



## Applications of Genetically Modified Tools to Safety Assessment in Drug Development

Hee Yeon Kay<sup>1</sup>, Hongmin Wu<sup>1</sup>, Seo In Lee<sup>2</sup> and Sang Geon Kim<sup>1</sup>

<sup>1</sup>College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University

<sup>2</sup>Daewon Foreign Language High School, WATCH21 Project Team, Seoul, Korea

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The process of new drug development consists of several stages; after identifying potential candidate compounds, preclinical studies using animal models link the laboratory and human clinical trials. Among many steps in preclinical studies, toxicology and safety assessments contribute to identify potential adverse events and provide rationale for setting the initial doses in clinical trials. Gene modulation is one of the important tools of modern biology, and is commonly employed to examine the function of genes of interest. Advances in new drug development have been achieved by exploding information on target selection and validation using genetically modified animal models as well as those of cells. In this review, a recent trend of genetically modified methods is discussed with reference to safety assessments, and the exemplary applications of gene-modulating tools to the tests in new drug development were summarized.

**Key words:** Gene modulation, Toxicological study, Knockout mice, Drug development

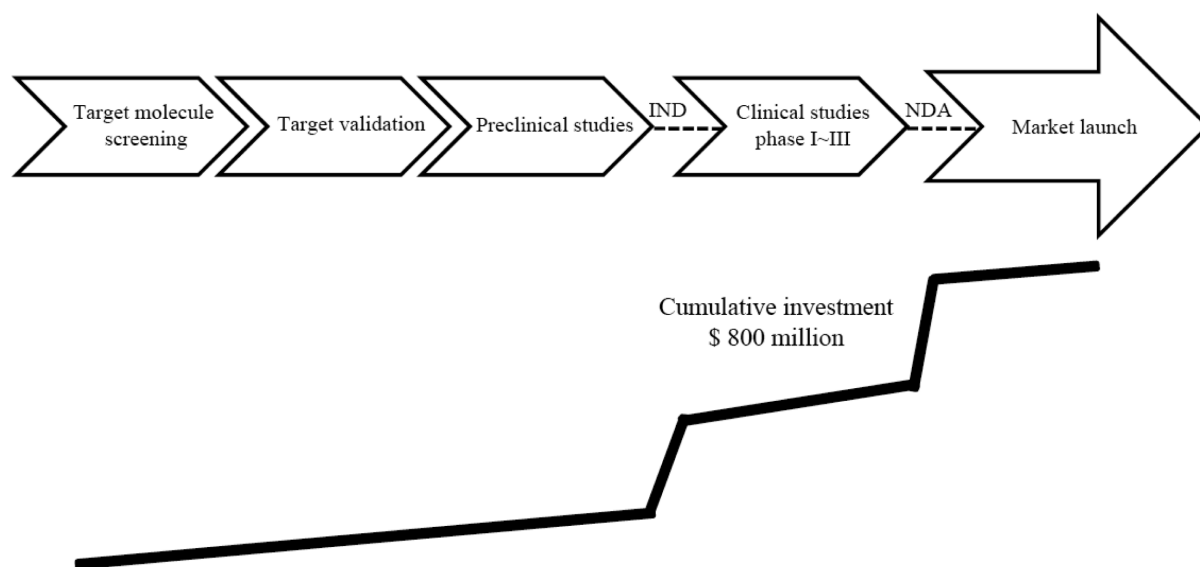
### IMPORTANCE OF TOXICOLOGY TESTS IN PRECLINICAL STUDIES

The purpose of pharmaceutical drug development is to discover safer and more effective drug. Preclinical development of a drug is the part of drug development occurring before the drug enters human trials. During this time, studies on the safety, activity, and mechanism of action are necessary. Preclinical toxicology study is a major strategy in the process of drug development for saving costs and is important for the prediction of effectiveness in human. Pharmaceutical companies can decrease the need for expensive and time-consuming clinical studies by doing good preclinical work (Ryan *et al.*, 2008). In this sense, drug developers continuously face increasing challenges in drug development from shorter product life cycles, higher global competition, and increased consumer demand. Enlarged candidate compound library due to advances in high-throughput screening system increases the cost and time of pharmaceutical process for new drug development. High attrition rate because of toxicity, lack of efficacy or poor pharmacokinetic profiles makes this situation worse. It usually takes a decade or longer to develop a drug and to reach

the market place. Moreover, the cost to identify and develop a therapeutically efficient drug is hundreds of millions of dollars (DiMasi *et al.*, 2003; Fricker, 2008). There are many new drugs investigated in cell or animal model systems every year that are found to be active in various ways. As summarized in Fig. 1, new drug development is an expensive, long-term and arduous task that requires 10 or more years' endeavor (Woodcock and Woosley, 2008). This begins from basic research including target molecular identification and target validation in laboratories. A couple of hundred target molecules that may be helpful for a specific disease are usually selected from thousands of molecules discovered during basic research. The next step will be preclinical studies to test several kinds of potential compounds on cells and animals and collect data in support of safety as well as efficacy. If the collected data is enough to suggest efficacy and safety on animals, a clinical study is conducted on humans usually for 5~10 years. However, few of these compounds can be translated successfully to human applications, which may be due to unacceptable toxicity, adverse effects, species variation in effect, and cost-benefit ratio in production (Lord *et al.*, 2006). Ultimately, the candidates that show robust efficacy and safety can be brought into the market. Having a standard set of procedures would make the process easier.

