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EFFICIENT CONDITIONAL INACTIVATION OF RDH10 REVEALS IMPORTANT ROLE OF RETINOIC ACID DURING LUNG BRANCHING MORPHOGENESIS

By

Nhut Quang Huy Tran

B.S Chemistry, Centre College, 2016

A Thesis

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Thesis Approved on

April 9th 2020

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ABSTRACT

EFFICIENT CONDITIONAL INACTIVATION OF RDH10 REVEALS IMPORTANT ROLE OF RETINOIC ACID DURING LUNG BRANCHING MORPHOGENESIS

Nhut Quang Huy Tran

April 9th 2020

Birth defects are complicated pathological processes. Many birth defects can be traced back to disrupted regulation of molecular signaling pathways during embryogenesis. One of the most important gene regulatory factors during embryo development is retinoic acid (RA). However, studying the specific roles of RA in each organ during embryogenesis in vivo is challenging. The Sandell laboratory has a conditional mutant mouse model that can potentially be used for studying the role of RA during embryogenesis by inducing stage-specific RA deficiency. This model allows *retinol dehydrogenase 10* (*Rdh10)*, a gene required for RA production, to be inactivated at a chosen time by exposing the embryos to the drug tamoxifen. However, in order for this conditional genetic inactivation model to be used for rigorous analysis of birth defects resulting from RA deficiency, it is essential to know the kinetics of *Rdh10* gene inactivation following exposure to the inducing drug tamoxifen. In order to characterize the conditional Rdh10 mutant model so that it can be useful to study the impact of RA deficiency on birth defects, I have determined the kinetics of the inactivation

Rdh10 upon exposure of the embryos to tamoxifen. My characterization of the conditional *Rdh10* inactivation kinetics was useful to other researchers, enabling them to use stage-specific *Rdh10* inactivation to identify new roles for RA in embryonic development, such as in formation of salivary glands and secondary palate. The characterization of the 6conditional *Rdh10* inactivation kinetics was also useful for my analysis of RA role in lung branching morphogenesis later. Using the stage-specific *Rdh10* inactivation model I identified that RA is important for lung branching after the formation of the lung buds. Gene expression analysis revealed that the defects in lung branching morphogenesis of *Rdh10* mutant embryos were not associated with mis-regulation of the known RA-regulated gene *Fgf10*, but were associated with overexpression of *Ctgf* and *Mgp*.

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CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Birth defects and genetic pathways

Birth defects are complicated pathological processes. These pathologies can be the result of a combination of genetic and environmental factors. Whether birth defects are caused by genetic or environmental factors, the immediate results of the changes are the mis-regulation of molecular signals that dictate the outcomes of embryonic development processes. In order to prevent the morphological defects from occurring, interventions based on understanding of the molecular actions are crucial. In fact, the knowledge about changes in molecular signaling cascades may be helpful for the development of therapies for abnormal tissue growth in embryos. In this study, we investigated the function of one molecular signaling pathway in regulating embryonic development of the lungs. We first tested the efficiency of a stage-specific mouse conditional mutant model for reduction of Retinoic Acid (RA) in developing mouse embryos during the second half of gestation. We then determined if early branching development of lungs requires RA signaling.

1.2 RA and Rdh10

Vitamin A is an essential micronutrient that cannot be produced in the human body. After it is absorbed, Vitamin A will go through multiple steps of metabolism to make Retinoic Acid (RA) (Metzler and Sandell 2016). RA is an important

signaling factor that plays a crucial role, along with other signaling factors, in regulating many processes in embryonic development and adult health (Duester 2008; Niederreither and Dollé 2008; Metzler and Sandell 2016). RA and related molecules, known as retinoids, have been increasingly used to aid therapy from treating pathologies such as cancers, autoimmune diseases to localized pathologies such as acne (Kligman et al. 1981; Abaza et al. 2017; Tobin et al. 2018; Xia et al. 2018; Tripathi et al. 2019).

Although, it is well acknowledged that RA is an important signaling factor, its role in the development of an embryo in vivo is not completely understood. In order to effectively utilize RA and retinoids for therapeutic purposes, it is crucial to understand the role of RA and retinoids in regulating tissue development and maintenance. One way to understand the action of retinoid molecules in cells and tissues is to remove or reduce the level of RA below normal concentration and observed the defects that result.

The gene *retinol dehydrogenase 10* (*Rdh10*) is essential for vitamin A metabolism into RA (Fig.1). During embryo development the RDH10 enzyme converts Retinol (vitamin A) into the retinoid intermediate Retinal, which is an essential rate-limiting step in production of RA. In fact, knocking out the function of *Rdh10* will result in developmental defects due to lack of RA in a developing embryo (Sandell et al. 2007: 10; Cunningham et al. 2011; Farjo et al. 2011; Sandell et al. 2012: 10; Metzler and Sandell 2016). However, studying the role of RA by inducing the complete absence of *Rdh10* in embryos proved to be challenging. Early development of heart and brain tissues requires RA, thus mice

with defects in early retinol metabolism owing to absence of *Rdh10* usually die at or before embryonic day 11.5 (E11.5) (Rhinn et al. 2011; Sandell et al. 2012). Because severe RA deficiency, owing to lack of Rdh10 or other disruption, causes early embryonic lethality, many important functions of RA during embryo development have not yet been studied.

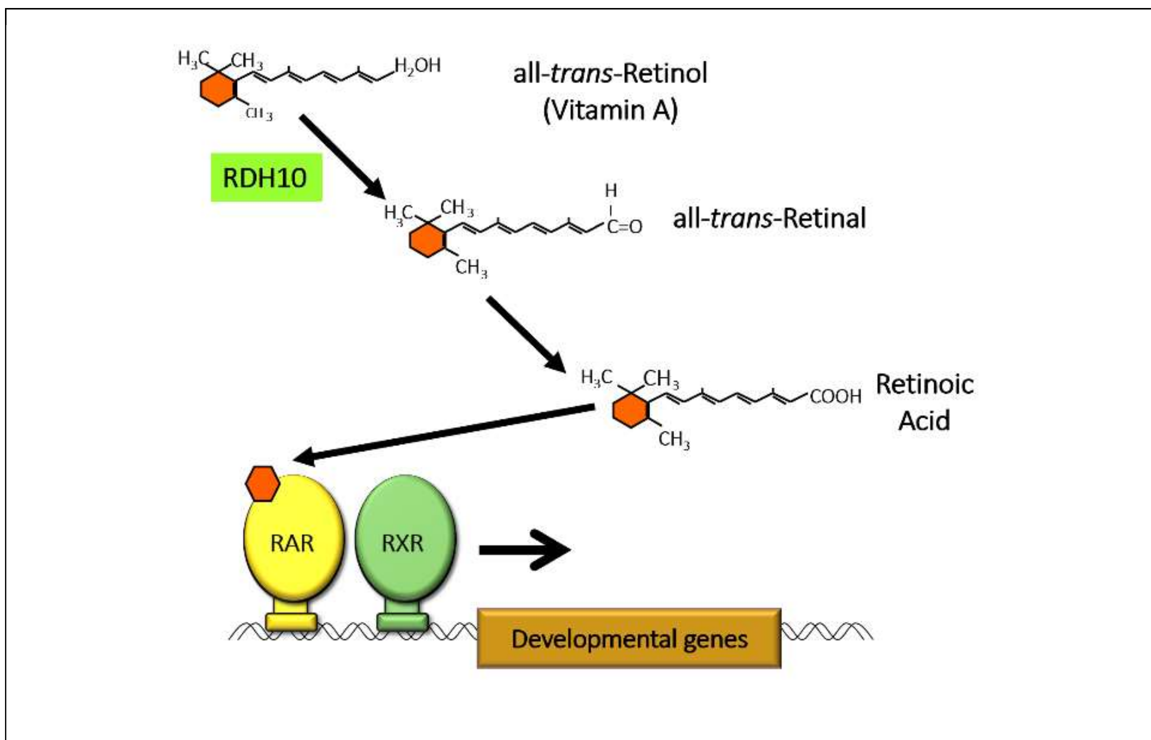

Figure 1. The *Rdh10* gene encodes an enzyme needed for embryonic metabolism of Vitamin A and production of RA, a signaling molecule that regulates gene transcription. RDH10 is required for metabolize retinoid precursor all-trans-retinol into the intermediate all-trans-retinal. All-trans-retinal is metabolized by other enzymes into the product RA. RA binds to RA receptors, nuclear transcription factors that regulate expression of developmental genes. Expression of many developmental genes depends upon presence or absence of RA to regulate activity of the nuclear RA receptor transcription factors.

