Reduced Expression of AP Endonuclease-1 Impairs Fetal Development and Leads to Embryonic Loss

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Abstract

Background: Apurinic/apyrimidinic endonuclease-1 (APE-1) is induced by the accumulation of reactive oxygen species (ROS) and plays multiple roles in response to oxidative stress including: regulation of redox-sensitive genes; inhibition of ROS accumulation; inhibition of apoptosis and; repair of abasic sites in oxidative DNA damage. While a complete APE-1 knockout (KO) is embryonic lethal [3,4], a novel hypomorphic (HM) mouse model was developed in which APE-1 expression was inhibited ~80%. The goal of this project was to test the hypothesis that reduced expression of APE-1 in HM embryos causes fetal loss during organogenesis.
Methods: MRI was used for in vivo assessment of fetal viability. Necropsies were performed at selected time points and samples were collected for DNA, protein, and histological analysis. PCR was used to determine genotype, and western blot was used to assess APE-1 expression.
Results: Breeding of heterozygous mice yielded ~50% of the HM offspring compared to what would be predicted by Mendelian genetics. Mating HM males with heterozygous (Het) females showed a higher failure:viable fetus ratio than heterozygous pairings (~3:5 vs ~1:8). HM mice were smaller than littermates at all gestation periods measured, and APE-1 expression was reduced. Analysis of embryonic/fetal failures is ongoing.
Interim Conclusions and Future Directions: Given the physical size of embryonic/fetal failures, challenges were encountered separating the conceptus from maternal uterine tissue. While MRI proved inadequate in resolving fetal size at key times, ultrasound may provide an alternative[2]. Additional samples and assays will be required to provide the power to support definitive conclusions. Histology and immunofluorescence has been performed, and will be assessed for tissue specific APE-1 expression and pathology.

Background

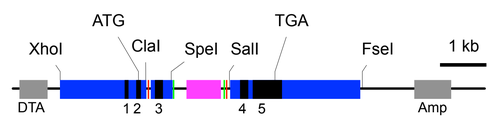

Fig 1. Hypomorph (HM) Mouse Model: An inducible *Apex1* KO construct was created by inserting loxP sites flanking part of the *Apex1* gene (floxed). The HM mouse was created by adding a neomycin resistance gene between exons 3 and 4 of the floxed *Apex1* gene which disrupted gene expression throughout the entire mouse. When the neomycin insert was removed through the FRT-FLP system, the HM phenotype disappeared, confirming that the neomycin insert impairs APE-1 expression.

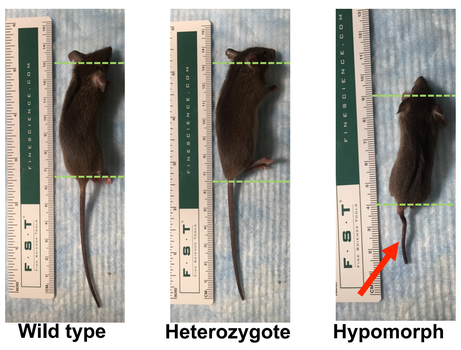

Fig 2. Phenotype of Adult Mice: The HM mouse is smaller, with varying degrees of kinked and rigid tails. In this litter, the body of the WT and Het are ~6.5 cm long. The HM is ~5 cm long.

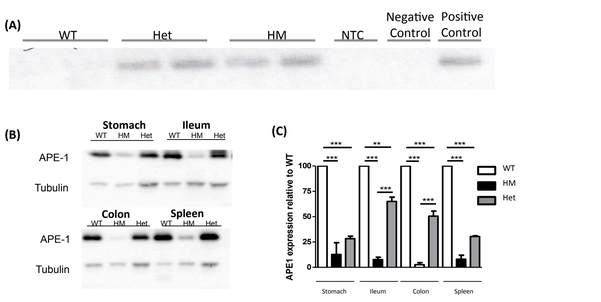

Fig 3. APE-1 Expression in HM Adults: The presence of the neomycin cassette was confirmed by PCR in the heterozygous and hypomorph animals while it was not present in the wild type animals (A). Decreased APE1 expression is seen in multiple tissues of the hypomorph (HM) mice while heterozygous (Het) mice were more variable but often intermediate compared to wild type (WT) as assayed by western blot (B). Panel (C) shows the summary of densitometry data from multiple mice. In HM mice there is an average of 92% reduction and in het mice there is 57% reduction throughout the analyzed tissues.

