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Genome-Wide RNAi Screen for the Discovery of Gene Function, Novel Therapeutical Targets and Agricultural Applications

Hua Bai

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1. Introduction

The phenomenon of double-stranded RNAs (dsRNAs)-mediated gene silencing or RNA interference (RNAi] was first discovered in nematode *Caenorhabditis elegans* by Andrew Fire and Craig Mello in 1998 [1]. This great discovery gives rise to a fast-growing field and leads to the identification of novel RNAi pathways by which small interference RNAs (siRNAs) regulate gene expression and gene functions. Collective evidence suggests that the RNAi pathway is conserved in many eukaryotes and this pathway can be triggered by either exogenous or endogenous small interference RNAs. Exogenous dsRNAs (e.g. a virus with an RNA genome) are typically required a membrane transporter for dsRNA uptake into the cytoplasm, while endogenous small interference RNAs (e.g. microRNAs) are encoded in the genome. The precursors of both dsRNA and microRNA are first cleaved into short interference RNAs by a ribonuclease III (RNaseIII) enzyme, Dicer. Then these short interference RNAs initiate RNAi process when interacting argonaute proteins in the RNA-induced silencing complex (RISC). The small interference RNAs normally consist of 20~30 nucleotides. They can repress the transcription of message RNAs containing homologous sequences by either post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS) [2].

In their *Nature* paper, Fire and Mello wrote: 'Whatever their target, the mechanisms underlying RNA interference probably exist for a biological purpose'. Indeed, it's been shown that there are numerous cellular and physiological functions linked to RNAi [1]. For example, viral defense has been proposed to be the primary function of RNAi in both plants and flies [3]. In plants, virus infection could trigger sequence-specific gene silencing [4].

Plant RNAi forms the basis of virus induced gene silencing (VIGS), proofed from the genetic links between virulence and RNAi pathways [5-6]. On the other hand, endogenous microRNAs (about 1000 microRNAs in human genome) [7] play essential roles in controlling cellular functions. For example, the early discovered microRNAs, such as *lin-4* and *let-7* of *C. elegans*, were identified to regulate developmental timing [8-9]. Following the identification of *let-7* in *C. elegans* and later in fruit flies *Drosophila melanogaster* (hereafter I will refer it as '*Drosophila*'), it is soon realized that *let-7* belongs to a conserved microRNA family in many species. Besides the regulation on development, many microRNAs have been found to control key physiological processes, such as lipid metabolism [10] and insulin sensitivity [11]. The dysregulation of microRNA may result in many human diseases. A mutation in the seed region of miR-96 causes hereditary progressive hearing loss [12]. Some microRNAs have also been linked to cancer [13].

Soon after the discovery of dsRNA-mediated RNAi in 1998, gene silencing through RNAi was quickly developed as a powerful tool or technique in functional genomics studies. Comparing to forward genetics tools (e.g. EMS-induced mutagenesis screens), RNAi is one of effective reverse genetic tools, especially for non-model organisms and mammalian systems in which genetics is difficult. The advantage of applying RNAi in function studies becomes even more apparent when the whole genome sequences of model organisms (e.g. *C. elegans* and *Drosophila*) were completed in the late 90's and early 2000's [14-15]. In the post-genome era, utilizing high-throughput platforms and innovative bioinformatics tools, many large-scale RNAi screens has been successfully applied for the discovery of novel gene function associated with many important aspects of biology such as signal transduction [16], cell proliferation [17], metabolism [18], host-pathogen interactions [19] and aging [20-21]. Through these genome-wide RNAi screens, we have gained new insights on novel players in many key biological processes and complexity of cellular signaling networks. Cell-based and *in vivo* RNAi screen has been extensively reviewed in the past [22-23].In this book chapter, I will focus on the recent development of high-throughput RNAi screen for functional analysis in cultured cells and *in vivo* systems, as well as its applications on functional genomics and the discovery of novel therapeutic drug and agricultural targets.

2. RNAi screen methods

Despite the challenges from off-target effects and false discovery during the data analysis, genome-wide RNAi screens have benefitted from improved RNAi delivery methods, automated high-content image system and robust statistic analysis [23]. In the following section, I will compare the different reagent delivery methods, read-out assays, and off-target effects in various systems and platforms. I will also provide several examples from recent studies using genome-wide RNAi screen in cultured cells.

