1c; the data

also revealed the concurrent down-regulation of miRNAs predicted to target these specific mRNA transcripts in Dicer KO adrenals.

Materials and Methods

Mice

Experiments involving live animals were performed in accordance with current and institutionally approved protocols and animal care guidelines. *Sf1-Cre^{high}* and *Sf1-Cre^{low}* mice were obtained and described previously [9,28]. Mice carrying the floxed *Dicer* allele (*Dicer1^{tm1Bdh}/J*) were purchased from The Jackson Laboratory (Bar Harbor, ME).

To obtain Sf1-Cre/Dicer$^{lox/lox}$ mice, Sf1-Cre/Dicer$^{+/lox}$ and Dicer$^{lox/lox}$ mice were mated together. Females from each mating pair were monitored for seminal plugs, and the morning of detection was designated as E0.5. Pregnant females were sacrificed and harvested at designated timepoints, and embryos were staged using Theiler staging criteria as described by the e-mouse Atlas Project (www.emouseatlas.org). Genotyping for the *Sf1-Cre* and *Dicer^{lox}* allele was performed on tail sections from both embryos and adult mice as previously described [28,29]. For long term observations of Sf1-Cre^{low}/Dicer$^{lox/lox}$ mice, control and knockout offspring were sacrificed at 6, 13, 18, 30, and 50 weeks of age. Adrenals were bilaterally excised, cleaned of excess fatty tissue, and weighed. Adrenal weights were normalized to the body weight of the animal from which they were isolated. Left adrenals were collected for histologic processing, and the contralateral glands were utilized for RNA isolation.

Dexamethasone suppression/ACTH stimulation test and plasma corticosterone RIA

50 week old Sf1-Cre^{low}/Dicer$^{lox/lox}$ (n=7) and 50 week old control mice (n=5) were subject to ACTH stimulation and tail vein blood collection prior to sacrifice as described previously [30]. Animals were intra-peritoneally injected (IP injected) with 5mg/kg body weight dexamethasone (Sigma-Aldrich, St. Louis, MO) at 1800 hours the night before and at 0800 hours the day of the assay. At 1000 hours, 1mg/kg body weight of ACTH [ACTH (1-24); Bachem, Torrance, CA] was IP injected. Blood was collected by venous tail vein puncture at 0, 15, 30, and 60 minutes post-ACTH injection.

Serum corticosterone levels in 50 week old knockout and control animals were measured by radioimmunoassay (RIA) using a ^{125}I RIA kit (MP Biomedicals, Solon, OH) using the manufacturer's instructions. Samples were run in triplicate and quantified using a Gammer Counter. All measurements were within the standard curve of the assay.

Adrenal histology, immunohistochemistry, and immunofluorescence

Tissues were fixed between 2-4 hours in 4% paraformaldehyde and then dehydrated in a series of graded ethanol solutions before paraffin embedding. 7μm sections were cut and placed on microscope slides for further manipulation.

Hematoxylin and eosin staining was performed by deparaffinizing tissue sections in xylene, then rehydrated in graded ethanol solutions of decreasing concentration. Slides were dipped in hematoxylin for 3 seconds, then

immediately transferred to deionized water and rinsed with running deionized water for several minutes. Slides were counterstained by a 3 second immersion in eosin, then rinsed and dehydrated in a series of ethanol and xylene baths, and finally mounted with Permount (ThermoFisher, Waltham, MA).

Immunohistochemistry was performed by processing tissue sections as described above. Following rehydration, slides were subjected to antigen retrieval by boiling in 10mM sodium citrate (ph 6) for 20 minutes. After cooling, slides were washed once in deionized water followed by 2 washes in Tris-buffered saline/0.1% Tween-20 (TBST, ph 7.5). Antibody staining was performed with VECTASTAIN ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Tissue sections were blocked in antibody diluent solution for 1 hour at room temperature, then incubated overnight at 4°C with anti-p21 (1:100, BD Pharmingen, San Diego, CA), anti-cleaved-caspase 3 (1:100, Cell Signaling, Danvers, MA), anti-Sf1 (1:1000, custom antibody), and anti-tyrosine hydroxylase (1:500, Pel-Freez Biologicals, Roger, AR). The following day, sections were washed 3 times in TBST, then 2 times in deionized water. Incubation with biotinylated secondary antibodies was performed for 1 hour at room temperature, and subsequent staining via 3,3'-Diaminobenzidine (DAB) was performed according to the manufacturer's instructions. DAB stained tissue sections were then counterstained with either diluted (1:10 deionized water) eosin or hematoxylin. Slides were finally mounted using Permount coverslip mounting medium, allowed to cure overnight at room temperature, and imaged using light microscopy.

Fluorescent labeled tissue sections were processed and cut as described above. Antigen retrieval was performed by boiling slides in 10mM citric acid (pH 6) for 30 minutes. After cooling to room temperature, tissue sections were blocked with PBS/2% non-fat dry milk/2% normal goat serum for 1 hour at room temperature. Sections were then incubated overnight at 4°C with anti-Sf1 (1:1000, custom antibody), anti-tyrosine hydroxylase (1:300, Millipore, Billerica, MA), and anti-PCNA (1:500, Santa Cruz, Santa Cruz, CA). The following morning, slides were washed 3 times in PBS, then 2 times in deionized water before incubation with Dylight™ 488 conjugated goat anti-rabbit or Dylight™ 549 conjugated goat anti-mouse (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour in the dark at room temperature. All antibodies were diluted in PBS containing 0.2% non-fat dry milk and 0.2% normal goat serum. Slides were washed again 3 times in PBS, followed by 2 washes in deionized water. The fluorescently labeled tissue sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma-Aldrich, St. Louis, MO). Coverslip mounting was performed using Tris-buffered Fluorogel (Electron Microscopy Sciences, Hatfield, PA), cured for 24 hours in the dark at room temperature, then visualized by fluorescent microscopy.

Quantitative Real-time PCR

Total RNA was isolated from the adrenals of control and Dicer KO animals were processed using either TRIzol reagent or the RNAqueous Micro kit (Life Technologies, Carlsbad, CA) to isolate total RNA. Up to 1µg of RNA was reverse

transcribed using the iScript system (Bio-Rad Laboratories, Hercules, CA) to generate cDNA. 1µL of the resulting cDNA was amplified with appropriate primers using Power SYBR Green PCR Master Mix and analyzed on an ABI 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA). Data analysis was performed using the $2^{-\Delta\Delta C(T)}$ method [31]. Gene expression was normalized to mouse β-Actin. Primers for each amplified gene are as follows:

β-Actin (*Actb*), Fwd 5'-CTAAGGCCAACCGTGAAAAG and
Rev 5'-ACCAGAGGCATACAGGGACA;

Sf1 (*Nr5a1*), Fwd 5'-TCCAGTGTCCACCCTTATCC and
Rev 5'-CGTCGTACGAATAGTCCATGC;

11β-hydroxysteroid dehydrogenase (*Cyp11b1*), Fwd 5'-GCCATCCAGGCTAACTCAAT and Rev 5'-CATTACCAAGGGGGTTGATG;

11β-aldosterone synthase (*Cyp11b2*), Fwd 5'-GCACCAGGTGGAGAGTATGC and Rev 5'-CCATTCTGGCCCATTTAGC;

ACTH receptor (*Mc2r*), Fwd 5'-TGGAAAAGTTCTCAGCACCAC and
Rev 5'-TCTTTGTGTGGAAGGATCTGG;

steroidogenic acute regulatory protein (*Star*),
Fwd 5'-TTGGGCATACTCAACAACCA and Rev 5'-ACTTCGTCCCCGTTCTCC;

cholesterol side chain cleavage enzyme (*Scc*), Fwd 5'-AAGTATGGCCCCATTTACAGG, and Rev 5'-TGGGGTCCACGATGTAAACT;

Dicer (*Dicer1*), Fwd 5'-GCAAGGAATGGACTCTGAGC and
Rev 5'-GGGGACTTCGATATCCTCTTC.

Microarrays

Timed matings were established to generate Sf1-Cre^{high}/Dicer$^{lox/lox}$ knockout embryos. At E15.5 and E16.5, pregnant females were sacrificed, and embryos were collected and staged according to the criteria referenced above. Adrenals from each embryo were micro-dissected and stored separately in RNAlater solution (Invitrogen, Carlsbad, CA) until genotyping confirmed Dicer KO status. The adrenals from control and Dicer KO littermates were pooled for 4 separate litters, resulting in a total of 4 control and 4 Dicer KO biological replicates at both E15.5 and E16.5 timepoints. Total RNA was isolated using the RNAqueous Micro kit (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol to preserve small RNA recovery. Isolated RNA was quantified on a Nanodrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE), and submitted to the University of Michigan Microarray Core Facility where samples were quality checked and finally analyzed with both Affymetrix Mouse 430 2.0 gene expression arrays and ABI miRNA OpenArrays.

Statistical Analysis

Microarray data were normalized using the robust multiarray average (RMA) algorithm [32]. MicroRNA data were normalized to the U6 rRNA value on the corresponding subpanel of the Openarray. For both types of arrays, differential gene expression between conditions was determined using the limma package by applying linear modeling followed by the empirical Bayes method to compute significance [33]. The resulting p-values were adjusted for multiple

testing by the Benjamini-Hochbberg method [34]. Genes with an absolute log2 fold change greater than or equal to 1.5 with an adjusted p-value of less than .05 were considered differentially expressed and statistically significant. For the DAVID analysis, each collection of differentially expressed genes was evaluated for gene-enrichment by submitting the Entrez Gene identifiers to DAVID and running the default analysis. Functional classifications with a false discovery rate (FDR) ≤ 5% were considered significant [35].

Results

Sf1-Cre^{low}/Dicer$^{lox/lox}$ mice survive up to 50 weeks and have no appreciable deficits in adrenal morphology or steroidogenesis

We generated knockout (KO) mice that lack Dicer expression in the adrenal cortex by breeding mice carrying the Sf1 promoter-driven Cre transgene and one copy of the floxed Dicer allele (*Sf1-Cre/Dicer$^{lox/lox}$*) with mice carrying homozygous floxed Dicer alleles (*Dicer$^{lox/lox}$*). The contrasting effects of the low and high Sf1-Cre transgenes have been described [9]. Briefly, the single-copy Sf1-Cre^{low} transgene (low driver) results in partial penetrance of Cre recombinase expression and a milder phenotype compared with the Sf1-Cre^{high} transgene (high driver) that contains five copies and results in full phenotypic penetrance. Because of the rapid and drastic nature of the phenotypes seen in other tissue specific Dicer knockout models, we chose to initially study Sf1-Cre^{low}/Dicer$^{lox/lox}$ (low driver Dicer KO) offspring in our study to determine whether a stochastic, partial deletion of *Dicer* could provide insight into its role in the long- term maintenance of the adrenal cortex.

Sf1-Cre^{low}/Dicer$^{lox/lox}$ mice were housed separately according to sex and followed for 6, 18, 30, and 50 weeks of age. Small cohorts of at least n=2 were sacrificed at each time point up 30 weeks, and the adrenals from these animals were analyzed. We were unable to detect any appreciable physiologic or histologic phenotype at the earlier timepoints (data not shown). We then concentrated our analysis on the 50 week Sf1-Cre^{low}/Dicer$^{lox/lox}$ mice by testing a larger cohort (n ≥ 5) for functional and gene expression perturbations. However,

we were unable to observe any appreciable differences in phenotype between 50 week old control versus low driver Dicer KO adrenals. Figure 3.1 illustrates representative hematoxylin and eosin stained sections from control and low driver Dicer KO adrenals at the 50 week timepoint, with additional to immunohistochemistry for the adrenocortical marker Sf1, and the medullary marker tyrosine hydroxylase. No appreciable structural changes in the adrenal such as capsular thickening or changes in cortical mass were observed. Likewise, there were no discernible differences in Sf1 or tyrosine hydroxylase staining as assessed by immunohistochemistry. Comparison of normalized adrenal weights between 50 week old control and low driver Dicer KO animals showed a slight trend in which the KO adrenals were on average slightly smaller in size than control adrenals, but this difference was not statistically significant. (Figure 3.2A) Prior to sacrifice, 50 week old control and low driver Dicer KO animals were subjected to an ACTH stimulation test to compare maximum steroidogenic output. This has been described in previous studies [30] as a method to measure adrenal function in vivo. We were unable to detect a significant difference between 50 week old control and low driver Dicer KO mice in ACTH-stimulated corticosterone output at 0, 15, 30, and 60 minutes post injection (Figure 3.2B). Therefore, 50 week old low driver Dicer KO adrenals maintained normal steroidogenic function. Finally, 50 week old control and low driver Dicer KO adrenals were compared by quantitative real-time PCR to determine whether any differences were detectable at the transcriptional level. Except for a decrease in *Dicer* transcript levels in 50 week old Dicer KO

adrenals, the relative expression of steroidogenic enzyme encoding transcripts remained constant between control and low driver Dicer KO adrenals (Figure 3.2C). In summary, no significant morphological or functional deficits were detected in the adrenals of 50 week old low driver Dicer KO mice compared to control animals. These results indicate that low driver Dicer KO adrenals can compensate for the loss of Dicer in a subpopulation of cortical cells, or perhaps Dicer function is not required in these cells. To further explore these possibilities, we then performed experiments aimed at determining the effects of *Dicer* deletion in all Sf1 expressing cortical cells by examining the adrenal glands in Sf1-Cre^{high} transgenic mice.

High driver (Sf1-Cre^{high}/Dicer$^{lox/lox}$) Dicer KO mice die shortly after birth

While we did not observe a significant phenotype in the adrenal glands of low driver Dicer KO animals as old as 50 weeks, the more robust expression pattern of Cre recombinase in the high driver Dicer KO animals resulted in a marked adrenal defect that proved to be lethal. Based on our breeding strategy, embryonic (E14.5-E18.5) and post-weaning (21 days post-parturition) offspring were expected to demonstrate Mendelian genotypic ratios in which 25% of progeny should have been positive for the Sf1-Cre^{high}/Dicer$^{lox/lox}$ genotype. However, high driver Dicer KO animals were not observed at weaning, and the expected Mendelian ratios for the Sf1-Cre^{high}/Dicer$^{lox/lox}$ genotype were seen only at the embryonic stages. Mortality among Sf1-Cre^{high}/Dicer$^{lox/lox}$ animals occurred 1-2 days post-parturition, and no Sf1-Cre^{high}/Dicer$^{lox/lox}$ offspring survived beyond

this timepoint. Therefore, Sf1-Cre^{high}/Dicer$^{lox/lox}$ offspring invariably died shortly after birth. This perninatal lethality in high driver Dicer KO animals is also supported by a previous report [36].