1.3 The need for better understanding of signals that regulate development of the lung

Congenital lung defects are serious medical problems that can lead to lethality in newborn. One of the most common birth defects that involves the lung is congenital diaphragmatic hernia. The molecular mechanism of the birth defect is reported to be a collection of affected genes and signaling pathway components including RA signaling pathways (Kardon et al. 2017). By studying the development of the lung, especially the molecular signaling cascade of the lung, we can utilize the knowledge to develop treatments that may either reduce the severity of the defects or prevent the abnormalities from occurring. In addition, by understanding how the lung is formed, in the future, people might be able to discover methods to promote the regeneration of damaged lung tissue. For example, by mimicking sequence of signals during embryonic lung development tissue, engineers can generate multipotent lung and airway progenitors from the lung epithelial cells of healthy people (Huang et al. 2014) and patient with cystic fibrosis (Mou et al. 2012) , creating a system to study the disease in vitro.

The development of the lung is divided into 5 stages: embryonic, pseudoglandular, canalicular, alveolar (Rackley and Stripp 2012). Out of the 5 stages, the alveolar stage occurs post-natal. At the embryonic stage, the lung buds are formed from endoderm. Early lung endoderm cells are multipotent until the second stage of the development where lung epithelium cells becomes more differentiated and branching of the lung buds occurs. Previously, signal pathways that regulate development of the lung have been identified, including TGF-β/bone

morphogenetic protein (TGF-β/BMP), Hedgehog (HH), WNT, and fibroblast growth factor (FGF) pathways (Herriges and Morrisey 2014). In fact, by manipulating these genes, other researchers were able to differentiate mice foregut endoderm into adult mice pulmonary epithelial cells (Longmire et al. 2012).

1.4 RA and lung development

It is documented that vitamin A deficiency, which leads to the disruption of RA signaling pathway, will result in abnormal lung development (Wilson et al. 1953; Chailley-Heu et al. 1999; Marquez and Cardoso 2016). The severity of vitamin A deficiency is directly correlated with the severity of lung developmental defects. While mild vitamin A deficiency can interfere with the maturation of the lung, severe vitamin A deficiency can lead lack of lung formation in rat (Wilson et al. 1953; Chailley-Heu et al. 1999).

In fact, it is found that during lung genesis, RALDH-2, an enzyme responsible for RA production is strongly expressed (Malpel et al. 2000). Previous studies have identified the function of RA in initiating the embryonic stage of the lung. RA is found to indirectly regulate the expression of *Fgf10* by influencing TGFβ pathway negatively and WNT pathway positively (Desai et al. 2006; Wang et al. 2006; Chen et al. 2007; Chen et al. 2010). RA is also found to promote the expression of *Shh* by increasing the ligand binding to the gene during the embryonic stage (Rankin et al. 2016).

In humans, genetic mutations that impair RA signaling can lead to syndromic defects that can involve multiple organs including the lung. Specifically, mutations of the STRA6 gene, which is involved in transport of Retinol into cells where it can be metabolized into RA, are associated with a syndrome characterized by lung hypoplasia known as PDAC or Matthew-Wood syndrome (Pasutto et al. 2007; Chassaing et al. 2013; Srour et al. 2013) .

Although there is strong evidence to support that RA concentration and RA signaling are tightly regulated at the initial lung development, namely the embryonic stage, the role and importance of RA during branching morphogenesis remains unclear and contradictory. It is reported that downregulation of RA occurs after the initial formation of the lung buds (Malpel et al. 2000). In fact, purposefully maintaining the RA level after the lung buds formation will lead to lung hypoplasia due to inability to form distal buds (Malpel et al. 2000; Chazaud et al. 2003; Mollard et al. 2003). It is shown that RA downregulation allows mouse developing lungs to reach the sacculation stage and the progenitor cells to become type I cells (Wongtrakool et al. 2003). These studies suggest that RA signaling must be reduced during the pseudoglandular stage of lung development when early branching growth occurs. However, there are other studies reports that mutant mice embryos with reduced levels of RA during the pseudoglandular stage develop lung hypoplasia (Wilson et al. 1953; Mendelsohn et al. 1994; Wang et al. 2006), suggesting that RA signaling is needed for branching growth. Thus, previous published studies of RA role in branching stage lung development are contradictory and unclear. However, since

there are more studies that show the correlation between lung hypoplasia and vitamin A deficiency(Wilson et al. 1953; Mendelsohn et al. 1994; Wang et al. 2006; Gavrilova et al. 2009), we hypothesize that RA has an important role in regulating lung branching morphogenesis.

1.5 Conditional mouse mutant Cre-lox technology.

To investigate the function of RA in lung development at the branching stage in vivo, we proposed to use a stage-specific *Rdh10* mutant mouse model that would overcome the problem of early lethality that occurs when *Rdh10* is completely absent (Rhinn et al. 2011; Sandell et al. 2012).

The stage-specific inactivation of *Rdh10* would make it possible for us to investigate the function of RA during the latter half of development, specifically after the heart has formed and blood circulation is functional. Many important development processes, such as formation of the secondary palate, salivary glands, and lungs occur during the in the second half of gestation.

To perform stage-specific gene knockouts, we decided to use Cre recombinase to delete an essential segment of the *Rdh10* gene. The essential gene segment, in this case *Rdh10* exon 2, is “floxed”. A floxed sequence is a gene sequence that has loxP sites inserted downstream and upstream. When Cre recombinase is present, the loxP sites will be targeted for excision and the intervening gene sequence will be deleted (McLellan et al. 2017). In order to perform a stage-specific knockout we used a version of Cre recombinase that can be induced by exposure to the drug Tamoxifen. The Tamoxifen-inducible Cre-ERT2 system has

been utilized and reported before as an successful system for inducible inactivation of a gene other than *Rdh10* (Ventura et al. 2007).

To use Cre-lox conditional mutant technology in a rigorous manner, it is important to define carefully the kinetics of excision. Because our studies are stage specific, identifying the moment when *Rdh10* is disabled became the foundation on which our experiments are carried out. Previously, the conditional inactivation model that utilize mice with *Rdh10* that has flox sequences has been investigated (Sandell et al. 2012). When applied the system to *Rdh10*^{flox}, the Cre-mediated excision of the *Rdh10*^{flox} allele was obtained (Kurosaka et al. 2017). However, the kinetics of the excision was not reported. In other words, we need to identify how long it takes for the *Rdh10*^{flox} to be excised using the Tamoxifen-inducible Cre-ERT2 system. It is vital for our experiment to be carried out at the exact timeline of embryonic development to obtain the most reliable outcomes.

1.6 Kinetics of Rdh10 knockout in the Rdh10$^{flox/delta}$; ERT2 Cre system

In order to measure how long it takes for the *Rdh10*^{flox} allele to be excised in *Rdh10*$^{flox/delta}$; ERT2Cre system, we need to measure the amount of *Rdh10* exon 2 (the excised sequence) that is present at different time points after the administration of the inducing drug Tamoxifen. To know how much *Rdh10* exon 2 is present, it is necessary to compare the level of exon 2 (the excised sequence) relative to some sequence that remains intact after the Cre excision. Therefore, to measure the amount of *Rdh10* exon 2 being excised, we compared

the amount of *Rdh10* exon 2 (the excised sequence) to the amount of *Rdh10* exon 3 (a neighboring sequence that remains intact after Cre activity). Because it is unlikely that Tamoxifen induction and Cre excision will occur instantaneously, we expect to observe a gradual decrease in the amount of exon 2 relative to exon 3 over time. Knowledge about the rate of exon 2 excision will allow us to reliably treat the embryos minimal uncertainty about the timing of stage-specific *Rdh10* inactivation.

To measure the amount of *Rdh10* exon 2 and *Rdh10* exon 3 in mouse embryos at different time points after Tamoxifen induction, I used qPCR. From this analysis I demonstrated that the inactivation of *Rdh10* in embryos reached near completion 48 hours after administration of Tamoxifen.

Once the efficacy of the inducible *Rdh10* mutant mouse model system was validated, I examined the lung phenotype of *Rdh10* mutant mouse embryos to identify the function of Rdh10-mediated RA signaling in lung development during branching growth at the pseudoglandular stage.

