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14. ABSTRACT

By virtue of their accumulated genetic alterations, tumor cells may acquire vulnerabilities that create opportunities for therapeutic intervention. We have devised a massively parallel strategy for screening short hairpin RNA (shRNA) collections for stable loss-of-function phenotypes. We assayed from 6000 to 20,000 shRNAs simultaneously to identify genes important for the proliferation and survival of five cell lines derived from human mammary tissue. Lethal shRNAs common to these cell lines targeted many known cell-cycle regulatory networks. Cell line-specific sensitivities to suppression of protein complexes and biological pathways also emerged, and these could be validated by RNA interference (RNAi) and pharmacologically. These studies establish a practical platform for genome-scale screening of complex phenotypes in mammalian cells and demonstrate that RNAi can be used to expose genotype-specific sensitivities. We are applying these methods to study the drug Bortezomib (Velcade). Velcade is the first targeted therapeutic to the proteasome approved by the FDA for treatment against multiple myeloma and is currently in phase II clinical trials for breast and lung cancers. We are identifying genes that mediate resistance against Velcade that could serve as potential drug targets.

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Introduction:

RNA interference (RNAi) is a conserved biological process in response to double-stranded RNA (dsRNA) [1]. DsRNAs are processed into short interfering RNAs (siRNAs), about 22 nucleotides in length, by the RNase enzyme Dicer. The siRNAs are then incorporated into a silencing complex called RISC (RNA-induced silencing complex), which identifies and silences complementary messenger RNAs. The most well characterized source of endogenous triggers for the RNAi machinery are the microRNA genes[2,3]. Numerous studies have demonstrated that, in animals, miRNAs are transcribed to generate long primary polyadenylylated RNAs (pri-miRNAs)[4,5]. Through mechanisms not yet fully understood, the pri-microRNA is recognized and cleaved at a specific site by the nuclear Microprocessor complex[6-10] to produce a ~70-90 nucleotide microRNA precursor (pre-miRNA) which is exported to the cytoplasm [11,12]. Only then is the pre-miRNA recognized by Dicer and cleaved to produce a mature microRNA. This probably involves recognition of the 2 nucleotide 3' overhang created by Drosha to focus Dicer cleavage at a single site ~22 nucleotides from the end of the hairpin[13].

This process can be programmed experimentally in order to repress the expression of any chosen gene. We have constructed shRNA libraries (shRNA-mir) that uses our advanced understanding of miRNA biogenesis. ShRNA-mirs are modeled after endogenous miRNAs, specifically contained in the backbone of the primary miR-30 microRNA[14]. We have produced and sequence-verified more than 200,000 shRNAs covering almost all of the predicted genes in the mouse and human genomes[15] and have used these libraries to study key aspects of breast cancer biology

Body:

Year 1 of this award was mainly devoted to the development of screening strategies and shRNA libraries (now termed the V2 library). This involved a large number of pilot scale studies to determine representation levels for library clones necessary for reproducibility amongst replicates and the development of computational pipelines that enable the analysis of screening data. This has been more fully described in the approved Year 1 report and will not be reiterated in detail herein.

In Year 2, we reported on both large-scale RNAi screens in breast cancer cells and on the development of tools that (now in long retrospect since the report is late) have proved foundational in the field, enabling others also to carry out similar large scale screens. In the final year of support from this grant, we built off efforts and preliminary results from those

screens to identify a suppressor of metastasis that is important in human breast cancers.

Our prior screens carried out both whole genome and genomic subset screens for lethal phenotypes. Lethality, though applicable in large scale, highly multiplexed applications, is a fairly non-specific phenotype. We wished to interrogate a much more specific, cancer-relevant phenotype, using a more limited subset of potential library targets. Here we took advantage of the expertise of CSHL colleague, Senthil Muthuswamy (who later became an EOH Scholar), who developed in vitro models of acinar development in which immortal but not transformed mammary epithelial cells are seeded under specific culture conditions as single cells. These proliferate into colonies into three-dimensional colonies that eventually undergo morphogenesis to produce a structure that resembles an acinus. As the colonies form, outer cells polarize, inner cells undergo apoptosis to form a hollow lumen, and a growth arrested, differentiated hollow structure is formed. Prior work, partly from Senthil's lab, had shown that oncogenic lesions or perturbations in cell polarity pathways could disrupt this orderly and stereotypic morphogenesis, providing a rationale for using this in vitro developmental model as a means to search for new breast cancer genes.