High driver (Sf1-Cre^{high}/Dicer$^{lox/lox}$) Dicer KO mice exhibit adrenal failure late in embryonic development

Due to the perinatal lethality of the high driver Dicer KO model, we performed detailed analyses of adrenal histology at various embryonic time points in these animals. E14.5 is the earliest time point in development where the adrenal has fully separated from adrenal-gonadal primordium, and medullary precursor cells have migrated from the neural crest into the adrenal. We were unable to detect significant histological changes at E14.5 (Figure 3.3B) between control and high driver Dicer KO adrenal glands. This implies that the fetal adrenal cortex in high driver Dicer KO animals undergoes normal specification and formation, and that Dicer loss in Sf1 positive cells is not initially detrimental to early adrenal development. In contrast, Sf1-null animals demonstrate marked developmental defects in the gonad and adrenal as early as E12.5 [7], illustrating the global necessity of Sf1 for proper adrenogonadal development. Adrenals from E16.5 high driver Dicer KO animals were still present compared with control adrenals, but cortical thickness was decreased in the sections that were analyzed. However, adrenals from KO animals still exhibited distinct cortical and medullary demarcations. The most significant phenotype in high driver Dicer KO animals occurred at E18.5. As shown in Figure 3.3B, adrenals from high driver

Dicer KO animals at this time point demonstrated a nearly complete absence of the cortex. Additionally, the overall size of the adrenal in Dicer KO animals was markedly smaller than control counterparts (Figure 3.3A). Interestingly, the adrenal medulla, which is derived from a separate cell lineage than the cortex, persisted in the high driver Dicer KO adrenals. Finally, an unidentified population of small, basophilic cells was observed between the medullary cells and the adrenal capsule.

To further characterize the phenotype seen in high driver Dicer KO adrenals, immunofluorescent antibody co-staining was performed on sections from control and Dicer KO adrenal sections as described in *Materials and Methods.* At the earlier time point of E14.5, both the adrenal cortex and medulla appear to be intact in both control and high driver Dicer KO adrenals, as evidenced by anti-Sf1 and anti-tyrosine hydroxylase staining (Figure 3.3C). However, as development progressed, cortical mass, as defined by Sf1-positive cells, began to decrease at E16.5. By E18.5, Dicer KO animals had very few residual Sf1 expressing cortical cells remaining in the adrenal gland. Again, tyrosine hydroxylase expressing medullary cells persisted at E18.5 in Dicer KO adrenals, despite the severe cortical failure that was observed.

In summary, high driver Dicer KO adrenals underwent normal early development, with adrenal-gonadal separation and formation of the fetal adrenal cortex, followed by infiltration of the developing cortex by neural crest derived medullary precursor cells by E14.5. However, beginning at E16.5, the Dicer KO adrenals exhibited a gradual loss of Sf1 positive cortical cells that accelerated

rapidly and led to the complete absence of the cortex by E18.5. This marked phenotype is incompatible with life, as evidenced by the perinatal lethality observed in high driver Dicer KO animals which do not survive beyond one or two days following live birth.

High Driver Dicer KO Adrenals Exhibit Increased dsDNA Damage and Apoptosis

We further investigated the adrenocortical destruction in high driver Dicer KO animals by examining differences in cortical proliferation or apoptosis versus control mice. In the adrenal gland, proliferating cortical cells are most abundant in the outer peripheral region of the cortex [37]. Anti-PCNA stained adrenal sections from control and Dicer KO animals were co-stained with anti-Sf1 to localize proliferating cells. Our results demonstrated that Sf1 expressing, proliferating cells localized to the sub-capsular cortical region were were present in the periphery of high Driver Dicer KO adrenals, suggesting that loss of Dicer in cortical cells did not significantly affect proliferation (Figure 3.4A).

We then addressed the question of whether Dicer loss in the adrenal cortex induced cell cycle arrest and apoptosis. Immunohistochemistry for Cdkn1a (p21) and cleaved-Caspase 3 indicated significant increases in the expression of these proteins in high driver Dicer KO adrenals (Figure 3.4B and Figure 3.4C). The expression pattern of p21 and cleaved-Caspase 3 in Dicer KO adrenals was limited primarily to the cortex, and could be detected as early as E14.5. This was surprising as we were not able to otherwise appreciate an

apparent phenotype in high driver Dicer KO adrenals at this early time point. Finally, phospho-gamma-H2A.X staining was present in high driver Dicer KO adrenals, indicating the presence of double stranded DNA damage (Figure 3.5).

In summary, high driver Dicer KO adrenals showed evidence of increased cell cycle arrest and apoptosis in the cortex, consistent with the induction of a DNA damage checkpoint and the aplastic cortical phenotype observed at E18.5.

High Driver Dicer KO Adrenals Demonstrate a Unique mRNA Expression Profile

Sf1-Cre^{high}/Dicer$^{lox/lox}$ high driver Dicer KO mice demonstrated marked aplasia of the adrenal cortex that began with increased cellular death at E14.5 and continued at a rapidly accelerating tempo until E18.5, at which time high driver Dicer KO adrenals had undergone nearly complete cortical failure. Whether this adrenal failure reflected dysregulation of miRNA biogenesis, or a cellular toxicity effect resulting from the accumulation of unprocessed pre-miRs is unclear. Further analysis on the high driver Dicer KO phenotype was therefore performed by assessing gene transcript expression in both control and KO adrenals over a 2 day time course just prior to the rapid cortical failure seen at E18.5.

E15.5 and E16.5 adrenals from control and high driver Dicer KO embryos were harvested and processed as described in *Materials and Methods.* Figure 3.6A shows a heatmap illustrating the differentially expressed gene transcripts in high driver Dicer KO adrenals compared with control adrenals at both the E15.5

and E16.5 time points. In E15.5 high driver Dicer KO adrenals, 10 up-regulated and 19 down-regulated transcripts were observed that were differentially expressed relative to control adrenals. Similarly, there were less up-regulated unique transcripts in E16.5 Dicer KO adrenals than unique down-regulated transcripts (19 up vs. 31 down). The majority of these differentially expressed genes overlapped between the E15.5 and E16.5 timepoints in Dicer KO adrenals as illustrated in Figure 3.6B.

Many of the differentially down-regulated gene transcripts in Dicer KO adrenals were related to steroidogenic pathways, with *Akr1d1* (a 5-beta reductase), and *Adh7*, (an alcohol dehydrogenase known to be expressed in the adrenal cortex), being the two most down-regulated transcripts in Dicer KO adrenals common to both E15.5 and E16.5 timepoints. Additionally, *Frzb*, a secreted Wnt antagonist, was also highly down-regulated in E15.5 and E16.5 Dicer KO adrenals. This result was consistent with data from our lab and others, which demonstrates a role of the Wnt/β-catenin signaling pathway in adrenal development and maintenance, and in the pathology of adrenocortical neoplasia [9,38].

When we compared all of the differentially up-regulated transcripts in high driver Dicer KO adrenals relative to control adrenals at both E15.5 and E16.5, numerous genes related to inflammatory or immune processes appeared to be over-represented in the data. We performed a DAVID (Database for Annotation, Visualization and Integrated Discovery) analysis which allowed us to identify enriched biological themes such as gene ontology (GO) terms and functionally

related gene groups. Comparison across time points and genetic background (control vs. Dicer KO) demonstrated that E16.5 high driver Dicer KO adrenals were particularly enriched for GO terms related to immune and inflammatory response pathway genes (Table 3.1). These data were consistent with an inflammatory process or a cell mediated immune response occurring in high driver Dicer KO adrenals that could either contribute to or be a consequence of the observed cortical cell death.

Interestingly, the most up-regulated transcript in high driver Dicer KO adrenals at both E15.5 and E16.5 timepoints was *Nr6a1*, or germ cell nuclear factor (*Gcnf*). There was concern this may have been an artifact due to the presence of approximately 10kb of the 3' end of *Nr6a1* on the BAC utilized to generate the Sf1-Cre transgene. To further investigate, we performed quantitative real-time PCR on E15.5 control and high driver Dicer KO adrenals. The up-regulation of *Nr6a1* in high driver Dicer KO adrenals was confirmed, as we observed at least 2-fold up-regulation of all 3 *Nr6a1* isoforms in Dicer KO adrenals with isoform 2 being the most robustly up-regulated (Figure 3.7A). Additionally, we compared *Nr6a1* transcript levels by quantitative real-time PCR in the adrenals of wild type animals and animals carrying only the Sf1-Cre^{high} transgene (Figure 3.7B). We observed a small but significant increase (up to 2 fold) in isoform 2 of *Nr6a1* in adrenals collected from animals harboring only the Sf1-Cre^{high} transgene. This suggested there may have been transcriptional leakage from the transgene, which contains approximately 10kb of the 3' end of the *Nr6a1* locus. Subsequent quantitative real-time PCR analysis performed on

cerebral and hepatic tissue isolated from mice bearing only the Sf1-Cre^{high} transgene supported the possibility that the transgene was leaking an incomplete 3' transcript of *Nr6a1* (Figure 3.7C). We observed a 2 to 5-fold increase in isoform 2 of *Nr6a1* in tissues isolated from animals carrying only the Sf1-Cre^{high} transgene. In comparison, the high driver Dicer KO animals that also carry the transgene demonstrated significantly higher levels of this particular isoform (10 to 16-fold) relative to wild type control animals that were not Dicer KO and did not have the Sf1-Cre^{high} transgene. Additionally, isoforms 1 and 3 of *Nr6a1*, which were also up-regulated in high driver Dicer KO adrenals, were not significantly changed in adrenals taken from animals bearing only the Sf1-Cre^{high} transgene. Taken together, these data confirm that *Nr6a1* was up-regulated resulting from loss of *Dicer* in the adrenal cortex. They also demonstrated transcriptional leakage of a partial, 3' transcript of *Nr6a1* from the Sf1-Cre^{high} transgene alone, which accounted for approximately 20-30% of the Nr6a1 up-regulation seen in Dicer KO adrenals.

Down-regulated miRNAs in High Driver Dicer KO Adrenals Are Predicted to Target Up-Regulated Gene Transcripts

In addition to performing Affymetric microarray analysis on high driver Dicer KO adrenals, we also profiled and compared miRNA expression for control and KO adrenals at E15.5 and E16.5. As expected, the differentially expressed miRNAs in Dicer KO adrenals relative to control were down-regulated to varying degrees (Figure 3.8A). Of these differentially expressed miRNAs, sixteen were

common among the E15.5 and E16.5 timepoints analyzed, and several including miR-21, have been implicated in the regulation of adrenal physiology [39] (Figure 3.8B). Of these down-regulated miRNAs seen at both E15.5 and E16.5, miR 34c, miR-21, miR-10a, and let-7d were among the most interesting candidates for future studies due to the large body of literature available regarding their function.

We cross-referenced the list of differentially expressed miRNAs with the list of differentially expressed gene transcripts in high driver Dicer KO adrenals at both E15.5 and E16.5 with the intent of identifying predicted miRNA-target mRNA pairs. Interestingly, the gene transcripts for *Nr6a1, Igdcc3, Acvr1c,* and *Greb1l* were consistently and repeatedly identified as targets for a small subset of miRNAs that were differentially expressed in common among E15.5 and E16.5 Dicer KO adrenals. Table 3.2 lists these miRNA-target miRNA pairs and the number of predicted sites in each gene target for a given miRNA. Let-7d, miR-10a, miR-202, miR-21, miR-674, and miR-362 were the six miRNAs in common between E15.5 and E16.5 Dicer KO adrenals, and it is important to reiterate that the four predicted gene targets listed above were also commonly up-regulated in Dicer KO adrenals at both time points analyzed. Finally, we compared the predicted binding sites for let-7 in the 3‘ UTRs of both mouse and human *NR6A1* and *ACVR1C.* As illustrated in Table 3.3, the seed sequences recognized by let-7 are strongly conserved between human and mouse for all of the predicted binding sites that were suggested by the TargetScan algorithm. In addition, these seed sequences were also found to be strongly conserved among

other vertebrate organisms (data not shown). This phylogenetic conservation of predicted let-7 binding sites among various species including between mice and humans supports the notion that these binding sites may indeed be functional. Similar analyses would need to be performed on the other predicted miRNA-mRNA interactions described here, and would help determine which predicted miRNA-mRNA interactions might be candidates for functional validation studies.

In summary, results from the arrays performed on E15.5 and E16.5 control versus high driver Dicer KO adrenals showed a unique gene expression profile. A number of other transcripts, most notably belonging to the genes *Nr6a1*, *Igdcc3*, *Acvr1c*, and *Greb1l* were also highly up-regulated in Dicer KO adrenal glands. Concurrent miRNA profiling suggested a strong correlation between these four differentially expressed genes and several down-regulated miRNA species. There was also data suggestive of an immune/inflammatory response, which may have been contributory to the apoptosis and ultimate aplastic phenotype observed in Dicer KO adrenal glands.

Discussion

In this study we generated mice that lacked the enzyme *Dicer* in the adrenal cortex utilizing a Cre-loxP excision system to selectively knock out *Dicer* in cells that expressed the steroidogenic regulator, Sf1. The first part of the study utilized Sf1-Cre^{low} transgenic mice, in which only a small percentage of cortical cells underwent Cre mediated excision of the floxed Dicer alleles. In contrast, the Sf1-Cre^{high} driver resulted in excision of floxed *Dicer* alleles in all Sf1 expressing cortical cells, and this mouse was used to determine the developmental effects of *Dicer* loss in the adrenal cortex.

Dicer ablation has been associated with cellular senescence and apoptosis or reduced proliferation in a number of biological models [40-43]. We hypothesized that generating Sf1-Cre^{low} Dicer KO mice might result in a protracted exhaustion of the adrenal cortex stemming from increased cellular turnover of the gland. Since Dicer knockout mice generated with the Sf1-Cre^{low} transgene did not exhibit any significant phenotype even up to 50 weeks of age, these data suggested that Dicer loss under the Sf1-Cre^{low} transgene was insufficient to produce a measurable effect in our animals, or the adrenals in these animals were able to physiologically compensate for the defect, as is seen in the contralateral adrenal following unilateral adrenalectomy [44]. In light of recent reports that describe heterozygous *DICER* mutations in humans resulting in a familial cancer syndrome, it would be interesting to see what effects, if any, may have resulted had Sf1-Cre^{low} Dicer KO animals been followed significantly beyond our experimental endpoint of 50 weeks.

We hypothesized that complete Dicer ablation in the developing adrenal cortex would result in failure of the tissue. In contrast to Sf1-Cre^{low} Dicer KO animals, Sf1-Cre^{high} Dicer KO animals exhibited severe adrenal aplasia at E18.5, despite normal fetal adrenal formation. There was a substantial delay between Cre-mediated *Dicer* excision at approximately E10-E11, and the onset of phenotypic changes in Dicer KO mice at E14.5-E16.5. This delay in phenotype onset may be due to varying half-lives of Dicer protein and/or mature miRNAs in the developing adrenal. A three to ten day delay has been reported between Cre-mediated Dicer excision and depletion of specific miRNAs in the developing mouse inner ear, and the possible persistence of other miRNAs has been observed long after Dicer ablation in Purkinje cells [45,46]. Another possibility for the delay between Dicer ablation and phenotype onset might be attributed to differing sensitivities of various tissues to miRNA mediated gene regulation. We observed increased apoptosis at E14.5 in Dicer KO adrenals, which coincides with the time at which the fetal cortex begins to transition to the adult cortex. It is known that the fetal adrenal cortex is eventually replaced by the adult adrenal cortex beginning at E14.5 [47]. Our lab has hypothesized that a small sub-population of fetal adrenocortical cells undergo a change in transcriptional programming as they populate the adrenal capsule and become adrenal stem/progenitor cells responsible for maintaining the adult adrenal cortex [5]. Such a transition would be predicted to require significant changes in gene transcription and expression, and could make the adrenal cortex more vulnerable to loss of miRNA mediated gene regulation in Dicer KO mice at this critical timepoint.

