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13. ABSTRACT (Maximum 200 Words)
An understanding of the cancer cell begins with knowledge of the genetic alterations that lead to neoplastic transformation. Much progress has been made in identifying areas of deletion, amplification and mutation in tumors. However, this is only part of the picture. Increasingly, we are learning that epigenetic changes, that is, changes in chromatin structure, are critically important in regulating cellular gene expression.

Recently, several labs have published manuscripts identifying RNA interference as being crucial for the establishment of such epigenetic changes in species as diverse as *Drosophila*, plants, and the fission yeast *S. pombe*. This knowledge presented a fantastic opportunity not only to study epigenetic changes, but to potentially *create* epigenetic changes by creating germline transgenic mice in which a target gene has been silenced by RNAi.

After the demonstration that RNAi in mammalian cells can be mediated by vectors encoding short, hairpin RNAs (shRNAs), we sought to develop a system by which to create transgenic mice using this technology. We demonstrate that a stable, heritable RNAi trigger in the form of a short hairpin was successfully passed through the mouse germline. As well as demonstrating a technique that can be applied to any gene of interest, we have created gene knock-down mouse models for the Neil-1 DNA glycosylase involved in DNA repair pathways, and for p53. These observations open the way to the use of RNAi as a complement to standard knock-out methodologies and provides a means to rapidly assess the consequences of suppressing a gene of interest in a living animal. The vision driving the creation of this technology was one of eventual RNAi-based therapeutics. One could particularly imagine silencing oncogenes in cancer cells to attenuate their tumorigenic tendencies.

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Introduction:

Last year I embarked on a study to create germline transgenic mice in which a target gene has been silenced by RNAi. The vision driving the creation of this technology was one of RNAi-based therapeutics. For example, one could particularly imagine silencing oncogenes in cancer cells to attenuate their tumorigenic tendencies. We were able to demonstrate that stable, heritable silencing could be achieved in mice expressing short hairpin RNAs from RNA polymerase III promoters. These hairpin RNAs are substrates for the Dicer enzyme and can be used to silence any gene of interest.

This technology provides a new method to rapidly assess the consequences of suppressing a gene of interest in a living animal. Silencing by RNAi can produce a loss of function similar to that achieved by standard knock-out technologies (a process that takes well over a year) in a matter of little more than a month. Specific silencing can be accomplished without constructing a complicated targeting vector, gene targeting in ES cells, and passing the targeted allele through the germline of a chimera. In addition, there is no need to breed to homozygosity because an RNAi trigger is a trans-acting, dominant silencing signal. In short, it is now possible to effectively silence a gene in a transgenic founder animal and observe a phenotype immediately. In addition, silencing of genes by RNAi is becoming increasingly versatile due to the ability to trigger silencing with tissue specific, inducible, and reversible systems. Together, these approaches promise to establish RNAi as a popular complement to standard knock-out methodologies. In addition, the sequencing of the mouse and human genomes has opened the door to the next logical step-- application of RNAi-silencing technology on a genome wide scale.

Body:

Last year's report discussed the creation of p53-deficient mice through the use of the abovementioned hairpin-based technology. The p53 hairpins that I have used have been validated by Hemann et al.[3], and shown to produce distinct tumor types *in vivo* when used in the context of reconstituted bone marrow. Although we could show that the hairpin was functional and prevented p53 expression in our hairpin mouse model—for example, cells made from the hairpin-expressing founder mice were unable to induce p53 expression upon DNA damage-- the founder mice were surprisingly tumor-free until death of natural causes.

In addition, all but one of the four founder animals was sterile, and we have had to use *in vitro* fertilization techniques to try and expand the colony. Despite a long period of monitoring transgenic animals, we have not yet encountered any increased tumorigenicity. This indicates that although we can suppress p53 expression to a large degree, perhaps even a small amount of expression is sufficient to prevent recapitulation of the p53-null mouse model phenotype. Thus, at face value, this particular hairpin may not be strong enough to function in this context. However, several studies have validated *in vivo* RNAi-silencing technology by demonstrating a recapitulation of a genetic null phenotype using hairpins (for example, Shinagawa et al. 2003). So, although the technology is viable and proven successful, the biology of p53 may be hindering success in this particular case.