1.7 The use of conditional Rdh10 mutation model for other studies

Once the kinetics of *Rdh10* inactivation were established, the *Rdh10* *Rdh10*$^{flox/delta}$; ERT2Cre system was useful for studying the role of RA in multiple systems in addition to the lung. Importantly, beyond my analysis of lung phenotypes, the study model was also utilized to define the role of RA in development of salivary glands (Metzler et al. 2018) and secondary palate . The

same argument can be made that the knowledge obtained can be helpful for medical intervention in cleft palate and salivary gland damage.

Salivary glands are very delicate organ which has limited repair mechanism and lack the ability to regenerate. Dysfunction of salary gland is a major clinic concern since it will affect not only patient's oral health but also systemic health and patient's quality of life (Deborah Greenspan 1996 Mar 1; Cassolato and Turnbull 2003; Navazesh and Kumar 2009; Sasportas et al. 2013; Villa et al. 2014). One possibility for future treatments of salivary gland dysfunction is to regenerate the defected glad tissue; the insights on the development of the salivary gland may one day aid the studies for treatment (Longmire et al. 2012; Patel and Hoffman 2014; Lombaert 2017). Dr. Melissa Metzler had successfully utilized the *Rdh10* stage-specific inactivation model to determine the crucial role of RA signaling in early salivary gland development (Metzler et al. 2018).

Cleft of the secondary palate are birth defects that impose substantial economic, physical, and social hardship on affected individuals and their families. Masters in Oral Biology students Regina Friedl and Swetha Raja utilized the *Rdh10* stage-specific inactivation model to identify that RA is crucial for enabling *in utero* mouth movement that is important for proper development of the secondary palate (Friedl et al. 2019).

CHAPTER 2 : RESULTS

2.1 Efficiency of Rdh10 inactivation following exposure of embryos to tamoxifen.

Previously, the conditional *Rdh10* inactivation model that utilizes mice with a floxed allele of *Rdh10* has been investigated (Sandell et al. 2012), and has be used in combination with the tamoxifen-inducible ERT2-Cre (Kurosaka et al. 2017), however, the kinetics of excision following tamoxifen exposure have not been quantified. To use Cre-lox conditional mutant technology in a rigorous manner, it is important to carefully define the kinetics of excision(Song and Palmiter 2018). For our mouse crossing scheme to generate conditional mutant embryos, one of the parent mice with the genotype $Rdh10^{flox/\mathrm{flox}}$; homozygous for *Cre* recombinase was crossed with a parent mouse with the genotype $Rdh10^{+/\Delta}$. In these crosses, the genotype of the resulting embryo offspring was either $Rdh10^{+/\mathrm{flox}}$ *cre* or $Rdh10^{flox/\Delta}$ *cre* (Fig. 2). The offspring that carried the genotype $Rdh10^{+/\mathrm{flox}}$ *cre* retain one wild type allele of *Rdh10* even after cre induction. The $Rdh\ 10^{+/\mathrm{flox}}$ *cre* embryos have no detectable phenotype and were used as controls. The offspring with genotype $Rdh10^{flox/\Delta}$ *cre* have no remaining functional alleles of *Rdh10* after cre induction. Therefore, the $Rdh10^{flox/\Delta}$ *cre* embryos were considered conditional mutants. It is worth noting that the pre-tamoxifen genotype $Rdh10^{+/\mathrm{flox}}$ (control) and $Rdh10^{flox/\Delta}$ (mutant) of the embryos

changes upon induction of cre by tamoxifen. *Rdh10*$^{+/flox}$ becomes *Rdh10*$^{+/\Delta}$ (control) and *Rdh10*$^{flox/\Delta}$ becomes *Rdh10*$^{\Delta/\Delta}$ (mutant).

Before exposure to tamoxifen and activation of cre recombinase, the control embryos have 2 alleles containing *Rdh10* exon 2, while the mutant embryos have 1 allele containing *Rdh10* exon 2. If activation of cre recombinase by tamoxifen causes 100% of the exon 2 of the *Rdh10* flox allele to be excised, then post-tamoxifen control embryos will have 1 allele containing *Rdh10* exon 2, and the mutant embryos will have no alleles of *Rdh10* exon2. Thus, by normalizing the amount of *Rdh10* exon 2 remaining in the mutant embryos post-tamoxifen relative to the control embryos post-tamoxifen, I can estimate the percentage of exon 2 that has been excised (Fig. 2).

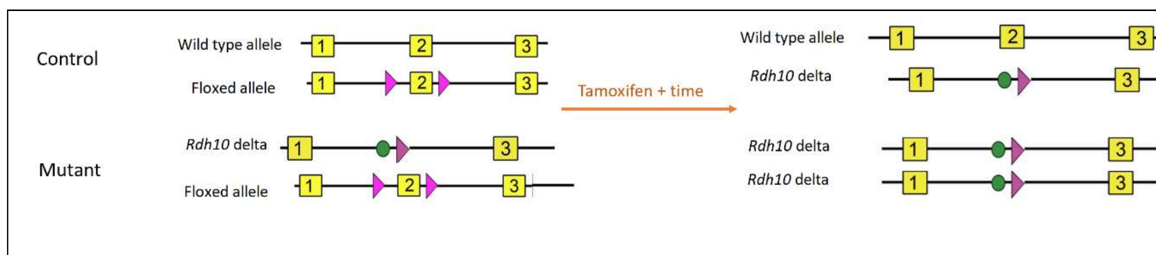

Figure 2. Genomic organization of *Rdh10* control and *Rdh10* conditional mutant embryos before and after treatment with tamoxifen to activate ERT2-Cre recombinase. Before the tamoxifen treatment, the control embryos have 2 functional alleles of *Rdh10*. The mutant embryos have 1 functional allele of *Rdh10*. After the treatment with tamoxifen and activation of ERT2-Cre recombinase, the control embryos will have one functional *Rdh10* allele. The mutant embryos will have zero functional *Rdh10.*

For this study, we utilized ERT2-Cre, a modified version Cre recombinase that is inducible by tamoxifen (Ventura et al. 2007). ERT2-Cre is normally is present in the cytoplasm where it does not have access to DNA, and remains inactive. When tamoxifen is present, ERT2-Cre is transported to the nucleus, where it can access the DNA and excise any floxed alleles. Upon the entry of the Cre recombinase into the nucleus, the enzyme acts on loxP sequences to excise exon 2 of the *Rdh10* gene, resulting in recombination of exon 1 to exon 3. As a result of exon 2 excision, the *Rdh10* allele becomes inactivated. In order to measure the kinetics of the inactivation of *Rdh10,* I compared the amount of exon 2 present in mutant embryos relative to control embryos at 24 and 48 hours after tamoxifen was administered to the pregnant mother mouse. Pregnant mice were given tamoxifen and E8.5 and E10.5. I collected the tissues of control and mutant embryos at 24 and 48 hours after tamoxifen treatment.

To detect any changes in the quantity of for *Rdh10* exon 2 post-tamoxifen, I look at the ratio between the quantity of for *Rdh10* exon 2 and *Rdh10* exon 3. In order to normalize the quantity of *Rdh10* exon 2 and *Rdh10* exon 3 present in the embryos, I used *Gapdh* as a house keeping gene. The *Gapdh* DNA sequences are not affected by the presence of tamoxifen or Cre recombinase. Using validated primer pairs for *Rdh10* exon 2, *Rdh10* exon 3, and *Gapdh*. I performed DNA qPCR in order to quantify the amount of *Rdh10* exon 2 relative to exon 3. With the help of Dr. Melissa Metzler, I carried out statistical analysis to quantify the the amount of *Rdh10* exon 2 remaining intact at 0, 24, and 48 hours post-tamoxifen exposure (Fig. 3).