The phenotype observed in the adrenal cortex of Sf1-Cre^{high} Dicer KO animals was caused in part by increased cell cycle arrest and apoptosis, resulting in the rapid perinatal death of affected offspring, and was consistent with adrenal failure [7]. As cited earlier, many of the previous tissue-specific Dicer loss of function studies report both an increase in apoptosis, and in some cases proliferative defects. Additionally, it is known that Dicer ablation in primary cell cultures results in the induction of a DNA damage checkpoint, and subsequent p19^{Arf}-p53 signaling, leading to increased cellular senescence [40]. The increased phospho-gamma-H2A.X staining (a marker for double stranded DNA damage) we observed in Sf1-Cre^{high} Dicer KO adrenals is consistent with this report. Adrenals from Sf1-Cre^{high} Dicer KO embryos also demonstrated a significant up-regulation of p21, a cell cycle inhibitor that in part mediates senescence, and cleaved Caspase-3, an apoptotic effector caspase.

The body of data generated by the numerous Dicer loss-of-function models show that despite tissue specific differences in observed phenotypes, there may be a common mechanistic element across Dicer knockout models. However, the exact mechanism responsible for the observed phenotypes is unknown, and several potential actions other than loss of miRNA mediated gene regulation could also be involved.

One possibility may be related to the accumulation of precursor miRNAs (pre-miRNAs) that result without the downstream processing provided by Dicer [26]. There is evidence for toxic effects associated with oversaturating the endogenous miRNA machinery [48]. Animals injected with shRNA vectors into

the liver exhibit toxic effects in addition to a decrease in the expression of several liver-specific miRNAs, [49] which appears to be a result of oversaturating the endogenous miRNA processing machinery. It is unknown what effect the analogous overabundance of immature miRNA species may have on cellular homeostasis.

Dicer is also reported to exhibit miRNA-independent cell survival functions, which may also be a contributing factor to consider in the context of Dicer loss of function phenotypes. A recent report by Kaneko et. al. demonstrates the necessity of Dicer in clearing *Alu* and *Alu*-like B1/B2 RNAs in the retinal pigmented epithelium (RPE) of humans and mice, respectively; loss of Dicer in these cells resulted in degeneration of the RPE and was not dependent on dysfunctional miRNA biogenesis [50]. Similarly, Dicer has been implicated in the silencing of centromeric chromatin and regulation of differentiation in mouse embryonic stem cells through a non-miRNA-dependent mechanism [26]. These reports support the possibility that not all Dicer loss-of-function model phenotypes may be necessarily due to impaired miRNA biogenesis, but could also be due to defects in other Dicer dependent pathways such as RNAi. There may also be other uncharacterized functions of Dicer that are unrelated to the RNAi or miRNA pathways.

Finally, Dicer in nematodes is reported to undergo specific cleavage at the C-terminus of the first RNase III domain in the presence of caspases, generating a truncated protein that has DNase activity [51] and produces 3‘ hydroxyl breaks in chromosomal DNA, leading to apoptosis. If a similar phenomenon also occurs

in mammals, it could provide a feed-foward loop in which upstream caspase activation promotes cell death by acting upon residual Dicer protein that may be undetectable in Dicer KO cells. Regardless of the mode of cell death that is induced by Dicer ablation, attempting to determine the specific effects and mechanism of Dicer ablation in the context of adrenal development is a complicated proposition, and is beyond the scope of this study.

The second half of this study profiled mRNA and miRNA expression in Sf1-Cre^{high} Dicer KO adrenals at E15.5 and E16.5 with the goal of identifying differentially expressed genes resulting from Dicer ablation in knockout adrenals prior to failure of the cortex. In addition, we aimed to identify inversely expressed miRNAs that could be potential regulators of down-regulated genes observed in Dicer KO adrenals. Results from the mRNA microarray showed significant down-regulation of numerous steroidogenic genes, in addition to the sonic hedgehog (Shh) transcript. The latter is intriguing as Shh signaling between subcapsular cortical cells and the adrenal capsule is believed to be closely involved in the maintenance of an adrenocortical progenitor cell population in the capsule [11,52,53]. At E16.5, there were substantially more differentially expressed gene transcripts in Dicer KO adrenals, suggesting a progressive disruption of gene expression resulting from Dicer ablation. Additionally, a surprising number of up-regulated transcripts at both E15.5 and E16.5 were associated with immune or inflammatory response processes. We performed a DAVID analysis on the array data, and in E16.5 KOs, there was a significant enrichment of GOTERMS among differentially expressed gene transcripts that supported this finding. However,

our histologic analysis of Dicer KO adrenals did not show signs of an active inflammatory process. Further study is needed to determine whether these gene changes are related to the phenotype seen in Dicer KO adrenals, or if they are indicative of some other processes resulting from Dicer ablation.

The most interesting result was the up-regulation of Nr6a1 (Gcnf) and Acvr1c (Alk7) in Dicer KO adrenals. These observations were of particular interest to us as they are implicated in developmental processes in other tissues and organs; they are also shown to be expressed in the developing adrenal gland at E14.5 by in situ hybridization [54], although their function in the embryonic adrenal is not known. Nr6a1 is reported to play important functions in germ cell and neuronal development [55], and is a paralog of Sf1, residing a mere 13kb downstream of Sf1 on chromosome 2 [56]. Despite the close proximity to *Sf1*, the expression pattern of Nr6a1 is relatively distinct, and an insulator defining a transcriptional boundary between *Sf1* and *Nr6a1* has been previously described [57]. Nr6a1 is transiently up-regulated following retinoic acid induced differentiation of embryonic stem cells [58], and is a potent transcriptional repressor of the stem cell pluripotency factor Oct4 [59]. It is also required for proper neural stem cell and germ cell development and differentiation [55]. The expression and function of Nr6a1 in the adrenal cortex is not well characterized, but it could be hypothesized to regulate the differentiation of adrenocortical cells from the population of progenitor cells thought to reside in the subcapsular region of the cortex. Further analysis would be required to

localize Nr6a1 expression in the normal embryonic adrenal, and to further characterize its function, if any, in adrenal development.

Acvr1c (Alk7) is a member of the TGF-beta receptor superfamily, and is a type I activin receptor, working in conjunction with type II activin receptors to transduce signaling of ligands through Smad proteins. Alk7 has a restricted expression pattern in contrast with the activin type IB receptor (Alk4), and is the preferred receptor for activin AB, activin B, and Nodal [60,61]. Nodal is a secreted ligand belonging to the TGF-beta superfamily, and is responsible for mesendoderm formation, node formation, and left-right patterning in the mouse [62,63]. It is also able to induce caspase-3 dependent apoptosis by activating Alk7 signaling in a variety of cell types, in addition to normal physiologic processes such as follicular atresia in the ovary and in trophoblast cells during placentation [64-68]. There are no published reports of Alk7 expression or function in the adrenal cortex, although its role in the ovary, an organ with a common development origin with the adrenal cortex, has been described [69]. It is possible that the caspase-3 mediated apoptosis seen in our Sf1-Cre^{high} Dicer KO adrenals may be mediated in part by the up-regulation of Alk7, and would be an interesting avenue for further study. Finally, there is evidence of activin/inhibin signaling as a regulator of adrenal-gonadal fate. It has been shown previously by our lab that inhibin KO mice develop gonadal sex-cord tumors, and when gonadectomized, adrenocortical tumors. These adrenocortical tumors display a change in cellular identity from adrenal to ovary, which is facilitated by a switch in the expression of the transcription factor Gata6 to Gata4. These transcription

factors define the normal adrenal and ovary, respectively [70]. While intriguing, it is unknown whether Alk7, although it functions as a receptor for certain activin family ligands, has a direct role in this previously observed phenomenon.

Interestingly, the literature provides circumstantial evidence of cross-talk between the Nr6a1, Alk7, and Wnt/B-catenin pathways. It is known that Nr6a1 represses the expression of Cripto1, an epidermal growth factor-Cripto1/FRL1/ cryptic (EGF-CFC) family growth factor that is capable of significantly enhancing Nodal mediated signaling through Alk7 [71]. In contrast, Cripto1 is a target of the canonical Wnt/β-catenin signaling pathway, and is activated by Lef/Tcf transcription factors [72]. Previous studies from our lab demonstrate that active β-catenin is present in the subcapsular cortical cells of the adrenal gland as early as E14.5, and these cells are believed to receive Wnt signals from the adrenal capsule [2]. Further study would be required to confirm the expression and potential interaction of these signaling pathways in the normal developing adrenal. If there proves to be some degree of interaction between these 3 signaling pathways, it would likely require a complex regulatory network to maintain homeostasis. In this regard, miRNAs would be a viable candidate for fine-tuning the relative expression levels of each gene, and perturbation of the miRNA biogenesis machinery could upset the fine balance required for normal adrenal development and homeostasis.

The miRNAs found to be significantly down-regulated in E15.5 and E16.5 Dicer KO adrenals provided several interesting avenues for further study. miR 34c, miR-21, miR-10a, and let-7d have all been significantly studied in the

literature, particular in aberrant physiological processes such as tumorigenesis. Let-7 is the second miRNA to be described after lin-4, and was discovered to regulate developmental timing in nematodes [73]. In addition, it is known to regulate the oncogenes RAS [74], HMGA2 [75], and MYC [76], and can regulate proliferation pathways in human cells [77]. The miR-34 cluster of miRNAs can act as a tumor suppressor downstream of p53, and promote cell cycle arrest, apoptosis, and senescence [78,79]. In contrast, evidence supports the role of miR-10a in retinoic acid induced differentiation of neuroblastoma cells [80], and the regulation of Bcl-6, a gene involved in the development of diffuse large B-cell lymphoma [81]. miR-21 is implicated in the regulation of aldosterone synthesis in the H295 human ACC cell line [39], and believed to promote tumor metastasis and tumorigenesis by targeting PTEN [82]. Although these miRNAs have been heavily studied in the context of cancer, the body of literature concerning them could be useful in elucidating their function in developmental processes, as the pathways responsible for organism development and the pathology of cancer often coincide.

We also compared differentially expressed miRNAs from E15.5 and E16.5 Dicer KO adrenals with differentially expressed mRNA transcripts using predictive algorithms in the TargetScan method in an attempt to identify miRNA-mRNA target pairs [83]. We found that Nr6a1 and Acvr1c were both overrepresented as predicted targets of a subset of significantly down-regulated miRNAs in both E15.5 and E16.5 Dicer KO adrenals, which consisted of the following miRNAs: let-7d, miR-10a, miR-202, miR-21, miR-674, and miR-362. These associations

were not likely due to random probability, and therefore suggest that following the loss of Dicer function, dysregulation of specific miRNAs may result in de-repression of Nr6a1 and Acvr1c. There is considerable literature on the function of let-7d and miR-10a as discussed above. miR-202 expression has been observed in mouse adrenals stimulated with ACTH [84], and in porcine adrenals associated with psychosocial stress [85]. However, there are very few reports regarding the function of miR-674 and miR-362, which have been reported to be altered in Huntington's Disease models [86], and correlated with certain melanoma subtypes [87], respectively. Similarly, little is also known about miR-202, although it has been reported to be expressed in the porcine adrenal following psycho-social stress [85], and is up-regulated in the circulating blood of early stage breast cancer patients [88]. When we compared the predicted let-7 binding sites in both Nr6a1 and Acvr1c, we found that these sequences were highly conserved between mouse, human and other organisms, suggesting these sites may be evolutionarily conserved to maintain functional miRNA-mRNA interactions. Further experiments are required to empirically confirm these miRNA-target associations, and to establish whether derepression of Nr6a1 and Acvr1c by these miRNAs is a result of the Dicer KO phenotype, or is contributory to it.

This study provided evidence for the requirement of Dicer in the developing adrenal cortex. It further built on the phenotypic observation seen here and previously [36] by using a bioinformatic approach to address potential underlying mechanisms at the transcriptional level by assessing differentially

expressed mRNA and miRNA transcripts in Dicer deficient adrenals. Although we were unable to define specific mechanistic aspects of Dicer ablation in the developing adrenal cortex, adrenal enriched miRNAs and potential target mRNAs were uncovered, providing several novel avenues of further study. Subsequent analyses of embryonic adrenal glands in the mouse would be more focused based on the data collected herein, allowing for the investigation of more specific hypotheses regarding Dicer and miRNA biogenesis in the developing adrenal gland.

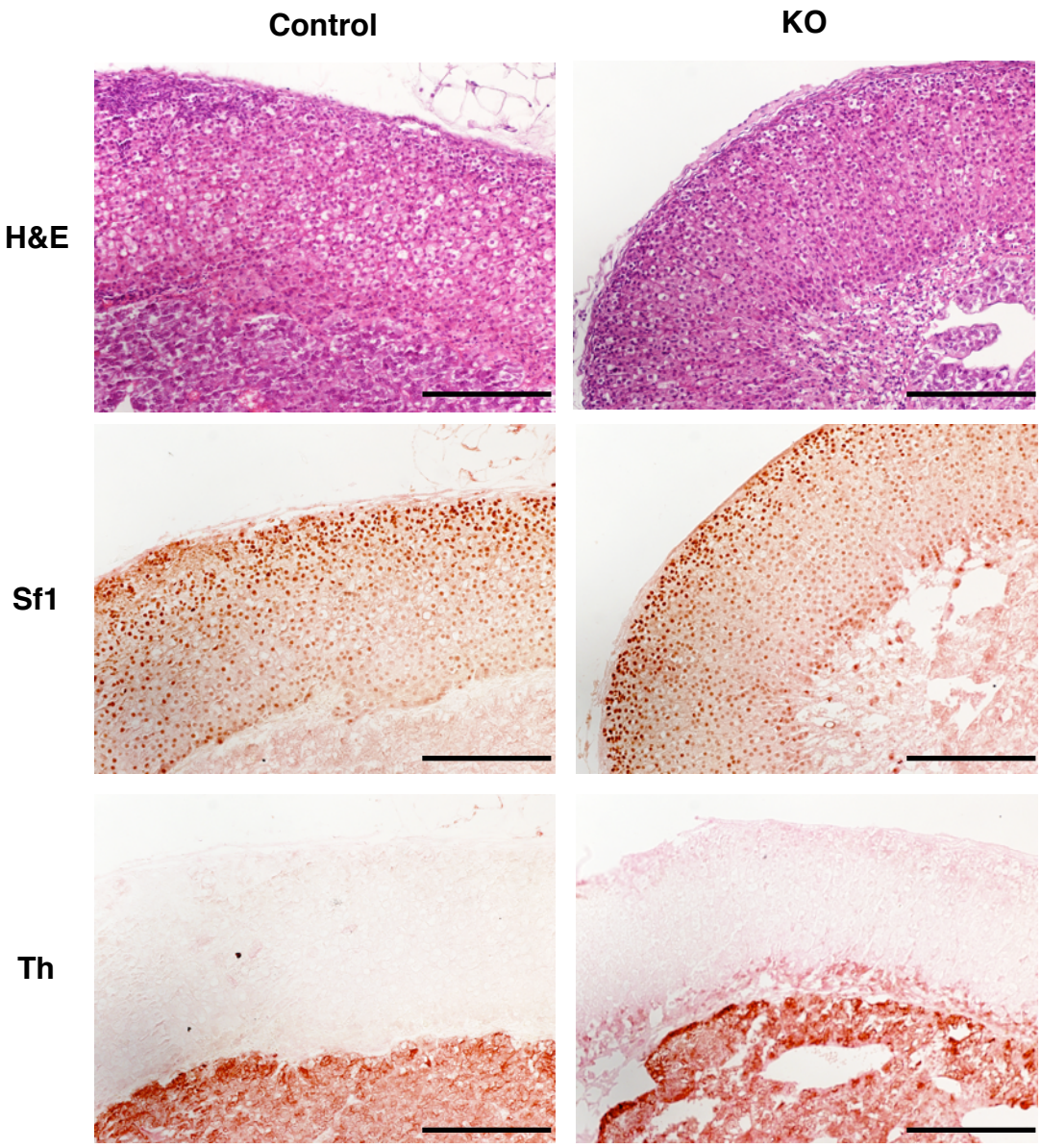


Figure 3.1 Histologic analysis of 50 week old control and Sf1-Cre^{low}/Dicer$^{lox/lox}$ (low driver) Dicer KO adrenals.

