The Role of Rb in Carrying a Successful Pregnancy in Mice

A Senior Honors Thesis

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By

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Abstract

Mutations in the Retinoblastoma (*RB*) tumor suppressor gene or disruptions in the RB-E2F signaling pathway have been identified in most human cancers. Homozygous deletion of *Rb* ($Rb^{-/-}$) in mice causes embryonic lethality by day 15.5 of gestation. Lethality of *Rb* ablation is not due to an intrinsic function of Rb in the fetus, but rather due to the severe disruption of placental architecture and function (Wu et. al, 2003). In the present study, we show that additional Rb function in maternal tissues other than the placenta is critical for fetal viability. Loss of one allele of *Rb* ($Rb^{+/-}$) in the mother resulted in increased fetal mortality upon exposure to the synthetic steroid dexamethasone during pregnancy. Liver-specific deletion of one or both conditional *Rb* alleles in mothers exposed to dexamethasone also resulted in fetal mortality, suggesting that the liver is an important site of pregnancy-associated Rb function. This study reveals a non-classical role for Rb in the detoxification of harmful substances and preservation of embryonic development during pregnancy.

Introduction:

Review of literature

Mutations in the *Retinoblastoma* (*RB*) tumor suppressor gene or disruptions in the RB-E2F signaling pathway have been identified in most human cancers (Harbour and Dean, 2007). Furthermore, studies in mice have shown that Rb is essential for embryonic

development (Jacks et. al, 1992). *Rb* mutant (*Rb*$^{-/-}$) embryos exhibit ectopic proliferation and apoptosis in the central nervous system (CNS) and have defective erythropoiesis, leading to lethality by embryonic day 15.5 (E15.5) (Jacks et. al, 1992, Lee et. al, 1992, Clarke et. al, 1992; Wu et. al, 2003). Loss of *Rb* specifically in the placenta severely disrupts the labyrinth architecture by inducing unscheduled proliferation of trophoblast cells (Wenzel et. al, 2007; de Bruin et. al, 2003). This leads to compromised placental vascularization and reduced placental surface area, which impair the exchange of waste and nutrients between mother and fetus, also resulting in lethality by E15.5 (Wenzel et. al, 2007; de Bruin et. al, 2003). Consistent with cell non-autonomous functions for Rb in maintaining fetal viability, *Rb*-deficient embryos supplied with a wild-type placenta did not exhibit the above neurologic and erythroid abnormalities thought to cause their demise. These embryos could be carried to term but die shortly after birth due to muscular and skeletal abnormalities (de Bruin et. al, 2003; Wu et. al, 2003).

Retinoblastoma is a childhood retinal cancer caused by mutation of the *RB* tumor suppressor gene and accounts for more than three percent of all cancers diagnosed under the age of 15. RB is widely known to control cell cycle progression by binding the E2F family of transcription factors, which regulate the expression of genes important for DNA replication and mitosis (Nevins, 2001). In humans, germline mutations resulting in a single dysfunctional copy of *RB* followed by the loss of the remaining allele in the retina lead to the development of hereditary retinoblastoma, which usually forms bilaterally. While the incidence of retinoblastoma has stabilized over the last thirty years, disease prognosis has significantly improved with advancing cancer treatments (Seregard et. al, 2003; Broaddus et. al, 2009). Fatality from retinoblastoma has drastically declined in

developed countries, leading to an increase in the number of retinoblastoma patients of childbearing age. However, no studies to date have examined the outcome of pregnancies from retinoblastoma survivors. Given that studies in mice have demonstrated the critical role of Rb in proper placental function (Wu et. al, 2003), we hypothesized that these patients might have difficulty carrying a pregnancy to term. To test this hypothesis in mice, we compiled and assessed litter size data from crosses between $Rb^{+/-}$ females and $Rb^{+/+}$ males as well as between $Rb^{+/+}$ females and $Rb^{+/-}$ males. However, we detected no significant difference in the average litter size of $Rb^{+/-}$ and $Rb^{+/+}$ mothers, shown in Figure 1 (Wenzel, unpublished data).

Problem Statement

We have shown that there was no difference in pregnancy outcomes between $Rb^{+/-}$ and $Rb^{+/+}$ mothers under normal conditions (Figure 1). Previous work has shown that inactivation of Rb and related pocket proteins by the Simian Virus 40 large T-antigen (SV40 T-Ag) in the mouse intestine resulted in decreased expression of genes encoding members of the cytochrome P450 family of enzymes (Sáenz-Robles et. al, 2007). We therefore hypothesized that under a persistent low level of toxin-induced stress $Rb^{+/-}$ mothers would have less successful pregnancies due to compromised *Cyp* induction. To test this hypothesis, we continuously administered the synthetic gluccocorticoid dexamethasone to pregnant $Rb^{+/-}$ and $Rb^{+/+}$ females in their drinking water starting from 10.5 days post-coitus and assessed the viability of their fetuses at E17.5 and post-natal day 0 (P0). Dexamethasone is an anti-inflammatory and a known inducer of several *Cyp* family genes (Tomilson et. al, 1997). Under these conditions, we found that the

percentage of fetal lethality was significantly higher in $Rb^{+/-}$ mothers than in $Rb^{+/+}$ mothers. Interestingly, we did not detect a difference in the rate of lethality between $Rb^{+/-}$ and $Rb^{+/+}$ fetuses from $Rb^{+/-}$ mothers, suggesting that fetal viability is critically dependent on maternal Rb function.

The liver is the major organ responsible for the detoxification of xenobiotics and endogenous substances. Detoxification occurs in two phases, with phase I mediated by the cytochrome P450 family of enzymes (CYPs) that catalyze the hydroxylation and demethylation of compounds destined for elimination from the body (Lindros, 1997). Known inducers of CYP expression and activity include caffeine, alcohol, and cigarettes, all of which have been shown to increase the likelihood of spontaneous abortions (Higdon et. al 2006, Rasch 2003). Interestingly, a polymorphism in *CYP1B1* was recently found to be associated with a higher risk of first trimester miscarriage (Karypidis et. al, 2006). These findings suggest that CYP expression and activity have an important role in maintaining healthy pregnancies. Phase II detoxification involves enzymes such as uridinediphosphate-glucuronosyltransferase (UGT), glutathione S-transferase (GST), and sulfotransferase (SULT) that catalyze the formation of phase I products into more water-soluble derivatives for renal elimination (Grant, 1991). Previous work has shown that inactivation of Rb and related pocket proteins by the Simian Virus 40 large T-antigen (SV40 T-Ag) in the mouse intestine resulted in decreased expression of a number of genes encoding enzymes that participate mostly in phase I detoxification (Sáenz-Robles et. al, 2007). Given that the liver is the predominant site of expression and activity of most CYP isoforms, using a conditional gene knockout approach in mice we asked whether liver-specific ablation of *Rb* would also result in the deregulated expression of

various members of the *Cyp* gene family. Under the same set of experimental conditions as the $Rb^{+/-}$ mouse, a mother with an ablation of one copy of *Rb* only in the liver has a significant rate of fetal lethality. However, lethality in the liver-specific deletion is not as high as the Rb knockout, pointing to additional tissues in which *Rb* may play a role during pregnancy. Since the *Cyp* genes are highly expressed in the liver, we hypothesized that detoxification levels may be affected.