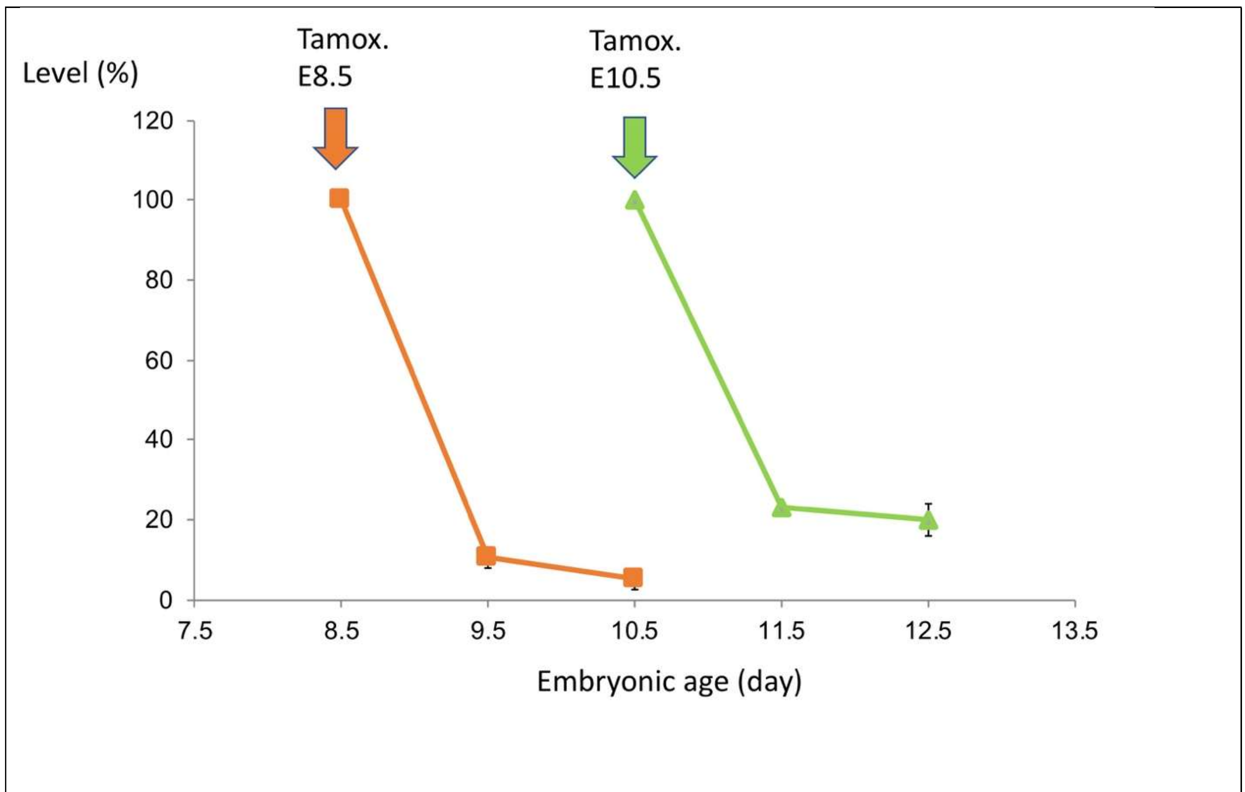

Figure 3. Analysis of kinetics of conditional *Rdh10* inactivation by DNA qPCR: percentage of *Rdh10* exon 2 remaining in mutant embryos post-tamoxifen. Level of *Rdh10* when the mutant embryos were exposed to tamoxifen at E8,5 is shown in red. Level of *Rdh10* when the mutant embryos were exposed to tamoxifen at E10,5 is shown in green. 24 hours post-tamoxifen at E8.5 (red line) shows 10.4% *Rdh10* exon 2 remained. 48 hours post-tamoxifen at E8.5 (red line) shows 4% *Rdh10* exon 2 remained. 24 hours post-tamoxifen at E8.5 (green line) shows 23.6% *Rdh10* exon 2 remained. 48 hours post-tamoxifen at E8.5 (green line) shows 20.1% *Rdh10* exon 2 remained. For the entire experiment, $n_{control}$ = 3; n_{mutant} = 3.

When tamoxifen was given to the pregnant mouse at E8.5, *Rdh10* exon 2 is 89.6% eliminated after 24 hours, and 96% eliminated at 48 hours. However, when tamoxifen was given to the pregnant mouse at E10.5, 76.7% and 79.9% of the *Rdh10* exon 2 was excised after 24 hours and 48 hours, respectively.

Despite the insufficiency of *Rdh10* exon 2 excision when tamoxifen is given to the mother at E10.5, the result from E8.5 shows that, *Rdh10* exon 2 is efficiently excised and most *Rdh10* in the mutant is inactivated. Therefore, the efficiency of tamoxifen-inducible *Rdh10* inactivation decreases after E10.5, possibly due to the increased of embryo size at the older gestational stage.

Since it takes 48 hours for most of the *Rdh10* to be inactivated, the tamoxifen treatment at E8.5 (T8.5) can effectively induces RA deficiency at E10.5. This finding that T8.5 induces *Rdh10* inactivation by E10.5 it demonstrates that the T8.5 model can be used to study the function of RA in embryos older than E10.5. Based on the results of the experiment, conditional mouse mutant Cre-lox technology might not be effectively used for every stage of the mouse embryonic development due to it insufficiency at later stages after E10.5, however it can be appropriately used for studying the effect of *Rdh10* and RA deficiency during organ morphogenesis. In fact, other lab member have successfully utilized the conditional mouse mutant Cre-lox technology in their investigation of *Rdh10* and RA in salivary and secondary palate formation (Metzler and Sandell 2016; Friedl 2019 May 1).

2.2 Is Rdh10 present in the lung after initiation stage?

Lung branching development begins after initiation of the lung bud at E9.5 (Marquez and Cardoso 2016). In order to investigate whether *Rdh10* function might be important for lung development after the initiation stage, we examined whether *Rdh10* is expressed after E9.5. In order to determine if *Rdh10* is present in lungs at E10.5, we collected lungs from mouse embryos that carry *Rdh10*-lacZ knock-in reporter allele (*$Rdh10^{Bgeo}$*) because the lacz gene is under the control of endogenous Rdh10 promoter (Sandell et al. 2012) at E10.5. We stained the isolated lung tissues for β-galactosidase activity produced by the lacZ gene of the *Rdh10* reporter construct. Staining revealed that we were able to detect expression of Rdh10 is expressed in the lung at E10.5 (Fig. 4). These data suggest that Rdh10 function may be important for development of the lung during early branching morphogenesis.

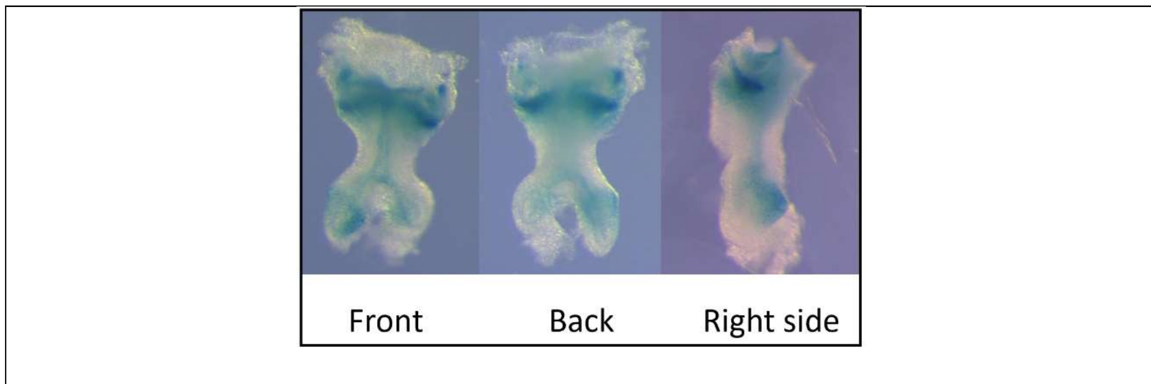

Figure 4. *Rdh10* gene is expressed in the lung at E10.5. Lungs of embryo carrying the *Rdh1*0Bgeo reporter were collected at E10.5 and stained for β-galactosidase activity to visualize *Rdh10* expression pattern. The presence of *Rdh10* gene transcription can be visualized by blue detection of beta-galactosidase activity.

The finding the *Rdh10* is present in the early pseudoglandular stage lung is important because if vitamin A is not metabolized in the branching lung, then it would be unlikely that RA essential for lung morphogenesis. Although presence of *Rdh10* in the E10.5 lung does not show that RA is essential for the lung morphogenesis, it suggests that RA is synthesized within the developing lung after the lung bud formation. These data prompted us to further investigate the role of *Rdh10* and RA in the lung after initiation E9.5 by administration of tamoxifen at E8.5 to inactivate RA production at E10.5.

2.3 Important role of RA during lung branching morphogenesis

RA is required for the initiation of lung at E9.5 (Marquez and Cardoso 2016), but there is contradicting evidence about the role of RA in a developing lung after the embryonic stage (Mendelsohn et al. 1994; Malpel et al. 2000; Wang et al. 2006). Because I had validated the efficiency of the conditional mutant mouse model of stage-specific *Rdh10* inactivation, I used the conditional mutant mouse model for investigating the role of RA during lung branching morphogenesis.