Hematoxylin and eosin staining, in addition to DAB staining for Sf1 and tyrosine hydroxylase, were performed as described in *Materials and Methods* to visualize changes in morphology. Scale bars: 100μm.

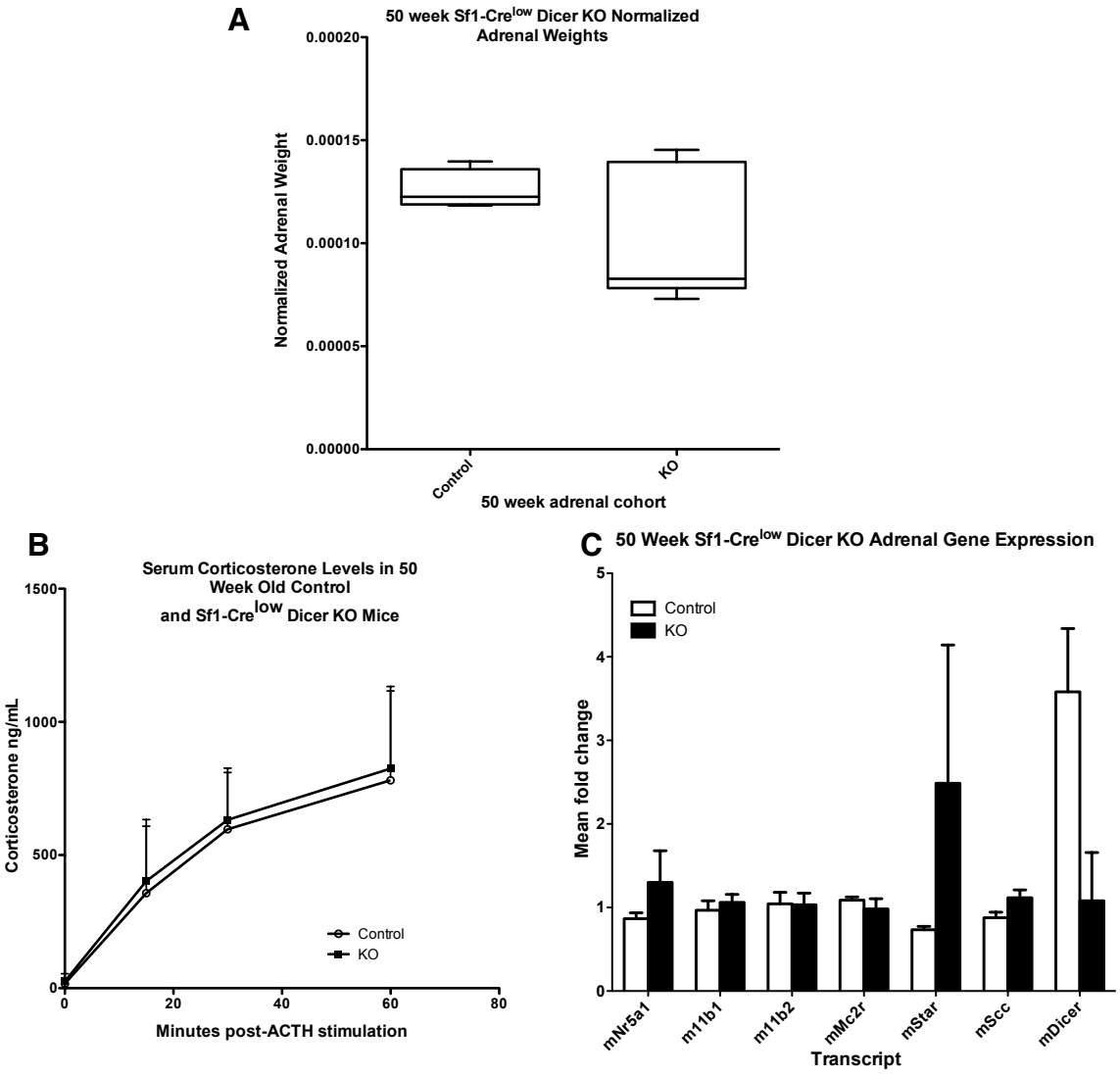


Figure 3.2. Functional analysis of Sf1-Cre^{low}/Dicer$^{lox/lox}$ Dicer KO adrenals.

(A) Adrenals weights of 50 week old control (n=4) and Sf1-Cre^{low}/Dicer$^{lox/lox}$ (n=6) adrenals normalized and expressed as a ratio to animal body weight. Horizontal lines indicate mean values, vertical lines indicate standard error. (B) Serum corticosterone following ACTH stimulation in 50 week old control and Sf1-Cre^{low}/ Dicer$^{lox/lox}$ animals. Blood samples were collected at 0, 15, 30, and 60 minutes post-ACTH injection, and corticosterone was measured by RIA. (C) Quantitative real-time PCR in total RNA isolated from 50 week old control and low driver Dicer KO adrenals. Fold changes for each sample were calculated relative to the mean dC(T) value of all the samples amplified for each gene. Open bars represent control adrenals, while solid bars represent low driver Dicer KO adrenals. All samples were run in triplicate.

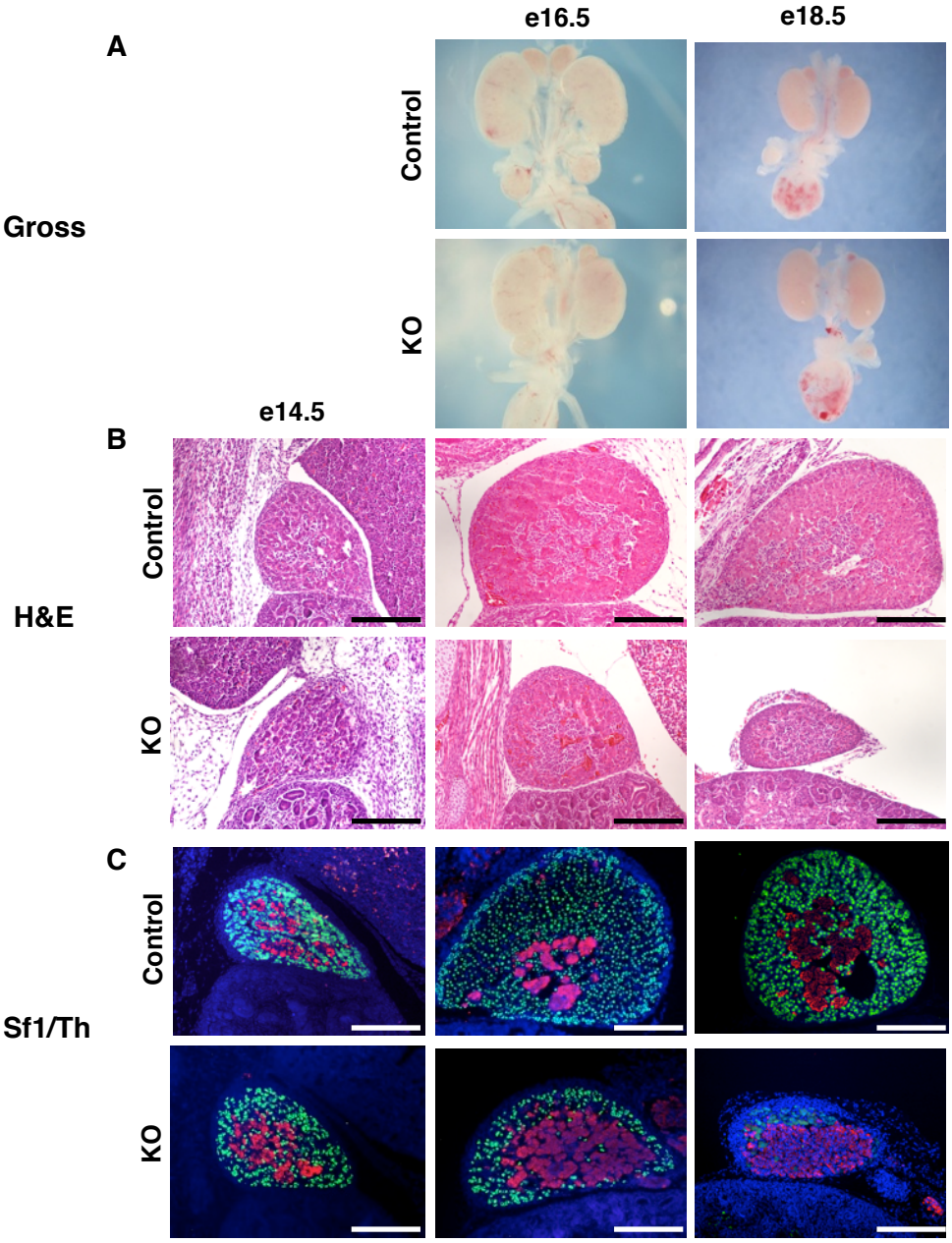
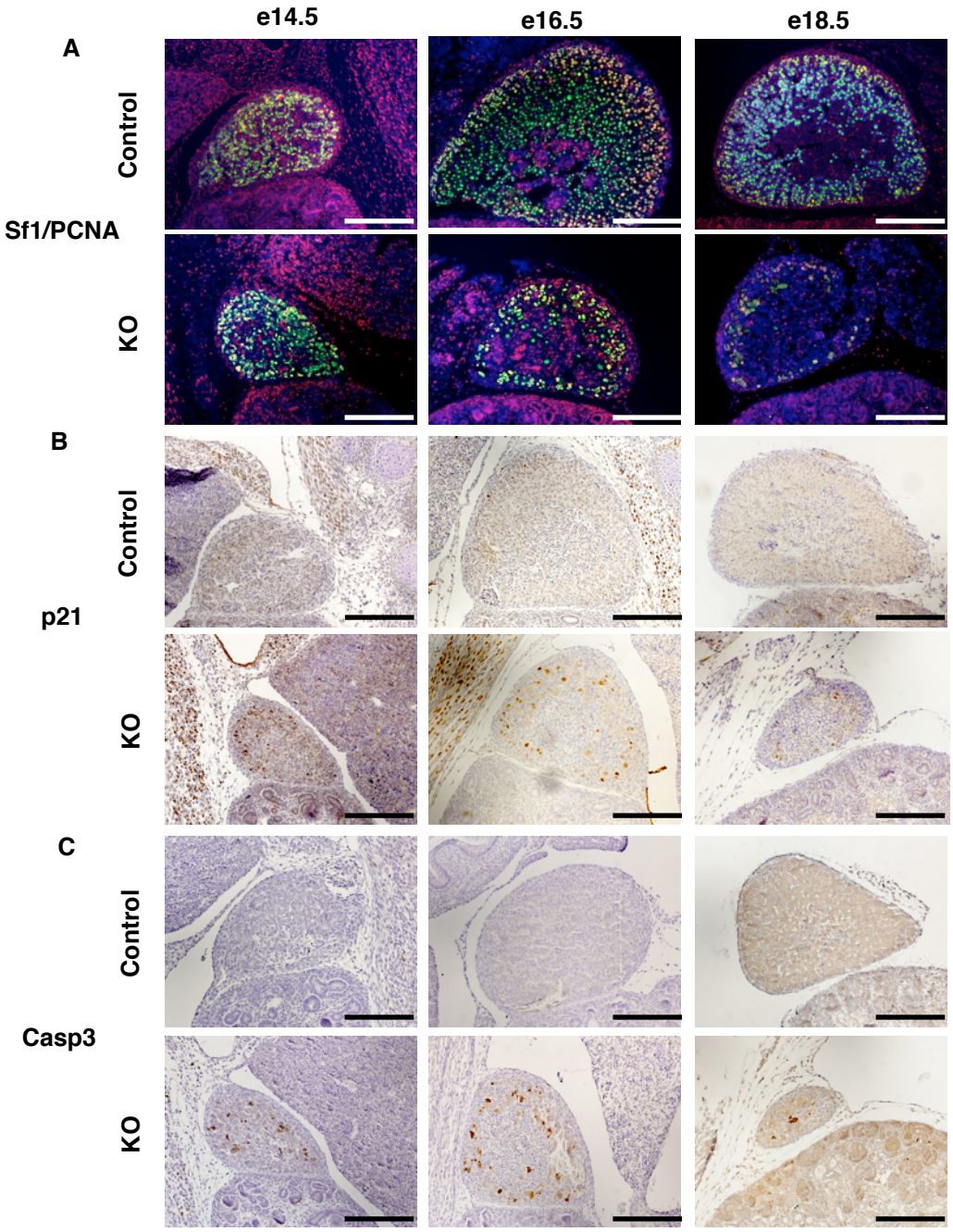


Figure 3.3. Phenotypic results of Sf1-Cre^{high}/Dicer$^{lox/lox}$ Dicer KO adrenals.

(A) Gross photos of E16.5 and E18.5 adrenals from control and high driver Dicer KO embryos. Photos from the E16.5 timepoint were taken at a 2x higher magnification than the E18.5 photos. (B) Hematoxylin/eosin staining of embryonic control and high driver Dicer KO adrenals at E14.5, E16.5, and E18.5. (C) Co-staining for Sf1 and tyrosine hydroxylase (Th) in control and high driver Dicer KO embryonic adrenals at E14.5, E16.5, and E18.5. Sections were counterstained with DAPI (blue) prior to visualization. Images were merged to show co-localization. Scale bars: 100μm.

Figure 3.4. Assessment for proliferation and cell cycle arrest/apoptosis in Sf1-Cre^{high} Dicer knockout adrenals.

