

Reverse Direction Method: A Possible Tool to Link Animal Models with Corresponding Human Diseases and Disorders

Rajeev Singh, Toru Atsumi, Hidenori Bando, Masaya Harada, Akihiro Nakamura, Moe Yamada, Jing-Jing Jiang, Hironao Suzuki, Koukiti Katsunuma, Takao Nodomi, Daisuke Kamimura, Hideki Ogura and Masaaki Murakami*

Laboratory of Developmental Immunology, JST-CREST, Graduate School of Frontier Biosciences, Graduate School of Medicine, and WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan

Abstract

Animal models are integral to our understanding of the cellular and molecular pathogenesis of human diseases and disorders. Functional genome-wide methods such as DNA microarray and RNA interference-based high-throughput screening have recently emerged as powerful tools for such studies. However, genomic results from animal models may not necessarily correspond to the pathogenesis in humans. Thus, there is a need for new methods that better correlate the data from these models with human disease and disorders. Here we describe the reverse direction method, which combines the *in vitro* data of genome-wide screening of animal models with the data from genome-wide association studies (GWAS) of human diseases and disorders to effectively link the results of the two. This review introduces the concept of the reverse direction method when applied to the study of the inflammation amplifier, a chemokine inducer in non-immune cells for the development of chronic inflammatory diseases.

Keywords: Reverse direction method; Inflammation amplifier; GWAS; Inflammation; Cytokines; NFkB; STAT

Introduction

One of the conventional approaches to understanding the molecular mechanisms of human diseases and disorders is forward genetic screening using animal models. Here, genomes are randomly and/or artificially mutated and then investigated for the disease phenotypes of interest. Although this approach has identified many genes related to human diseases and disorders, it is inadequate in describing the pathogenesis of a majority of chronic inflammatory diseases that are multi-factorial and polygenic in nature. The full genome sequence of many organisms including mouse and human are now available. This information has built a foundation for the establishment of several high-throughput approaches that can investigate the function of each gene. Indeed, recent advances in mammalian RNA interference (RNAi) and short hairpin RNA (shRNA) technologies and DNA microarray have made it possible to conduct genome-wide analysis of the pathological processes in animal models [1]. However, the genomic responses of these models do not necessarily correspond to the pathogenesis in humans for example due to different compensatory mechanisms [2-5]. Thus, alternative methods that can better correlate the data of animal and human studies are needed.

We have recently established the reverse-direction method, which involves a series of steps for such a purpose, and applied it to the inflammation amplifier, a chemokine inducer in non-immune cells that promotes inflammation by the local accumulation of various immune cells [6]. As a first step, we performed high throughput genome-wide screening using a mouse shRNA library and a mouse DNA microarray. Then we combined these genome-wide data with the data of single nucleotide polymorphisms (SNPs) associated with human diseases and disorders using databases including genome-wide association studies (GWAS). Next we identified the genes involved in the regulation of the inflammation amplifier. Gene lists carrying positive regulators and targets of the inflammation amplifier contained significantly high numbers of genes related to various human diseases and disorders including not only autoimmune diseases, but also metabolic syndromes, neurodegenerative diseases, and other inflammatory diseases including

allergies and atopic dermatitis [7]. These findings demonstrate that the reverse-direction method can comprehensively identify genes that relate to human diseases and disorders using the experimental results from disease models like those of genome-wide screenings.

In this review, we illustrate how the reverse direction method opens up new experimental avenues to appropriately correlate the pathophysiological responses in animal disease models with human diseases and disorders. Moreover, application of this approach is exemplified by the epiregulin-ErbB1 pathway, a positive regulator of the inflammation amplifier, and is enhanced in human chronic inflammatory diseases like rheumatoid arthritis, multiple sclerosis, atherosclerosis, and chronic allogeneic lung graft rejection.