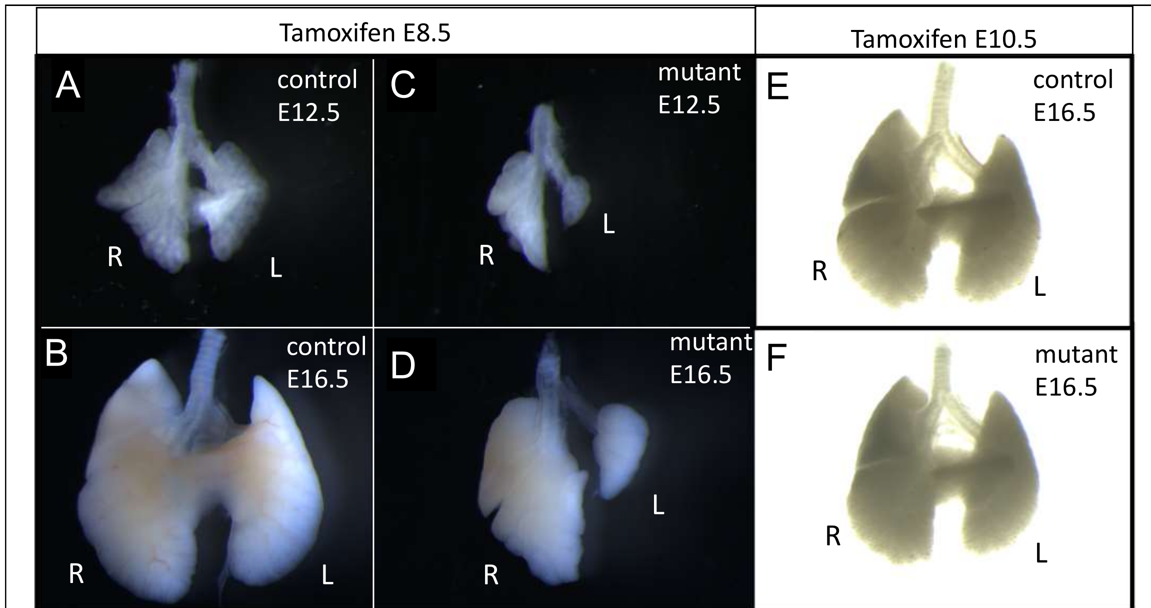

Figure 5. Lungs of mutant embryos are small and hypoplastic when exposed to tamoxifen at E8.5 but not when exposed at E10.5. (A-D) Following tamoxifen treatment at E8.5, lungs of mutant embryos (B, D) are noticeably smaller and hypoplastic relative to the control littermate embryos (A, C). Lungs of control embryo treated with tamoxifen at E8.5 and harvested at E12.5 (A) and 16.5 (B) have normal growth and morphology. (C-D) Lungs of mutant embryo treated with tamoxifen at E8.5 and harvested at E12.5 (C) and E16.5 (D) are noticeably smaller than control littermates. (E,F) Treatment with tamoxifen at E10.5 does not cause a noticeable lung phenotype in mutant embryos. Lungs mutant embryo treated with tamoxifen at E10.5 and harvested at E16.5 (F) are grossly normal and similar in size to their control littermate embryos (E). L, left lung; R, right lung.

In order to detect whether RA deficiency alters lung branching morphogenesis, I looked at the overall morphology of embryonic lungs with and without lung morphogenesis. Rdh10 control and mutant embryos were exposed to tamoxifen at E8.5 and harvest at E12.5 and E16.5. Since it took 48 hours to knock out most of the *Rdh10*, the inactivation of RA production occurred ~ E10.5, which allowed lung initiation to occur at E9.5. In *Rdh10* mutant embryos mild lung hypoplasia observed at E12.5, which were more obvious and apparent at E16.5 (Fig. 5, A-D). In contrast, when the embryos was exposed to tamoxifen at E10.5 and harvested at E16.5, no defects in the lungs of the mutant embryos were observed (Fig.5, E, F).

In order to quantify the lung defects observed in the lungs of the mutants, I first compare the number of branching buds between the mutant and the control lungs by counting the number of visible buds under light microscopes. However, the experiments was difficult to execute since the buds were difficult to visualize distinguish. With the help of Dr. Melissa Metzler and Dr. Lisa Sandell, we came up with an alternative method to quantify the difference in lung morphology by performing whole mount immuno-staining of the embryonic lungs to label all lung epithelium. Immunostained lung specimens were then imaged whole mount by confocal microscopy to allow visualization and quantification of the lung epithelium.

To analyze defects in early branching growth of the lungs, tamoxifen was given to the pregnant mice at E8.5, and embryos were harvested at E10.5, and E12.5. Both the lungs from the control and the mutant embryos were collected and

whole mount immuno-stained to label lung epithelium. Whole mount confocal microscopy was then performed to allow analysis of the morphological defects of the mutant lungs in 3 dimensions (Fig. 6, A-D). In order to quantify the difference between the control and the mutant lungs, we compare the volume of the mutant lung epithelium with the volume of the control lung epithelium. The volume analysis reveals that the lungs of mutant embryos are statistically smaller that the lungs of control embryos at E10.5 and E12.5 (Fig. 6, E-F). The difference in volume between the mutant lungs and the control lungs was more severe in sample collected from the embryos at E12.5 relative to samples collected at E10.5 (Fig. 6, E-F). Therefore, it is shown that the severity of the hypoplasia in the mutant lungs increases as the embryos grow. The lung hypoplasia of the mutant embryos is more severe for the left lung than that of the right lungs (Fig. 6, F).

The finding that the severity of the hypoplasia in the mutant lungs increases as the embryos grow between E10.5 and E13.5 is important because it helps us to confirm that RA does play a role in lung morphogenesis after the initiation stage at E9.5 (Marquez and Cardoso 2016).

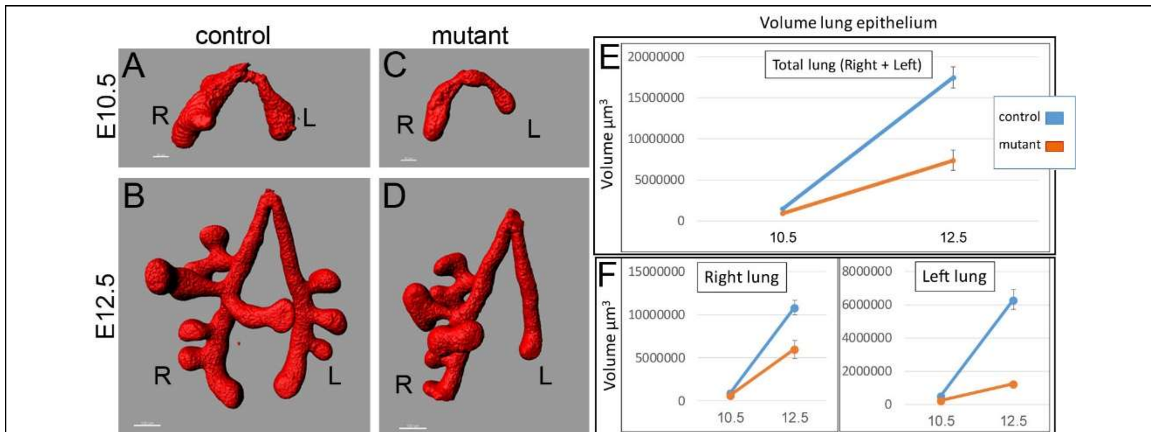

Figure 6. Lung epithelium volume of E8.5 tamoxifen-treated mutant embryos increases more slowly than that of controls embryos between E10.5 and E12.5, demonstrating RA is required during early branching growth. (A-D) Volume rendering of immuno-stained epithelia of lungs of control and mutant littermate treated with tamoxifen at E8.5. (A-B) Lungs of control embryo have normal size and morphology (A) relative to lungs of mutant embryo (B) which are slightly smaller at the E10.5 initial bud stage of development. (C-D) By E12.5 the growth defect of mutant lungs is more apparent. Control embryo lungs have normal size and morphology (C) relative to lungs of mutant embryo (D) which are noticeably smaller, particularly in the left lung. (E) The growth defect of mutant lungs becomes more severe between E10.5 and E12.5. Between E10.5 and E12.5 the volume growth of mutant lungs is much smaller than the volume growth of control littermate embryos. The slower growth of the mutant lungs is evident from analysis of whole lung volume. (F) The lung volume growth defect is more pronounced for the left lung than for the right lung. E10.5 lungs, $N_{control}$ = 5, $N_{mutants}$ = 4, $N_{control}$ =4, $N_{mutants}$ = 6. R, right lung; L, left lung

2.4 Lung growth phenotype is sensitive to developmental stage when Rdh10 is inactivated

After demonstrating that Rdh10-mediated RA production was important for lung development at the early pseudoglandular stage, I next sought to determine whether *RDH10* activity was also needed during later stage branching growth. Although we are aware that tamoxifen-induced inactivation of *Rdh10* in the mutant embryos is less efficient at when administered at E10.5 (79.9% inactivation at 48 hours, we decided to carry the experiment to determine if near elimination of *Rdh10* at later stages impacts lung development. To address that question, I compared the morphology mutant lungs relative to the control lungs when the embryos were exposed to tamoxifen at E9.5, or when they were exposed to tamoxifen at E10.5. In each case, lungs were examined at E12.5.