Significance

Inactivation of RB through direct mutations of the *RB* gene or deregulation of other components in the RB tumor suppressor network has been identified in most human cancers (Harbour and Dean, 2007). In this work, we propose a novel link between RB and CYP expression and activity that appears to be independent of RB's role in cell cycle control. This link could alter the approach to treatment of retinoblastoma patients, who are predisposed to the development of other types of cancer including small cell lung carcinoma and osteosarcoma (Hawkins, 1986). Precisely how RB impacts CYP levels is currently not known, but one possible mechanism could be through direct regulatory control of *CYP* transcription. A decrease in the level of functional RB could potentially produce a decrease in CYP function, which includes metabolizing drugs used for the treatment of cancer. As a result, the effective dose of chemotherapeutics given to retinoblastoma or other cancer patients must be adjusted accordingly in an effort to minimize the morbidity associated with most chemotherapeutic regimens.

A second significant contribution of this work is towards an understanding of the underlying genetic basis of miscarriage and other pregnancy-associated complications.

With advancing treatments, more children with hereditary retinoblastoma survive to adulthood. If these survivors choose to have children, they are more incidents of maternal-fetal detoxification, calling into question how functional this detoxification is. Also, identifying a possible compromised detoxification could lead towards prevention of certain miscarriages and other pregnancy disorders in retinoblastoma patients. Perhaps more importantly, this would represent the first of many genes that are likely involved in this process and that might be related to pregnancy disorders in humans.

Materials and Methods:

Albumin-cre mice

Cre-recombinase, or *cre* (*causes recombination*), is a bacteriophage-derived enzyme that promotes site-specific (the 34-base-pair *"lox"* sequence) recombination of DNA (Sauer and Henderson, 1988). The tissue-specific expression of *cre* is achieved by linking the *cre* gene to a cell type-specific promoter. Generation of the transgenic *Albumin-cre* (*Alb-cre*) mice has been previously described (Magnuson and Postic, 2000; Postic et. al,., 1999). These transgenic mice express *cre* specifically in the hepatocytes, and *Alb-cre*-mediated deletion of different conditional alleles has been demonstrated to be nearly 100% at 6 weeks of age (Magnuson and Postic, 2000; Postic et. al, 1999). The conditional *Rb* allele (Rb^{LoxP}) harbors two *lox* sequences flanking exon 19 of the gene. Previous work from the Knudsen Lab has shown that deletion of the Rb^{LoxP} allele was efficiently mediated by *Alb-cre* by approximately 4 weeks of age (Mayhew et. al,., 2005).

Mice and genotyping

Rb$^{LoxP/LoxP}$;*Alb-cre*$^{+/-}$ mice were obtained from the Knudsen Lab. *Rb*$^{+/-}$ (Jacks et. al,. 1992) and *Rb*$^{LoxP/LoxP}$;*Alb-cre*$^{+/-}$ mice were bred into and maintained on a pure FVB background. The mice were housed under standard conditions on a 12:12 hr light-dark cycle and controlled temperature (21 ^{o}C - 23 ^{o}C). The mice were given food and water *ad libitum*. Mice and embryos were genotyped by PCR (*Rb*$^{+/-}$: 3'-GCCTGAAGAACGAGATCAGCAGC-5', 3'-GCATCTGCATCTTTATCGCAGCAG-5', 3'-CCCATGTTCGGTCCCTAG-5'; *Rb*$^{LoxP/LoxP}$: 5'-GGCGTGTGCCATCAATG-3', 5'-CTCAAGAGCTCAGACTCATGC-3'; *Alb-cre:* 5'-GCGGTCTGGCAGTAAAAACTATC-3', 5'- GTGAAACAGCATTGCTGTCACTT-3'). Females were mated with males and then housed in groups of three or four. At day E10.5 of pregnancy (vaginal plug found the morning after mating is considered day E0.5), mice were either injected intraperitoneally (IP) with dexamethasone every other day or given filtered water with 0.005 mg/mL dexamethasone *ad libitum* until day of delivery. Glucose readings were taken four times throughout pregnancy of a subset of mice that received IP delivery of dexamethasone.

Assessment of fetal viability

Postnatal day 0 (P0) Pups were determined as alive, dead, or compromised on the basis of breathing and heartbeat. Pups were counted as compromised if they appeared blue and hypoxic, had an irregular heartbeat, or were gasping for air. A subset of pregnant females given oral dexamethasone was sacrificed on day E17.5 and fetal viability was observed. Fetuses were counted as alive if they were breathing, moving, or had a heartbeat. Dead embryos did not breathe, move, or have a heartbeat.

IP and oral delivery of dexamethasone

Dexamethasone (Sigma) was stored at 4º C and kept out of direct light. Pregnant dams received IP injections of dexamethasone suspended in sterile filtered corn oil at a concentration of 0.05 mg/kg every other day beginning at day E10.5 until birth of pups. Sterile needles were used and mice were weighed just before injection to determine dose. Water-soluble dexamethasone (Sigma) was stored at 4º C and kept out of direct light. Pregnant dams were given 0.005 mg/mL dexamethasone in drinking water *ad libitum* beginning at day E10.5 until harvest at day E17.5.

Real time-PCR

The liver, kidney, intestine, and lung were removed from pregnant dams at day E17.5. Total RNA was isolated from tissues after homogenization in TRIzol reagent (Life Technologies). RNA was used to generate cDNA using Superscript III reverse transcriptase (Invitrogen). Real time-PCR (RT-PCR) was performed using the BioRad iCycler PCR machine. A 25µl final reaction volume contained 0.25µl of cDNA template, 100 nM primers, and 1x SYBR Green Reaction Mix (BioRad). Reactions were performed in triplicates for each sample and relative gene expression determined by normalizing against *GAPDH* expression.

Data analysis

Chi-squared test was performed to test significance. Lethality data from $Rb^{+/+}$ males crossed with $Rb^{+/+}$ and $Rb^{+/-}$ females as well as data from $Rb^{+/-}$ males crossed with $Rb^{+/+}$

females was sent to the OSU Center for Biostatistics and pooled by experiment. A logistic regression clustering was run to generate an odds ratio for each group.