2.1. Cell-based RNAi screen

Genome-wide RNAi screen in cultured cells or primary cells provides an opportunity to systematically interrogate gene function. Now large-scale RNAi screens have been routinely

performed in *Drosophila* and mammalian cultured cells, as well as in primary cells. RNAi screen with *Drosophila* and mammalian cells has already led to important discovery in a wide variety of topics, including signal transduction, metabolism and cancer [22]. In general, a cell-based RNAi screen involves four major steps: [1]. RNAi library selection; [2]. Incubation of appropriate cell lines with RNAi reagents that are pooled or individually arrayed into 96- or 384-well plates; [3]. After additional treatments (if applicable), cells are subjected to the automated plate reader to quantify the specific readout (e.g. changes in cell morphology or fluorescence and luminescence signals from study targets); [4]. High-content image data analysis.

RNAi library and reagent delivery methods. Since the completion of *C. elegans* genome sequencing in 1998 [14], more and more eukaryotic genomes have been sequenced, which makes it possible to produce whole-genome RNAi libraries for functional genomics studies. Typically, long dsRNAs are used for RNAi screen in *Drosophila* cells, while synthetic siRNAs or vector-based short hairpin RNAs (shRNAs) are commonly used for mammalian cells [24]. Several *Drosophila* cell lines (e.g. S2 and Kc167) can directly take up dsRNA without the help of transfection reagents, which provides great advantages in high-throughput RNAi screens [25-26]. For mammalian cells, RNAi reagents are transient transfected into the cells. Therefore, not only the variation of transfection efficiency among cell lines and experimental replicates will affect the RNAi knockdown effects, but also the doubling time will affect the duration of gene silencing. Compared to synthetic siRNA method, vector-based shRNA technology combining with viral delivery methods provides robust gene silencing for a longer period of time. Besides, vector-based shRNA approaches make it possible to build renewable and cost-effective RNAi libraries.

Now RNAi libraries are available for many model organisms, especially *Drosophila* and mammalian cells (Table. 1). These RNAi libraries normally contain the collection of RNAi reagents (dsRNAs, siRNAs and shRNAs) for all annotated genes in the genome. In the RNAi libraries, typically there are several different dsRNAs or siRNAs corresponding to each gene. For example, The GeneNet™ Human 50K siRNA library from System Biosciences contains 200,000 siRNA templates targeted to 47,400 human transcripts (~4 different siRNA sequences per transcript) (http://www.systembio.com/rnai-libraries). In *Drosophila* DRSC 2.0 RNAi collection, there are 1-2 dsRNAs per gene, including genes that encode proteins and non-coding RNAs (http://www.flyrnai.org). Beside genome-wide libraries, many pathway libraries or sub-libraries are also available for silencing specific signal pathways or multi-gene families (e.g. kinases & phosphatases library, G protein-coupled receptor library, apoptosis & cell cycle library, etc.).

Read-out assays. Together with luminescent and fluorescent reporter-based image analysis and improved high-content image processing packages (e.g. CellProfiler [27] , various read-outs are used in RNAi screens, including the expression changes in target genes or proteins, post-translation modification, metabolic processes, and changes in sub-cellular localization patterns. Most of these read-outs are based on the measurement of the intensity of luminescent and fluorescent reporters. Although RNAi screens for cell morphology (e.g. cytoskeletal organization and simple cell shape) have been previously reported [28-30],

complex cell shape and structure-based read-outs still remain problematic. Frequently, additional treatments are performed before the read-out assays in RNAi screens. These treatments can be drugs, pathogens, or stress inducers [31-33].

Name	Species	Type	Link
Genome-wide RNAi libraries			
Drosophila RNAi Screen Center	Fruit fly	dsRNA	www.flyrnai.org/
DKFZ Genome RNAi	Fruit fly	dsRNA	www.genomernai.org
Open Biosystems	Human, mouse	siRNA , shRNA	www.openbiosystems.com/RNAi
Sigma	Human, mouse	siRNA , shRNA	www.sigmaaldrich.com
SBI	Human, mouse	siRNA , shRNA	http://www.systembio.com/rnai-libraries
Pre-defined or custom RNAi libraries			
Ambion	Human, mouse	siRNA , shRNA	www.invitrogen.com/sirna
Qiagen	Human, mouse	siRNA , shRNA	www.qiagen.com
Dharmacon	Human, mouse	siRNA , shRNA	www.dharmacon.com
The Netherlands Cancer Institute (NKI)	Human	shRNA	www.lifesciences.sourcebioscience.com/

Table 1. List of RNAi libraries used in cell cultures

Off-target effects. False positive or false negative results are commonly associated with high-throughput studies, including genome-wide RNAi screens [34]. The false discovery in RNAi screens can be caused by instrument errors, statistical noises, low knock-down efficiency and off-target effects of RNAi reagents. Off-target effects of RNAi reagents usually include: [1]. A general interference on endogenous RNAi pathway; or [2] Sequence-dependent effects on the expression of non-target genes. In order to minimize off-target effects, it is suggested to perform sufficient replication experiments and choose two or more RNAi reagents that target different regions of the coding sequences. Sequence-dependent off-target effects can be avoided by selecting sequences that do not contain 19 or more base pairs of contiguous nucleotide identity to other genes in the genome [34-35].

