AD\_\_\_\_\_

Award Number: DAMD17-02-1-0346

TITLE: Scanning the Human Genome for Novel Therapeutic Targets for Breast Cancer

PRINCIPAL INVESTIGATOR: Greg J. Hannon, Ph.D.

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory Cold Spring Harbor, NY 11724

REPORT DATE: April 2006

TYPE OF REPORT: Final

## PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

## DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

R	EPORT DOC		Form Approved OMB No. 0704-0188				
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.							
1. REPORT DATE (DD	-MM-YYYY)	2. REPORT TYPE		3.	DATES COVERED (From - To)		
4. TITLE AND SUBTIT	LE	IIIa		5	a. CONTRACT NUMBER		
Scanning the Hum	an Genome for No	vel Therapeutic Tar	gets for Breast Cano	cer			
					DAMD17-02-1-0346		
				50	2. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Greg J. Hannon, Ph.D.				50	I. PROJECT NUMBER		
				50	e. TASK NUMBER		
E-Mail: <u>hannon@c</u>	<u>shl.edu</u>			51	5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)				8.	8. PERFORMING ORGANIZATION REPORT NUMBER		
Cold Spring Harbor Laboratory Cold Spring Harbor, NY 11724							
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS U.S. Army Medical Research and Materiel Command Fort Detrick Manyland, 21702-5012			S(ES)	10	). SPONSOR/MONITOR'S ACRONYM(S)		
				1'	11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited							
13. SUPPLEMENTARY	NOTES						
14. ABSTRACT:							
No Abstract Provided							
15. SUBJECT TERMS Cancer Biology, Genetics, Synthetic Lethality, Apoptosis							
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC		
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	23	<b>19b. TELEPHONE NUMBER</b> (include area code)		

# **Table of Contents**

Cover	
SF 2982	2
Table of Contents	3
Introduction4	ŀ
Body5	5
Key Research Accomplishments2	20
Reportable Outcomes2	: <b>0</b>
Conclusions1	6
References17	7

### INTRODUCTION

The broad goal of this project was to develop genome-wide RNAi approaches in mammals and to apply these to the discovery of new therapeutic targets for cancer. Specifically, we have generated and continue to build a library of short hairpin RNA expression constructs (shRNA) that will ultimately correspond to every gene in the human and mouse genomes. These are presently available as a public resource and used internally to screen for genes that are essential to the survival of breast cancer cells but which are dispensable for the survival of normal cells. A subset of these might prove suitable as therapeutic targets for breast cancer therapy. During the course of funding, two things have become clear. First, although they were not in place at the time of submitting this application, we have largely developed the technologies necessary to pursue the above goal. Second, funding in the Innovator award fell far short of that necessary to achieve the goal. Relevant to the last point, we have been able to leverage the Innovator award with several other funding sources to create a program, which was more suited to meeting the proposed goals.

## BODY

Progress toward developing the technology necessary for genome-wide RNAi in mammals (these were funded in part by the Innovator award and also by a P01 from the NCI)

### General introduction to the work supported under the Innovator award

### Studies on the mechanism of RNAi

All of the technology described above was built upon studies of the RNAi mechanism. While these studies are funded by an R01, they benefit from the Innovator award, and it is acknowledged as general support for the P.I. We have made progress relevant to this goal, understanding in more depth the biochemistry of the RNAi pathway and working to apply this knowledge to the improvement of RNAi as a tool. Progress relevant to this effort are listed in the Reportable Outcomes section.

### RNAi in mammals

When the application that led to this award was originally submitted, RNAi was know to work only in invertebrate systems. Use of RNAi as an experimental tool was common only in C. elegans and in Drosophila. Arguably, the discovery of Dicer and its connection to microRNAs by my laboratory and the elucidation of the structure of siRNAs by Tuschl and colleagues started a revolution in experimental biology by creating means to silence the expression of any gene in cultured cells or in animals. The development of stable silencing technologies

was supported in part by this award, and these are now in widespread use within the breast cancer community. Indeed, in my recent service on the BCRP Integration Panel, I noted that a substantial fraction of the applications that we reviewed used the technologies that we developed with BCRP support. Manuscripts relevant to the development of these tools are listed in the Reportable Outcomes section.

#### Genome-wide RNAi libraries

Through our efforts and those of others, there have emerged two major methods for triggering RNAi in mammalian cells. These are transient silencing using siRNAs or stable or transient silencing using shRNAs. Both of these approaches have been validated in numerous publications. Since before the inception of this award, our goal has been to create a technology that would allow a single postdoctoral fellow in any reasonably well funded laboratory to scan every gene in the mammalian genome for those relevant to a specific phenotype. By building tools rather than by studying a specific biological question in breast cancer, we felt that we could maximize our impact. Now that the tools are largely built, we are returning to our initial goal of applying them to breast cancer biology. However, I firmly believe that putting the technologies that we have created in the hands of others will magnify the effects of the Innovator award.

Specifically, we have sought to create genome-wide RNAi libraries. Such libraries would contain elements capable of initiating an RNAi response in a transient or stable manner against every gene in the mouse or human genomes. In considering how to construct such genome-wide RNA libraries for human and mouse, we evaluated carefully the two available options. Our choice of the encoded artificial microRNAs (shRNAs) rather than siRNAs reflects several factors. First and foremost, shRNA expression constructs can be propagated and thus provide a limitless supply of material for public distribution. Second, many phenotypes, especially those relevant to breast cancer, require examination of cells over a long time frame. Third, shRNAs offer the flexibility to examine the consequences of silencing both in vitro and in vivo. Generation of the library has proceeded as a phased project with funding coming in part from the Innovator award and in part from other public and commercial sources (NCI, Merck, Oncogene Sciences, Genetech). No funding mechanism has been permitted to place any restrictions on library distribution.