Results:

Haploinsufficiency of *Rb* is sufficient to negatively impact pregnancy when challenged with dexamethasone

We hypothesized that *Rb* potentially mediates detoxification through the *Cyp* genes, which were not activated under the previous ideal environment. To induce *Cyp* expression, the anti-inflammatory steroid dexamethasone was chosen to simulate a low level of constant detoxification of dietary and environmental toxins. A small initial experiment in wild-type mice (n=6) determined the concentration of dexamethasone required to adversely affect but not completely terminate the pregnancy. Wild-type mice were also injected with only sterile filtered corn oil as a control. No lethality from corn oil was observed. A dose of 0.05mg/kg dexamethasone was chosen because it gave a low level (~10%) of lethality in the wild-type mice.

Since dexamethasone can stimulate diabetes-like symptoms in mice (Wong et. al, 1971), glucose readings were taken with a standard glucometer from the tail vein of a small subset of mice (n=3) to ensure that any negative effects observed in the $Rb^{+/-}$ and $Rb^{+/+}$ females would not be due the potential hypoglycemic effects of dexamethasone. Glucose readings were taken before the first dose of dexamethasone at E10.5, then at E15.5, and finally after the pups had been delivered (E19.5-E21.5) from $Rb^{+/-}$ and $Rb^{+/+}$ mice. Glucose was measured in non-pregnant $Rb^{+/-}$ and $Rb^{+/+}$ mice as control. There was no significant difference in the blood glucose levels between the two genotypes.

$Rb^{+/-}$ and $Rb^{+/+}$ mothers were bred with $Rb^{+/+}$ males and injected IP every other day with dexamethasone beginning at E10.5 (vaginal plug = E0.5). At birth, pups were categorized as dead, alive, or compromised. While a low level of lethality was observed from $Rb^{+/+}$ mothers, offspring from $Rb^{+/-}$ mothers showed a marked increase in fetal lethality at birth (35/182, 19.23%) as compared to offspring from $Rb^{+/+}$ mothers (7/117, 5.98% $p<0.01$), suggesting a novel role for Rb in regulating fetal viability (Figure 2). Moreover, a significant number of $Rb^{+/-}$ offspring showed signs of compromise such as a decrease in size and oxygenation, appeared to be gasping for air (21/182, 11.54%, $p<0.001$), and died shortly after birth. These effects were not observed in offspring from $Rb^{+/+}$ mothers. The compromised pups were included with the dead pups when calculating odds ratios, since this was not a truly successful pregnancy (56/182, 30.77%). When combined with the percentage of dead pups, the odds ratio was 6.98 (95% confidence interval: 3.09-15.80) with a p-value <0.001. The odds of a pup being born compromised or dead are almost seven times greater from the $Rb^{+/-}$ mother than from the $Rb^{+/+}$ mother, suggesting that Rb has a potential role in determining a successful pregnancy.

_Rb_ function in the embryonic portion of the placenta does not contribute to fetal lethality.

The placenta is highly vascularized and is critical for the exchange of waste and nutrients between mother and fetus. Although the placenta is often thought to be part of the mother, the majority of this tissue is actually derived from the embryo, and thus reflects the genotype of that embryo. Previous research has shown that $Rb^{-/-}$ fetuses can be

rescued to birth by supplying them $Rb^{+/+}$ placenta (Wu et. al, 2003). Also, complete placental ablation of *Rb* by the placenta-specific *Cyp19-cre* results in a large reduction in the surface area of the placenta and disruption of the vasculature (Wenzel et. al, 2007). We hypothesized that if two copies of functional *Rb* are required in the placenta for a successful pregnancy, then we would see a larger percentage of $Rb^{+/-}$ pups dying as compared to $Rb^{+/+}$ pups when looking at the genotypes of embryos from $Rb^{+/-}$ females. Here we show that the loss of one allele of *Rb* in the embryonic portion of the placenta is not sufficient to determine fetal lethality, pointing to a maternal and not embryonic role for *Rb* in determining a successful pregnancy.

The genotypes of offspring from $Rb^{+/-}$ mothers injected IP with dexamethasone were analyzed. From $Rb^{+/-}$ females crossed with $Rb^{+/+}$ males, there is a 50% chance the offspring would be $Rb^{+/-}$. Therefore, any one dead or compromised offspring should have a 50% chance of being $Rb^{+/-}$. We calculated that in our breeding, each pup had a 53% of being heterozygous. Out of all unsuccessful pregnancies from $Rb^{+/-}$ mothers, heterozygous offspring accounted for 67.5% of dead and compromised offspring (27/40, $p>0.05$). However, there was no statistically significant difference in lethality or compromise between $Rb^{+/-}$ and $Rb^{+/+}$ offspring, suggesting that the loss of one *Rb* allele in the embryonic portion of the placenta does not contribute to fetal lethality (Figure 3).

Hepatic and intestinal *Cyp* expression is not affected by maternal loss of *Rb* function

The liver is one of the essential organs for detoxification. We collected maternal livers after delivery treated with dexamethasone and isolated RNA from the livers for analysis of the level of *Cyp* expression by RT-PCR. We hypothesized that any change in *Cyp*

expression would reflect a difference in the metabolic capacity of the liver of $Rb^{+/-}$ and $Rb^{+/+}$ mothers. We measured the expression levels of *Cyp1a1, 3a25*, and *2d10*, which were previously shown to be down-regulated with the ablation of Rb function in the mouse intestine (Sáenz-Robles et. al, 2007). Dexamethasone is also a known inducer of *Cyp* genes including *Cyp3a25*, the mouse homologue of *CYP3a4* associated with detoxifying prescription drugs (Grant, 1991). The expression of *Gstt1* gene encoding a phase II enzyme was also analyzed.

RNA was isolated from maternal intestines in an effort to reproduce the result of *Cyp* down-regulation observed with the T-Ag-mediated intestinal inactivation of Rb. Failing to reproduce the *Cyp* down-regulation previously observed in the intestine could be due to different methods of Rb inactivation. Simian virus 40 Large T Antigen, or *SV40TAg*, inactivates the protein by binding Rb and related proteins p107, p130, and the tumor suppressor p53 (Ali and DeCaprio, 2001). Since Rb is a suppressor, the *E2F* binding target of *Rb* is free to stimulate transcription much like in the *Rb* knockout mouse. Still, other proteins in the Rb family are affected which are present in the knockout. Furthermore, the previous down-regulation of the *Cyp* genes was observed with a complete inactivation of Rb unlike in the heterozygous $Rb^{+/-}$ mouse.

Although the *Cyp* genes were up-regulated by the dexamethasone compared to mice without drug, there was no significant difference between expression levels of detoxification enzymes in $Rb^{+/-}$ and $Rb^{+/+}$ mothers in the liver or the intestine (Figure 4). It is possible that the *Cyp* detoxification genes do not play a role in the fetal lethality observed. However, there are numerous *Cyp* genes as well as multiple genes involved in the phase II detoxification pathway which were not analyzed.