Discovery of the Inflammation Amplifier

The development of several complicated diseases including autoimmune diseases involves a complex biological process that often begins with inflammation, particularly the chronic form. Inflammation is mediated by a variety of soluble factors, including cytokines and chemokines. The major cytokines involved in inflammation are interleukin (IL)-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-12, IL-17, interferons, transforming growth factor- β , and tumor necrosis factors (TNF) α and β [8]. The increasing understanding of the role of cytokines in inflammation has led to a new generation of therapeutics, termed anti-cytokine biological therapy. One of the most successful anti-cytokine biological therapies has targeted TNF α with a humanized anti-TNF α antibody that has proven effective for many

*Corresponding author: Masaaki Murakami, Innovation Center Building A, Room 401, 2-1, Yamada-oka, Suita, Osaka, Japan 565-0871, Tel: +81-6-6879-4856, Fax: +81-6-6879-4706; E-mail: murakami@molonc.med.osaka-u.ac.jp

Received July 08, 2013; Accepted August 20, 2013; Published August 27, 2013

Citation: Singh R, Atsumi T, Bando H, Harada M, Nakamura A, et al. (2013) Reverse Direction Method: A Possible Tool to Link Animal Models with Corresponding Human Diseases and Disorders. Int J Genomic Med 1: 106. doi:10.4172/2332-0672.1000106

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rheumatoid arthritis patients [9]. Similarly, anti-IL-6 receptor antibody therapy is also effective [10]. It is also known that both TNF α and IL-6 are genetically linked to various diseases and disorders including rheumatoid arthritis.

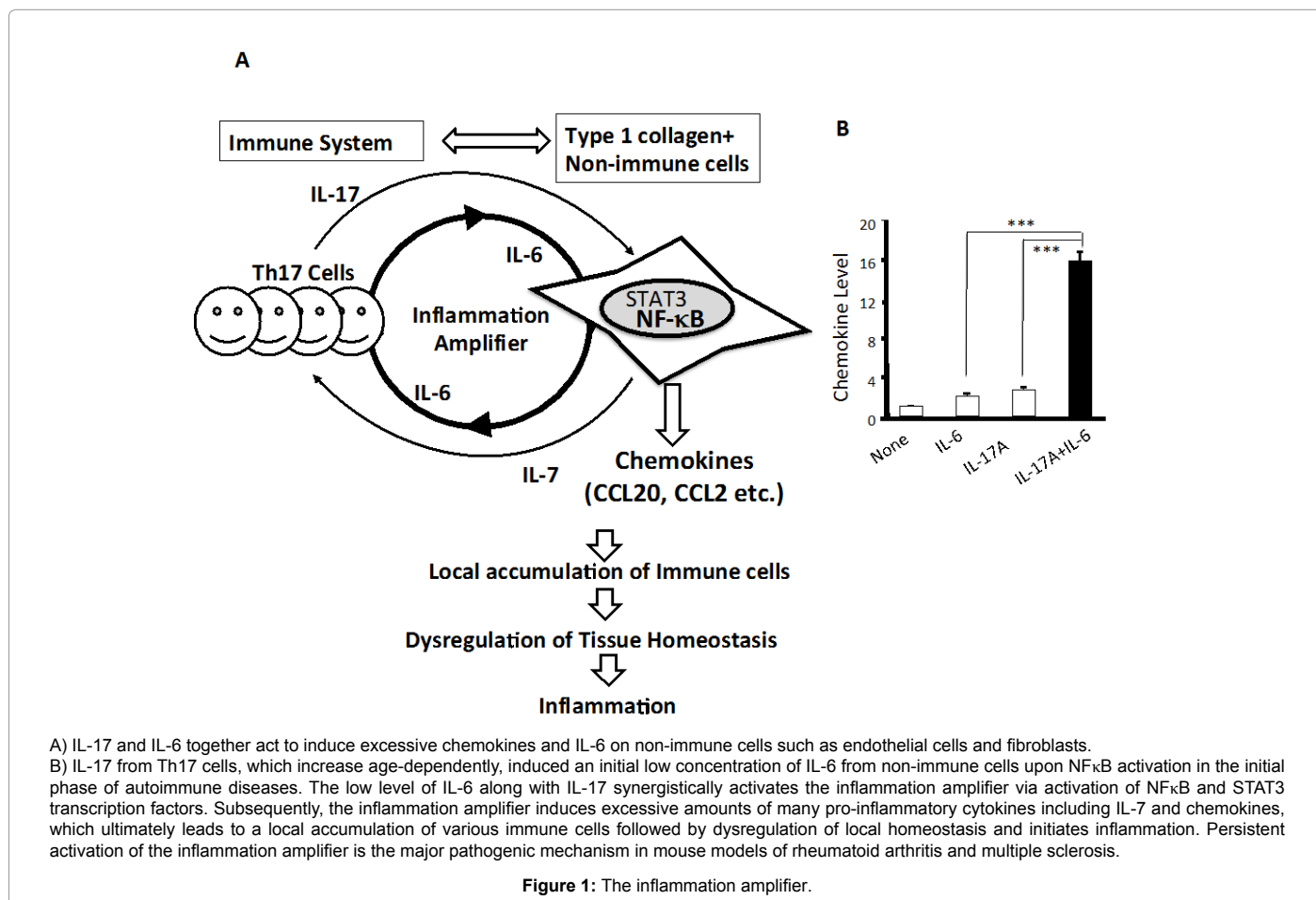
On the other hand, chemokines are a group of about 50 molecules that play a critical role in the development of inflammation via a local accumulation of immune cells [11]. We recently discovered a chemokine inducer in non-immune cells, which we named the inflammation amplifier and describes an amplification loop involving an NF κ B signal together with STAT3 activation followed by a local production of excessive chemokines. The inflammation amplifier is critical for the dysregulation of tissue homeostasis (Figure 1A) [12-16,6]. It is fueled by an IL-6 signal in the presence of IL-17, which induces enhanced activation of NF κ B and enormous amounts of inflammatory chemokines in type I collagen+ non-immune cells such as fibroblasts, endothelial cells, epithelial cells, and astrocytes *in vivo* and *in vitro* (Figure 1B). Indeed, activation of NF- κ B and STAT3 regulates the activation status of the inflammation amplifier, which is essential for the development of autoimmune diseases in mouse disease models including rheumatoid arthritis and multiple sclerosis [6]. Moreover, the inflammation amplifier is also critical for chronic graft rejection responses in a mouse model of tracheal transplantation [14]. In humans, activation of the inflammation amplifier is evident in autoimmune diseases and atherosclerosis as well as lung tissues around the lesions of chronic allogeneic lung graft rejection [7,13,14]. Thus, the inflammation amplifier is a ubiquitous phenomenon which