Tamoxifen was administered to the pregnant mice at E9.5 or E10.5 and embryos were collected at E12.5. Lungs were isolated from embryos by microdissections, and lung epithelium was immuno-stained whole mount with fluorescently antibody. Confocal microscopy of whole mount specimens was performed to render the volume of the lung epithelium. In contrast to growth defect observed in the lungs from mutant embryos exposed to tamoxifen at E8.5, no apparent growth phenotype was detected in the lungs of mutant embryos exposed to tamoxifen at E9.5 or E10.5 (Fig.7). Analysis of volume of epithelium from lungs of mutant embryos compared to control embryos revealed no statistically significant difference in volume. These data may indicate that that RA is needed only at a very specific stage during lung branching morphogenesis. Because the kinetic

analysis demonstrated that it took 48 hours to inactivate the *Rdh10* in mutant embryos when tamoxifen is given to the mother at E8.5, we could interpret that RA is crucial for lung branching from E10.5 to E11.5, approximately 24 hours after lung bud initiation, but not at later time points. Alternatively, since the kinetic analysis demonstrates that tamoxifen at E10.5 does not yield complete inactivation of *Rdh10*, it is possible that the low level of *Rdh10* that remains is sufficient to prevent lung morphogenesis defects in the mutant mice. Because we did not perform the kinetic analysis on embryos treated with tamoxifen at E9.5, we do not know whether the lack of lung defects in mutant embryos is due to a precise stage requirement of RA, or because some *Rdh10* remains activated.

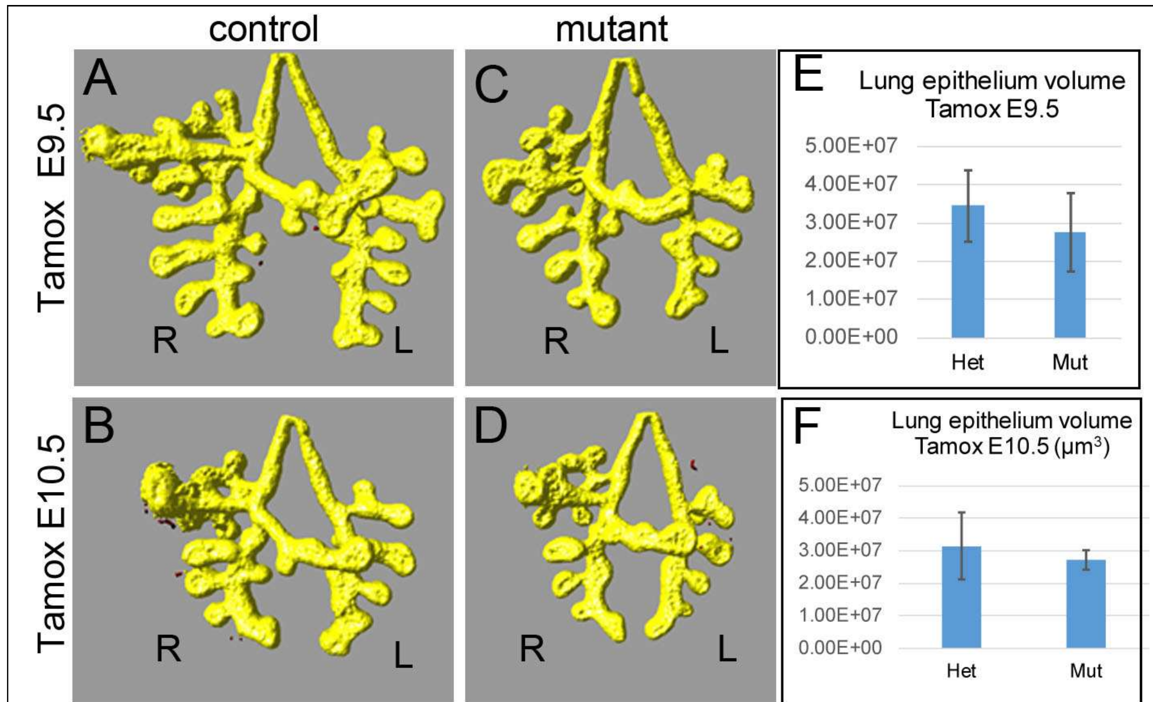

Figure 7. Inactivation of *Rdh10* by treatment with tamoxifen at E9.5 or E10.5 does not impair lung growth. (A-B) E12.5 lungs of control (A) and mutant (B) embryos treated with tamoxifen at E 9.5 have similar growth and branching as assessed by immunostaining of epithelium and whole mount confocal microscopy. (C-D) E12.5 lungs of control (C) and mutant (D) embryos treated with tamoxifen at E 10.5 have similar growth and branching as assessed by immunostaining of epithelium and whole mount confocal microscopy. (E-F) Analysis of E12.5 lung epithelium volume following tamoxifen treatment at E9.5 (E) or E10.5 (F) reveals no significant difference between control and mutant embryos. (F). For tamoxifen at E9.5, $N_{controls}$ = 4; $N_{mutants}$ = 6. For tamoxifen at E10.5, $N_{control}$s = 4; $N_{mutants}$ = 4.

2.5 qPCR reveals extracellular matrix genes overexpressed in hypoplastic lungs of Rdh10 mutant embryos

Since we know that RA plays an important role during lung morphogenesis, we sought to investigate some of the candidate genes known to be important for lung morphogenesis. For this experiment, we tested whether RA deficiency leads to mis-regulation of *Fgf10* expression, a growth factor known to be important for lung development. Dysregulation of *Fgf10* due to RA deficiency has been reported to be responsible for lung agenesis during the bud initiation stage (Wang et al. 2006; Chen et al. 2007; Chen et al. 2010). We therefore want to the test if the same mechanism is responsible for the lung defects in mutant embryos. In addition to *Fgf10*, we also measured expression of *Mgp,* and *Ctgf,* two extracellular matrix genes that must be properly regulated for lung development. *Mgp* is expressed in a developing rat lung especially at the mesenchymal cells at the tip of the distal buds (Gilbert and Rannels 2004). Over-expression of *Mgp* causes defects in lung development and poor pulmonary angiogenesis (Yao et al. 2011). *Ctgf* holds a very important role in the proliferation and maturation of fibroblast in the lung (Yang et al. 2015).

It is shown that over-expression of *Ctgf* can disrupt normal post-natal lung development and is correlated with lung fibrosis (Wu et al. 2010; Lipson et al. 2012). Moreover, it has been reported that *Ctgf*-null mice will die from respiratory failure after birth (Baguma-Nibasheka and Kablar 2008). Because these three genes are known cause defects in lung development if they are mis-regulated, we measure expression of each in *Rdh10* control and mutant lung tissues (tamoxifen E8.5) via qPCR.

Lung samples from the mutant and the control embryos treated with tamoxifen at E8.5 were collected at E11.5 by microdissection and processed for RNA extraction, purification and cDNA synthesis. Using primers with validated efficiency between 90% and 110%, we ran qPCR for *Fgf10, Mgp, Ctgf,* and the house-keeping gene *Actb*, which is used to normalize RNA/cDNA levels for each sample (Fig. 8).