Average *Cyp* and *Gstt1* levels were similar between genotypes. However, there were large variations between maternal *Cyp* expression in both wild-type and $Rb^{+/-}$ animals. Deviation from the average expression levels from wild-type mice were graphed against overall percentage of unsuccessful pregnancy, the combination of compromise and lethality, in each $Rb^{+/-}$ mouse. Since lethality and compromise varied between mother, it was hypothesized that the lower the *Cyp* expression, the higher the percentage of unsuccessful pregnancy in each litter would be. There was no statistically significant correlation between *Cyp* expression change and pregnancy success (Figure 5). Interestingly, the larger the deviation in *Cyp* genes *1a1* and *3a25,* whether up or down-regulated, from the wild-type average expression of these genes (change of 0) in the heterozygous mouse, the less fetal lethality was observed in the litter.

Oral dexamethasone significantly negatively impacts pregnancy in $Rb^{+/-}$ mothers

To assess whether the differences seen in fetal lethality were due to the dexamethasone and not due to mechanical injury to the fetus or placenta, oral dexamethasone was chosen as a preferred method of challenging pregnant dams with toxic stress. The dexamethasone used had an added phosphate group which made the compound more water-soluble. A second dose experiment was performed in $Rb^{+/-}$ mice with four separate doses to determine the concentration of water-soluble dexamethasone needed to confer approximately the same level of fetal lethality observed in the IP injections.

$Rb^{+/-}$ and $Rb^{+/+}$ mothers were crossed with wild-type males and separated and housed in groups of five after plugging. At day E10.5 of pregnancy, the mice were administered dexamethasone through drinking water *ad libitum* in a dark water bottle to

ensure the dexamethasone would not be degraded by exposure to light. The mice were allowed to progress to delivery, and fetal lethality was observed. At birth, offspring were determined as either dead or alive on the basis of movement, breathing, and heartbeat after decapitation. No compromised offspring were observed.

Rb$^{+/-}$ mothers again gave birth to a significantly higher number of dead pups than did *Rb*$^{+/+}$, confirming previous results and validating oral dexamethasone as a practical delivery method. There was an overall increase in lethality compared to the injections. However, the relative differences between the two genotypes were similar to results obtained from IP injections, with wild-type mothers having a lower lethality (17/124, 13.71%) than *Rb*$^{+/-}$ mothers (68/128, 53.13%, Figure 6). The odds ratio was very close to our previous results, being that a pup born from a *Rb*$^{+/-}$ mother is 7.13 times more likely to be dead than a pup born from a *Rb*$^{+/+}$mother (95% confidence interval: 2.10-24.28, p-value = 0.002). These results suggest that *Rb* plays a significant role in determining a successful pregnancy.

Fetal lethality is maternally derived

To further the hypothesis that fetal lethality correlated with maternal haploinsufficiency of *Rb*, three breedings of mice were used: *Rb*$^{+/+}$ males were crossed with *Rb*$^{+/+}$ and *Rb*$^{+/-}$ females as well as *Rb*$^{+/-}$ males crossed with *Rb*$^{+/+}$ females. Mothers from all three groups were administered oral dexamethasone in drinking water beginning at day E10.5. At day E17.5, the mice were harvested and fetal lethality was observed to ensure that any differences would not be because of difficulty during or just after delivery.

Consistent with previous experiments, $Rb^{+/-}$ mothers had a significantly higher percent of fetal lethality than the $Rb^{+/+}$ mothers (Figure 7). Compared with previous data collected upon delivery of offspring, there was an overall decrease in lethality. This suggests that some of the previously observed deaths among offspring might either be due to factors other than the dexamethasone, such as maternal neglect at birth or difficulty during delivery, or that some deaths occur late in pregnancy. Harvesting the mothers instead of collecting the pups at delivery demonstrated that embryonic lethality occurred at all stages of pregnancy. Dead embryos ranged from small reabsorptions to fully developed embryos the same size as live pups. Also, there were no instances in which all deaths were from a single side of the uterine horn, suggesting that lethality is not due to a failure within a single ovary.

Offspring from both $Rb^{+/+}$ males crossed with $Rb^{+/+}$ females and from an $Rb^{+/-}$ male bred with an $Rb^{+/+}$ female had similar low levels of lethality (12/129, 9.3% and 7/100, 7% respectively). Under the same experimental conditions, an $Rb^{+/-}$ female crossed with a $Rb^{+/+}$ male gave birth to a significantly higher number of dead embryos (49/127, 38.6%), furthering the hypothesis that it is the maternal role of Rb which determines a successful pregnancy. It is 6.3 times more likely that a dead pup will be born from an $Rb^{+/-}$ mother compared to an $Rb^{+/+}$ mother (95% confidence interval: 2.67-14.05, $p<0.001$). This odds ratio is similar to previous results, although lower because of observing lethality early in the pregnancy.

_Rb_ function is essential in the maternal liver for a successful pregnancy

Using an Albumin-*cre* transgene, *Rb* was deleted in the maternal liver. Three subsets of mice were used, $Rb^{+/+}$-*cre*, $Rb^{+/LoxP}$-*cre* and $Rb^{LoxP/LoxP}$-*cre*. $Rb^{+/+}$-*cre* was used as a wild-type control for potential *Albumin-cre* induced toxicity. All three genotypes were bred with $Rb^{+/+}$ males and separated after becoming pregnant. At day 10 of pregnancy, mice were given oral dexamethasone through drinking water, and harvested at E17.5 to observe fetal viability. Pups were determined to be alive based of movement, breathing, and heartbeat at decapitation. No compromised pups were observed.

$Rb^{+/+}$-*cre* mothers crossed with $Rb^{+/+}$males had a similar level of lethality (15/146, 10.3%, Figure 8) compared to lethality from wild-type mothers observed in previous experiments (12/129, 9.3%), demonstrating that any observed toxicity in other subsets of mice was not due to the *Albumin-cre*. A significant level of fetal lethality was observed in offspring from $Rb^{+/LoxP}$-*cre* mothers, essentially heterozygous for *Rb* only in the liver (43/120, 35.8%, $p<0.005$). If *Rb* is to play a crucial role during pregnancy, it must be in the liver. The lethality was slightly less than previously observed from the $Rb^{+/-}$ mothers, (49/127, 38.6%) pointing to possible additional tissues in which Rb is essential during pregnancy.

Surprisingly, the $Rb^{LoxP/LoxP}$-*cre* subset of mice did not have a higher percentage of lethality than did the $Rb^{+/LoxP}$-*cre* mothers. The percentage of unsuccessful pregnancy was in between that of the $Rb^{+/+}$-*cre* and $Rb^{+/LoxP}$-*cre* mice (11/49, 22.5%). It is possible this difference is because of the small sample size (n=5). If *Rb* were mediating a detoxification pathway in the liver affected by the loss of one allele, potentially a complete ablation of Rb could force a shift to an alternative mechanism. However, until

the mechanism by which Rb affects pregnancy is discovered, the answer remains uncertain.