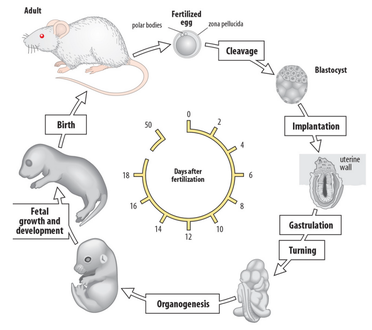

Fig 4. Progress of Fetal Development: Embryo implantation occurs around day 5.5, with gastrulation and neurulation occurring subsequently in one day increments. Organogenesis begins around day 10.5. The average gestational length is 21.5 days. Mice are nocturnal animals, thus timepoints are measured in 0.5 day increments, with the first day after conception measured as day 0.5.
(http://www.mun.ca/biology/desmid/brian/BIOL3530/DEVO_03/ch03f21.jpg)

Specific Aims

Aim 1: Determine the genotype and phenotype of fetal mice and embryonic/fetal losses.
Aim 2: Determine the critical window during development in which fetal/embryonic loss is occurring.
Aim 3: Compare the expression of APE-1 protein in fetal mice and its correlation to fetal losses.
Aim 4: Analyze resorptions for signs of cellular stress or pathology.

Methods

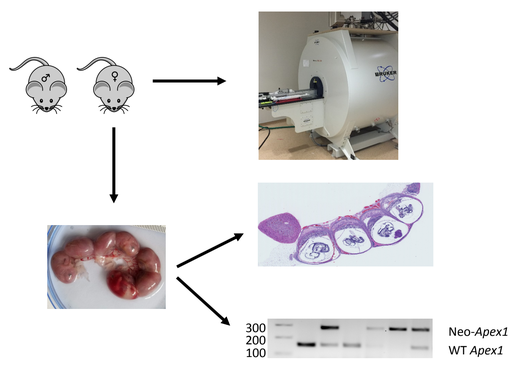

Fig 5. Methods: Heterozygous (Het) C57BL/6 X 129 females were bred with Hypomorph (HM) and Het males, with one or two females per male. HM females were not successful with either HM or Het males. One pregnant female was imaged using 11 Tesla MRI to determine if embryonic/fetal loss was observable in vivo. Serial necropsies were performed to stage embryonic/fetal loss and tissue was collected for histological and molecular analysis. PCR was used to determine genotype and western blots were used to assess protein expression. Immunofluorescence and histology were performed but have not yet been analyzed.

Summary

- The frequency of Hypomorphic (HM) offspring is ~50% less than predicted in Het/Het matings (Fig 6).
- Phenotypic differences in fetuses are consistent with observations in live mice (Figs 2, 6, 8).
- APE-1 expression in fetal HM mice (Fig 10) is impaired as is seen in live pups (Fig 3).
- Fetal resorptions are visible by necropsy between GD 12.5-19.5 (Fig 8, 9), and are increased in HM/Het mating compared to Het/Het mating (Fig 9).

