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14. ABSTRACT:
Tuberous Sclerosis Complex (TSC) patients develop tumors of the brain, kidney, skin and heart upon loss of either the TSC1 or TSC2 gene, and we are interested in elucidating early molecular events that contribute to loss of TSC2 and in understanding how TSC2 heterozygosity might contribute to this process. Our lab uses the Eker rat, which possesses an inactivating retroviral insertion in one Tsc2 allele (Tsc2Ek/+), as a model to better understand how Tsc2 heterozygosity contributes to cancer susceptibility. The goal of this award was to determine whether Tsc2 haploinsufficiency generated a mutator phenotype in target tissues in vivo that possibly contributed to early events in tumorigenesis within TSC2+/- individuals and establish an in vitro model of Tsc2 haploinsufficiency. We were able to successfully establish an in vitro method of depleting Tsc2 expression, and we are performing experiments to analyze mutation frequency and spectra in vitro in the presence or absence of Tsc2 expression. In addition, our preliminary results indicate that rats heterozygous for Tsc2 have a higher mutation frequency in vivo as they age compared to wild-type rats.

15. SUBJECT TERMS
Big Blue rat, Eker rat, haploinsufficiency, mutation frequency, mutation spectra, mutator phenotype, TSC, TSC2, tuberin

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Introduction

Tumors (brain, kidney, skin and heart) of Tuberous Sclerosis Complex (TSC) patients follow Knudson's 2-hit model for tumorigenesis whereby germline defects in one allele of either the *TSC1* or *TSC2* gene is accompanied by somatic inactivation of the remaining wild type allele (1,2). Our lab uses the Eker rat, which possess an inactivating retroviral insertion in one *Tsc2* allele ($Tsc2^{Ek/+}$), as a model to better understand how *Tsc2* heterozygosity contributes to cancer susceptibility (3). The Eker rat is highly susceptible to spontaneous and chemically induced renal tumors, and our lab is interested in elucidating early molecular events that contribute to loss of tuberin function and in understanding how *TSC2* heterozygosity might contribute to spontaneous and chemically induced cellular transformation and tumor progression.

The purpose of this Concept Award was to determine whether haploinsufficiency of the *Tsc2* gene generated a mutator phenotype in target tissues before the development of tumors. This could provide a mechanism for acquisition of additional genetic alterations that possibly contributed to early events in tumorigenesis in $TSC2^{+/-}$ individuals. To test this hypothesis, our lab obtained a licensing agreement from Stratagene to cross Eker rats with Big Blue rats™ that carry biallelic copies of the λLIZ shuttle vector, producing Big Blue/Eker (BB/Ek) and Big Blue/wild type (BB/WT) F1 rats. These F1 animals permit analysis of the effect of a defective *Tsc2* allele on the mutation frequency and spectra in rats that carry the λLIZ shuttle vector. Mutation frequencies and spectra can be analyzed using bacteriophage lytic growth to detect mutations within the cII region of λLIZ or blue-white screening of bacterial colonies to detect mutations in the *lac* region of the λLIZ transgene.

The scope of this project included determining whether Eker rats developed a mutator phenotype in organs prone to tumorigenesis spontaneously or in response to treatment with carcinogens. In addition, part of this project aimed to establish an *in vitro* model for Tsc2 haploinsufficiency leading to a mutator phenotype.

Body

Aim 1: Determine whether Tsc2 haploinsufficiency results in a mutator phenotype as a result of increased oxidative damage to DNA.

Task1. Analysis of mutation frequency and mutation spectra in DNA from kidney OSOM of rats that have been treated with TGHQ (known to increase oxidative damage to kidney OSOM).

Task2. Analysis of mutation frequency and mutation spectra in DNA from kidney tissue of rats treated with $KBrO_3$.