A more detailed description of the technology with some introduction regarding the underlying biology for reference follows. This has been taken from a recent Perspective that we assembled for Nature Methods to describe the tools that we built to the scientific community. All of these tools have been built as part of an ongoing collaboration with Steve Elledge (Harvard).

#### Introduction to RNAi

RNAi is an evolutionarily conserved, sequence-specific gene-silencing mechanism that is induced by double-stranded RNA (dsRNA). Each dsRNA silencing trigger is processed into 21-25bp dsRNAs called small interfering RNAs (siRNAs)1,2,3 by Dicer, a ribonuclease III family (RNase III) enzyme4. Resulting small RNAs enter the RNA-induced silencing complex (RISC), which uses a single stranded version of the small RNA as a guide to substrate selection1,3,5,6. Perfect complementarity between the substrate and the small RNA leads to target RNA cleavage by an RNAseH-like active site within an Argonaute protein that forms the core of RISC7,8. For each small RNA, the two strands of the Dicer product are treated differently. The strand with the less stable duplex at its 5' end is incorporated preferentially into RISC9.10. This realization has led to rational designs for effective siRNA sequences and yielded substantial improvements in both the efficiency and reliability of RNAi.

MicroRNAs are a class of endogenous double-stranded RNAs that exert their effects through the RNAi pathway. More than a decade elapsed between the discovery of the first microRNA (miRNA), C. elegans lin-411,12, and the achievement of at least a superficial understanding of the biosynthesis, processing and mode of action of this class of non-coding regulatory RNAs13,14. MicroRNAs are transcribed by RNA polymerase II as long primary polyadenylated transcripts (pri-miRNAs)15,16. Through mechanisms that have yet to be discovered, the pri-miRNA is recognized and cleaved at a specific processing site by the RNase III family enzyme, Drosha, in the context of the Microprocessor complex, to produce a miRNA precursor (pre-miRNA) of approximately 70-90 nucleotides (nt)17-21, 39. The pre-miRNA has a 2-nt 3' overhang at one end17. This distinctive structure of is recognized by the Exportin-5/Ran-GTP heterodimer, and the pre-miRNAs is shuttled to the cytoplasm22,23. Only then is the miRNA precursor recognized and processed by Dicer into a mature miRNA, using the 3' overhang as a guide to site-specific cleavage at the second processing site24,25. We have build upon our ever deepening understanding of the biology of the RNAi and microRNA pathways to construct several large-scale libraries of artificial microRNAs that cover the majority of genes in the mouse and human genomes. Libraries covering the rat genome are currently under construction (G.J.H. S.J.E. and R. Gibbs, unpublished). Initial designs were based upon a simple hairpin structure that mimicked an intermediate in the microRNA maturation pathway26,27, the premiRNA; however, more recent and more effective designs are based upon modified primary microRNA transcripts28,29. In this review, we discuss the construction and features of our second-generation shRNAmir library with some reference to the first-generation library.

#### micro-RNA-based shRNA libraries

A miR-30-based shRNA expression cassette

The discovery of endogenous triggers of RNAi suggested that RNAi might be induced in mammalian cells by synthetic genes that express mimics of these naturally occurring regulatory molecules. Several groups have tested this approach by expressing artificial miRNAs in the form of short hairpin RNAs (shRNAs) with stems of varying lengths between 19 to 29 nt and loops of from 4 to 15 nt24,26,27,30,31. Artificial miRNAs can be expressed from both RNA polymerase II and III promoters resulting in silencing to varying degrees.

At present there is no clear consensus as to the most effective manner to present synthetic miRNAs to the RNAi pathway. However, after extensively testing different design strategies, our results indicate that expression of the artificial microRNA in the context of a natural microRNA primary transcript provides the highest levels of mature microRNA in RISC and generally effective silencing. These structures can be transcribed by either RNA polymerase II or RNA polymerase III promoters, although in the context of an integrated genomic copy, the former provided the most consistent results. This permits the use of standard expression strategies for artificial microRNAs and allows access to all inducible and tissue-specific expression systems that have been developed for protein expression. Given previous reports of artificial microRNAs based upon miR-3015-17,28,29,32,33 and our own attempts to express artificial microRNA from several precursors, we chose to model our library design upon miR-30 (Fig. 1). Previous studies have indicated that the presence of sequences flanking the native microRNA is essential for its efficient processing. Therefore we designed each shRNAmir in a carrier such that it is flanked by ~ 125 bases 5' and 3' of the pre-miR-30 sequence.



### Selection of shRNA sequences

The artificial microRNAs used in our second-generation library are designed based upon a proprietary algorithm developed by Rosetta Inpharmatics (Ge et al., unpublished). In general, these designed were reverse-engineered based



upon tests of several thousand siRNAs. Overall, the designs follow the thermodynamic rules suggested by analysis of endogenous microRNAs and by studies of siRNA incorporation into RISC. However, additional positional biases

are also introduced by the algorithm. Second generation libraries were designed to include six different shRNAmir constructs to each known and predicted genes of the human (34,711 genes) and mouse (32,628) genomes.

#### Vector Backbone

We constructed a second-generation library shRNA vector, pSM2 (Fig. 2), in which the shRNA-mir expression cassette can be packaged in a self-inactivating murine stem cell virus (MSCV). As with our first-generation shRNA library vector, pSM1 (Fig. 2), the expression of the small RNA is driven by the U6 promoter along with the U6 snRNA leader sequence, which is lies between the promoter and the 5' end of the miR-30 flanking region. An RNA polymerase III termination signal is inserted immediately after miR30 cassette. This arrangement was determined empirically in transient transfection experiments to give effective silencing.

To facilitate the tracking of shRNAs in complex pools, a randomly generated 60 nt DNA 'barcode' region was created downstream of the miR-30 cassette such that individual hairpin RNAs are identified through their hybridization to custom barcode to oligonucleotide microarrays. One of the key design features of pSM2, unlike its predecessor, is the ability to swap library inserts (miR30 cassette, barcode, and the microbial selection marker) into recipients plasmids containing other promoters (e.g. Pol II promoters for tissue-specific or inducible expression) by mating-assisted, genetically integrated cloning (MAGIC)27,29.