Recent cell-based RNAi screen studies. Genome-wide RNAi screens have been primarily conducted in both *Drosophila* and mammalian cultured cells (Reviewed in [22]. These screens are involved in studies on a variety of biology processes, such as signal transduction, metabolism, cancer, stem cells. The cell-based RNAi screens have yielded tremendous amount of novel discoveries and greatly promoted our understanding on many basic biological processes, molecular functions and complexity of cellular networks. New findings from genome-wide RNAi screens have led to the identification of novel components of canonical signaling transduction pathways, such new players of ERK pathway [16] and phosphorylation networks regulating JUN NH2-terminal kinase (JNK) pathway [36]; the role of *S1pr2* gene (Sphingosine-1-phosphate receptor 2) in insulin

signaling [37]; novel modulators of p53 pathway [38]; and key genes that are essential for the proliferation of cancer cells [17]. Recently, an integrative approach with RNAi screen and whole genome structural analysis identified IKBKE kinase as a breast cancer oncogene [39]. Beside its application in studying signaling pathways, cell-based RNAi screens are also performed to understand the cellular responses to pathogens. For example, recent RNAi screens identified novel host factors that are required for dengue virus propagation [40] and influenza virus replication [41]. I will discuss more detail on some of genome-wide RNAi screens in section. 3.

2.2. *In vivo* RNAi screen

Many complex phenotypes, such as aging, cannot be tested in a cell-based assay, thus *in vivo* functional analysis is required. *In vivo* RNAi screen is one of such approaches to study gene function at an organism level. *C. elegans* and *Drosophila* are two model organisms that are commonly used in *in vivo* RNAi screens. Although *ex vivo* RNAi screens have been done by introducing shRNA-transfected cells into mice [42-43], direct *in vivo* RNAi screen in mice is still under development. Most importantly, *in vivo* RNAi makes it possible for gene function studies in species lacking classical genetic tools, even including species without whole genome sequences (Usually next-generation sequencing is used to identify gene coding sequences and to facilitate RNAi reagent design and production).

In vivo genome-wide RNAi screen was first reported in *C. elegans* [44]. In *C. elegans*, dsRNA-mediated RNAi effects are systemic and heritable [1], although gene knockdown is less efficient in the nervous system than in other tissues. *In vivo* genome-wide RNAi screens have been performed in the studies of aging [20-21, 45-46], metabolism [47] and microRNA pathways in *C. elegans* [48]. It is relatively easy to deliver dsRNA into *C. elegans*. Typically, dsRNAs are introduced to worms by soaking the animals in dsRNA solution [49], by injection of dsRNA [1], or by feeding the animals dsRNA-expressing bacteria [44]. The last method is commonly used to generate genome-wide RNAi libraries. These *C. elegans* RNAi libraries are now available from Ahringer lab RNAi collection and Open Biosystems (Table. 2).

In contrast, dsRNA feeding does not appear to work for gene silencing in *Drosophila*, while RNAi via injection of dsRNA is effective only in certain embryonic stages. Therefore, transgenic RNAi approach has been developed to express a double-stranded 'hairpin' RNA from a transgene. In *Drosophila*, RNAi is cell-autonomous, so that gene silencing can be easily performed in tissue- and spatial-specific manner by using the binary GAL4/UAS expression system. Currently, there are three groups that have generated genome-wide transgenic RNAi *Drosophila* stains [50] (Table. 2). These transgenic RNAi stains are all expressing inverted-repeat hairpin RNAs once crossing to appropriate Gal4 drivers. Recently, it's been shown that small hairpin RNAs (~19 nt) can trigger stronger gene inactivation than long hairpin RNAs. Therefore new constructs expressing small hairpin RNAs were generated to produce second generation of transgenic RNAi *Drosophila* stains at Harvard medical school (Table. 2). In the past several years, a number of genome-wide RNAi screens in *Drosophila* have been conducted to study the major signaling pathways [51], as well as many important disease models [52-55].