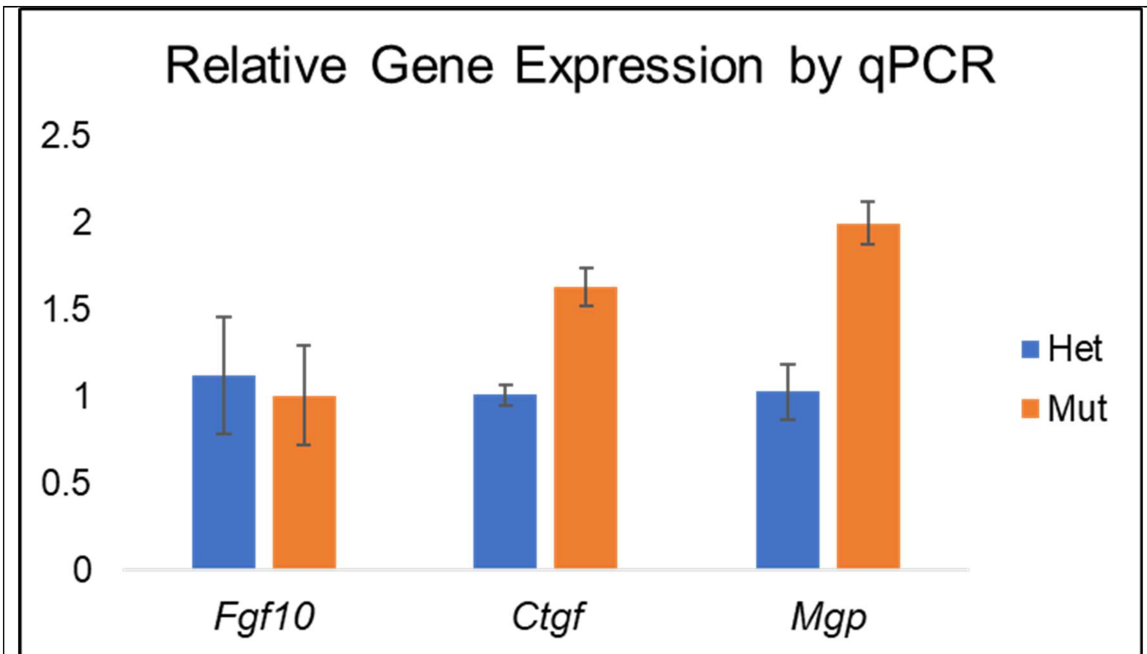

Figure 8. *Ctgf* and *Mgp* are up-regulated, but *Fgf10* is unchanged, in mutant lungs relative to control lungs after tamoxifen treatment at E8.5. There is no significant difference between the expression of *Fgf10* in the lungs from the control embryos (Het) and the lungs from the mutant (Mut) embryos. *Mgp*, and *Ctgf* are significantly overexpressed in the lung of the mutant embryos compared to the lung of the control embryos. *Ctgf,* $N_{control}$=4, N_{mutant}=6 samples; *Fgf10* and *Mgp*, $N_{control}$=3, N_{mutant}=3, ** $p \leq 0.005$

The results showed no change in the level of *Fgf10* expression in the lungs of mutant embryos compared to control embryos. However, we did detect that both *Mgp,* and *Ctgf* are significantly overexpressed in the lungs of mutant embryos compared to the control embryos. These data show that the hypomorphic lung phenotype of the *Rdh10* mutant embryos treated with tamoxifen at E8.5 is not associated with altered expression of the growth factor *Fgf10*, but is associated with upregulation of two extracellular matrix genes *Ctgf* and *Mgp*.

CHAPTER 3 : DISCUSSION

I have shown that the conditional *Rdh10* floxed mice in combination with the tamoxifen-inducible ERT2-Cre can be used to inactivate *Rdh10* in embryos to study the role of RA in embryonic development. Importantly, by measuring the amount of the floxed *Rdh10* exon 2 present at different time points after administration of tamoxifen, I have showed that *Rdh10* can be inactivated at a chosen embryonic stage ~ 48 hours after tamoxifen treatment. The efficiency of the *Rdh10* inactivation is nearly 100% when tamoxifen is administered at E8.5, but decreases as the age of the embryo increases. The data validate that *Rdh10* floxed mice in combination with the tamoxifen-inducible ERT2-Cre is a useful genetic system to study the effect of RA deficiency around embryonic day E10.5. E10.5 is a very important time point given that many organs begin to develop occur at this embryonic age. However, the model might be inappropriate for studying the role of RA in vivo in later stages of embryonic development due to lower efficiency of *Rdh10* inactivation. The mechanism of such change in the kinetics of the model remains unclear. Future studies are needed to investigate how the changes in the model’s kinetics occurs so that the model may be beneficial for studies that look at the role of RA in embryonic development after E10.5 in vivo.

The value of having validated the *Rdh10* conditional mutant model is demonstrated by its use in two recently published studies, one study identified the role of RA in the formation of secondary palate (Friedl et al. 2019), and one study that identified the essential role of RA in the initiation for submandibular salivary glands (Metzler et al. 2018).

In this study, we used the mutant mice to identify a new function of RA in regulating lung development during early branching morphogenesis. RA has been known to be important for lung bug initiation at E9.5 (Marquez and Cardoso 2016). However, no previous study identified that RA was needed during early branching growth of the lungs. Because we were able to detect lung defects upon causing RA deficiency at E10.5 (Tamoxifen E8.5), we conclude that the RA continues to play an important role in lung after the initiation stage and into the branching morphogenesis. In contrast to the E8.5 treated embryos, we did not detect lung hypoplasia in mutant lungs when we exposed the embryos to tamoxifen at E9.5 and E10.5. The lack of lung phenotype in the later-treated embryos could mean that the role of RA in lung branching growth is strictly limited to the earliest stages of branching growth, before E12.5. Alternatively, the lack of phenotype in the later-treated mutant embryos might be due to incomplete *Rdh10* inactivation or the presence of another enzyme able to help produce RA at that stage.

In addition to examining the impact of RA deficiency on lung morphology, we also have investigated molecular changes associated with the lung defects. Using qPCR to measure expression of candidate genes involved in lung development,

we have identified two genes that are mis-regulated upon RA deficiency in the *Rdh10* mutant lungs. Surprisingly, the level of *Fgf10* is not influenced by the lack of RA after E8.5, unlike the earlier lung bud initiation stage, where *Fgf10* is known to be regulated by RA. Our data show that, although RA is known for regulating the levels of *Fgf10* in the lung during the lung bud formation at E9.5 (Wang et al. 2006; Chen et al. 2007; Chen et al. 2010), RA does not regulate the morphogenesis of the lung after the initiation stage via *Fgf10* pathways.

Our analysis identified two new genes, *Ctgf* and *Mgp*, that are altered in lungs of RA deficient embryos. Both *Ctgf, and Mgp* are genes encoding extracellular matrix proteins, and both were overexpressed in the RA deficient lungs. Although the overexpression of *Ctgf* is not reported to cause pulmonary hypoplasia, it is known to cause of pulmonary fibrosis (Lipson et al. 2012; Yang et al. 2015). So far there is no reports on overexpression of *Ctgf* will lead to lung hypoplasia neonatally. However, since *Ctgf* positively correlates with pulmonary fibrosis (Wu et al. 2010; Lipson et al. 2012), which is the result of over deposition collagen in the lung leading to the organ inability to expand early deposition of *Ctgf* might be the cause for neonatal lung hypoplasia. While *Ctgf* null rat embryos suffers from lung hypoplasia (Baguma-Nibasheka and Kablar 2008), it might be possible that the overexpression of *Ctgf* might lead to the same lung defects. Our new finding that RA is required for reducing *Ctgf* in lungs of embryos suggests that future studies may look at RA as a therapeutic agent to reduce *Ctgf* levels for treating pulmonary fibrosis in lung injuries associated with elevated Ctgf expression.

Mgp has also been linked to lung morphology in previous studies. In rat lungs cultured *ex vivo*, elimination or inhibition of *Mgp* disrupts lung branching morphogenesis (Gilbert and Rannels 2004). Transgenic mice with excess *Mgp* develop smaller lungs and have reduced branching of the pulmonary vasculature (Yao et al. 2011). Our new analysis identifying that lung defects of RA deficient mouse embryos are associated with elevated *Mgp* strengthens the evidence that *Mgp* plays a role in lung biology. may disturb lung branching growth. Despite there are no reports showing that over expression of *Mgp* directly correlate to pulmonary hypoplasia, it is reported that *Mgp* over expression leads to the development of pulmonary vasculature (Yao et al. 2011). The fact that we observe an overexpression of *Mgp* and lung hypoplasia suggests there might be a correlation between vasculature and lung branching.

In addition to the analysis of the role of Rdh10 and RA in lung branching morphogenesis, a major contribution of this project is the validation of a mouse genetic model that can be used in inactivate *Rdh10* and reduce RA signaling at E10.5. The model can also be applied to other organ systems allowing deeper understanding about the role of RA and *Rdh10* in embryogenesis. However, it is undeniable that additional analysis can improve the usefulness of the model. For example, since my study did not quantify the efficiency of *Rdh10* inactivation when tamoxifen was introduced to the embryos at E9.5, I do not know how the kinetics of *Rdh10* inactivation at that stage. Thus, the lack of mutant lung defects observed when tamoxifen was introduced at E9.5 must be interpreted with caution.