The aforementioned work represents Task II of my statement of work (Analyze the success of this method of gene silencing in recapitulating phenotypes of classical knockouts). Although the *in vivo* results were disappointing, the training and experience I gained in the past year has been invaluable.

As Task I is complete, and Task II is coming to an end, I have also been working on trying to decipher the mechanism by which the silencing occurs *in vitro* and *in vivo*. To

this end, I have been working with mouse knockouts of Argonaute2, a member of a multigene family involved in RNAi-mediated silencing. Argonaute proteins, along with siRNAs, are core members of the RNA-induced silencing complex, or RISC. Recent work has helped to reveal that the multiple Argonaute proteins present in mammals are both biologically and biochemically distinct, with a single mammalian family member, Ago2, being responsible for siRNA-directed mRNA cleavage activity[4]. This is precisely the cleavage activity that the hairpin-expressing, trangenic mouse silencing relies on. Ago2 is essential for mouse development and cells lacking Ago2 are unable to mount an experimental RNAi response. The revelation that a cryptic RNAseH domain lies within Argonaute proteins supports a model in which Argonaute contributes mRNA cleavage activity to RISC, providing the catalytic engine for RNAi.

A fuller understanding of the molecular mechanisms of Argonaute protein function will enable tailoring of hairpin silencing technology with the end result of making it more effective. For example, what we now know about the structure of Argonaute proteins gives us clues as to where both the siRNA and target mRNA lie in the protein. This information can perhaps enable us to smartly design siRNAs and shRNAs targeting a gene of interest. The relative expression levels of Ago2 protein *in vivo* in different tissues may also help us to predict which tissues might be more susceptible to hairpin silencing than others. In short, the more we know about the cell's own toolbox of gene silencing, the more we can realize its full potential.

Key Research Accomplishments:

- Using the p53 knockdown mice for further confirmation of the efficacy of a generally applicable technique for creating germline transgenic mice in which a target gene has been silenced by RNAi
- Investigating components of the RNA silencing machinery in order to more fully understand the mechanisms by which the silencing via RNA hairpins occurs.

Reportable Outcomes:

Manuscripts:

Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ. Argonaute2 is the catalytic engine of mammalian RNAi. Science. 2004 Sep 3;305(5689):1437-41.

Presentations:

Invited Speaker, Graduate School, University of Utrecht, June 2005

Poster, Diverse Roles of RNA in Gene Regulation, Keystone Symposium, January 2005

Poster, Germ Cells, Cold Spring Harbor, October 2004

Conclusions:

Perhaps the next logical step following the advent of this technology is to pursue large-scale applications. Sequencing of the mouse and human genomes has provided us with a wonderful tool to study genome organization, genetic instability, and polymorphisms. In addition, the identification of all genes encoded in the human genome provides the opportunity for large-scale systematic gene silencing using small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs). The recent development of siRNA and shRNA expression libraries in our lab and others provides the opportunity for both forward and reverse genetic screens. Our lab has constructed a library comprising approximately 28,000 shRNA expression cassettes targeting 9610 human and 5563 mouse genes[5]. Libraries such as this allow the use of RNAi technology to assign function to cancer genes, and to identify the molecular pathways these genes affect, in normal and transformed cells. Undoubtedly, use of these libraries in cancer studies will expand the body of knowledgee necessary to develop new, and also improve existing, cancer therapies.

Thus, as I look to the future, I hope to become increasingly involved in the fine tuning and application of this technology. As a direct extension of my previous work with hairpin mouse models, I am interested in the hairpin library especially as it pertains to translating the technology into *in vivo* mouse studies. As we continue to learn more about the molecular mechanisms behind the silencing, the technology can be continually improved. I believe that the *in vivo* studies will be invaluable, as they represent one step closer to treatment of human patients in the future.

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