For example, a genome-wide obesity gene screen revealed hedgehog signaling as one of major adipose tissue regulators [18], while genome-wide Parkinson's disease modifier screen identified novel *Park* and/or *Pink1*-interacting genes [56]. In the following section, I will discuss some of these RNAi screens in more detail.

Name	Species	Type	Link
Ahringer lab RNAi Library at Geneservice	Nematode	Bacterial clone	www.lifesciences.sourcebioscience.com/
Open Biosystems	Nematode	Bacterial clone	www.openbiosystems.com/RNAi/
Transgenic RNAi project at Harvard Medical School	Fruit fly	Long dsRNA, Short shRNA	www.flyrnai.org/TRiP-HOME.html
Vienna Drosophila RNAi Center	Fruit fly	Long dsRNA	stockcenter.vdrc.at
NIG-FLY	Fruit fly	Long dsRNA	http://www.shigen.nig.ac.jp/fly/nigfly/

Table 2. List of RNAi libraries used for *in vivo* systems

2.3. Advantage and limitation of cell-based and *In vivo* RNAi screens

Unlike forward genetic screens where mutations are randomly generated, RNAi screens provide a fast way to link phenotypes of interest to a precise gene. Beside, RNAi screens are generally performed in a genome-wide scale which brings us a comprehensive view of gene functions. Both cell-based and *in vivo* RNAi screens are highly effective and less labor-intensive on the discovery of gene functions when compared to traditional mutagenesis screens. Furthermore, cell-based and *in vivo* RNAi can be applied to study gene functions in species lacking classical genetics tools.

In the past decade, genome-scale *in vitro* RNAi screens have been successfully applied for gene discovery and understanding fundamental biological processes and cellular signal pathways. A variety of RNAi libraries for cell-based RNAi screens have been developed for both invertebrate and vertebrate systems. Nowadays cell-based RNAi screens are relatively less expensive, and have become a fast and user-friendly platform for functional genomics studies. On the other hand, complex phenotypes that cannot be analyzed in cell-based RNAi screens, are normally directly studied *in vivo*. When compared to forward genetic screens where mutations are occurred in every cell and many mutations lead to developmental defect or lethality, *in vivo* RNAi screens can be performed in various developmental stages and different tissues. This is especially useful when adult-specific functions of target genes are studied and these genes are essential for the development. Currently, most of *in vivo* RNAi screens are conducted in *C. elegans* and *Drosophila* due to the availability of a tremendous amount of resources and advanced genetic tools. In contrast, *in vivo* RNAi screen in mice is still in early stage.

3. Application of RNAi screen

Genome-wide RNAi screen has greatly advanced our understanding on many fundamental biology problems, from signaling transduction pathways to complex phenotypes. Furthermore, the results from RNAi screen can be used to design future theroputic drugs and crop protection reagents. In the following section, I will discuss several applications using RNAi screen approaches.

3.1. Deciphering cellular signaling pathways and complex traits

RNAi is one of the most powerful tools in functional genomics studies. Genome-wide RNAi screens have accelerated our understanding of basic biological functions and cellular signal networks, as well as the novel modulators of diseases. Follow-up experiments are usually performed to confirm the screen results and further study the underlying molecular mechanisms of identified genes or pathways. *In vivo* RNAi screens have also been conducted to study complex traits, such as aging. *C. elegans* is the primary model organism used in longevity gene screen, not only because high-throughput RNAi experiments are relatively easy to do in *C. elegans*, but also because of its short lifespan [57].

3.1.1. Understanding signaling pathways

Our understanding on canonical signal pathways is rapidly evolving and many new components or modulators are being identified with the help from improved technologies, including genome-wide RNAi screen. In the past decade, RNAi screens have been applied for deciphering many classical signal pathways, such as Notch, Wnt, and ERK signalings. Receptor tyrosine kinases (RTKs) are probably one of most critical protein families that regulate development, cell proliferation and growth. One of RTK families, insulin signaling plays important roles in controlling metabolism and growth. Disrupted insulin signaling leads to many human diseases, such as diabetes. To facilitate the underlying mechanism of diabetes and identify novel components and modulators of the insulin signaling pathway, a RNAi screen was conducted using 3T3-L1 adipocytes [37]. About 313 obesity and diabetes related genes were selected in the RNAi screen. The release of free fatty acid (FFA) was used as a read-out, since insulin-dependent FFA release is an indicator of insulin resistance. This screen showed that RNAi against 126 candidate genes resulted in significant changes of FFA release. After future filtering, *S1pr2* gene was identified as one of key regulators of insulin signaling. Increased plasma insulin levels were detected in male *S1pr2 -/-* knockout mice, suggesting there is a potential link between *S1pr2* and insulin resistance [37].