Discussion:

Conclusion:

The *Rb* gene was the first tumor suppressor identified, but it is still not completely understood. New data has shown that *Rb* potentially mediates such diverse pathways as detoxification (Sáenz-Robles et. al, 2007). Here we demonstrate the first known role of *Rb* during pregnancy. We show that the loss of one copy of *Rb* is sufficient to negatively affect pregnancy under the administration of the gluccocorticoid dexamethasone. However, it is possible that there are many potential toxins that could affect the pregnancy of someone with a mutation in *Rb*.

Interestingly, it is maternal *Rb* function and not the fetal function that causes the observed lethality. It is only when the mother has an ablation of *Rb* that significant fetal lethality is observed. Also, the genotype of the offspring does not correlate with an unsuccessful pregnancy. Furthermore, we pinpoint the maternal liver as one of the organs in which *Rb* plays a role during pregnancy. Although the liver is an essential organ for detoxification, we found no statistically significant difference between the expression levels of those genes responsible for detoxification.

Future Directions:

This project has answered many questions about the link between *Rb* and pregnancy while leaving others unanswered. The next project will determine if specific mutations in

the gene observed in retinoblastoma are sufficient to impact the viability of the pregnancy. Four common mutations will be chosen that mimic changes in the *Rb gene* in human retinoblastoma, and the experiment will be repeated. If fetal lethality is still observed from pregnant dams with the mutations, not only will *Rb* be linked to pregnancy and the disease retinoblastoma, but also potentially to other human cancers. *Rb* is mutated in approximately 30% of all human cancers, and changes in the *Rb* pathway have been discovered in many more (Harbour and Dean, 2007). Furthermore, differences in the lethality between the distinct mutations might elucidate the pathway by which *Rb* affects pregnancy.

The largest limitation of this project was the inability to discover which genes are affected during the observed lethality. This could either be because we only examined a small subset of the *Cyp* genes, or that they are not involved. A microarray would be the most efficient way to examine a large number of genes at one time, and document up or down regulation. Finally, discovering the complete pathway by which this fetal lethality occurs will be a major project in the near future.

a)

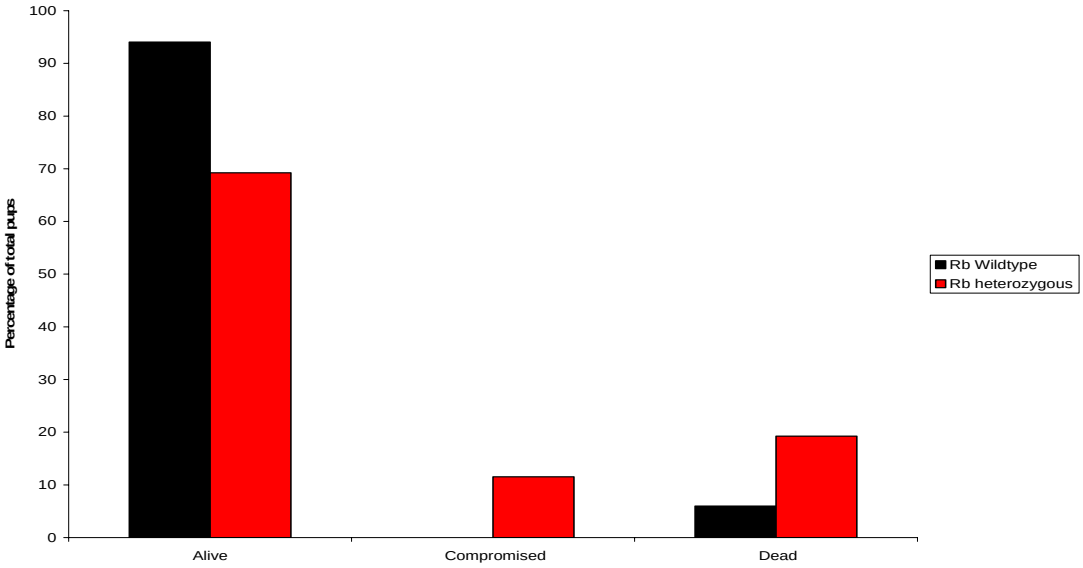
	$Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀		$Rb^{+/-}$ ♂ x $Rb^{+/+}$ ♀		$Rb^{+/-}$ ♂ x $Rb^{+/-}$ ♀	
	H	W	H	W	H	W
Offspring	241 *(230)*	219 *(240)*	91 *(87.5)*	84 *(87.5)*	671 *(692)*	367 *(346)*
Ratio	1: 1.100457 *(1:1)*		1: 1.08333 *(1:1)*		1: 1.828338 *(1:2)*	

b)

	$Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀	$Rb^{+/-}$ ♂ x $Rb^{+/+}$ ♀	$Rb^{+/-}$ ♂ x $Rb^{+/-}$ ♀
Total #	535	144	1120
Average	7.039474	8.470588	6.256983

Figure 1: a) Schematic of compiled data from null maternal Rb, null paternal Rb, and intercross. There is no significant difference between the observed and expected frequency of passing the mutation. Observed *(expected).*
b) Average litter size from compiled data. There is no statistical significance between the crosses. The reduced litter size between the $Rb^{+/-}$ x $Rb^{+/-}$ mice can be accounted for by the lethality of Rb mutants at embryonic day 15.

IP injections of dexamethasone, birth



a)