contributes to the development of various inflammatory diseases and disorders in humans.

Genome Wide Screenings of the Inflammation Amplifier

Because the inflammation amplifier is a critical molecular mechanism for inflammation in animal models, we hypothesize that its continuous activation is responsible for chronic inflammation not only in animals, but also in humans as well. In other words, the sites of inflammation amplifier activation define the regions of a wide range of chronic inflammatory diseases and disorders, including autoimmune diseases, metabolic syndromes, neurodegenerative diseases, and other inflammatory diseases in humans.

Lentiviral libraries having short hairpin RNA (sh-RNA) provide a powerful approach for targeting all the genes in a mouse or human cell [17]. There are at least several advantages to using shRNA libraries over siRNA ones, including the long time effect, the convenience in using gene-deficient cells, and easier application to *in vivo* studies. Importantly, lentiviral vectors infect non-dividing cells, which means they can be used to efficiently suppress gene expressions *in vivo*. Furthermore, lentiviral shRNA libraries, when combined with a large number of cell-based assays, provide a unique opportunity for high-throughput discovery of the genes responsible for complex cellular phenotypes *in vitro*. We have recently applied these approaches to understanding the molecular mechanism that activates the inflammation amplifier.



We performed systemic screenings using a shRNA library that contains about 65500 lentiviruses having shRNA and covers about mouse 16000 genes. Each lentivirus was infected in to a mouse endothelial cell line, BC1, in the presence of IL-17 and hIL-6, which are stimulators of NFkB and STAT3, respectively, and measured by observing IL-6, which is a marker for the inflammation amplifier and cell viability. In total, 1289 genes were identified as positive-regulator

candidates of the inflammation amplifier activation [7] (Murakami et al., 2013). Since we applied stringent screening criteria (which included significant thresholds for cell viability as well as the suppression rate of the assayed cytokines by one or more clones of shRNA), the false-positive rate in our shRNA-based screen was low (<30%). Thus, it is reasonable to assume that at least 70% of the candidate genes (about 900 genes) from the functional shRNA screen are involved in the