### The MAGIC system

MAGIC is a gene transfer system designed to work exclusively *in vivo*34. While originally designed for transfer of open reading frames, we adapted it for use in our shRNA system. For use in with MAGIC, the pSM2 vector was designed with a conditional origin of replication (R6K $\gamma$ ) and a transfer system for the fertility factor F'. In the presence of an F' with a functional tra operon, the transfer origin can mobilize pSM2 for exchange from one strain into a second strain. Additionally, the pSM2 mir-barcode-CmR region is flanked by I-Scel sites. The recipient vector, whether it be a standard plasmid or a lentivirus vector, must also contain two I-Scel sites flanking a negative selectable marker, PheS294G. Outside of the I-Scel sites in the recipient lies homology to the 5' of mir30 on one side and homology to the sequences just after the CmR gene on the other side. The PheS gene encodes a mutant phenylalanine tRNA synthetase which can charge tRNAPhe with phenylalanine or an altered version, chloro-phenylalanine (CI-Phe) if the latter is supplied in the medium. CI-Phe is toxic to cells if incorporated into proteins and is not utilized by the wild-type PheS protein. Thus the mutant PheS is a dominant, negative selectable marker. Recombinational shRNA gene transfer occurs when pSM2 is mated into the strain containing the recipient vector of interest, typically an ampicillin resistant vector containing a non-conditional pUC origin. In the recipient strain the I-Scel restriction enzyme is induced and cleaves both pSM2 and the recipient. The resulting linear molecules can be recircularized by homologous recombination between the liberated mir30-CmR fragment and the recipient vector from which the PheS294G gene was excised by I-Scel. Only a correct recombination event will give rise to a replication competent vector that contains the Amp marker plus the Cmr marker and that lacks pheS. It should be noted that the pSM2 plasmid has a conditional origin of replication. Thus, it cannot replicate in the recipient strain and therefore any cell that becomes Cmr must have transferred the resistance cassette to the non-conditional origin on the ampicillin resistant recipient plasmid to create the desired recombinant. This is illustrated below in figure 3. The advantages of this system is that whole libraries of shRNAs and their barcodes can be transferred into different vector backbones with different markers. different promoters or in combination with other genes without the need for conventional cloning, lending great versatility to the existing libraries.



Library construction

DNA oligonucleotide templates for each second generation silencing trigger consists of common 5' and 3' miR-30 regions flanking a 22 nt sense and a 22 nt antisense target sequence that is separated by the 19 nt miR30 loop (Fig. 1a). These shRNAmir templates are designed to be conveniently amplified by universal primers (Fig. 1b). The amplicon encoding each artificial microRNA is inserted between XhoI and EcoRI restriction sites that are flanked by pri-miR-30 sequences.

The cloning strategies used for creating first and second generation libraries were notably different. Rather than using conventional methods for the generation of large and complex libraries of defined nucleic acids, we employed a more cost effective approach by synthesizing oligonucleotides on printed microarrays. Though there are several available technological approaches to synthesizing DNA microarrays, we chose and validated inkjet synthesis (Agilent Technologies, Inc.) as it provided the most accurate production of the long oligonucleotides (96 nt) required for our application. PCR amplification of ammonium hydroxide-cleaved templates yielded an average of ~25 % to >60% of perfect shRNAs, using a combination of thermostable, proofreading polymerases and PCR enhancing agents. Oligonucleotides corresponding to more than 32,000 known and predicted genes in human (> 195,000) and mouse (>187,000) were synthesized on more than 21 glass slide microarrays, each capable of carrying 22,000 to 24,000 oligo probes per array.

Functional Category	DescriptionHuman GenesHumanMouse GenesTryin the categoryshRNA clonesin the category		Mouse Genes in the category	Mouse shRNA clones
Apoptosisd	587	2525	584	1834
Cancer	871	3793	937	3005
Relevantg Cell Cycle	536	2646	505	1815
Checkpoint	124	650	121	434
DNA Repair	118	620	139	413
DNA Replication	240	1168	258	841
Enzymes	3010	12928	2934	9703
GPCRc	690	2632	716	2132

#### Table 1. A sampling of second-generation library by functional groups.

Kinasesa	625	3163	579	2440
Phosphatasesb	210	933	198	725
Proteasesc	470	1761	467	1384
Proteolysisf	305	1778	286	1021
Signal Transductionc	2724	11250	2690	8660
Trafficking	488	1993	483	1560
Transcriptionc	841	3576	799	2604

To maximize cloning efficiency, we inserted the pSM2 backbone into lambda phage flanked by loxP sites. This was first used to create a high complexity library of barcoded cosmids comprising ~ 108 independent clones. This pool then served as the vector population for library construction. shRNAmir inserts were amplified from cleaved chip oligonucleotides in pools of ~22,000. These were inserted between the XhoI and EcoRI sites in barcoded  $\lambda$ -pSM2. More that 106 independent clones were routinely obtained for each pool. Phage populations containing the shRNAmir inserts were used to infect BNN132, a bacterial strain that expresses Cre recombinase. Exision of the shRNAmir plasmid from the phage occurred with ~50% efficiency (comparison of plaque forming versus colony forming units). Each pool was then entered into a customized, high throughput sequencing pipeline.