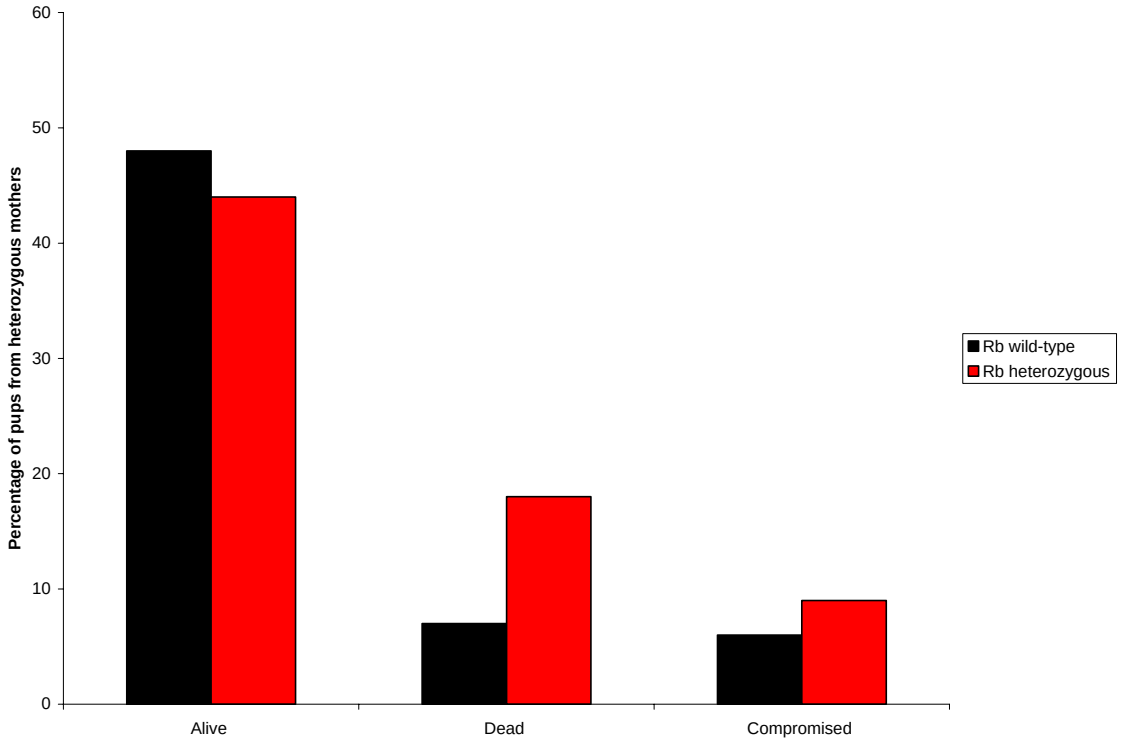
b)

	$Rb^{+/+}$ ♂ x $Rb^{+/+}$ ♀	$Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀
Alive	110	126
Dead	7* *(16.4)*	35 *(25.6)*
Comp.	0# *(8.2)*	21 *(12.8)*
Total	**117**	**182**

Figure 2: a) Graph of percentages viable offspring, fetal lethality, and compromise vs. genotype of mothers injected with dexamethasone. $Rb^{+/-}$ mothers are represented with the red bar, while $Rb^{+/+}$ mothers are represented in black. There is a significant increase in the percent of fetal lethality from heterozygous mothers.
b) Table with offspring death and compromise from the two maternal genotypes. There were 35/182 dead offspring from $Rb^{+/-}$ mothers, as compared to 7/117 dead from $Rb^{+/+}$ mothers ($p<0.01$). Expected values are in italics. * $p<0.01$. # $p<0.001$.

IP injections of dexamethasone, birth. Lethality from $Rb^{+/-}$ mothers

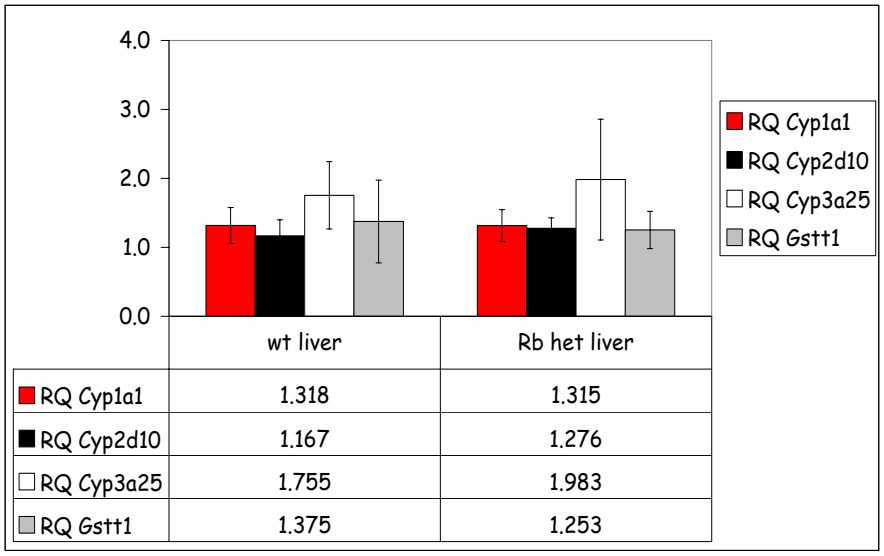
a)



b)

	$Rb^{+/+}$ offspring	$Rb^{+/-}$ offspring
Alive	48	44
Dead	7 *(11.7)*	18 *(13.3)*
Comp.	6 *(7)*	9 *(8)*
Total	**61**	**71**

Figure 3: a) Graph of percentages of live, dead, and compromised births from $Rb^{+/-}$ mothers categorized by genotype of offspring. There is no statistical significance between the genotypes.
b) Table with offspring death and compromise from heterozygous mothers. There were 13/61 unsuccessful pregnancies that were $Rb^{+/+}$ compared to 27/71 that were $Rb^{+/-}$. This was not statistically significant.



	wt liver	Rb het liver
RQ Cyp1a1	1.318	1.315
RQ Cyp2d10	1.167	1.276
RQ Cyp3a25	1.755	1.983
RQ Gstt1	1.375	1.253

Figure 4: Schematic of the average *Cyp* and *Gstt1* levels in the livers of $Rb^{+/+}$ (wt liver) and $Rb^{+/-}$ (Rb het liver) mothers. Expression levels were normalized to wild-type control. There is no significant difference in the expression levels, indicating that Rb does not function as a mediator for these genes in the liver.