One of RTK downstream effectors is ERK signaling pathways. Misregulated RTK/ERK signaling leads to developmental disorder and many human diseases (e.g. cancer). A recent RNAi screen study using *Drosophila* cell lines has identified 331 regulators of ERK pathway, suggesting a number of integrated signal pathways in the regulation of fine-tuned ERK signaling [16]. In this study, fluorescently-conjugated phospho-ERK antibodies were used to monitor the changes of phosphorylated ERK upon insulin stimulation, which is the first time that a RNAi screen uses post-translation modification as a read-out assay.

3.1.2. Identification of longevity genes

Aging is one of the complex traits that are controlled by a large number of genes or the interaction between multiple genes/pathways. The first longevity pathway, insulin/ IGF-1 pathway, was identified in *C. elegans* in early 90's [58]. Following studies have shown that TOR (Target of Rapamycin) [59] and AMP kinase signaling [60] are also involved in longevity regulation. To explore other potential longevity assurance genes/pathways, two systematic RNAi screens for longevity genes were independently conducted at almost the same time in *C. elegans*. [45-46]. Both groups used the Ahringer bacterial RNAi libraries, although they chose different worm strains in the RNAi screen. Initially, the maximum lifespan of each RNAi clone was monitored, due to the tremendous work on the large-scale lifespan screen. In one screen, 89 genes were identified to be involved in lifespan regulation. These candidate genes encode diverse biological functions, including metabolism, mitochondrial functions, signal transduction, protein turnover, and so on. In contrast, 29 genes were identified in another screen. Although both groups are able to re-discover the genes in insulin/IGF-1 signaling, only three newly identified genes are shared by these two screens. This may be due to the different worm stains used in these two screens, plus high level of false positive/negative hits and off-target effects. Knockdown of these three genes led to robust lifespan extension [57].

Although genome-wide RNAi screens for longevity genes have not been reported in other species, large-scale genetic screens were performed recently. A screen of 564 single gene deletion strains was conducted to identify longevity genes in budding yeast [59]. Deletion of 10 genes led to extended replicative lifespan. Among them, many genes are involved in TOR pathway, suggesting a link between nutrient sensing and longevity. Recently, a large-scale misexpression screen for *Drosophila* longevity genes was reported. In this screen, a total of 15 longevity genes were identified, including genes involved in autophagy, mRNA synthesis, intracellular vesicle trafficking and neuroendocrine regulation [61]. With more longevity gene screens from other species, a cross-species comparison of these large-scale screens may provide us a list of conserved genes/pathways in regulating longevity.

3.2. Identification of therapeutic drug targets

A number of genome-wide RNAi screens have led to the identification of novel modulators of human diseases. RNAi screens have become an effective tool to identify and validate drug targets and to enhance novel drug discovery. On the other hand, RNAi-based therapies have been developed to target viral infection, cancer, cardiovascular disease and neurodegenerative diseases using specific shRNAs, although we should always keep in mind that there are some drawbacks and concerns of this technology, such as off-target effects, activation of endogenous RNAi pathways, individual genetic variation.

Traditional cancer drug discoveries still focus on a handful of known oncogenes. It remains a key challenge in identifying new targets. Application of genome-wide RNAi screens in novel target discovery greatly enhanced cancer drug discovery. In the past few years, several RNAi screens were performed using cancer cell lines to explore the essential genes

that are required for survival and proliferation of cancer cells. One of these studies identified more than 250 genes are essential for the proliferation of cancer cells [17]. In the same study, four genes were implicated in the response of cancer cells to tumoricidal agents (e.g. imatinib). Several similar screens on drug sensitivity have led to the identification of cancer-associated genes, e.g., *ACRBP*, *TUBGCP2*, and *MAD2* in breast cancer [62]. Combining RNAi screen and other genomic tools (e.g. SNP array, aCGH, and SAGE data analysis), a recent study identified IKBKE kinase as a breast cancer oncogene [39]. This discovery could lead to the development of pharmaceutical inhibitors that block activity of IKBKE kinase in breast cancer.