As with the first generation library, all pSM2 clones are sequenced-verified and stored individually in multiwell format. Depending upon the individual array synthesis, between 25 and 50% of all clones contained the correct shRNAmir sequence. Sequencing progress was monitored dynamically for each pool, and sequencing was halted when accumulation of new clones dropped to an unacceptable rate. At this point, sequences that were already identified were withdrawn from the library and arrays were synthesized to produce new shRNAmir oligonucleotide populations for cloning and sequencing. Each time the number of sequence-verified shRNAmir for a given gene exceeded three, the remaining oligonucleotides were also withdrawn from the synthesis queue. Iterative synthesis of more than 70 custom microarrays has thus far yielded 87,283 verified human shRNAmir clones and 76,896 verified mouse shRNAmir clones (see Library at a Glance). Summaries of coverage for specific functional groups are given in Table 1. All second-generation shRNA clones are made available to the research community through Open Biosystems Inc. (Huntsville, AL) as soon as they are sequence-verified and pass through Open Biosystems quality control.

### Validation of shRNAmir designs

Our initial studies indicated that longer hairpin structures (~29 nucleotide stems) were more effective triggers than those containing shorter stems of 19 nt to 21 nt24. However, all of these structures were designed around the pre-miRNA, which is an intermediate in microRNA biogenesis. When advances in our basic understanding of microRNA biology revealed in initial upstream processing step, we sought to compare the relative potency of artificial microRNAs designed around expression of the pre-miRNA to those modeled on the primary microRNA transcript. Direct comparison of small RNA production by northern analysis showed that a significantly higher amount of small RNAs (22 nt) are produced with shRNAmir designs (modeled on the primary miRNA. Overall, approximately 12 times more small RNA was incorporated into RISC when the same mature sequence was produced from pSM2 as compared to pSM1.

In order to successfully apply siRNA design rules to shRNAs, it was crucial to predict precisely the mature small RNA that is generated from pSM2 by Dicer and Drosha processing. Initial designs were based upon studies of Dicer and Drosha processing sites in native miR-30 and on extensive biochemical experimentation with processing of artificial microRNAs both in vitro and in vivo. We verified assumptions inherent in shRNAmir design by directly mapping the 3' ends of a number of small RNAs generated from shRNAmir vectors. We were able to infer the 5' end of mature small RNAs by counting 22 nucleotides from mapped 3' ends of both the guide and passenger strands of shRNAmir constructs (Fig. 1a). For some synthetic hairpins that have been tested, we could not determine the cleavage site definitively as our studies indicated that mature products could vary by a single base at their 3' ends (Fig. 1a). Perhaps this reflects a genuine heterogeneity in Drosha cleavage as also been observed with Dicer substrate processing in vitro and for mature microRNAs in vivo. Thus in target sequence selection for design of shRNAmirs, we chose target sequences that gave similar thermodynamic profiles even if processing sites were translocated by a base.

### Library validation

To test the performance of the first- and second-generation shRNA libraries in a biological context, we chose an assay for which we could expect to recover numerous shRNAs targeting a known biological process. We focused on an assay that could report, in a quantitative manner, proper function of the 26S proteasome. The proteasome is a major non-lysosomal protease in eukaryotic cells and its function can be detected in an assay in which the mouse ornithine decarboxylase (MODC) gene, a well-known target of the proteasome, containing a PEST domain, directs degradation without the need for ubiquitination35. A green fluorescent protein (Zs green) is fused to the MODC degron such that any

disruption of proteasome function can be detected by the accumulation of fluorescence. Thus we sought to identify shRNAs that could compromise proteasome function. Our goal was to examine the performance of both versions of our shRNA libraries using the same approach.

A collection of approximately 7000 first-generation library plasmids, targeting 4873 genes, were individually transfected into HEK293T cells along with the MODC reporter and a transfection control (DsRed). We recovered close to 100 RNAi constructs that resulted in the increase of ZsGreen fluorescence above background. Twenty-two of these corresponded to 15 proteasome subunits, and all scored positively in a secondary screen involving only these 22 hairpins and 33 non-scoring proteasome hairpins from the first round. An additional 14 shRNAs targeting proteasome directly also scored in a more focused assay. In all, the positively-scoring hairpins targeted mostly the 19S base subunits including 5 out of 5 ATPases and the 2 largest nonATPases. We found that targeting of the 19S lid and the 20S core produced lower hit rates. All positive hairpins showed measurable suppression by western blotting and fluorescent microscopy 27.

In order to compare performance of the second-generation library to the first, we chose 53 shRNAs (24 first generation and 29 second generation hairpins), targeting 13 different genes involved in proteasome function and tested their individual ability to effect accumulation of ZsGreen in transient assays. We found that the majority of second-generation shRNAmirs were as potent as the best hairpins identified in the screen of the first generation library. There was a good correlation between disruption of proteasome function and reduction in mRNA levels as measured by quantitative RT-PCR. This indicated that, on average, the second shRNAmirs performed substantially better than first-generation shRNAs modeled on pre-miRNAs. To test the second-generation library in the context of a screen, a set of 515 human kinase shRNAmirs containing 47 shRNAmirs directed against proteasome subunits was assayed for effects on proteasome function. We found that 34 of the 47 proteasomal hairpins scored positive and 10 other shRNAmirs that were not previously linked to proteasome function also scored. In a secondary screen with these 44 candidates, all but 5 shRNAs (nonproteasomal) scored upon retesting 29.

### Vectors and shRNA expression

It is now known that miRNAs are predominantly transcribed by RNA polymerase II. Direct comparison of various RNA polymerase III promoters (U6, H1, and tRNA-val) and RNA polymerase II promoters (CMV and MSCV-LTR) with a highly potent shRNAmir in transient assays showed little difference in suppression. With a less efficient hairpin, however, the CMV and U6 promoters demonstrated the highest level of suppression, but performed equally well. Based upon these results, we chose to retain the U6 promoter for the expression of library shRNAmirs. For long-term suppression, pSM2 can efficiently produce stable populations by random integration of library cassettes into target genomes 36.