CHAPTER 4: METHODS

4.1 Mouse strains

For the experiments of this study the *Mus musculus* laboratory mice were used. The used *Rdh10* mutant strains is described previously((Sandell et al. 2012). Mutant *Rdh10* alleles were obtained from *Rdh10*$^{Bgeo/+}$ embryonic stem cells from the trans-NIH Knockout Mouse Project (KOMP) Repository, a National Center for Research Resources - National Institutes of Health (NCRR-NIH)-supported strain repository (www.komp.org; email service@komp.org). For the experiments described in this study, the *Rdh10*$^{flox/flox}$ and *Rdh10*$^{delta/+}$ mice were bred extensively to FVB/NJ such that their background is mixed with a significant contribution of FVB/NJ. Genotyping of all *Rdh10* alleles and transgenes, from tissue samples collected was done by the commercial genotyping service Transnetyx. The mutant and transgenic mouse strains used in this study are as follows: *Rdh10*$^{flox/flox}$, *Rdh10*$^{delta/+}$ (both mixed background, primarily FVB/NJ) (Sandell et al. 2012), Cre-ERT2 *Gt(ROSA)26Sor*$^{tm1(cre/ERT2)Tyj}$ (mixed background) (Ventura et al. 2007). *Rdh10*$^{Bgeo/+}$ (Sandell et al. 2012) sas also used to stain for *Rdh10* expression in lung tissues.

All animal experiments described in this study were approved by the Institutional Animal Care and Use Committee at the University of Louisville and were treated according to the approved protocol.

4.2 Genetic crosses and conditional Rdh10 inactivation by tamoxifen administration

Embryos were generated by mating mice and observing when a vaginal plug is present as a sign of successful mating. Appearance of vaginal plug is considered E0.5. For all experiments in this study, the mating cross was performed to produce control and mutant embryos within the same litter. Homozygous *Rdh10$^{flox/flox}$*; Cre–ERT2/Cre-ERT2 mice were crossed with *Rdh10$^{delta/+}$* mice. Litters produced by such crosses have 50% of 'control' embryos with a pre-tamoxifen genotype of *Rdh10$^{flox/+}$* 'control', which are heterozygous for the haplosufficient wild-type allele of *Rdh10*. The remaining 50% embryos from such a cross are referred as 'mutant' and have a pre-tamoxifen genotype *Rdh10$^{delta/flox}$* ', which are heterozygous for a deleted allele and a conditional floxed allele of *Rdh10*. All embryos were heterozygous for Cre-ERT2. For all time-mated animals, a single dose of tamoxifen was administered at a chosen time (E8.5, E9.5 or E10.5) via maternal oral gavage. Cre-ERT2 excision of *Rdh10* exon 2 converts the *Rdh10^{flox}* allele into the *Rdh10^{delta}* allele in all tissues of the embryo. The genotype of the control embryos that are *Rdh10$^{flox/+}$* pre-tamoxifen are converted to heterozygous *Rdh10$^{delta/+}$* post-

tamoxifen. The mutant embryos genotype are *Rdh10*$^{flox/delta}$ pre-tamoxifen and are converted to *Rdh10*$^{delta/delta}$ genotype post-tamoxifen.

Each pregnant dam was administered an oral gavage dose of 5 mg of tamoxifen plus 2 mg progesterone in 250 µl of corn oil. For all time-mated animals, a single dose of tamoxifen was administered at a chosen time (E8.5, E9.5 or E10.5) via maternal oral gavage.

4.3 Analysis of conditional Rdh10 exon 2 excision kinetics by qPCR

To determine the efficiency of the excision at E8.5 of the *Rdh10* exon 2, tamoxifen was applied at E8.5 to 2 pregnant mice. One litter was harvested 24 hours post-tamoxifen and the other was collected was collected 48 hours post-tamoxifen. For the E8.5-treated embryos, whole embryos were collected for qPCR analysis of *Rdh10* exon 2 excision. To determine the efficiency of the excision at E10.5 tamoxifen was applied at E10.5 to 2 pregnant mice. The one litter was harvested 24hours post-tamoxifen and the other was collected was collected 48hours post-tamoxifen. For the E10.5 treated embryos, whole embryos were too big for the RNA extraction columns therefore, only the heads of these embryos were used. Levels of *Rdh10 exon 2, Rdh10 exon 3* and *Gapdh* were quantified by qPCR. DNA was extracted from the tissue using DNeasy kit (Qiagen). For normalization of DNA levels between samples, *Gapdh* was used as normalization control.

All qPCR was performed on an ABI 7500, using SYBR Select Master Mix (Thermo Fisher Scientific). expression was quantified using the ΔΔCT method (Livak and Schmittgen, 2001), and the Student's t-test was used to determine statistical significance. The efficiency of all primer pairs used was validated to be between 90% and 110%. *Gapdh* (F) 5′-ACAGTCCATGCCATCACTGCC-3′, (R) 5′-GCCTGCTTCACCACCTTCTTG-3′; *Rdh10 exon 2* (F) 5'-GGCAGGCATTACAGTGTTAGA-3', (R): 5'-CCACGTCACAAGTGTAGGTAAA-3'; *Rdh10 exon 3* 5'-(F) AGGCCTTTCTTCCAACGATG-3', (R): 5'-CAACTCCAGCAGTGCTGAAC-3';

4.4 Analysis of gene expression in embryonic lung tissues by qPCR

Lungs of control and mutant embryos treated with tamoxifen at E8.5 were isolated from E11.5 embryos by microdissection. RNA was extracted using a Qiagen RNeasy micro kit. For normalization of RNA levels between samples, *Actb* was used as normalization control. All qPCR was performed on an ABI 7500, using SYBR Select Master Mix (Thermo Fisher Scientific). Expression was quantified using the ΔΔCT method (Livak and Schmittgen, 2001), and the Student's t-test was used to determine statistical significance. The efficiency of all primer pairs used was validated to be between 90% and 110%.

*Actb (*F): 5'-GGCTGTATTCCCCTCCATCG-3', *(R):* 5'-CCAGTTGGTAACAATGCCATGT-3', *Fgf10 (F): 5'-*TTTGGTGTCTTCGTTCCCTGT-*3', (R): 5'-*TAGCTCCGCACATGCCTTC-*3'*; *Ctgf* (F): 5'-GGGCCTCTTCTGCGATTTC-3', (R): 5'-ATCCAGGCAAGTGCATTGGTA-

3'; *Mpg* (F):5' GGCAACCCTGTGCTACGAAT-3', (R): 5'-CCTGGACTCTCTTTTGGGCTTTA-3'.

4.5 Lung morphology phenotype

Lungs from post-tamoxifen mouse embryos (E12.5, or E16.5.) were isolated by microdissection and imaged by white light microscopy using a Leica stereomicroscope.

4.6 Immunostaining, imaging and analysis of lung epithelium volume

Lungs from post-tamoxifen mouse embryos (E10.5 or E12.5) were isolated by microdissection. Immunostaining whole-mount tissues was performed as previously described (Abashev et al. 2017), with the assistance of Dr. Melissa Metzler. To label all epithelium, anti-E-cadherin primary antibody was used (Invitrogen #13-1900) Fluorescently conjugated secondary antibody Alexa Fluor 546 was used at 1:300. Fluorescently immunostained embryos were imaged by confocal microscopy on a Leica SP8 confocal microscope.

Lung volume was determined by measuring E-cadherin signal using IMARIS (Bitplane AG). The lung immunofluorescent signal was measured specifically (omitting the esphagus signal). Volume was determined by Signal Intensity Sum for each sample. Statistical analysis was performed using the Student's *t*-test.

4.7 X-gal staining of* Rdh10^{Bgeo} *reporter embryos

Tissue from embryos containing either the *Rdh10^{Bgeo}* reporter were stained with an X-gal substrate as previously described (Wright et al. 2015). To perform whole-mount tissue staining, tissue samples were fixed in 2% paraformaldehyde/0.2% glutaraldehyde from 45 to 90 minutes on ice cold condition, incubated with Rise Solution A (Millipore, BG-6-B) for 30 min at room temperature, and then incubated with Rise Solution B (Millipore, BG-7-B) for 15 min at 37°C. Tissue was placed in X-gal solution [Stain Base Solution (Millipore, BG-8-C)+1 mg/ml X-gal (Sigma-Aldrich, B4252)] overnight in a light-protected area.

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