Results

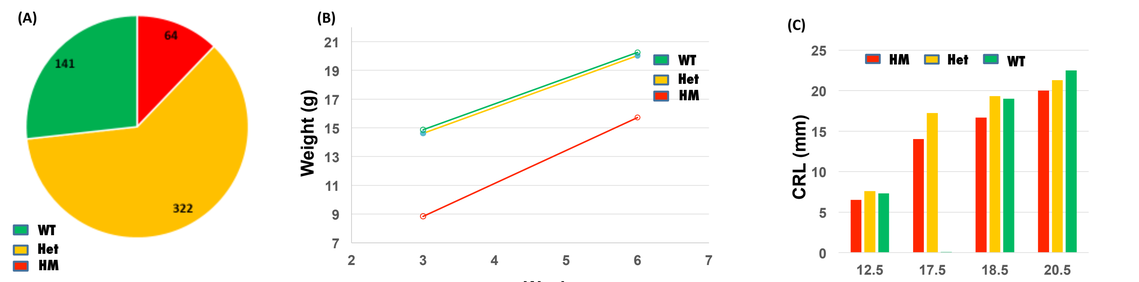

Fig 6. Genotype and Phenotype: Analysis of over 500 pups from Het/Het matings over the past 18 months showed ~50% fewer pups with the HM genotype than expected (A). A subset of mice were weighed at weeks 3 and 6 (n= 6 WT, 5 HM, 11 Het) (B). The HM mouse showed decreased average weight at both timepoints compared to WT and Het littermates. Fetal mice were measured by crown-rump length (CRL) at necropsy and on histology (C). The HM mouse showed decreased CRL at all gestational stages.

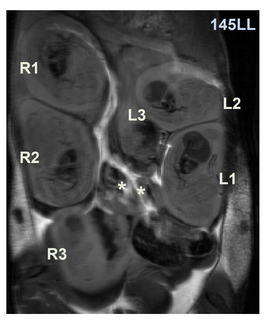

Fig 7 MRI: Imaging was performed on a Het/HM mating at Gestation Day (GD) 15.5 and 17.5. This image (GD 15.5) shows six fetuses, with one visibly smaller than the others in all sections (L3). At necropsy (Fig 8), this litter was observed to have five viable pups, and three failed conceptuses with two distinct appearances. The larger of the fetal failures was visible on MRI (L3), but distinguishing the smaller resorptions on MRI was not obvious (indicated by *). It was determined that MRI was not practical for identifying fetal/embryonic failure.

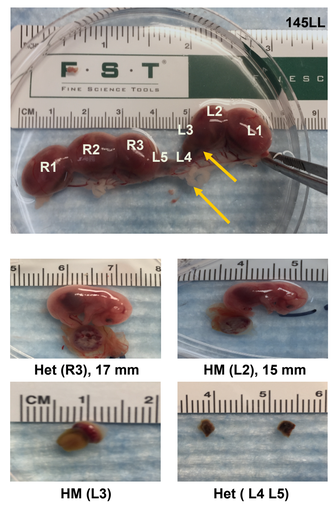

Fig 8. Necropsy: Fetal development was estimated by dam weight gain and confirmed by crown-rump lengths (CRL)[1]. Embryonic/fetal failure was determined by gross observation. Depending on the stage of fetal development, different samples were taken for molecular analysis. Fetuses were labeled for uterine horn (R or L) and numbered in series from the ovary (proximal = 1). When minimal tissue was present, DNA was prioritized over protein, and molecular analysis was prioritized over histology. In some instances, fetal tissue was not completely separated from maternal uterine tissue, clouding molecular analysis of those samples.

Dam ID	GD	Viable	Failure	Dam/ Sire
138LL	9.5	10	0	Het/Het
145R	10.5	8	0	Het/Het
138L	11.5	13	0	Het/Het
147R	12.5	5	3	Het/**HM**
149L	12.5	10	1	Het/Het
145LL	17.5	5	3	Het/**HM**
140R	18.5	7	2	Het/Het
149LR	19.5	4	3	Het/Het
143R	20.5	6	0	Het/Het
143L	21.5	7	0	Het/Het
147L	21.5	2	1	Het/**HM**

Fig 9. Embryonic/Fetal Loss Rates: Eleven necropsies were performed at various gestational ages. Females bred with **HM** males had fewer viable fetuses and more fetal/embryonic failures compared to Het/Het matings at similar gestational stages. The total number of visually healthy fetuses was higher at earlier gestational development suggesting that not all conceptuses bound for resorption were grossly identifiable. Most fetal failures were identified between GD 12.5 – GD 19.5, suggesting a critical timeframe for embryonic/fetal loss.