Suppression of gene expression at single copy facilitates screening of shRNAs in pools. This is true for both positive selections and for negative selections using barcode hybridization for tracking depleted clones. Initial experiments aimed at generating gene knockdown using single copy integrants of pSM2 were highly variable using the U6 promoter. We therefore began to explore other promoters to drive miR-30 expression. CMV-miR-30 which had been shown by others and ourselves to work in transient assays, did not fare well at single copy in a range of cell lines tested. However, extensive testing led to two strategies that allowed much higher penetrance knockdown at single copy. We found that including a substantial spacer sequence, GFP in the initial case, between CMV and miR-30 greatly increased its ability to knockdown gene expression36. The underlying biochemical reason why the spacer leads to increased knockdown efficiency is not presently clear. We also found that the non-self inactivating LTR (SIN) version of pSM2, MSCV, was also highly efficient at single copy gene knockdown when miR-30 was driven from the LTR promoter37. This is likely due both to the broad spectrum of cell types in which the MSCV LTR is active and to the 1.8 kB packaging signal serving as a *de facto* spacer sequence. These findings have led to much more efficient vectors for expression of shRNAmir libraries that should allow routine, high efficiency knockdown of gene expression and efficient barcode-based screening.

Understanding the interplay between the spacer region and the shRNAmir in combination with pollI promoters allowed us to take advantage of existing conditional promoter technologies to generate conditional knockdown vectors. We have developed a series of Lentiviral vectors with both constitutive and conditional promoters based on the various Tet-repressor regulated systems36. These are the PRIME vectors, which have been demonstrated to allow efficient regulated gene knockdown even at single copy. The PRIME series also has additional spacer fragments in place of GFP to allow their use with screens that already use GFP as a reporter. Similar series of vectors based upon conditional promoters inserted into MSCV-based SIN vectors have also been reported and validated.

## Outlook : Barcode versus arrayed screening formats

The second generation Hannon-Elledge libraries are presently available in arrayed formats suitable for high throughput DNA preparation, plasmid transfection, virus production and well-by-well screening approaches. However, the use of such methods is beyond the budgets of all but the most well funded laboratories. At the core of our philosophical approach toward the generation of the tools described herein was to democratize the use of RNAi as a genome-wide tool. Therefore, we have been developing strategies that take advantage of pooled collections of sequence-verified shRNAs. Of course, the use of such

pools in positive selection schemes is easy to envision. However, we have also developed methods that permit the discovery of shRNAs that become either enriched or depleted from a population in response to a specific stress using barcode microarrays to measure relative abundance of individual constructs within a mixed population. Current protocols allow the simultaneous screening of up to 10,000 individual constructs using arrays that report for approximately 85% of all members of the population. The behavior of constructs is consistent with biological replicates in outgrowth experiments displaying correlation coefficients in the range of .85-.95 (Silva et al, in prep). We therefore feel that in the near future, the use of highly parallel tools for forward genetics in mammalian cells will become accessible to a substantial fraction of the community.

### Summary and future directions

The Innovator award has provided key support for the development of broadbased technologies that are now in widespread use within the breast cancer community. Thus far, through Open Biosystems, we have distributed more than 1,000,000 shRNA constructs to laboratories worldwide. While their application is not specifically restricted to breast cancer, the impact within the breast cancer community can be seen both in the literature and in the grant applications that are being seen by the BCRP.

My own laboratory remains dedicated to our original goal, the application of these genome-wide resources to identifying new targets for breast cancer therapy. We are presently trying to raise philanthropic support for a large program to scan for genetic susceptibilities of roughly 60 breast cancer cell lines. By correlating specific lethality profiles, determined by barcode microarrays, with mutation, copy number polymorphism and expression pattern data that are already available, we hope to find correlations between specific genetic alterations in tumor and sets of genetic susceptibilities. This would lead to the simultaneous identification of new targets and instruction for how best to apply drugs targeting them. Beyond, the initial search, we plan extensive pre-clinical evaluation of targets in orthotopic mouse models. Over the past few years, we have gained substantial experience in mammary stem cell biology and in mammary fat pad reconstitution. Bv orthotopic transplantation of human tumor cell lines or through experiments based upon tumors arising from engineered mouse mammary epithelial stem cells, we can evaluate targets using first inducible RNAi strategies developed with the support of this award. Moreover, we have begun to develop systemic RNAi delivery strategies that will allow us to test potential targets, essentially using synthetic RNAs as drug mimics. While our goal is not explicitly to develop RNAi-based therapies within this program, these could arise as a secondary consequence of our preclinical work.

Overall, I believe that we have used the support from the Innovator award wisely and have enabled important new approaches to the eradication of breast cancer – at least as a life threatening disease. Of equal importance from my own perspective is that this award and my exposure to the program has caused us to focus our efforts on the breast cancer problem and to dedicate ourselves to fulfilling the original long-term goals of the innovator award, despite the fact that its funding has come to an end.

## References

1. Zamore, P. D., Tuschl, T., Sharp, P.A. & Bartel, D.P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33 (2000).

2. Hamilton, A. J. B., D.C. A species of small antisense RNA in

posttranscriptional gene silencing in plants [see comments]. *Science* **286**, 950-952 (1999).

3. Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G.J. An RNA-directed nuclease mediates posttranscriptional gene silencing in Drosophila cells. *Nature* **404**, 293-296 (2000).

4. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366 (2001).

5. Hammond, S. M., Boettcher, S., Caudy, A.A., Kobayashi, R. & Hannon, G.J. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**, 1146-1150 (2001).

6. Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P. & Sharp, P. A. Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev.* **13**, 3191-3197 (1999).

7. Liu, J. et al. Argonaute2 Is the Catalytic Engine of Mammalian RNAi. *Science* **305**, 1437-1441 (2004).

8. Song, J.-J., Smith, S. K., Hannon, G. J. & Joshua-Tor, L. Crystal Structure of Argonaute and Its Implications for RISC Slicer Activity. *Science* **305**, 1434-1437 (2004).