Since we felt that this type of model would not lend itself well to a genome-wide approach, we chose to focus our efforts on areas of copy number alteration in human cancers. We constructed a sub-genomic shRNA library targeting genes resident in focal deletions present in epithelial cancers, drawing from a dataset, which comprised 271 tumors, 104 of which were breast cancers. We interrogated only focal deletions in order to keep the number of genes manageable. We created shRNAs corresponding to all the genes in 36 regions that met our criteria, introduced these into MCF10A cells and monitored acinar development with the modified cell lines. We found that cells expressing shRNAs targeting one gene, cyfip1, resident in a focal deletion on chromosome 15, produced abnormal acini. Rather than appearing as symmetrical, size limited structures, foci from cyfip1 knockdown cells appeared disorganized with a morphology that resembled bunches of grapes rather than individual spheres. Although they did seem to form a hollow lumen, there appeared to be defects in polarization.

Examination of human tumor specimens indicated a loss of cyfip1 expression as a common event, with up to 31% of breast cancers showing decreased cyfip1 immunostaining. Similar loss of expression was also seen in colon, lung, and bladder cancers, wherein loss also correlated with the degree of invasiveness. Considered together, our results implicated cyfip1 as a possible regulator of epithelial morphogenesis and thereby of cancer progression through

control of the invasive phenotype.

Several different roles had been ascribed to cyfip1. These included interactions with an RNA binding protein, FMR1, and regulatory roles in actin cytoskeletal dynamics through participation in the WAVE complex. We were able to produce phenotypes similar to those seen with cyfip1 knockdown using shRNAs targeting other WAVE components but not by knockdown of FMR1, pointing to the WAVE complex as the mediator of cyfip1 cancer-relevant phenotypes. This led us to characterize the detailed outcomes of WAVE disruption on cell adhesion and cell behavior, which are described in detail in the paper cited below.

Importantly, we were able to show functionally in a model of progression from carcinoma in situ to invasive carcinoma that cyfip1 knockdown could accelerate the transition to invasive disease. Moreover, we could show a similar correlation with transition to the invasive phenotype in human cancer samples, with cells at the invasive front selectively showing loss of cyfip1 expression.

Considered together, these findings strongly implicate cyfip1 as an invasion suppressor in breast and other epithelial cancers and further validate the utility of RNAi-based screening procedures as a route toward understanding cancer pathways.

Key Research Accomplishments:

- The production of genome-wide shRNA libraries (partial support)
- The creation of multiplexed screening methods that enable genome-wide searches for tumor-specific genetic dependencies
- The application of these methods to several breast cancer cells lines
- The identification of pathways and genes upon which those cell lines selectively depend
- Development of an assay that enables RNAi screening for regulators of proper acinar development
- The use of this assay to screen genes present in focal deletions in human cancer via the development of a custom sub-genomic shRNA library
- The identification of cyfip1 as an invasion suppressor gene and the establishment of its relevance in human cancer.

Reportable Outcomes:

Minisymposium Talk:

Siolas, D., Chang K, Silva J, Rollins F, Powers S, Parker J, Hannon GJ. (2007) High throughput

RNA interference barcode screens as a tool for discovering gene function. *American Association for Cancer Research Conference* Minisymposium Presentation, Los Angeles, California, USA

Awards:

Siolas, D. (2007) AACR-WICR Brigid G. Leventhal Scholar Award in Cancer Research Award. American Association for Cancer Research, Philadelphia, PA, USA.

Publications:

Silva JM, Marran K, Parker JS, Silva J Golding M, Schlabach MR, Elledge SJ, Hannon GJ, Chang K. Profiling essential genes in human mammary cells by multiplex RNAi screening. *Science*. 2008 Feb 1;319(5863):617-20.

Silva JM, Ezhkova, E., Silva, J, Heart, S., Castillo, M., Campos, Y., Castro., V., Bonilla, F., Cordon-Cardo, C., Muthuswamy., S. K., Powers, S., Fuchs., E., and Hannon, G. J. Cyfip1 is a putative invasion suppressor in epithelial cancers. (2009) Cell 137:1047-1061.

Conclusions:

We have developed highly sophisticated RNAi platforms and corresponding screening paradigms that can identify genetic dependencies of cultured cancer cells and have used these also to identify breast cancer-relevant suppressors of progression to the invasive phenotype.

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