Eker rats were successfully crossed with Big Blue rats that carry biallelic copies of the λLIZ shuttle vector, generating enough F1 animals (both BB/Ek and BB/WT) to perform the treatments described in Task 1 and Task 2 of this Aim. After initially obtaining results that showed BB/Ek rats had a higher mutation frequency (as measured by bacteriophage lytic growth) than BB/WT rats after treatment with TGHQ, we were unable to reproduce these results and found that TGHQ did not have an impact on the mutation frequency of the cII region of the λLIZ transgene in either wild-type or Eker rats that contained the λLIZ shuttle vector. This was quite surprising, since TGHQ was previously reported to increase 8-oxo-dG levels in Eker rats (4); however, it should be noted that we never tested our F1 rats by an independent mechanism for increased ROS upon TGHQ treatment. Therefore, the research was delayed, and we requested an extension of this award (~September 2006). Since the TGHQ treatments used for detection of 8-oxo-dG were much shorter (3 days) than our treatments (2-3 weeks), we chose not to analyze these samples for signs of ROS. Instead, we are analyzing genomic DNA from animals used for Task 2 by HPLC to detect 8-oxo-dG, a DNA lesion and ROS indicator. If an increase in 8-oxo-dG can be detected in $KBrO_3$-treated animals compared to control rats, then we will proceed with the analysis described in Task 2. Since there have also been reports showing that mutation frequency differences can be detected within the lac region but not in the cII region of the λLIZ shuttle vector in response to the same treatment (5), we are also going to analyze mutation frequency and spectra of the lac region (by blue-white screening of bacteria) from the same genomic DNA of animals used for Task 1 and Task 2 of this Aim.

Aim 2: Determine whether the mutator phenotype is specific for tissues at risk for tumorigenicity in heterozygous individuals.

Task 1. Analysis of mutation frequency and mutation spectra using DNA from target tissues (kidney and spleen) and non-target tissues (lung and liver) in rats aged-out over one year.

Task 2. To analyze mutation frequency and spectra using DNA from target tissues (kidney and spleen) and non-target tissues (lung and liver) of BSO-treated rats.

F1 rats (both BB/EK and BB/WT) were treated as described in Task 1 and Task 2. For task 1, rats were aged to 2, 4, 8, and 12-months and then sacrificed. For task 2, two-month old rats were treated with low or high doses of BSO for three weeks, and then sacrificed. Preliminary data indicates that 8-month old Eker rats have a higher spontaneous mutation frequency in DNA isolated from kidney tissue than 8-month old wild-type rats (see Appendix, Figure 1);

however, other non-target tissues have not been analyzed yet and this data has not been reproduced and confirmed.

Because we could not reproduce results in Aim 1 (described above), we have not yet analyzed the mutation frequency or spectra from BSO-treated animals. Instead, we are analyzing genomic DNA from animals used for Task 2 by HPLC to detect 8-oxo-dG, similar to Aim 1, Task 2. We also plan to analyze mutation frequencies by both the cII detection method as well as the blue-white screening method (mutations within the lac region of λLIZ) using the genomic DNA of animals from Task 2 of this Aim.

Aim 3: Establish an in vitro system to explore mechanisms by which TSC2 haploinsufficiency results in a mutator phenotype in human cells.

Task 1. Analyze mutation frequency and mutation spectra using DNA from Big Blue rat fibroblast treated with Tsc2 siRNA.

Begin Task 2. Transfect HEK293 and HeLa cells with chemically synthesized TSC2 siRNA molecules and analyze repression of tuberin expression.

We tested three different chemically synthesized siRNA in Big Blue rat fibroblasts and found one chemically synthesized siRNA capable of repressing tuberin protein levels (see Appendix, Figure 2). However, no differences in spontaneous mutation frequency (within the cII region) could be detected after 3-days. We plan to challenge cells with KBrO3, and BSO to determine whether we can induce a difference in mutation frequency (of both the cII region and the lac region of the λLIZ transgene) with these compounds. However, it is possible that three days will not be long enough (enough cell divisions) to be able to observe mutations within this system. An alternative method would be to generate a stable Big Blue rat fibroblast cell with siRNA for TSC2 that would allow longer growth of cells (more replication cycles), allowing more time for cell divisions and incorporation of mutations.

In addition, we have tested several chemically synthesized siRNA molecules for human TSC2 in HEK293 and MCF-7 cells and are also capable of repressing tuberin expression in human cell lines (data not shown), so Task 2 of this Aim has been completed. However, it is also possible that we will have to generate stable cell lines in the human cells for TSC2 siRNA in order to analyze mutagenesis in the human cell line system.