9.. Schwarz, D.S. et al. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**,

199-208 (2003).

10. Khvorova, A., Reynolds, A. & Jayasena, S.D. Functional siRNAs and miRNAs exhibit strand

bias. Cell 115, 209-16 (2003).

11. Lee, R.C., Feinbaum, R.L., and Ambros, V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75, 843-854 (1993).

12. Wightman, B., Burglin, T.R., Gatto, J., Arasu, P., & Ruvkun, G. Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during Caenorhabditis elegans development. *Genes Dev.* 5, 1813-1824 (1993).

13. Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function.

Cell 116, 281-97

(2004).

- 14. He, L. & Hannon, G.J. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev*
- *Genet* **5**, 522-31 (2004).
- 15. Lee, Y. et al. MicroRNA genes are transcribed by RNA polymerase II. *Embo J* **23**, 4051-60
- (2004).
- 16. Cai, X., Hagedorn, C.H. & Cullen, B.R. Human microRNAs are processed from capped,
- polyadenylated transcripts that can also function as mRNAs. *RNA* **10**, 1957-66 (2004).
- 17. Lee, Y. e. a. The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415-419 (2003).
- 18. Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F. & Hannon, G.J. Processing of primary
- microRNAs by the Microprocessor complex. Nature 432, 231-5 (2004).
- 19. Landthaler, M., Yalcin, A. & Tuschl, T. The Human DiGeorge Syndrome Critical Region
- Gene 8 and Its D. melanogaster Homolog Are Required for miRNA Biogenesis. *Curr Biol* **14**,
- 2162-7 (2004).
- 20. Han, J. et al. The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* **18**,
- 3016-27 (2004).
- 21. Gregory, R.I. et al. The Microprocessor complex mediates the genesis of microRNAs.
- Nature **432**, 235-40 (2004).
- 22. Yi, R., Qin, Y., Macara, I.G. & Cullen, B.R. Exportin-5 mediates the nuclear export of pre-
- microRNAs and short hairpin RNAs. Genes Dev 17, 3011-6 (2003).
- 23. Lund, E., Guttinger, S., Calado, A., Dahlberg, J.E. & Kutay, U. Nuclear export of microRNA
- precursors. Science 303, 95-8 (2004).
- 24. Siolas, D. et al. Synthetic shRNAs as potent RNAi triggers. *Nat Biotechnol* **23**, 227-

- 25. Song, J.J. et al. The crystal structure of the Argonaute2 PAZ domain reveals an RNA
- binding motif in RNAi effector complexes. Nat Struct Biol 10, 1026-32 (2003).
- 26. Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. & Conklin, D.S. Short hairpin
- RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* **16**, 948-58.

(2002).

- 27. Paddison, P.J. et al. A resource for large-scale RNA-interference-based screens in
- mammals. *Nature* **428**, 427-31 (2004).

<sup>31 (2005).</sup> 

- 28. Zeng, Y., Wagner, E.J. & Cullen, B.R. Both natural and designed micro RNAs can inhibit the
- expression of cognate mRNAs when expressed in human cells. *Mol Cell* **9**, 1327-33. (2002).

29. Silva, J.M. et al. Second-generation shRNA libraries covering the mouse and human genomes. *Nature Genetics* 37(11):1281-8 (2005).

- 30. Westbrook, T.F. et al. A genetic screen for candidate tumor suppressors identifies REST.
- *Cell* **121**, 837-48 (2005).
- 31. Kim, et al. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol.* 23, 222-226 (2004).
- 32. Chen, C.Z., Li, L., Lodish, H.F. & Bartel, D.P. MicroRNAs modulate hematopoietic lineage
- differentiation. Science 303, 83-6 (2004).
- 33. Zeng, Y. & Cullen, B.R. Sequence requirements for micro RNA processing and function in
- human cells. Rna 9, 112-23 (2003).
- 34. Li, M.Z. & Elledge, S.J. MAGIC, an in vivo genetic method for the rapid construction of
- recombinant DNA molecules. Nat Genet 37, 311-9 (2005).
- 35. Ghoda, L., Sidney, D., Macrae, M. & Coffino, P. Structural elements of ornithine
- decarboxylase required for intracellular degradation and polyamine-dependent regulation. Mol
- Cell Biol 12, 2178-85 (1992).
- 36. Stegmeier, F., Hu, G., Rickles, R.J., Hannon, G.J. & Elledge, S.J. A Lentiviral microRNA-
- based System for Single Copy Pol II Regulated RNAi in mammalian Cells. *Proc Natl Acad Sci*
- *USA*, 102, 13212-13217 (2005).
- 37. Dickins, R.A. et al. Probing tumor phenotypes using stable and regulated synthetic
- microRNA precursors. Nat Genet, 37, 1289-1295 (2005).
- 38. Zender L. et al. Identification and Validation of Oncogenes in Liver Cancer Using an
- Integrative Oncogenomic Approach. Cell, 125,1253-1267 (2006).
- 39. Han J. et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* 125, 887-901 (2006).

## KEY RESEARCH ACCOMPLISHMENTS

- Development of strategies for gene silencing in mammals
- Construction of genome-wide shRNA libraries for mouse and human
- Development of powerful genetic screening strategies to identify new potential targets.