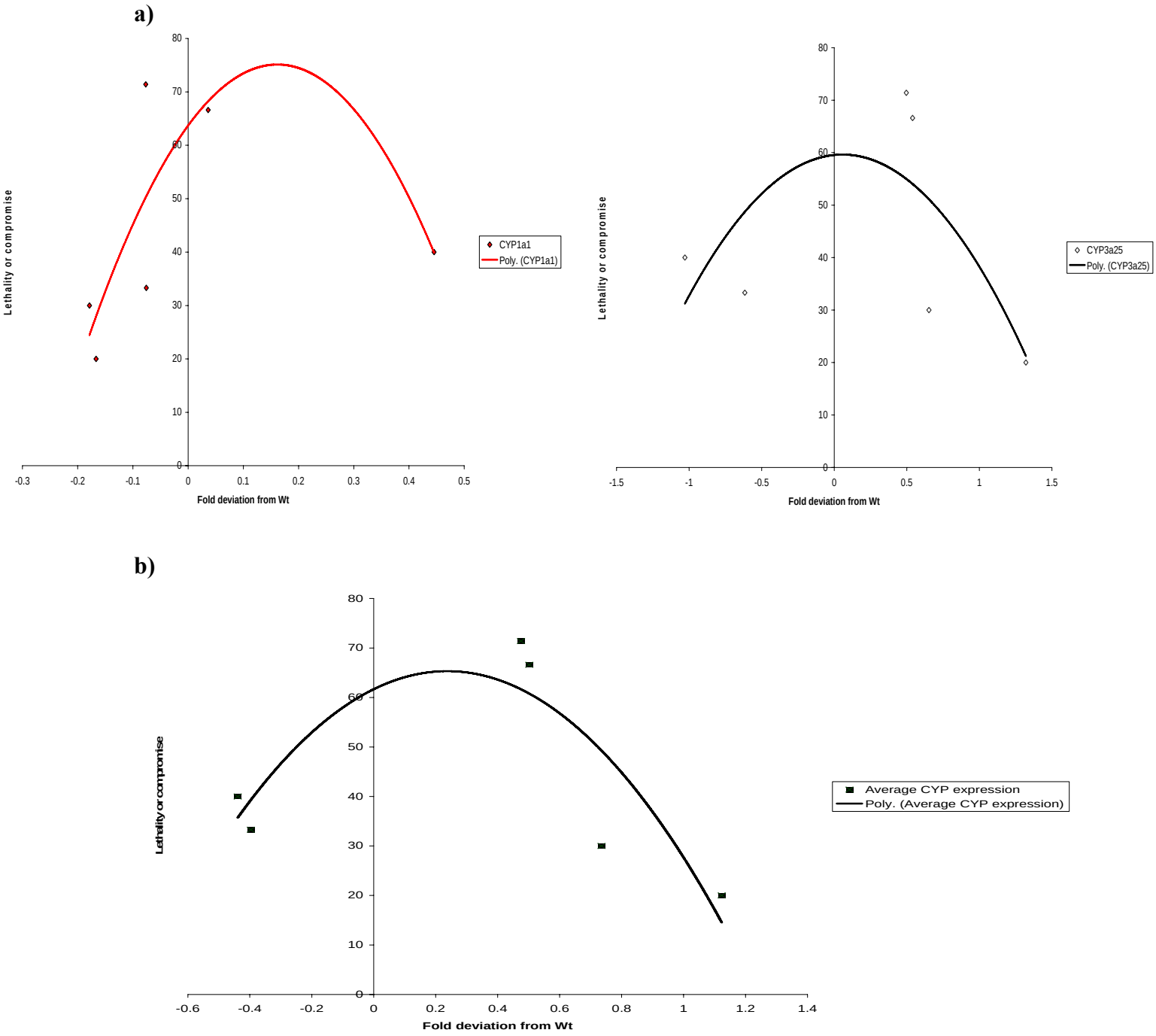
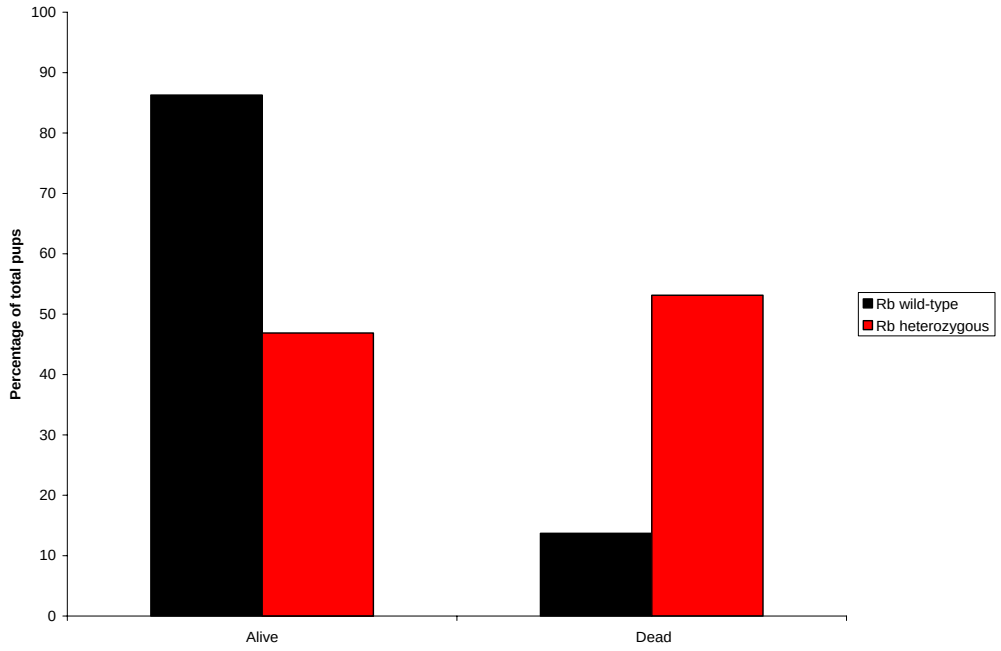


Figure 5: a) *Cyp1a1* and *3a25* expression levels were subtracted from the average $Rb^{+/+}$ fold expression to generate fold deviation, which is plotted against percent of lethality and compromise in the litter. The greater the deviation, the less lethality and compromise observed in the litter.

b) Expression of *Cyp1a1*, *2d10*, and *3a25* were averaged and subtracted from the average $Rb^{+/+}$ *Cyp* expression to generate fold deviation, plotted against lethality and compromise of the litter. The same trend is observed, though not significant.

Oral dexamethasone, birth



a)

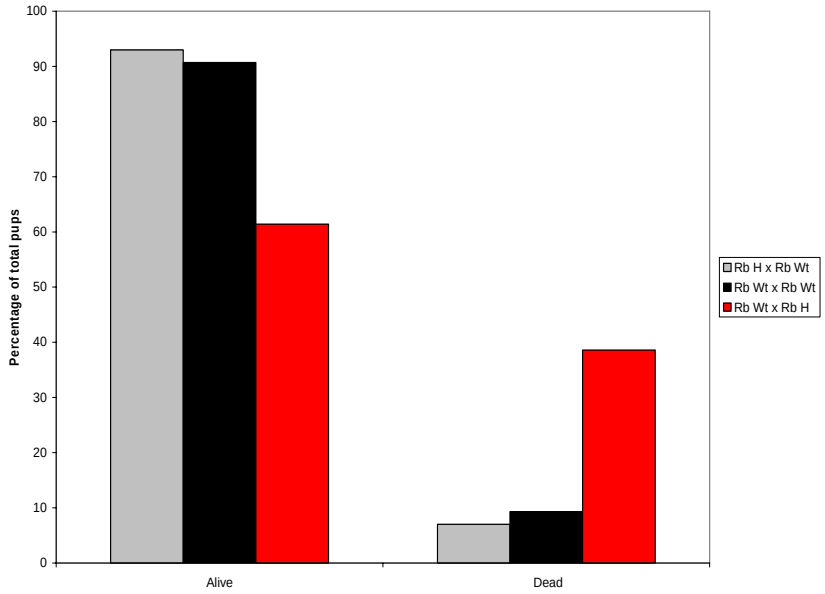
b)

	$Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀	$Rb^{+/+}$ ♂ x $Rb^{+/+}$ ♀
Alive	60	107
Dead	68 *(43.2)*	17* *(41.8)*
Total	**128**	**124**

Figure 6: a) Graph of percentages viable offspring and fetal lethality vs. genotype of mothers under administration of oral dexamethasone. $Rb^{+/-}$ mothers are represented with the red bar, while $Rb^{+/+}$ mothers are represented in black. This confirmed previous results as well as using oral dexamethasone as a delivery method.
b) Table with offspring death and compromise from the two maternal genotypes. There were 68/128 dead offspring from $Rb^{+/-}$ mothers, as compared to 17/124 dead from $Rb^{+/+}$. Expected values are in italics. * $p<0.01$.