Key Research Accomplishments

- Successfully bred Big Blue and Eker rats; treated F1 progeny as described in Aims 1 and 2
- Performed phage method of mutation detection (cII region of λLIZ transgene) for Aims 1-3
- Set up *in vitro* TSC2 haploinsufficiency system in Big Blue rat fibroblasts and human cells as described in Aim 3
- Generated preliminary data that aged Eker rats ($Tsc2^{Ek/+}$) have a higher mutation frequency than $Tsc2^{+/+}$ rats.

Reportable Outcomes

N/A

Conclusion

Although we were able to cross Eker rats with Big Blue rats and successfully treat the F1 rats as described in Aims 1 and 2 and generate an *in vitro* method of depleting tuberin expression, we were unable to reproducibly use the phage method of mutation detection (cII region of λLIZ transgene). However, our data from Task 1 of Aim 2 (Eker rats have higher mutation frequency than wild-type rats) cause us to be optimistic that Eker rats have a higher mutation frequency than wild-type rats. For this reason, the research was delayed and we previously filed for an extension on this grant. We will likely have to verify that our treatments are working by detection of ROS and use both the phage method (cII mutation detection) along with the blue-white method of mutation detection (lac region of λLIZ transgene) for future experiments before being able to definitively say whether there are differences in the mutation frequency or spectra that are due to Tsc2 haploinsufficiency.

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Appendices

N/A

Supporting Data

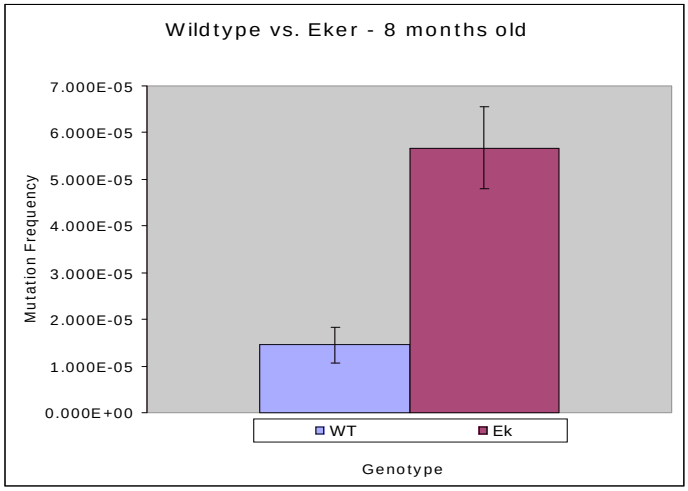

Figure 1. Spontaneous mutation frequency of DNA from kidney of 8-month old WT and Eker rats (n=2). The mutation frequency of DNA from WT animals fell within the normal DNA mutation frequency of 1-2 X 10^{-5} (as reported by Stratagene, Inc.), but the mutation frequency of Eker rat DNA was much higher than the normal frequency.

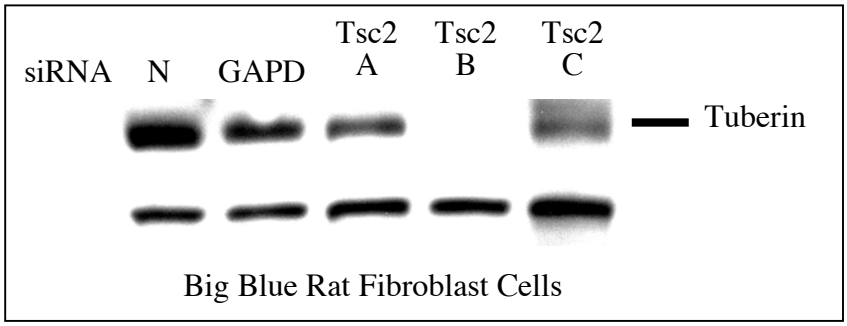

Figure 2. Suppression of tuberin protein levels by siRNA. Three chemically synthesized TSC2 siRNAs were transfected into Big Blue rat fibroblast cells for 48-hours. Western blot analysis using an anti-tuberin antibody revealed that one of the siRNA molecules (Tsc2 siRNA-B) was capable of ablating tuberin expression. Similar results were seen at 72-hours post-transfection. A non-specific band is also shown to indicate equal loading.