(A) Co-staining for Sf1 and PCNA in control and high driver Dicer KO adrenals at E14.5, E15.5, and E16.5. Sections were counterstained with DAPI (blue) prior to visualization. Images were merged to show co-localization. (B) Immunohistochemistry with DAB for p21 (Cdkn1a), a cell cycle inhibitor. Dicer KO adrenals appear to have increased p21 staining compared to control adrenals. Tissues were counterstained with hematoxylin diluted 1:10 in deionized water. (C) Immunohistochemistry with DAB for cleaved Caspase3, an apoptotic marker. Dicer KO adrenals demonstrate significantly higher cleaved Caspase3 staining relative to control adrenals. Tissues were counterstained with hematoxylin diluted 1:10 in deionized water. Scale bars: 100μm.


e14.5
e16.5
e18.5
A
Control
Sf1/PCNA
KO
B
Control
p21
KO
C
Control
Casp3
KO

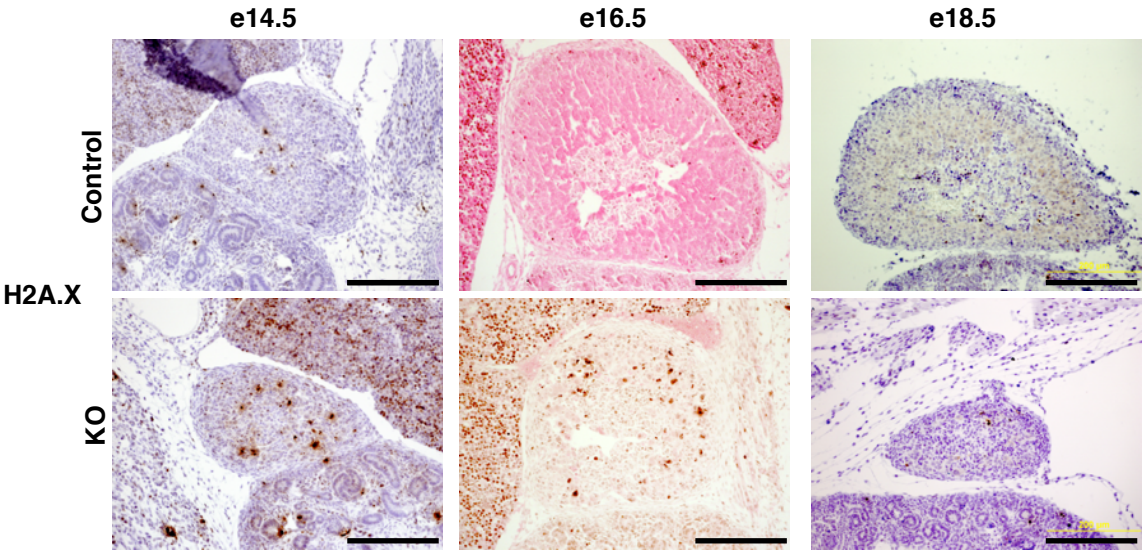
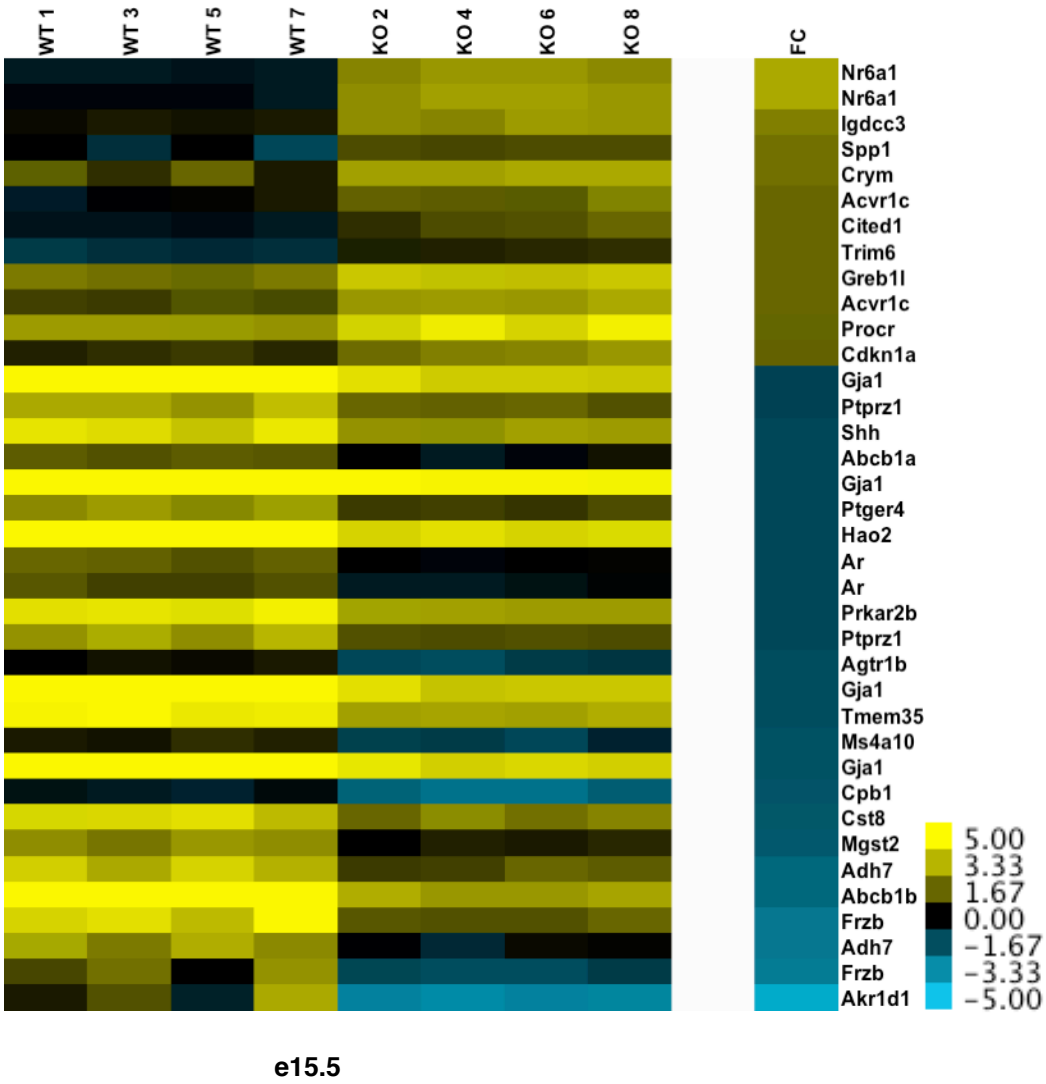


Figure 3.5. H2A.X staining in Sf1-Cre^{high} Dicer KO adrenals.

Immunohistochemistry for gamma-H2A.X in Sf1-Cre^{high} Dicer KO adrenals at E14.5, E16.5, and E18.5. Scale bars: 100μm.

A

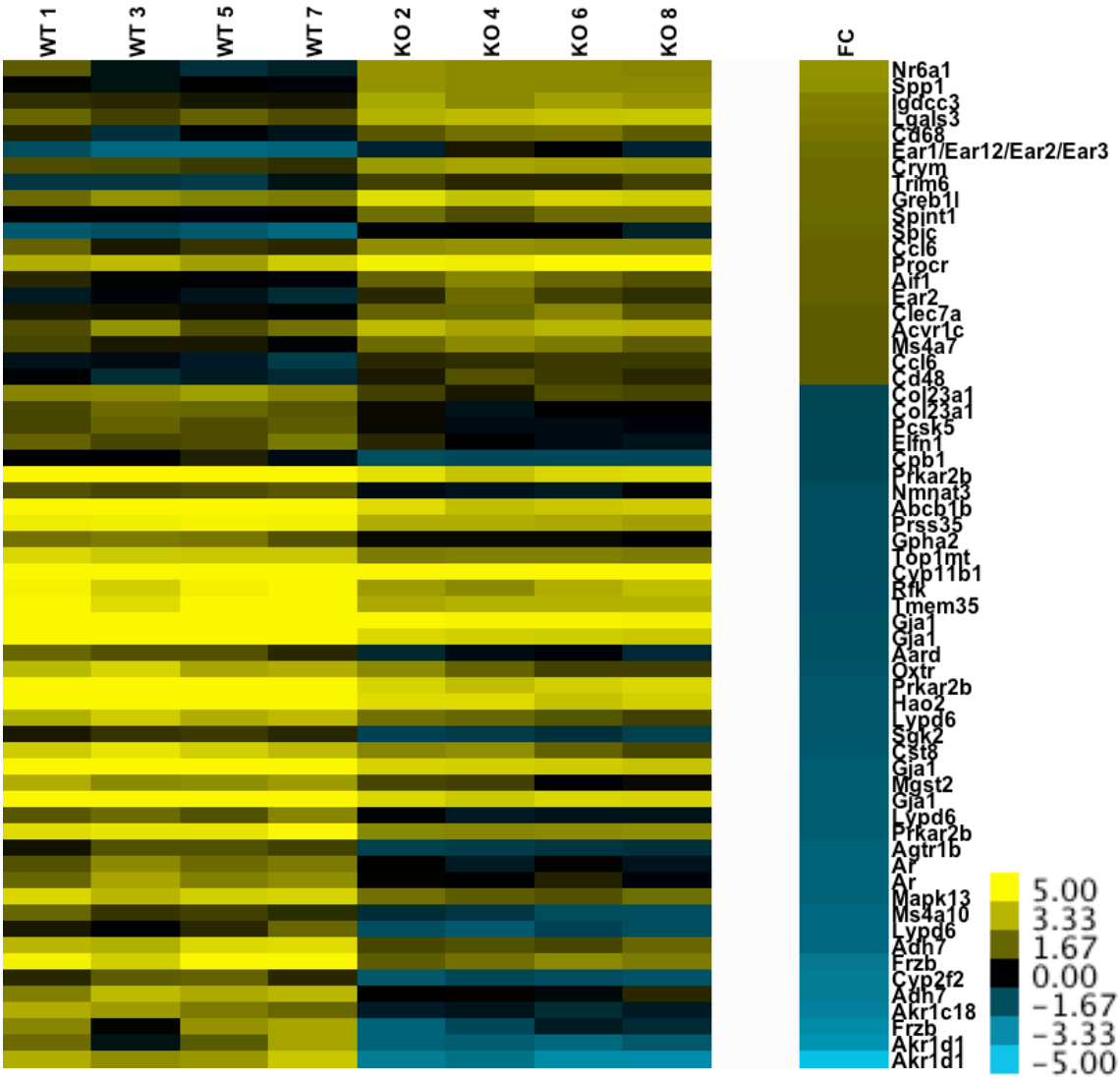


e15.5

Figure 3.6. Affymetrix gene expression data from control and Sf1-Cre^{high} Dicer KO adrenals at E15.5 and E16.5.

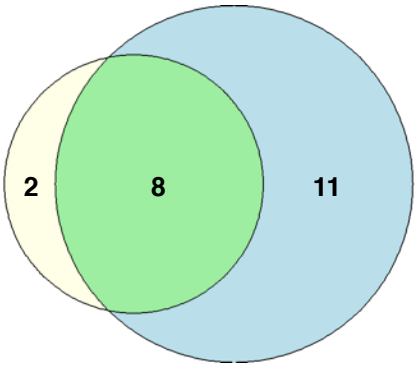
Heatmaps illustrating differentially expressed genes in Dicer KO adrenals versus control adrenals at (A) E15.5 and (B) E16.5. To narrow down differentially expressed genes, probe-sets were filtered by excluding those with a false discovery rate (FDR) of ≥ .05, and a log fold change of ≤ 1.5 or ≥ -1.5. Yellow bars indicate an increase over the mean chip intensity, and blue indicates a decrease over mean intensity. (C) Venn diagram illustrating common differentially expressed genes in E15.5 (light yellow) and E16.5 (light blue) Dicer KO adrenals. **Special thanks to Katherine Gurdziel, MS, for her significant contributions to the analysis and organization of the data depicted.**

B


WT 1
WT 3
WT 5
WT 7
KO 2
KO 4
KO 6
KO 8
FC
Nr6a1
Spp1
Igdcc3
Lgals3
Cd68
Ear1/Ear12/Ear2/Ear3
Crym
Trim6
Greb1l
Spint1
Spic
Ccl6
Procr
Aif1
Ear2
Clec7a
Acvr1c
Ms4a7
Ccl6
Cd48
Col23a1
Col23a1
Pcsk5
Efn1
Cpb1
Prkar2b
Nmnat3
Abcb1b
Prss35
Gpha2
Top1mt
Cyp11b1
Rfk
Tmem35
Gja1
Gja1
Aard
Oxtr
Prkar2b
Hao2
Lypd6
Sgk2
Cst8
Gja1
Mgst2
Gja1
Lypd6
Prkar2b
Agtr1b
Ar
Ar
Mapk13
Ms4a10
Lypd6
Adh7
Frzb
Cyp2f2
Adh7
Akr1c18
Frzb
Akr1d1
Akr1d1
5.00
3.33
1.67
0.00
-1.67
-3.33
-5.00

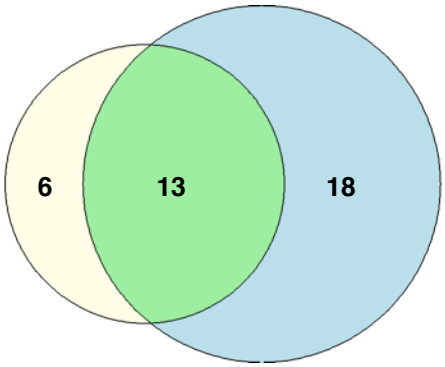
e16.5

C



e15.5 e16.5 KO UP

Acvr1c
Crym
Greb1l
Igdcc3
Nr6a1
Procr
Spp1
Trim6



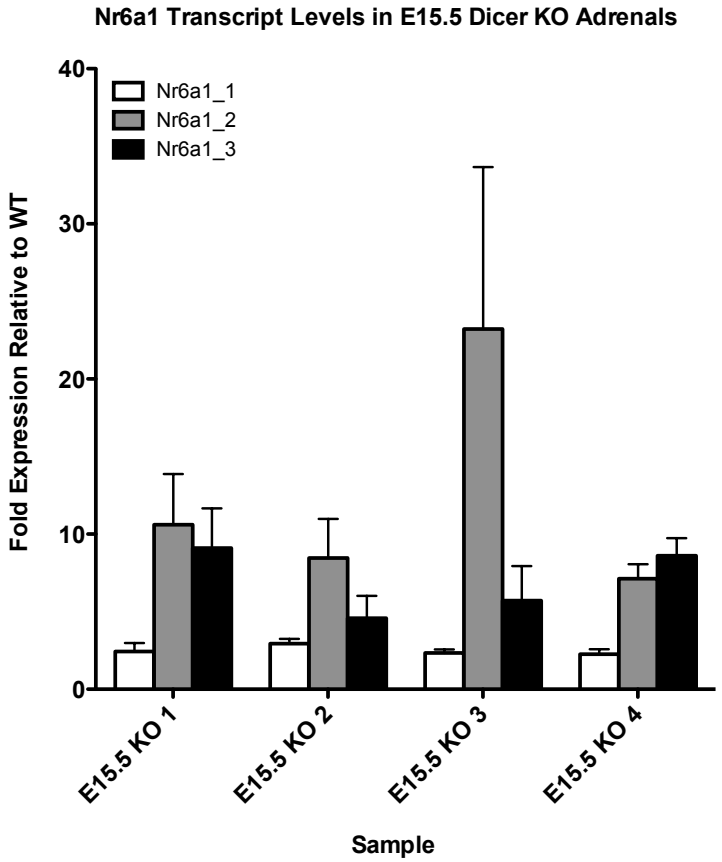
e15.5 e16.5 KO DOWN

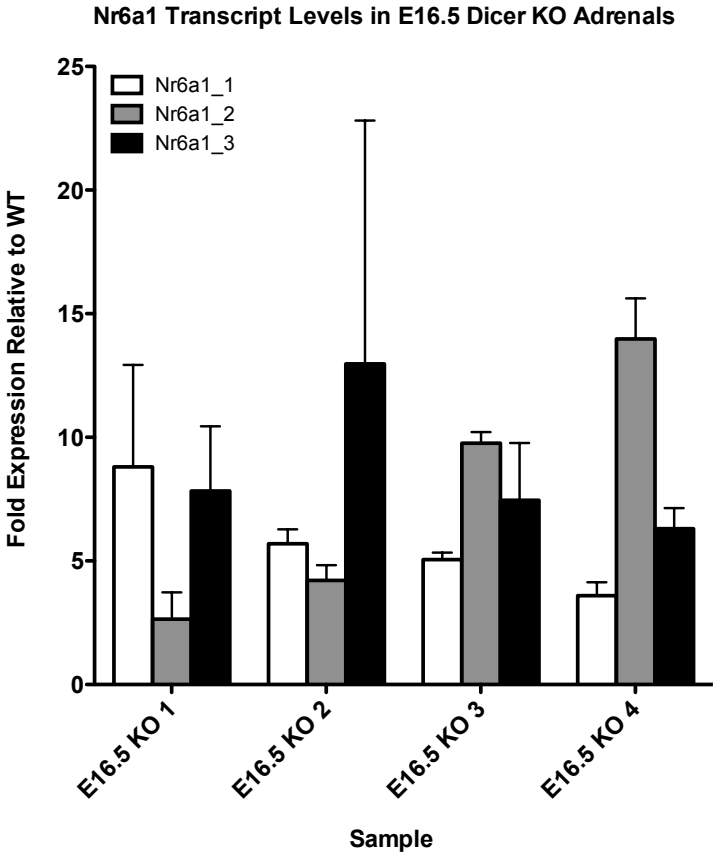
Abcb1b
Adh7
Agtr1b
Akr1d1
Ar
Cpb1
Cst8
Frzb
Gja1
Hao2
Mgst2
Ms4a10
Prkar2b

Figure 3.7. Quantitative real time PCR to confirm Nr6a1 expression in Sf1-Cre^{high} Dicer KO adrenals and Sf1-Cre only tissues.