Oral dexamethasone, day 17.5 of pregnancy

a)



b)

	$Rb^{+/-}$ ♂ x $Rb^{+/+}$ ♀	$Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀	$Rb^{+/+}$ ♂ x $Rb^{+/+}$ ♀
Alive	93	117	78
Dead	7	12	49
Total	**100**	**129**	**127**

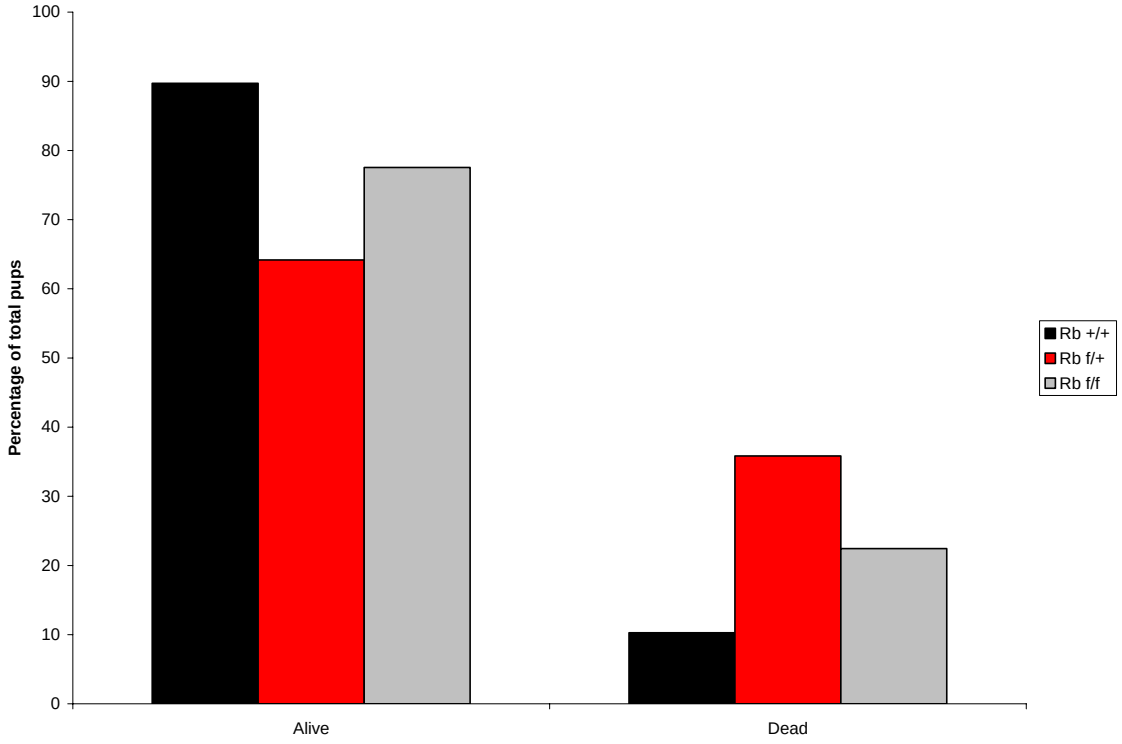
c)

Group 1	Group 2	Odds Ratio	95% Confidence Interval	p-value
$Rb^{+/+}$ ♂ x $Rb^{+/+}$ ♀*	$Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀	6.13	2.67-14.05	<0.001
$Rb^{+/+}$ ♂ x $Rb^{+/+}$ ♀ *	$Rb^{+/-}$ ♂ x $Rb^{+/+}$ ♀	0.73	0.26-2.05	0.555
$Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀	$Rb^{+/-}$ ♂ x $Rb^{+/+}$ ♀*	8.34	4.20-16.61	<0.001

Figure 7: Graph of percentages viable offspring and fetal lethality vs. genotype of fathers and mothers under administration of oral dexamethasone. $Rb^{+/-}$ fathers crossed with $Rb^{+/+}$ mothers are represented with the grey bar (7% lethality), $Rb^{+/+}$ fathers crossed with $Rb^{+/+}$ mothers are represented in black (9.3% lethality), and $Rb^{+/+}$ fathers crossed with $Rb^{+/-}$ mothers are in red (38.6% lethality).
b) Table with offspring death and compromise from the three possible crosses.
c) Table summarizing the pairwise comparisons using logistic regression with clustering on litter. The odds of a pup being born dead is 6 times greater for $Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀compared to $Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀ ($p<0.001$) and the odds of dying is over 8 times greater in the $Rb^{+/+}$ ♂ x $Rb^{+/-}$ ♀ compared to the $Rb^{+/-}$ ♂ x $Rb^{+/+}$ ♀. *Reference group for odds ratio.

Oral dexamethasone, day 17.5 of pregnancy

a)



b)

	***Rb*$^{+/+}$ ♂ x *Rb*$^{+/+}$*; Alb-cre* ♀**	***Rb*$^{+/+}$ ♂ x *Rb*$^{+/LoxP}$*; Alb-cre* ♀**	***Rb*$^{+/+}$ ♂ x *Rb*$^{LoxP/LoxP}$*; Alb-cre* ♀**
Alive	131	77	38
Dead	15	43	11
Total	**146**	**120**	**49**

Figure 8: Schematic of percentage of successful pregnancy and fetal lethality under administration of oral dexamethasone. *Rb*$^{+/+}$*; Alb-cre* mothers are represented in black, *Rb*$^{LoxP/+}$*; Alb-cre* in red, and *Rb*$^{LoxP/LoxP}$; *Alb-cre* in grey.
b) Table with offspring death and compromise from the three possible crosses. A much higher percent of deaths came from the *Rb*$^{LoxP/+}$; *Alb-cre* group than did the *Rb*$^{+/+}$; *Alb-cre* (35.8% vs. 10.9%* $p<0.01$). The Rb$^{LoxP/LoxP}$-Cre group had a lower lethality than the *Rb*$^{LoxP/+}$; *Alb-cre*, but twice that of the *Rb*$^{+/+}$*Alb-cre*. (22.4%).

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