## **REPORTABLE OUTCOMES**

- Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. and Conklin, D.S. (2002) Short hairpin RNAs (shRNAs) induces sequence-specific silencing in mammalian cells. Genes & Dev., 16: 948-959.
- McCaffrey, A., Meuse, L., Pahm, T.T., Conklin, D.S., Hannon, G.J., and Kay, M.A. (2002) RNA interference in adult mice. Nature 418: 38-39.
- Hannon, G.J., (2002) RNAi. Nature, 418: 244-251.
- Paddison, P.J. and Hannon, G.J. (2002) RNA interference: the new somatic cell genetics? Cancer Cell, 2: 17-23.
- Caudy, A.A., Myers, M., Hannon, G.J., and Hammond, S.M. (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. Genes & Development 16: 2491-2496.
- Carmell, M., Xuan, Z., Zhang, M. and Hannon, G. J. (2002) The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes & Development 16: 2733-2742.
- Silva, J., Hammond, S. M. and Hannon, G. J. (2002) RNAi : A promising approach for antiviral therapy? Trends in Molecular Medicine 8 : 505-508.
- Seger, Y. R., Garcia-Cao, M., Piccinin, S., Lo Cunsolo, C., Doglioni, C., Blasco, M. A., Hannon, G. J. and Maestro, R. (2002) Transformation of normal human cells in the absence of telomerase activation. Cancer Cell 2: 401-413.
- Hemann, M.T., Fridman, J., Zilfou, J.T., Hernando, E., Paddison, P.J., Cordon-Cardo, C., Hannon, G.J., Lowe, S.W. (2003) An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes *in vivo*. Nature Genetics, 33: 396-400.
- Carmell, M.A., Zhang, L., Conklin, D.S., Hannon, G. J., and Rosenquist, T. A. (2003) Germline transmission of RNAi in mice. Nature Structural Biology 10: 91-92.
- Denli, A. M. and Hannon, G. J. RNAi : an ever-growing puzzle. Trends Biochemical Sci. 28:196-201.
- Narita, M., Heard, E., Narita M., Lin, A. W., Hearn S., Spector, D. L., Hannon, G. J. and Lowe, S. W. (2003) Rb-mediated heterochromatin formation and silencing of E2F targets during cellular senescence. Cell 113(6):703-716.
- Caudy A. A., Hammond, S. M., Denli, A. M., Silva, J. M., Myers, M., Ketting, R., Hannon, G. J., and Plasterk, RH A micrococcal nuclease homolog in RNAi effector complexes (2003) Nature 425:411-414.

- Bernstein, E., Kim, S. Y., Carmell, M., Murchison, E., Alcorn, H., Li, M., Mills, A., Elledge, S. J., Anderson, K. V., Hannon, G. J. Dicer is essential for mouse development. (2003) Nature Genet. 35:215-217.
- Paddison, P. J. and Hannon, G. J. siRNAs and shRNAs : skeleton keys to the human genome? (2003) Curr. Opin. Mol. Ther. 5:217-224.
- Song J., Liu, J., Tolia, N. H., Schneiderman, J., Smith, S. K., Martienssen, R. A., Hannon, G. J. and Joshua-Tor, L. (2003) The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nature Structural Biology 10:1026-1032.
- Silva, J., Sachidanandam, R. and Hannon, G. J. Free energy lights the path toward more effective RNAi. (2003) Nat. Genet. 35:303-305.
- Svoboda, P., Stein, P., Anger, M., Kato, Y., Bernstein, E., Hannon, G. J. and Schultz R. M.(2004) RNAi-mediated repression of transposons-derived sequences in preimplantation mouse embryos. Development 1;269 (1):276-285
- Gupta, S., Schoer, R. A., Egan, J. E., Hannon, G. J. and Mittal V. (2004) Inducible, reversible and stable RNA interference in mammalian cells. Proc. Natl. Acad. Sci. 101:1927-1932..
- Paddison, P. J., Silva., J. M., Conklin, D. S., Schlabach, M., Li, M., Aruleba, S., Balija, V., O'Shaughnessy, A., Gnoj, L., Scobie, K., Chang, K., Westbrook., T., Cleary, M., Sachidanandam, R., McCombie, W. R., Elledge, S. J. and Hannon, G. J. (2004) A resource for large-scale RNAi based screens in mammals. Nature 428:427-431.
- Carmell, M. A. and Hannon, G. J. (2004) RNAse III enzymes and the initiation of gene silencing: just PAZzing through? Nature Structural and Molecular Biology 11:214-218.
- Silva, J. M., Mizuno, H., Brady, A., Lucito, R. and Hannon, G. J. (2004) RNAi microarrays : High-throughput loss-of-function genetics in mammalian cells. Proc. Natl. Acad. Sci., Apr 27;101(17):6548-6552
- Hannon, G. J., Conklin, D.S. (2004) RNA interference by short hairpin RNAs expressed in vertebrate cells. Methods Mol. Biol. 257:255-266.
- Murchison, E. P. and Hannon, G. J. (2004) miRNAs on the move : miRNA biogenesis and the RNAi machinery. Curr. Opin. Cell Biol.June;16(3):223-229
- Du, J. and Hannon, G. J. (2004) Suppression of p160ROCK bypasses cell cycle arrest following Aurora-A/STK15 depletion. Proc. Natl. Acad. Sci June 15;101(24):8975-8980.
- Hemann, M.T., Zilfou, J.T., Zhao,Z., Burgess, D.J., Hannon, G.J. and Lowe, S.W. (2004) Suppression of tumorigenesis by the p53 target PUMA. Proc Natl Acad Sci June 22;101(25):9333-9338.
- He, L., and Hannon, G.J. (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet July;5(7):522-531
- Paddison, P.J., Caudy, A.A., Sachidanandam, R. and Hannon, G.J. (2004) Short hairpin activated gene silencing in mammalian cells. Methods Mol Biol. 265:85-100
- Caudy, A.A. and Hannon, G.J. (2004) Induction and biochemical purification of RNAinduced silencing complex from Drosophila S2 cells. Methods Mol. Biol. 265:59-72
- Hannon, GJ, Rossi JJ (2004) RNAi: unlocking the potential of the human genome. Nature 16;431(7006):371-8 Review

Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ (2004) Processing of primary microRNAs by the Microprocessor complex. Nature 11;432 (7014):231-5