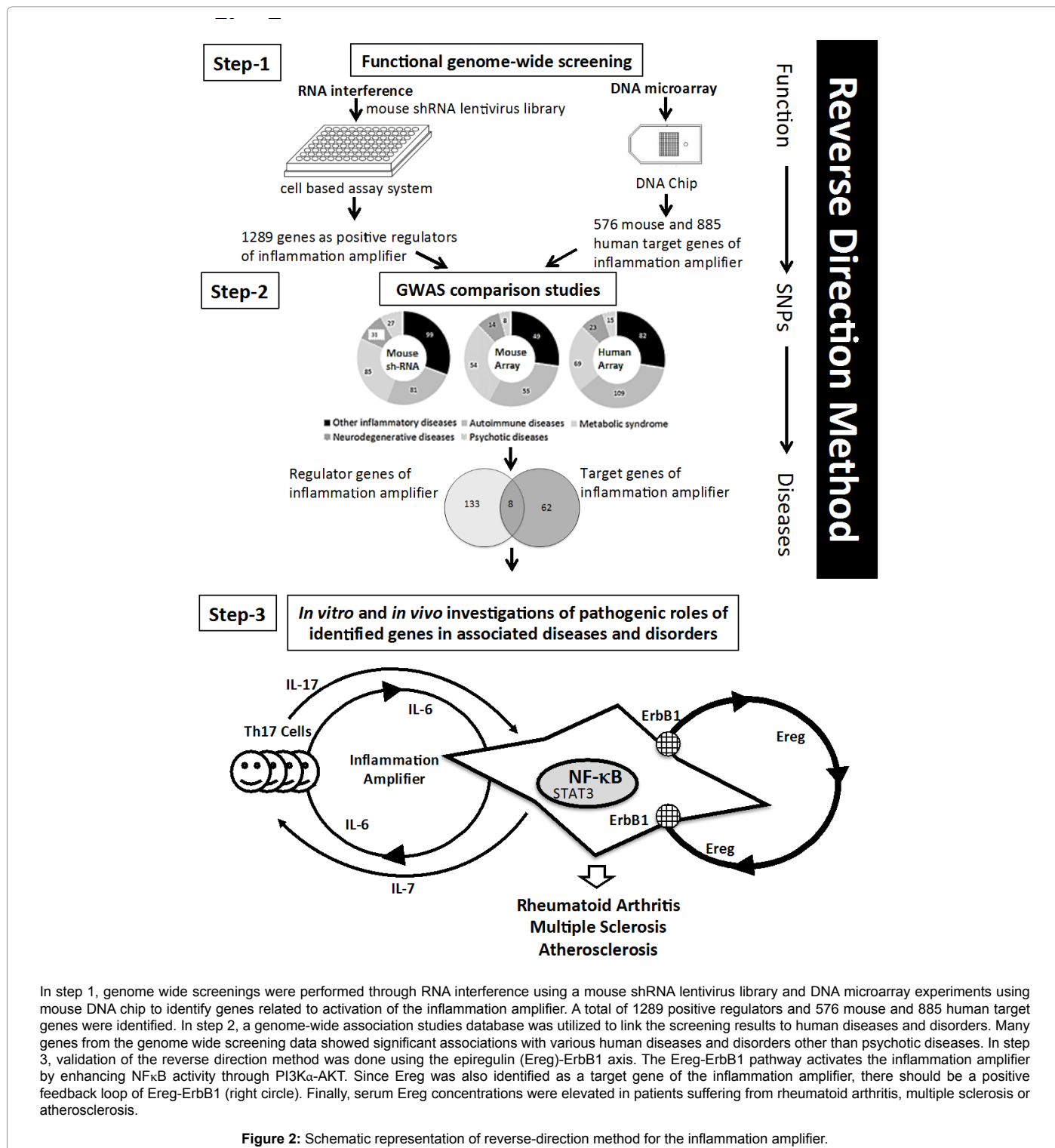


Figure 2: Schematic representation of reverse-direction method for the inflammation amplifier.

activation of the inflammation amplifier. Along with this screening, we also performed DNA microarray analysis in mouse and human fibroblastic cells in the presence of IL-17 and hIL-6 to identify target genes of the inflammation amplifier, finding 576 mouse and 885 human target genes. Although we have identified various genes linked to pathological processes like the inflammation amplifier via animal models, it is still possible that the genomic responses of these models may fail to correspond to the pathogenesis in humans. Therefore, we developed a method that could directly link the results of the animal models to human cases.