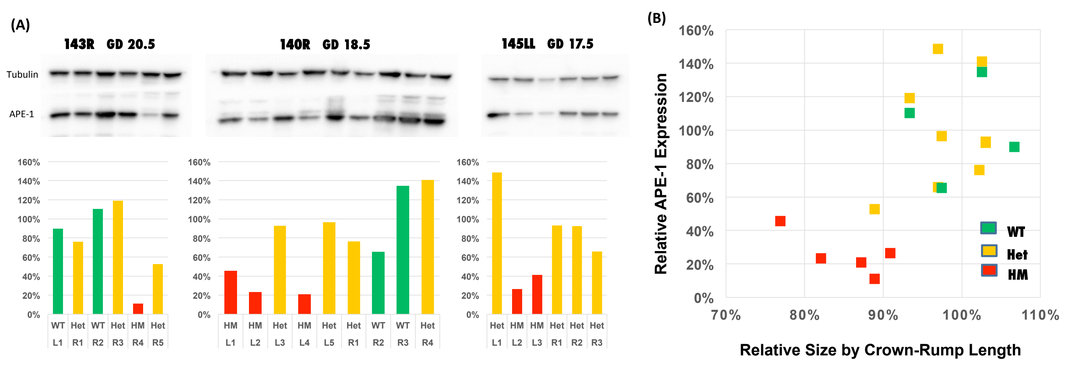

Fig 10. APE-1 Expression Relative to Fetal Size: APE-1 expression was measured by western blot and compared to the average WT expression in each litter (Het if no WT were present) (A). Compared to littermates, HM mice had reduced expression of APE-1 protein at all GD measured. Het mice had variable expression and were less distinct from WT expression. Plotting relative APE-1 and CRL shows that HM mice are both smaller and have less APE-1 expression when compared to Het and WT littermates ($p<0.05$ Pearson correlation of coefficient) (B). Data included all mice from 143R, 140R, and 145LL with both western blot and CRL measurements.

Discussion and Future Directions

The expected fetal resorption rate in WT mice is 10%[2]. The increased rates of resorption observed in the HM mouse are consistent, although less penetrant, with the reported embryonic lethality of the KO model[3,4]. The timing and stage of observed resorptions suggest multiple mechanisms are at play, and at least some resorptions are triggered early during organogenesis, and others may occur later. This may be attributable to varying levels of APE-1 expression fetus-to-fetus, or changing expression during pregnancy and from tissue-to-tissue.

The smaller size phenotype observed in weaned HM mice was also observed in utero. Recent RNA seq data from adult HM tissue suggests that APE-1 has a role in metabolism, and the reduced size of HM mice may reflect impaired metabolic efficiency during embryonic development, some of which could reach a threshold and contribute to embryonic/fetal failure. A specific tissue impacted during organogenesis was not identified, and current results cannot determine if embryonic/fetal failure is due to a specific target of oxidative damage, impaired metabolic function, or some combination of factors.

The gradient of APE-1 expression among HM in litter cohorts suggests that the HM mouse has variable expression of APE-1 in any given mouse. Differences in the number of fetuses per uterine horn suggests an environmental factor contributes to their viability. Future studies will help to identify the interaction between genes and the fetal environment.

References

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2. Flores et al., 2014. 'Early detection and staging of spontaneous embryo resorption by ultrasound biomicroscopy in murine pregnancy', *Reprod Biol Endocrinol*, 12: 38.
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Acknowledgments

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