- Siolas D, Lerner C, Burchard J, Ge W, Linsley PS, Paddison PJ, Hannon GJ, Cleary MA (2004) Synthetic shRNAs as potent RNAi triggers. Nat Biotechnol. 2:227-31
- Liu, J., Carmell, M.A., Rivas, R.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., and Hannon G.J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science 305:1437-1441
- Song, J.J., Smith, S.K., Hannon, G.J., and Joshua-Tor, L. (2004) Crystal structure of Argonaute and its implications for RISC Slicer activity. Science 305:1434-1437
- Cleary, M., Kilian, K., Wang, Y., Bradshaw, J., Ge, W., Cavet, G., Paddison, P. J., Chang, K., Sheth, N., Leproust, E., Coffey, E. M., Burchard, J., Linsley, P. and Hannon, G. J. (2004) Production of complex libraries of defined nucleic acid sequences using highly parallel in situ oligonucleotide synthesis. Nature Methods, 18:1(3):241-248.
- Paddison, P. J., Cleary, M., Silva, J. M., Chang, K., Sheth, N., Sachidanandam, R. and Hannon, G. J. (2004) Cloning of short hairpin RNAs for gene knockdown in mammalian cells. Nature Methods 1:163-167.
- Silva, J., Chang, K., Hannon, G. J. and Rivas. F. V. (2004) RNA-interference-based functional genomics in mammalian cells : reverse genetics comes of age. Oncogene 23:8401-8409.
- Dike S, Balija VS, Nascimento LU, Xuan Z, Ou J, Zutavern T, Palmer LE, Hannon GJ, Zhang MQ, and McCombie WR. (2004) The mouse genome: Experimental examination of gene predictions and transcriptional start sites. Genome Research 14:2424-2429
- Rivas FV, Tolia NH, Song JJ, Aragon JP, Liu J, Hannon GJ, Joshua-Tor L. (2005) Purified Argonaute2 and an siRNA form recombinant human RISC. Nat Struct Mol Biol. 12:(4):340-9
- He., L., Thomson, J. M., Hemann, M. T., Mu, D., Powers, S., Hernando-Monge, E., Goodson, S., Cordon-Cardo, C., Lowe, S. W., Hannon, G. J. and Hammond, S. M. A microRNA polycistron as a potential human oncogene. Nature 2005; Jun 9;435(7043):828-33
- Westbrook, T. F., Martin, E. S., Schlabach, M. R., Leng, Y., Liang, A. C., Feng, B., Zhao, J. J., Roberts, T., Mandel, G., Hannon, G. J., DePinho, R., Chin, L. and Elledge, S. J. A genetic screen for candidate tumor suppressors identifies REST. Cell 2005; Jun 17;121(6):837-48.
- Liu, J., Valencia-Sanchez, M.A., Hannon, G. J. and Parker, R. MicroRNAdependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol. 2005; Jul;7(7):719-23
- Silva JM, Li M, Chang K, Golding M, Rickles R, Siolas D, Hu G, Paddison PJ, Schlabach MR, Ge W, Sheth N, Bradshaw J, Burchard J, Cavet G, Sachidanandam R, McCombie WR, Cleary MA, Elledge SJ, and Hannon GJ. Second-generation shRNA libraries to the mouse and human genomes. Nat Genet 2005 Nov;36(11):1281-8
- Murchison EP, Partridge JF, Tam O, Cheloufi S, and Hannon GJ. Characterization of Dicer-deficient Murine Embryonic Stem Cells. Proc. Natl Acad. Sci 2005; Aug 23;102(37):13212-7

- Stegmeier F, Hu G, Rickles RJ, Hannon GJ and Elledge SJ. A lentiviral microRNAbased system for single-copy polll regulated RNAi in mammalian cells. Proc Natl Acad Sci 2005 Sep 13:102(37):13212-7
- Elledge SJ, Hannon GJ. An open letter to cancer researchers. Science. 2005 Oct 21;310(5747):439-41.
- Dickins RA, Hemann MT, Zilfou JT, Simpson DR, Ibarra I, Hannon GJ and Lowe SW. Conditional tumor suppression through expression of synthetic microRNA precursors. Nat Genet. 2005 Nov;37(11):1289-95.
- Qi Y, Denli A, Hannon GJ. Biochemical specialization within Arabidopsis RNA silencing pathways. Mol Cell 2005 Aug 5;19(3):421-8
- Qi Y and Hannon GJ. Uncovering RNAi mechanisms in plants: Biochemistry enters the foray. FEBS Lett. 2005 Oct 31;579(26):5899-90
- Elledge SJ, Hannon GJ. An open letter to cancer researchers. Science. 2005 Oct 21;310(5747):439-41
- Mason DX, Keppler D, Zhang J, Jackson TJ, Seger YR, Matsui S, Abreo F, Cowell JK, Hannon GJ, Lowe SW, Lin AW. Defined genetic events associated with the spontaneous in vitro transformation of E1A/Ras-expressing human IMR90 fibroblasts. Carcinogenesis. 2006 Feb;27(2):350-9
- Liu J, Rivas FV, Wohlschlegel J, Yates JR, Parker R, and Hannon GJ. A role for the P-body component GW182 in microRNA function. Nat Cell Biol. 2005 Dec 7: (12):1161-6
- Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of Translation and mRNA degradation by miRNAs and siRNAs. Genes Dev. 2006 Mar 1;20(5):515-24.
- Golding MC, Long CR, Carmell MA, Hannon GJ, Westhusin ME. Supression of prion Protein in livestock by RNA interference. Proc Natl Acad Sci. 2006 Apr 4;103(14):5285-90.
- Andl T, Murchison EP, Liu F, Zhang Y, Yunta-Gonzalez M, Tobias JW, Andl CD, Seykora JT, Hannon GJ, Millar SE. The miRNA-Processing Enzyme Dicer is Essential for the Morphogenesis and Maintenance of Hair Follicles. Curr Biol. 2006 May 5 [Epub ahead of print] PMID:16682203