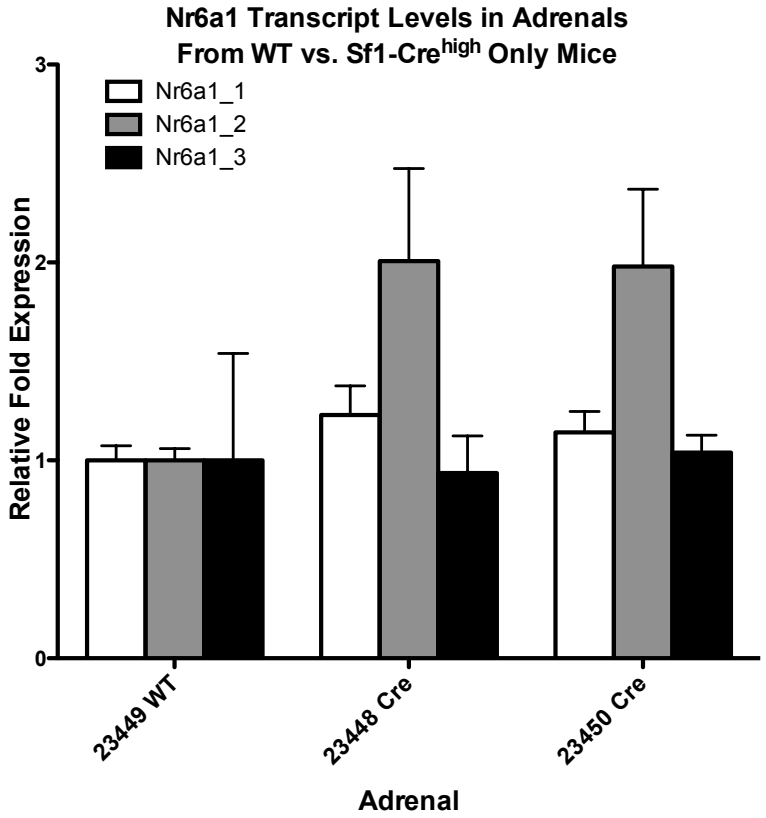
(A) Expression of the 3 Nr6a1 transcript isoforms in 3 individual samples of E15.5 and E16.5 Sf1-Cre^{high} Dicer KO adrenals relative to control adrenals. Isoform 2 (Nr6a1_2) is the most highly expressed at E15.5 by up to 20 fold; isoform 3 (Nr6a1_3) is also up-regulated relative to control adrenals but to a much lesser degree. At E16.5 isoform 1 (Nr6a1_1) expression is up-regulated in Dicer KO adrenals, in addition to isoforms 2 & 3. (B) Comparison of Nr6a1 expression in adrenals from adult mice expressing only the Sf1-Cre^{high} transgene (Cre), relative to wild type (WT) animals. Expression of isoform 2 seems to suggest leakage of a 3' transcript from the transgene. (C) Additional Nr6a1 expression data in adult adrenal, brain, and liver from mice expressing only the Sf1-Cre^{high} transgene. Expression is relative to corresponding tissue from wild type (WT) animals (not shown).

A


Nr6a1 Transcript Levels in E15.5 Dicer KO Adrenals
Nr6a1_1
Nr6a1_2
Nr6a1_3
Fold Expression Relative to WT
40
30
20
10
0
E15.5 KO 1
E15.5 KO 2
E15.5 KO 3
E15.5 KO 4
Sample


Nr6a1 Transcript Levels in E16.5 Dicer KO Adrenals
Nr6a1_1
Nr6a1_2
Nr6a1_3
Fold Expression Relative to WT
25
20
15
10
5
0
E16.5 KO 1
E16.5 KO 2
E16.5 KO 3
E16.5 KO 4
Sample

B


Nr6a1 Transcript Levels in Adrenals
From WT vs. Sf1-Cre^high Only Mice
Nr6a1_1
Nr6a1_2
Nr6a1_3
Relative Fold Expression
0
1
2
3
23449 WT
23448 Cre
23450 Cre
Adrenal

C

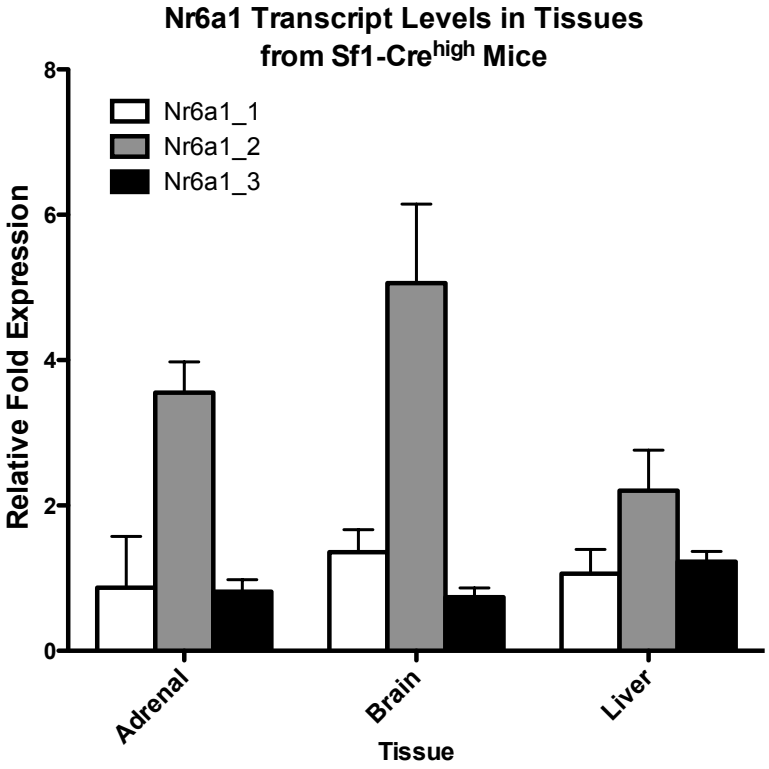
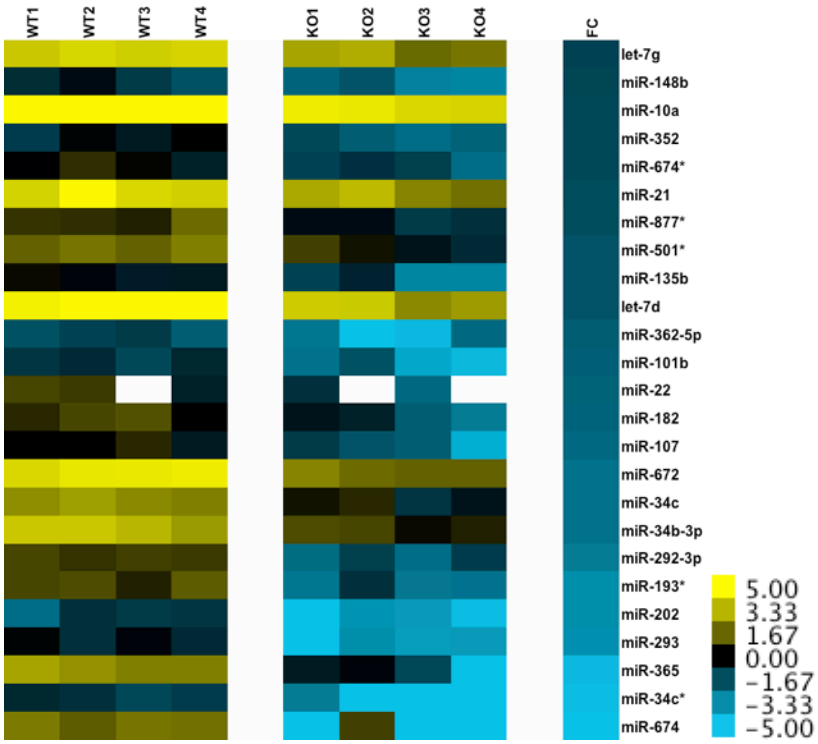

Nr6a1 Transcript Levels in Tissues
from Sf1-Cre^high Mice
Nr6a1_1
Nr6a1_2
Nr6a1_3
Relative Fold Expression
0
2
4
6
8
Adrenal
Brain
Liver
Tissue

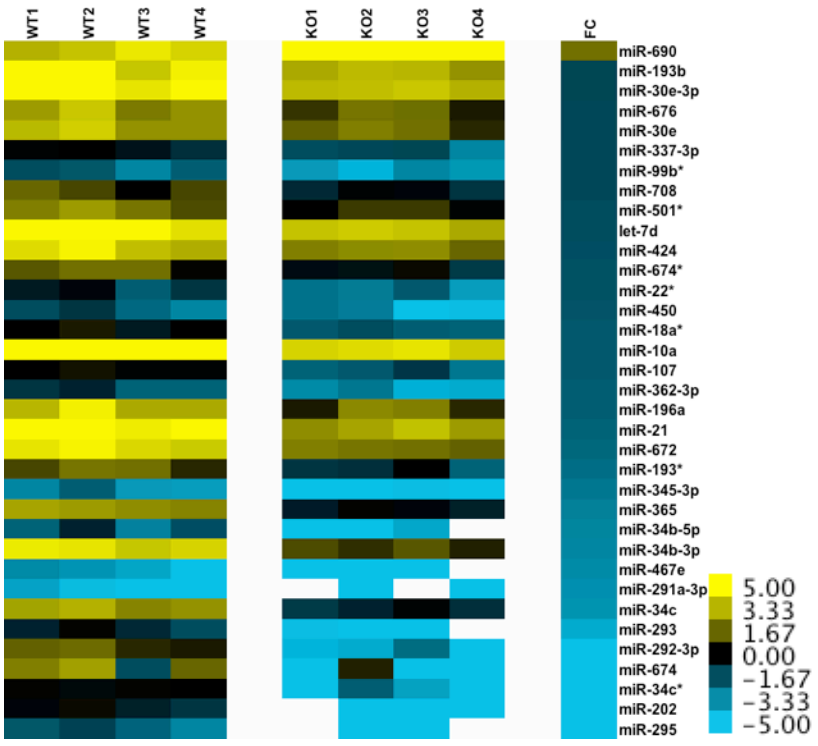
Figure 3.8. Differentially expressed miRNAs in Sf1-Cre^{high} Dicer KO adrenals.

OpenArray assays for rodent miRNA chips were utilized for differential miRNA analysis as described in *Materials and Methods*. (A) Differentially expressed miRNAs at both E15.5 and E16.5 in Dicer KO adrenal relative to control adrenals were filtered to select only those whose expression was changed significantly with a p value of ≤ .05. Only miRNAs with a fold change of ≥ 1.5 or ≤ -1.5 were included. (B) Venn diagram illustrating commonly down-regulated miRNAs at both E15.5 and E16.5 in Dicer KO adrenals. **Special thanks to Katherine Gurdziel, MS, for her significant contributions to the analysis and organization of the data depicted.**

A

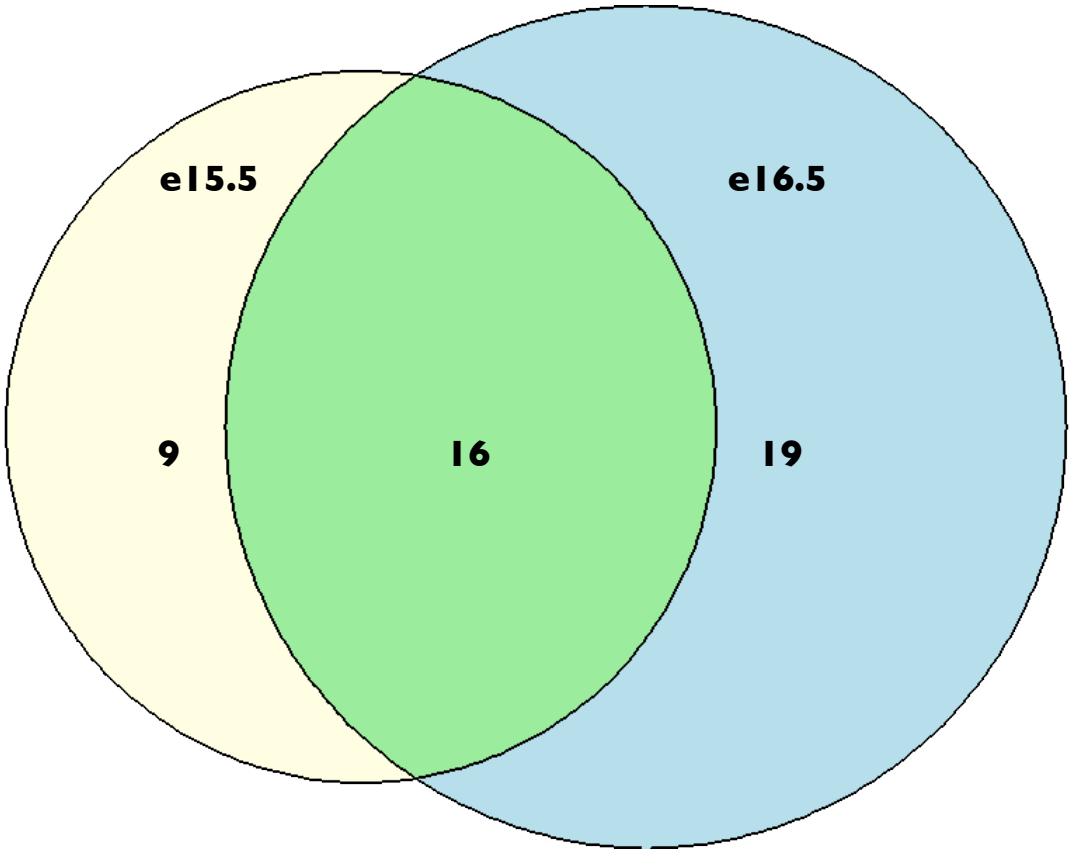

WT1
WT2
WT3
WT4
KO1
KO2
KO3
KO4
FC
let-7g
miR-148b
miR-10a
miR-352
miR-674*
miR-21
miR-877*
miR-501*
miR-135b
let-7d
miR-362-5p
miR-101b
miR-22
miR-182
miR-107
miR-672
miR-34c
miR-34b-3p
miR-292-3p
miR-193*
miR-202
miR-293
miR-365
miR-34c*
miR-674
5.00
3.33
1.67
0.00
-1.67
-3.33
-5.00

e15.5


WT1
WT2
WT3
WT4
KO1
KO2
KO3
KO4
FC
miR-690
miR-193b
miR-30e-3p
miR-676
miR-30e
miR-337-3p
miR-99b*
miR-708
miR-501*
let-7d
miR-424
miR-674*
miR-22*
miR-450
miR-18a*
miR-10a
miR-107
miR-362-3p
miR-196a
miR-21
miR-672
miR-193*
miR-345-3p
miR-365
miR-34b-5p
miR-34b-3p
miR-467e
miR-291a-3p
miR-34c
miR-293
miR-292-3p
miR-674
miR-34c*
miR-202
miR-295
5.00
3.33
1.67
0.00
-1.67
-3.33
-5.00

e16.5

B



E15.5 & E16.5 Down-regulated miRNAs

miR-674*	miR-34b-3p
miR-10a	miR-292-3p
miR-21	miR-193*
miR-501*	miR-202
let-7d	miR-293
miR-107	miR-365
miR-672	miR-34c*
miR-34c	miR-674