Metabolic syndrome, such as obesity, can increase the risk of developing cardiovascular disease and diabetes. However, the underlying molecular mechanisms are far from clear. To understand how adiposity is regulated in *Drosophila*, an *in vivo* genome-wide RNAi screen was reported recently. In this study, transgenic RNAi lines corresponding to 10,489 distinct open reading frames were used in RNAi screen. Tissue-specific gene inactivation for 500 candidates identified from the first screen was further tested. This study reveals hedgehog signaling as one of major adipocyte regulators [18]. To test whether hedgehog signaling plays any role in mammalian adipose tissue, mutant mice were generated to activate hedgehog in adipocytes. Activation of hedgehog in mice adipose tissues resulted in a dramatic loss of white fat compartments (but not brown) by directly blocking differentiation of white adipocytes. These results support the idea that white and brown adipocytes are derived from distinct precursor cells. Interestingly, glucose tolerance and insulin sensitivity remained normal in mutant mice. This suggests that modulating hedgehog signaling can reduce lipid accumulation in white adipose tissue, while maintain a fully functional brown adipose tissue. Since it has been suggested that functional brown adipose tissue represents a potent therapeutic target for obesity control, novel adiposity regulators (e.g. hedgehog signaling) will be developed as obesity drug targets in the near future.

3.3. Agricultural applications

Initially, it's believed that systemic RNAi is a unique feature in worms, until this idea was tested in insect species other than *Drosophila*. The first systemic RNAi in insects was reported in the red flour beetle, *Tribolium castaneum* [63]. Injection of dsRNA for bristle-forming gene, *Tc-achaete-scute* (*Tc-ASH*), resulted in bristle loss phenotype. Following this study, it was discovered that systemic RNAi via dsRNA injection works in many insect species, including mosquitoes [64], honey bees [65], aphids [66], termites [67], etc. Therefore RNAi became a useful tool in functional genomics studies in many non-model insect species, especially those economically important ones.

In 2007, two breakthrough studies described the technology on pest control through feeding transgenic plant expressing dsRNA [68-69]. It is the first evident to show RNAi can be used as a potential pest control strategy for crop protection and feeding RNAi works in certain insect group just like worms. In these studies, initially a list of potential target genes were chosen and dsRNAs against target genes were synthesized *in vitro* and mixed with artificial

diet. RNAi for several target genes results in larval growth arrest and lethality. Next, transgenic plant was engineered to produce dsRNA against genes whose inactivation results in strong RNAi response. Such genes include V-type ATPase A in western corn rootworm and cytochrome P450 (CYP6AE14) in cotton bollworm. These results provide strong evidence to support the feasibility of using RNAi in pest control and crop protection. Recently, feeding RNAi was also demonstrated in termites [67]. Feeding on cellulose disks soaked with dsRNA against digestive cellulose enzyme and hexamerin storage protein caused reduction in termite fitness and increased mortality. This study opened a new way for termite control using feed RNAi technology combining with a bait system. Although developing RNAi-based pest control approach is still at early stage and it is not as effective as current crop protection technology (e.g. *Bacillus thuringiensis* (Bt) toxin), RNAi will provide an alternative strategy for the future pest management.

3.4. A case study: Large-scale GPCR RNAi screen for novel pesticide target discovery

The G protein-coupled receptors (GPCRs) belong to the largest superfamily of integral cell membrane proteins and play crucial roles in physiological processes including behavior, development and reproduction. About 1-2% of all genes in an insect genome code for GPCRs. Whole genome sequencing identified about 200 GPCRs in *Drosophila* and 276 GPCRs in African malaria mosquito, *Anopheles gambiae*. Currently, there is not a commercial insecticide that targets GPCR. The red flour beetle, *T. castaneum* is one of the worldwide stored product pests. The genome of *T. castaneum* has been sequenced in 2008 [70], which offers great opportunities for the studies on functional genomics and the identification of targets for pest control. In one recent study [71], 111 non-sensory GPCRs were annotated from the beetle *T. castaneum* genome. To discover potential GPCRs as pesticide targets, a large-scale RNAi screen was performed by injecting dsRNA into developing larvae. The outline of this study is shown in Figure. 1. In this study, eight GPCRs were found involved in larval growth, molting and metamorphosis. The identified GPCRs may serve as potential insecticide targets for controlling *T. castaneum* and other related pest species.