The Reverse-Direction Method: Linking Data of Animal Disease Models to Human Diseases and Disorders

Recently, genetic epidemiology has produced enormous amounts of GWAS data in various human diseases and disorders that combined with SNPs have successfully predicted an individual's susceptibility to these pathologies [18]. Normally, current genetic studies first identify the SNPs in patients using GWAS data and then focus on validating the data by other genetic and/or functional experiment datasets. We, however, reversed the process where biologically functional candidate genes of the inflammation amplifier were identified first by systemic genome-wide screenings in a mouse model and then compared with GWAS data to identify which of these genes can potentially regulate the development of human diseases and disorders. Therefore, we termed this approach the reverse-direction method (Figure 2) [7]. Application of this method identified many genes that associated with human disease and disorders and corresponded with activation of the inflammation amplifier, including not only autoimmune diseases, but also many human diseases that can be classified as metabolic syndromes or neurodegenerative diseases (Figure 2). These results suggest that the reverse direction method is a better way for obtaining quantitative evidence of links between animal disease models and human diseases and disorders than the more common method for individual genes described above or genetic approaches without functional screenings. Furthermore, these findings also suggest that the reverse direction method may be a useful tool for establishing a global view of human disease associations by correlating mass screening data of animal models with GWAS data.

The Epregrulin-ErbB1 Pathway Activates the Inflammation Amplifier and Associates with Several Inflammatory Diseases in Human

We selected the ErbB1 (EGFR) gene to evaluate the effectiveness of the reverse direction method, as this gene is a candidate positive-regulator and/or target of the inflammation amplifier. Importantly, ErbB1 is genetically associated with several human inflammatory diseases [7]. ErbB1 is a growth factor receptor with a tyrosine kinase domain that transduces a signal after binding with its ligands including epregrulin [19]. *In vitro* knock down of EGFR or epregrulin in mouse endothelial cells and fibroblasts significantly suppresses the expression of the target chemokines and pro-inflammatory cytokines of the inflammation amplifier. On the other hand, activation of fibroblast cells with exogenous epregrulin enhances the expression of the target genes of the inflammation amplifier. Mechanistic analysis showed that the epregrulin-ErbB1 axis positively regulates the activation of the inflammation amplifier by enhancing NFκB activity through the PI3Kα-AKT pathway. Importantly, epregrulin expression is also enhanced by the inflammation amplifier and the epregrulin-ErbB1 pathway itself, showing a positive feedback loop of epregrulin in non-immune cells that accelerates the NFκB signal, particularly in the

presence of the inflammation amplifier (Figure 2). *In vivo* blockade of the ErbB1-epregrulin pathway suppresses the development of cytokine-induced arthritis in F759 mice and a multiple sclerosis mouse model, experimental autoimmune encephalomyelitis. Moreover, the epregrulin-ErbB1 axis is not only critical for inflammation amplifier activation in human cells *in vitro*, but serum epregrulin concentrations are increased in patients suffering from rheumatoid arthritis, multiple sclerosis, or atherosclerosis [7]. Furthermore, NFκB, STAT3, and ErbB1 are all activated in the epithelial basal cells of bronchi in human lung tissues that showed chronic rejection inflammation responses after allogeneic lung transplantation [13]. Together, these findings confirm that activation of the inflammation amplifier, which was originally discovered in mouse disease models, is also associated with various human diseases and disorders, thus demonstrating the reverse direction approach bridges data from animal models with human diseases and disorders. While promising, this method does have some limitations, however, as the genome wide screenings are generally time consuming, especially when using shRNA or siRNA libraries. Moreover, it may be more difficult to apply this method to certain types of diseases like sensory diseases, because of poor detect ability or quantification. The experimental systems, which can be easily quantified in *in-vitro* systems just like the inflammation amplifier, are better suited for samples on the reverse direction method.

Conclusions

Animal disease models are instrumental for the study of disease mechanisms and testing therapeutic strategies against various human diseases and disorders. Evolving technologies in genomics, proteomics, metabolomics, and epigenomics have improved our understanding of diseases pathogenesis *in vivo* when using these models, as too have development of the next generation of genetic association databases for common human diseases and disorders. However, the mechanistic findings from animal models do not always correspond with the pathogenesis of human diseases, especially complex multi-gene-associated diseases including autoimmune diseases, metabolic syndromes, neurodegenerative diseases, etc. The reverse direction method is an alternative method that shows promise in correlating the results from animal models with human diseases and disorders. As a case study, we show success of the reverse direction method for studying the fundamental molecular mechanism(s) of the inflammation amplifier *in vivo* and *in vitro*.

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