A

Term	Percentage	Fold Enrichment	p-value
Innate immune response	2.840909091	5.218794008	9.72E-07
Positive regulation of immune response	3.598484848	5.200886382	2.27E-08
Activation of immune response	2.272727273	5.194520548	1.78E-05
Immune effector process	3.03030303	4.727288541	1.36E-06
Positive regulation of immune system process	4.545454545	4.337172496	7.13E-09
Regulation of cytokine production	3.03030303	4.28516803	4.71E-06
Positive regulation of response to stimulus	3.787878788	4.002945942	6.00E-07
Regulation of cell activation	3.03030303	3.818194591	1.92E-05
Cell activation	4.734848485	3.783272079	4.72E-08
Leukocyte activation	3.787878788	3.399762307	6.92E-06
Immune response	7.954545455	3.31964052	2.48E-11
Response to wounding	5.492424242	3.111223402	2.07E-07
Cell surface	4.734848485	2.831265284	8.55E-06

Table 3.1. DAVID enrichment analysis illustrating differentially expressed genes enriched for specific GOTERMS.

Enriched GOTERMS in E16.5 Dicer KO adrenals relative to E16.5 control (A). Enriched GOTERMS in E16.5 versus E15.5 Dicer KO adrenals (B). FDR ≤ .05 and fold changes ≥ 1.5 were used as cut-offs. **Special thanks to Katherine Gurdziel, MS, for her significant contributions to the analysis and organization of the data depicted.**

B

Term	Percentage	Fold Enrichment	p-value
Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	5.93220339	12.58148148	1.80E-05
Adaptive immune response	5.93220339	12.58148148	1.80E-05
Inflammatory response	10.16949153	8.052148148	2.36E-07
Response to wounding	12.71186441	6.526416907	5.22E-08
External side of plasma membrane	8.474576271	6.193778482	3.07E-05
Cell surface	11.86440678	5.856674473	6.22E-07
Immune response	14.40677966	5.449304081	5.78E-08
Carbohydrate binding	9.322033898	5.36160223	3.33E-05
Defense response	12.71186441	5.055059524	1.17E-06

e15.5 Differentially Expressed miRNAs			e16.5 Differentially Expressed miRNAs		
miRNA	**Predicted Targets**	**Number of Predicted Sites**	**miRNA**	**Predicted Targets**	**Number of Predicted Sites**
let-7g	Nr6a1	4	let7d	Nr6a1	4
let7d	Igdcc3	2		Igdcc3	2
	Acvr1c	1		Acvr1c	1
	Greb1l	1		Greb1l	1
miR-10a	Nr6a1	1	miR-10a	Nr6a1	1
miR-202	Igdcc3	2	miR-202	Igdcc3	2
	Acvr1c	1		Acvr1c	1
	Greb1l	1		Greb1l	1
	Nr6a1	1		Nr6a1	1
miR-21	Acvr1c	1	miR-21	Acvr1c	1
miR-674	Acvr1c	1	miR-674	Acvr1c	1
miR-182	Acvr1c	1	miR-30e	Nr6a1	2
miR-101b	Acvr1c	1	miR-362	Greb1l	1
	Greb1l	1			
	Nr6a1	1			
miR-362	Greb1l	1			

Table 3.2. Predicted targets of differentially expressed miRNAs.

Differentially expressed miRNAs in E15.5 and E16.5 Dicer KO adrenals were cross referenced with differentially expressed genes for predicted mRNA-miRNA interactions based on target predictions algorithms from Targetscan (targetscan.org). Some miRNAs have more than one predicted gene target, and some predicted targets have multiple potential miRNA binding sites in the 3' UTR.

Special thanks to Katherine Gurdziel, MS, for her significant contributions to the analysis and organization of the data depicted.

Predicted let-7 binding sites in the Nr6a1 3' UTR

```
        ....340.......350.......360.........370.......380..
Mmu     CUAGAUGAGUAUUUCCAUAAUGUUGACAAA-GACAACUACCUCAAUGGAA
Hsa     GCAGAUGACUAUUUCCAUAAUGUUGACAAA-GAUGACUACCUCAAUGGAA

        .........1230......1240......1250.........1260....
Mmu     CAUUAAACUACCUCAUGUUUCUAAGGGCUAGGCUGCU---GCU--CUGCA
Hsa     CUUUAAACUACCUCAGGUUCCUAAUGGCCCGGCUGCU---ACC-UCUGCA

        ......3830.......3840......3850......3860.......38
Mmu     GA-CUACCUCUUGAG-UGACAUCCUGGUCGAUUCCUCUCU-GAGGAGUCC
Hsa     AA-CUACCUCUUAAG-CGACAUCCUGGUCGAUUCCUCUCU-GAGGAAUCC

        .....4310..........4320.........................4
Mmu     CUCUGCUACCUCC----CACA-----------------CUU-GC-CUGC
Hsa     CUCUGCUACCUCC----CAC------------------CCU-GC-CAGC
```

Predicted let-7 binding sites in the Acvr1c 3' UTR

```
        ..100.......110.............................120....
Mmu     GUUCUACCUCAAG---------------GAUGACU---C--ACUACAGU
Hsa     GUUCUACCUCAAA---------------GAUAAGA---C--AGUACAGU
```

Table 3.3 Predicted let-7 binding sites in Nr6a1 and Acvr1c 3' UTRs.

Comparative sequence analysis between mouse and human 3' UTRs based on TargetScan (www.targetscan.org) miRNA-mRNA target prediction algorithms. The four predicted let-7 sites in the 3' UTR of mouse (Mmu) *Nr6a1* are shown and compared with human (Hsa) *NR6A1*. let-7 seed sequences are highlighted in red. Additionally, the single predicted let-7 binding site in both human and mouse *ACVR1C* are also shown, with the seed sequence highlighted in red. The predicted binding sites for both genes are tightly conserved between human and mouse, in addition with other vertebrates.

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CHAPTER 4

Summary and Future Directions

Summary

Dicer is an RNase III enzyme necessary for the maturation of miRNAs, which fine tune the expression of target genes by repressing their translation at the post-transcriptional level. Loss of Dicer in various biological models results in a down regulation of mature miRNAs, which in turn is predicted to disrupt the normal, homeostatic expression levels of countless target genes. In vitro models of Dicer loss have demonstrated increased activity of cell cycle inhibitors leading to failure to proliferate, senescence and apoptosis. In other models involving stem cells, the lack of Dicer has led to impaired regulation of pluripotency and differentiation. Furthermore, miRNA independent functions for Dicer have been described, adding another level of complexity to Dicer mediated regulatory functions in the cell. In vivo, tissue-specific Dicer loss of function results in developmental failure of the targeted tissue or organ system that is consistent with in vitro observations. Although it would appear loss of miRNA biogenesis due to Dicer inactivation is the primary reason for the phenotypes seen in Dicer knockout models, recent evidence suggests miRNA independent function of Dicer may also play a role. miRNAs are also implicated in human disease, and DICER expression is often perturbed in human tumors, and genetic

haploinsufficiency of the gene has been implicated in familial tumor syndromes. Additionally, many specific miRNAs have been implicated in the tumorigenesis of human cancers by their ability to regulate cellular processes such as differentiation, proliferation, and apoptosis. This work addresses the developmental effects of Dicer loss in the embryonic adrenal cortex, and the correlation between miR-483-3p and IGF2 in adrenocortical carcinoma.

Chapter 2 confirms the observation that in a high percentage of sporadic human ACC cases, IGF2*,* an adrenocortical mitogen, is highly up-regulated, presumably due to defects at the 11p15.5 locus. In conjunction with IGF2 up-regulation in ACC, the miR-483 locus residing in the 2nd intron of *IGF2* was concurrently over expressed in human ACCs, and the IGF2 expressing human ACC cell line, NCI-H295. This result suggested that the H295 cell line may serve as a useful in vitro model in which to study the effects of IGF2 and miR-483-3p function in the tumorigenesis of ACC. We designed and constructed several molecular tools in an attempt to perturb miR-483-3p expression in vitro and identify putative gene targets in the context of ACC. First, a miR-483-3p expression vector was constructed that was able to express miR-483-3p at similar levels seen in the H295 cell line. We also showed that the mature miRNA from this expression vector was able to function as an siRNA towards a luciferase reporter designed to respond to miR-483-3p. We also demonstrated that endogenous miR-483-3p in H295 cells had a similar inhibitory effect on the luciferase reporter. We also constructed a miR-483-3p “sponge” designed to be stably expressed at high levels and act as a competitive inhibitor for miR-483-3p.

Unfortunately the results of this sponge were inconclusive, hampered in part by the lack of validated miR-483-3p targets that could be used as a gold standard to determine the efficacy of the sponge. Cellular growth of H295A and H295R cells transduced with the miR-483-3p sponge was slightly affected, as there were fewer viable cells at the endpoint of each experiment compared with controls. This may have indicated that the sponge needed further optimization to be more efficacious, or that miR-483-3p does not affect cellular proliferation as we had hypothesized. We also determined that nine predicted miR-483-3p target genes are significantly down-regulated in primary human ACCs that express high levels of endogenous IGF2. Of these predicted targets, IGF1 and EGR1 were of particular interest as IGF1 can act as an adrenal mitogen, and EGR1 is described as a tumor suppressor. We assessed mRNA levels of IGF1 and EGR1 in H295 cells transduced with the miR-483-3p sponge, and observed a modest increase in EGR1 transcript levels, but inconclusive changes in IGF1 transcript levels. These two predicted targets would be ideal candidates for further target validation and additional study in the context of ACC. The molecular tools described in Chapter 2 showed promise, but it became readily apparent that with the emergence of commercial reagents designed for miRNA research, construction of such tools for routine studies would be relatively inefficient from a financial and labor perspective. Furthermore, independent research groups were were able to describe potential mechanistic functions of miR-483-3p by reporting a putative target, BBC3/PUMA. This miRNA-target association is consistent with the aggressive nature of ACC, as PUMA functions as a pro-apoptotic regulator. It

also supports the theory that miR-483-3p co-expression with IGF2 may synergize to drive a more aggressive form of ACC compared to those that do not express high levels of IGF2.

Chapter 3 describes the results of generating a tissue specific Dicer KO mouse. We utilized both the Sf1-Cre^{low} and Sf1-Cre^{high} transgenic mice to compare the effects of a continual, stochastic model of Dicer inactivation that persisted through adulthood (Sf1-Cre^{low}*)*, and a complete model of Dicer inactivation in the adrenal cortex that occurred in early development (Sf1-Cre^{high}*)*. Use of the Sf1-Cre^{low} transgene to knock out Dicer in a subset of adrenocortical cells did not result in an appreciable phenotype over the course of 50 weeks. This suggested that the adrenal cortex was able to compensate for any deleterious effects resulting from a continuous, stochastic pattern of Dicer inactivation. Another possible explanation is that Dicer and miRNA biogenesis is not as important for the maintenance of the adrenal cortex versus adrenocortical development. In contrast, Sf1-Cre^{high} Dicer KO mice underwent a dramatic failure of the adrenal cortex that began as early as E14.5 and rapidly progressed over the subsequent 4 days. The adrenals in Sf1-Cre^{high} Dicer KO mice initiated normal development, as the presence of adrenal medullary cells implied that coalescence of the fetal cortex had occurred. However, our results show that at approximately E14.5, the cortex began to fail, and resulted in the complete loss of cortical cells. Unsurprisingly, these animals died shortly after birth as a result of apparent adrenal failure. Analysis of embryonic Sf1-Cre^{high} Dicer KO mice revealed increased DNA damage, as evidenced by an increase in H2A.X

staining, and the up regulation of the cell cycle arrest and apoptotic markers, p21 and cleaved Caspase-3, respectively. In contrast, Sf1-PCNA co-immunofluorescence indicated that proliferation remained unchanged. Together, these result indicate that the E14.5 time point may be host to an important developmental milestone that is sensitive to Dicer expression or miRNA biogenesis. As previously cited in Chapter 3, E14.5 has been shown to be the developmental time point at which the fetal cortex begins to regress, and Sf1 expression through the fetal adrenal enhancer is abrogated. It is possible that this critical transition point from fetal to adult cortex is particularly sensitive to perturbations in miRNA biogenesis. Alternatively, if the source of adrenal stem/progenitor cells indeed comes from Sf1 expressing fetal cortical cells as hypothesized, then the inactivation of Dicer in these cells may prevent them from completing this transition, resulting in the loss of the adrenal stem/progenitor cell pool. This is an interesting possibility as it has been demonstrated that Dicer inactivation can adversely affect stem cells by resulting in the down regulation of Oct4. Additional studies are required to further pursue this hypothesis.