In this GPCR RNAi study [71], 111 annotated *T. castaneum* GPCRs were classified into four different families based on conserved domain prediction program: Class A, Rhodopsin-like receptor; Class B, Secretin receptor-like; Class C, Metabotropic glutamate receptor-like and Class D, Atypical GPCRs. In summary, there are 74 Rhodopsin-like GPCRs, 19 Secretin receptor-like GPCRs, 11 Metabotropic glutamate receptor-like GPCRs, and 7 Atypical GPCRs. Rhodopsin-like GPCR family contains 20 biogenic amine receptors, 42 peptide receptors, four glycoprotein hormone receptors and one purine receptors.

A large-scale GPCR RNAi screen was then conducted by injecting dsRNA for 111 *T. castaneum* GPCRs into one-day-old final instar larvae. Mortality and development defects of dsRNA injected insects were recorded every 2-3 days until adult eclosion. This screen identified 12 GPCRs that effect growth and development. Among 12 GPCRs identified there

are biogenic amine receptor (TC007490/D2R), peptide receptors (TC013945/CcapR, TC012493/ETHR, TC004716 and TC006805), and protein hormone receptors (TC008163/bursicon receptor and TC009127/glycoprotein hormone-like receptor). Silencing of genes coding for four GPCRs (TC012521/stan, TC009370/mthl and TC001872/Cirl) in Class B and two GPCRs (TC014055/fz and TC005545/smo) in Class D also caused severe mortality (Table. 3). DsRNA-mediated knockdown for eight GPCRs caused more than 90% mortality after dsRNA injection. Interestingly, RNAi for one of the GPCRs, dopamine-2 like receptor (TC007490), resulted in high lethality during early larval stage. In *Drosophila*, dopamine-2 like receptor (D2R) is one of the genes highly expressed in head and brain (http://www.flyatlas.org/) and D2R RNAi flies with reduced D2R expression show significantly decreased locomotor activity (Draper et al. 2007). Since TC007490/D2R RNAi beetles died during the larval stage, TC007490/D2R might be playing a critical role in the growth and development of beetle larvae by modulating neuronal development and locomotor activity as reported in *D. melanogaster*. Collectively, the RNAi screen in *T. castaneum* has provided useful information and it has also been proven to be a nice model system for future pesticide screen.
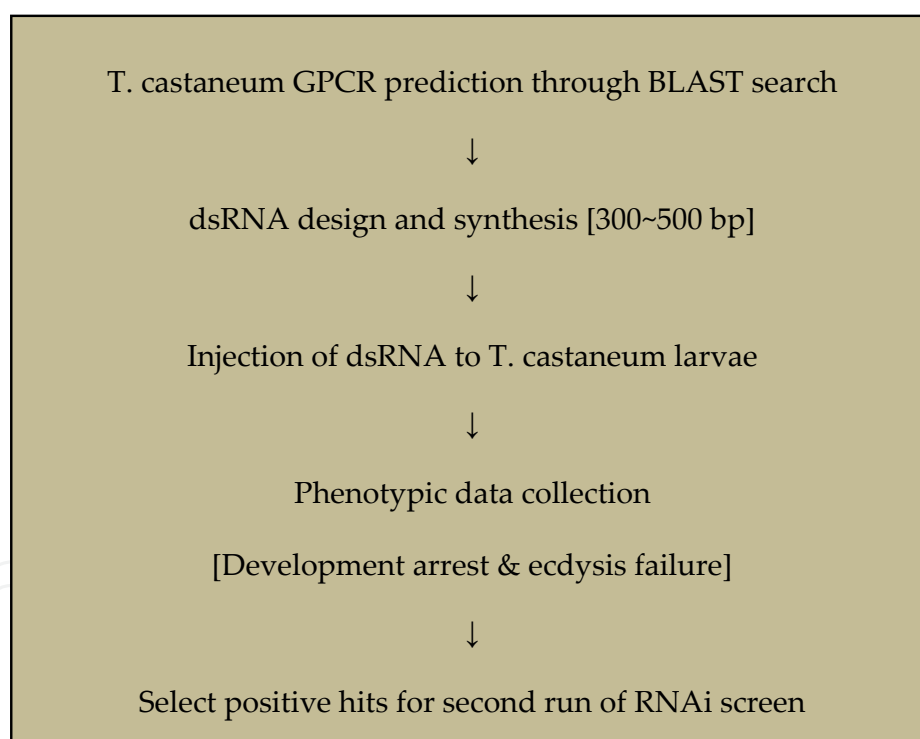

T. castaneum GPCR prediction through BLAST search

↓

dsRNA design and synthesis [300~500 bp]

↓

Injection of dsRNA to T. castaneum larvae

↓

Phenotypic data collection

[Development arrest & ecdysis failure]