The second goal of this in vivo project involved profiling the differentially expressed mRNA and miRNA transcripts in Sf1-Cre^{high} Dicer KO adrenals versus control adrenals. We profiled four biological replicates each for Dicer KO and control adrenals, then compared differentially expressed mRNA and miRNA transcripts between the two experimental groups. Unsurprisingly, numerous steroidogenic enzymes were down regulated in Dicer KO adrenals. We also observed and confirmed through DAVID analysis that a significant number of

genes associated with an inflammatory or immune response were up regulated as well. The up-regulation of these immune and inflammatory pathway genes could play a role in the phenotype seen in Dicer KO adrenals, which was characterized by the expression of cell cycle arrest and apoptotic protein markers. The most intriguing results, however, were the observation that Nr6a1 and Acvr1c were highly up-regulated in Sf1-Cre^{high} Dicer KO adrenals. These genes have not been functionally described in the adrenal cortex previously, but have been shown to be important in regulating development and differentiation in other organ systems. For example, Nr6a1 regulates the pluripotency and differentiation of stem cells by repressing the pluripotency marker Oct4, and has also been implicated in the differentiation of neural progenitor cells and the regulation of germ cells. Acvr1c is a type I activin receptor that transduces signals from the Nodal ligand, which helps regulate developmental patterning in the mouse. The potential roles of these two genes in adrenal development are promising avenues for subsequent study. We also profiled the differentially expressed miRNAs in Dicer KO adrenals compared to controls. As expected, nearly all of the differentially expressed miRNAs in Dicer KO adrenals were down regulated, presumably due to the loss of Dicer activity. There was significant overlap in down regulated miRNAs between E15.5 and E16.5 Dicer KO adrenals, with 16 commonly down regulated miRNAs. Of these, miR-34c, miR-21, miR-10a, and let-7d were among the most interesting candidates for follow up study given the considerable amount of literature that exists regarding their function. As discussed previously in this work, let-7 is known to regulate the

oncogenes RAS, HMGA2, and MYC, and can regulate proliferation pathways in human cells. Similarly, the miR-34 cluster of miRNAs can act as a tumor suppressors downstream of p53, and promote cell cycle arrest, apoptosis, and senescence. In contrast, evidence supports the role of miR-10a and miR-21 as oncogenic miRNAs, as they are often up regulated in human cancers, and in the case miR-21, is believed to promote tumor metastasis and tumorigenesis by targeting PTEN. Finally, we asked whether the down regulated miRNAs in Dicer KO adrenals were predicted to target any up regulated mRNA transcripts. We matched the list of up regulated mRNA transcripts from E15.5 and E16.5 Dicer KO adrenals with down regulated miRNA transcripts using the TargetScan algorithm to determine predicted miRNA-target associations. We discovered that a small subset of down regulated miRNAs in Sf1-Cre^{high} Dicer KO adrenals were consistently predicted to target Nr6a1 and Acvr1c. These miRNAs were as follows: let-7d, miR-10a, miR-202, miR-21, miR-674, and miR-362. Whether the up regulation of these two genes is a direct result of or in response to Dicer loss is still unknown, and further study is required to validate the predicted miRNA-mRNA interactions. These data demonstrate that Dicer loss in the developing adrenal cortex resulted in a unique transcriptional and miRNA profile which may reflect the underlying mechanism of adrenocortical failure seen in Sf1-Cre^{high} Dicer KO mice.

Future Directions

The results from Chapter 2 confirmed the expression of IGF2 and miR-483-3p in ACC, and provided possible gene targets of miR-483-3p for subsequent study. Identifying the gene targets of miR-483-3p would be a significant advancement in understanding how this miRNA affects the process of tumorigenesis in ACC. As discussed previously, IGF1 and IGF2 are both expressed in the normal adult human adrenal, whereas IGF2 is predominantly expressed in the embryonic and cancerous adrenal gland. Could there be a regulatory mechanism by which IGF2 expression indirectly (through miR-483-3p) inhibits IGF1? What are the consequences of IGF1 down regulation, if any, in the context of ACC? Furthermore, the fact that EGR1 is also a predicted miR-483-3p target is of great interest considering the role of EGR1 as a tumor suppressor gene that is often down regulated in a number of human cancers. It is therefore a reasonable hypothesis that low EGR1 expression seen in primary human ACCs is a contributory factor in ACC. Validation of IGF1 and EGR1 as targets of miR-483-3p could be performed using the now widely available molecular tools to perturb endogenous miR-483-3p and the H295 cell line could be utilized as a model system in which to pursue this goal. Knockdown of miR-483-3p could be accomplished by transfecting synthetic oligos designed to competitively inhibit miR-483-3p, and the resulting changes in IGF1 and EGR1 transcript levels can be measured using standard methods such as quantitative real-time PCR. Protein could also be measured using Western blot techniques. In contrast, the effects of miR-483-3p over expression on IGF1 and EGR1 could

be studied in the SW13 cell line, which does not express high levels of endogenous IGF2 or miR-483-3p. Over expression of miR-483-3p in such a system would be predicted to have an opposite effect on IGF1 and EGR1 transcript levels. Finally, target validation would have to be confirmed using an exogenous GFP or luciferase reporter construct bearing the 3' UTR of either IGF1 or EGR1. If the predicted target sequences in the UTRs of both genes are responsive to miR-483-3p and responsible for target inhibition, we would expect to see decreased reporter activity relative to control reporter constructs in which the predicted target sequences are mutated such that they no longer recognize miR-483-3p. The results of these experiments would complement what is known about the previously validated miR-483-3p target, BBC3/PUMA.

Although BBC3/PUMA has been reported to be a target of miR-483-3p in human ACCs, the fact that miRNAs can bind to multiple target mRNAs implies there should be other target genes for miR-483-3p that have yet to be identified. Identification of additional targets, particularly in the context of ACC, could be accomplished using a combination of bioinformatic and molecular techniques to empirically determine miRNA-target associations. Knock down of endogenous miRNA could be accomplished using synthetic inhibitory oligos specific for miR-483-3p. Cultured H295 cells subject to miRNA knockdown could then be subject to microarray or RNA sequencing analysis to determine the effects of miR-483-3p knock down on mRNA expression. Transcripts shown to be up regulated in response to miR-483-3p knock down could then be further scrutinized as potential miR-483-3p targets by analyzing the 3' UTRs for putative

binding sites. Additionally, such an experiment could provide insight into the physiologic roles of miR-483-3p. For example, if miR-483-3p inhibition resulted in changes to proliferative, metastatic, or steroidogenic capacity of H295 cells, these data could be taken into consideration when filtering down potential miR-483-3p targets by placing greater weight on target genes known to regulate these physiologic processes. Then, bioinformatic algorithms such as those provided by TargetScan would serve to narrow down the list of targets based on putative miR-483-3p binding sites in the 3' UTR of candidate genes. The most likely candidate genes could be tested in-vitro using molecular tools similar to those described in Chapter 2 to confirm miR-483-3p mediated repression of the target at both the mRNA and protein level.

Evidence suggesting that miR-483-3p can be directly regulated by the Wnt/β-catenin signaling pathway has been recently published. Given that many cases of ACC are characterized by inappropriately increased Wnt/β-catenin signaling, a logical question to pursue would be whether this signaling pathway intersects with the up regulation of IGF2/miR-483-3p that is also seen in many ACC cases. First, the binding of canonical Wnt/β-catenin transcription factors such as TCF/LEF to the miR-483-3p locus could be confirmed with chromatin immunoprecipitation, and the transcriptional complex defined. This could be performed in the H295 cell line, which harbors an activating mutation in β-catenin, or heterologous cell lines treated with lithium to activate the Wnt/β-catenin pathway. Mutation of the putative TCF/LEF binding sites would help confirm these results. Additionally, the effects of inhibiting Wnt/β-catenin

signaling via inhibitors such as cardamonin or the over expression of APC on miR-483-3p expression can also be assessed. Finally, published data suggests miR-483-3p negatively regulates β-catenin, implying a self regulating feedback loop. While this would initially appear counter intuitive, it is possible that mutated β-catenin in the context of ACC is insensitive to miR-483-3p mediated inhibition because mutations in the 3' UTR of β-catenin prevent the binding of miR-483-3p. It would be interesting to determine if the β-catenin transcripts in primary human ACCs harbor 3' UTR mutations, rendering itheminsensitive to miR-483-3p mediated repression. Genomic and RNA sequencing of the β-catenin locus would help provide insight into this possibility.

Lastly, further analysis on miRNA expression in ACC could be performed by assessing the miRNA profiles of ACCs versus normal or adenomatous adrenals. There are several published studies that pursue this avenue of study, but are somewhat limited by small tumor samples sizes. Nevertheless, these reports support the observation made in this thesis that products from the miR-483 locus are generally up regulated in ACCs versus adrenal adenomas or normal adrenals. Our laboratory, in collaboration with Dr. Arul Chinnaiyan at the University of Michigan, has recently begun a large scale miRNA and mRNA profiling study consisting of a significantly larger cohort of primary human ACCs. This would allow for a comprehensive study of differentially expressed miRNAs and mRNA targets that may be involved in the tumorigenesis of ACC. Because miRNAs can act as both tumor suppressors or oncogenes, highly up-regulated or down-regulated miRNAs in ACC may suggest that important target genes are

dysregulated as a result. Large scale bioinformatic analysis to identify predicted miRNA-mRNA networks could allow for more detailed classification of ACCs and prognostic indicators. Ultimately, it is hoped that these large scale profiling studies will help shed light on the pathophysiology of ACC, and provide clues to biochemical or molecular weaknesses in ACC that may be used as targets for new and more effect therapies.

Chapter 3 discussed the results of Dicer inactivation in the developing mouse adrenal cortex, and data from those studies showed that loss of Dicer in the embryonic adrenal resulted in the developmental failure of the adrenal cortex. This failure became evident as early as E14.5, the time point at which the fetal cortex begins to be displaced by the definitive cortex. Because the Sf1-Cre transgene used in these studies is active in both the fetal and definitive cortex, it would be of interest to determine whether it is the fetal or definitive cortex that is most suscpetible to Dicer inactivation. This could be accomplished in part by generating conditional *Dicer* KO mice using FAdE-Cre transgenic mice, in which the Cre transgene is driven specifically by the FAdE promoter of *Sf1*. These mice are already available to our lab, and if FAdE-Cre Dicer KO mice recapitulate the phenotype seen in Sf1-Cre Dicer KO mice, it would be reasonable to hypothesize that Dicer activation might primarily affect the fetal cortex, particularly as the adrenal begins to transition from fetal to definitive cortex. It is hypothesized that a small number of fetal adrenocortical cells are destined to become the adrenal stem/progenitor cells, which ultimately maintain the definitive adrenal cortex. If this is true, and Dicer loss of function primarily affects fetal cortical cells, it could

be speculated that this would negatively affect the specification of adrenal stem/progenitor cells from fetal cortical cells. Such a scenario would be consistent with the cortical failure seen in Sf1-Cre^{high} Dicer KO mice in which the adrenal cortex fails to maintain itself beyond the E14.5 time point. To determine the specific effects of Dicer inactivation in the definitive cortex would most likely require the use of an inducible Cre transgene. The majority of tissue specific *Dicer* knockout mouse models involves the use of Cre transgenes under the control of developmental transcription factors. This results in *Dicer* loss of function during developmental stages of the organism during which tissues undergo significant molecular and physiologic change. Unfortunately, the Sf1 definitive adrenal enhancer (DAdE) is still being characterized by our lab, and a definitive cortex specific Cre transgene has yet to be developed. In lieu of this, the use of an inducible Sf1-Cre transgene under the control of exogenous tamoxifen, for example, might provide a means to study Dicer ablation in the definitive cortex. However, timing the tamoxifen administration and subsequent Cre-mediated excision to coincide with the emergence of the definitive cortex during embryogenesis would be difficult, and it would be likely that fetal cortical cells would also be affected. However, this system would be a useful tool for studying the effects of Dicer inactivation in a fully developed organ in which the definitive cortex predominates. Because many of the existing publications detailing tissue specific Dicer inactivation involve developmental models, it is difficult to determine if Dicer inactivation is affecting the differentiation and specification of developing tissues, or the maintenance of established tissue.

Would Dicer ablation in a fully developed, adult adrenal result in complete failure of the cortex as was observed in this thesis? Or would it simply cause increased cellular turn over of the adrenal cortex, which could remain in relative compensatory homeostasis? A Dicer KO model under the control of an inducible Cre transgene may provide insight into the role of Dicer and miRNA biogenesis in the physiology and self renewal of the adult adrenal cortex.

In the second aim of Chapter 3, we assessed Dicer KO adrenals at E15.5 and E16.5 for differentially expressed miRNAs and mRNAs. The goal was to profile transcriptional changes resulting from Dicer inactivation in these adrenal glands, identify potential miRNAs involved in adrenal development, and to determine any potential miRNA-mRNA associations. The unexpected observation of Nr6a1 and Acvr1c up regulation in Sf1-Cre^{high} Dicer KO adrenals is an avenue for further investigation. As the expression of these genes has not been detailed in the adrenal, it would be important to first confirm mRNA and protein expression in both wild type and Sf1-Cre^{high} Dicer KO embryonic adrenals. Both of these genes are implicated in developmental processes, and the ultimate question is whether they have a similar function in the embryonic adrenal cortex. Northern blot analysis from Genepaint (www.genepaint.org) demonstrates the presence of Nr6a1 and Acvr1c RNA in the adrenal cortex at E14.5. This is the timepoint at which the adrenal begins to transition from fetal to definitive cortex, and therefore, raises the question if Nr6a1 or Acvr1c are somehow involved in this process. Our data shows the loss of Dicer and miRNA biogenesis appears to de-repress the expression of these genes, and could be

evidence supporting the hypothesis that Nr6a1 and Acvr1c are expressed at E14.5 in the developing adrenal, but then must subsequently be down regulated for normal development to proceed. This could be confirmed by assessing the expression of these genes over an embryonic time course to elucidate the temporal-spatial expression of Nr6a1 and Acvr1c. Should the expression of these genes in the developing adrenals be confirmed, functional studies could be initiated by generating adrenal specific mouse knock out models with the Sf1-Cre and conditional floxed allele mice.

Confirmation of the differentially expressed miRNAs in Sf1-Cre^{high} Dicer KO adrenals would need to be confirmed. We observed in our results sixteen down regulated miRNAs that were common to both the E15.5 and E16.5 time points. Of these, let-7d, miR-10a, miR-21, and miR-34c would be viable candidates to initially pursue in subsequent studies due to the large amount of literature that is available regarding their function. The role of these miRNAs in adrenal development is an interesting avenue to pursue. What is the function of these miRNAs in the developing adrenal cortex, and is the phenotype in Dicer KO adrenals attributable to their down regulation? Confirmation of the expression of these miRNAs in the developing adrenal can be accomplished through TaqMan based quantitative real time PCR assays, and if necessary, Northern blot techniques to localize the spatial expression in the developing adrenal cortex. This could aid in the identification of specific miRNAs whose expression is required for proper adrenal development. Additionally, the miRNA arrays we performed could also be extended to include the E18.5 time point in wild type

adrenals, giving us an additional data point that may aid in elucidating the temporal expression of specific miRNAs in the developing adrenal cortex.

Finally, our data demonstrated that six common miRNAs in both E15.5 and E16.5 Dicer KO adrenals were consistently predicted to target Nr6a1 and Acvr1c, two genes which were highly up regulated in knockout adrenals. These miRNAs were: let-7d, miR-10a, miR-202, miR-21, miR-674, and miR-362. TargetScan prediction algorithms demonstrate putative binding to phylogenetically conserved sequences in the 3' UTRs of these genes, but empirical evidence of binding and repression would have to gathered. Bioinformatic analyses to align and compare the 3' UTRs of Nr6a1 and Acvr1c could be performed initially to identify common sequence motifs that might suggest conserved binding sites for these six miRNAs. Then, using techniques and tools discussed in Chapter 2, target validation could be performed in an in vitro system in which GFP or luciferase reporter constructs engineered to contain the 3' UTR of putative miRNA targets could be tested against exogenously expressed miRNAs of interest. Lastly, it would be interesting to determine the regulatory networks that control the expression of these miRNAs predicted to target Nr6a1 and Acvr1c. Are they co-transcribed with other protein expressing genes, or do they respond to transcription factors downstream of canonical signaling pathways such as Wnt/β-catenin or Shh which have both been implicated in adrenal organogenesis? Such a question could be investigated by taking a bioinformatic approach to determine if there are response elements or binding sites in the promoter or regulatory region of these miRNAs that might

respond to signaling pathways known to be active in the developing adrenal. If so, in vitro perturbation of the suspected signaling pathway or transcription factor(s) should result in a concomitant change in the expression of the miRNA in question. The results from these studies would not only represent a significant contribution to the field of adrenal development, but to other areas of organogenesis.