↓

Select positive hits for second run of RNAi screen

Figure 1. The outline of GPCR RNAi screen in *T. castaneum*

Beside mortality, RNAi for eight GPCRs also resulted in severe developmental arrest and ecdysis failure, including recently characterized bursicon receptor [72]. Interestingly, the majority of insects injected with TC007490/D2R dsRNA was not able to molt to the pupal stage and died during the larval stage. Only, a few larvae injected with TC007490/D2R dsRNA were able to reach quiescent stage (a non-feeding prepupal stage, about 96 hr after ecdysis into final instar), suggesting that this gene may play an important role during larval

growth and development rather than molting and metamorphosis. In contrast, most of the insects injected with TC001872/Cirl dsRNA entered the quiescent stage and died during this stage. About 40% of the insect injected TC001872/Cirl dsRNA were able to molt to the pupal stage and eventually died during the early pupal stage. The majority of insects injected with TC012521/stan dsRNA was not able to complete adult eclosion and died during pharate adult stage. Interestingly, TC014055/fz and TC009370/mthl RNAi caused an arrest in both larval-pupal and pupal-adult ecdysis, suggesting that they may play important roles in the regulation of ecdysis behavior. In contrast, insects injected with TC009370/mthl dsRNA were arrested at the late phase of larval-pupal and pupal-adult ecdysis. The majority of insects injected with TC005545/smo dsRNA died during the early pupal stages without showing any ecdysis defects.

The GPCRs identified in this study [71] could be served as potential pesticide targets, which can be used in small molecule screen, or the development of RNAi-based pesticides. Among the identified GPCRs, many of them belong to classic GPCR families, e.g. biogenic amine receptors (TC007490 /D2R and TC011960/5-HTR) and neuropeptide receptors (TC009127/glycoprotein hormone-like receptor). These GPCRs, which are activated by small molecules, can be used as potential tar-gets for novel pesticide development. On the other hand, it may not be possible to apply small molecule ligands for pest management through targeting identified atypical GPCRs (e.g. TC014055 / fz and TC005545 / smo) whose ligands tend to be larger proteins. However, it should be possible to develop a RNAi-based pest control strategy through ingestion of specific dsRNA targeting atypical GPCRs as well as classical GPCRs [73].

Class	Official ID	First Screen		Second Screen	
		Larva Mortality	Pupa Mortality	Larva Mortality	Pupa Mortality
/	malE [#]	6.7%	0.0%	2.4%	2.4%
Class A Rhodopsin-like	TC007490	64.3%	35.7%	100.0%	0.0%
	TC008163	10.0%	90.0%	21.2%	75.8%
	TC009127	50.0%	16.7%	40.0%	0.0%
	TC006805	0.0%	62.5%	9.1%	54.5%
	TC013945	0.0%	100.0%	42.1%	52.6%
	TC012493	20.0%	60.0%	*	*
	TC004716	0.0%	41.7%	38.9%	22.2%
Class B Secretin receptor-like	TC001872	55.6%	44.4%	68.4%	31.6%
	TC009370	0.0%	90.0%	42.9%	57.1%
	TC012521	0.0%	90.0%	31.6%	68.4%
Class D Atypical GPCRs	TC014055	0.0%	100.0%	60.0%	40.0%
	TC005545	0.0%	92.3%	46.7%	53.3%

[#]: *E. coli malE* gene is used as a negative control.

Table 3. Summary of RNAi for 12 GPCRs in *T. castaneum*. Asterisk indicates that RNAi for TC012493 was not carried out at the second screen.

4. Conclusion

Genome-wide RNAi screen is a powerful technique for studying gene functions, deciphering complex phenotypes, and identifying novel drug targets. It opens up a whole new field that allows researchers to explore new modulators in classical signaling pathways, new mechanisms underlying basic biological functions, and new drug targets of human diseases. An increasing number of genome-wide RNAi screens have been successfully conducted for all kinds of novel discoveries. Although the off-target effects and other false discovery issues still remain, RNAi screen technique will be greatly improved as the development of new RNAi libraries and image detection instruments. Most importantly, as our understanding of RNAi pathway continues to grow, we will be able to design more specific and effective RNAi tools for genome-wide RNAi screen. There is no doubt that, through genome-wide RNAi screens, we will gain more insights into complex signaling networks and molecular mechanism of diseases in the near future, which will eventually lead to the discovery of novel therapeutic drug and crop protection reagents.

Author details

Hua Bai
Department of Ecology and Evolutionary Biology, Brown University, USA

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