Before clinical trials, consistent efforts are made over a long period of time, including preliminary laboratory inves-

Correspondence to: Sang Geon Kim, College of Pharmacy, Seoul National University, Sillim-dong, Kwanak-gu, Seoul 151-742, Korea  
E-mail: [sgk@snu.ac.kr](mailto:sgk@snu.ac.kr)



**Fig. 1.** The general steps of new drug development and investment.

tigation and animal experiments. The aim of clinical trials is to provide a definitive answer on treatment approach to prevent, detect, or treat diseases, or to improve care for patients (Paul *et al.*, 2005). Clinical trials take place in phases after laboratory studies. A phase I trial is the first step in testing safety and tolerability of a new drug in healthy volunteers (O'Quigley *et al.*, 1990). In this trial, researchers find the best way to give people the new drug (i.e., tablets, capsules or injectables), and search for the appropriate frequency and dose. In phase I trial, small groups of people with a disease are treated with certain dose of a widely pre-studied new drug at a certain frequency. Among the groups of patients, dosage is gradually increased to find the maximal dose that will not cause harmful side effects. This process identifies safe and appropriate doses for phase II trial. During phase II trials, researchers evaluate whether the investigational drug benefits patients at the dose employed.

Before starting a clinical trial, preclinical toxicology is required to accurately evaluate the efficacy of a new drug and provide critical information for clinical trials (Grieshaber and Marsoni, 1986). Preclinical toxicology analyzes the toxic effect of a substance on cells and animals, and establishes safe starting dose and dose escalation parameters for phase I trial in human volunteers. Thus, preclinical toxicology studies include determination of acute, subacute, and chronic toxicity, carcinogenicity, mutagenicity, teratogenicity, and effects on the reproductive system. In this process, study aims to obtain information on toxic effect as much as possible so that good preparations can be made for further study.

Safety dosage still be tested during phase II trials, and the therapeutic effect on a particular disease come into be evaluated (Simon, 1989). In phase II trials, using the safety dos-

age found in phase I trials, the new drug candidate is given to groups of patients in question. Only agents that work effectively against the disease and are safe for patients in a phase II trial can enter a phase III trial. In general, people who participate in phase II trials have other treatments, but those treatments have not been effective. Usually, patients who have ever taken a cure before are not allowed to take part in these trials. As a general rule, phase II trials own no more than 100 participants.

Nowadays, pharmacogenomics is a powerful tool for the prediction of pharmacological and toxicological effects based on drug response in different genetic patients, and is increasingly incorporated into phase II trials (Rioux, 2000). Pharmacogenomic technology deals with genetically determined variations in how individuals respond to drugs and designs rational treatment for patients to make sure maximum efficacy with minimal adverse effects. This technology is likely to be among the first clinical applications of the Human Genome Project, and have impacts on the clinical practice of medicine. Progress has been made towards the incorporation of pharmacogenomics information into clinical practice. For example, these include genetic testing for thiopurine methyltransferase variant alleles in patients prior to mercaptopurine administration, and for UGT1A1\*28 in patients prior to administration of irinotecan therapy (Marsh, 2007). However, increasingly recognized limitations still exist in this approach. Particularly, low event rates in phase II trial that include less than 100 participants can make inaccurate response-genotype relationship. More importantly, clinical phenotype is usually caused by coordinated expression of thousands of genes, which also limits the application of pharmacogenomics. Thus, there is still a long way to go before pharmacogenomics achieve the goal of individual-

ized selection of drug treatment (Pusztai, 2007).

After phase II treatment shows positive results, phase III can be taken on. Phase III trials compares the newly found treatment to the “standard treatment” (the most classical treatment, based on results of past research) in order to know whether the new treatment surpasses standard treatment. Every volunteer in phase III trials has the same chance of being assigned to one of two or more arms. The participants are randomly assigned to groups, which is referred as randomization (Peto *et al.*, 1976). Many people choose to get their first treatment in a phase III trial. The type of participant varies, depending on the kind of question being asked about a particular disease. In general, phase III trials have hundreds to thousands of participants to determine whether the differences in the effectiveness and safety of the newly found treatments are true. If the drug passes all of these clinical phases, it can be submitted to regulatory authority as a new drug application. The proportion of investigational new drug applications that succeed has been estimated to be less than 50% in phase I and II studies (Caldwell *et al.*, 2001).

Even once clinical trials have begun, further preclinical studies can be performed to answer developing questions. Clinical toxicology, a branch of toxicology, mainly focuses on the diagnosis including toxicological analysis and the treatment of intoxications and poisonings, chemical-induced diseases, environmental and hazardous material exposures, and other toxicological emergencies (Descotes and Testud, 2005). Especially, clinical toxicology is important in promoting drug development, because a concrete plan on counteracting toxicological effects and reducing the body burden of various poisons needs to be established. Individual patients show different tolerabilities to medications, which leads to different responses. So even at standard doses, a drug may have toxic adverse events in some patients, but fail to reach the expected therapeutic effect in others. Genetic differences in drug response may explain inter-individual variations occurring in therapeutic response and toxicity (Weinshilboum, 2003). For example, ~20% of the populations in industrialized countries reveal immunoglobulin E antibodies against physiological components, which may cause allergic reactions (Valenta, 2002). The aim of an improved therapy is the preparation of an individual composition of the components according to the condition of patients. For this reason, the standardization and characterization of the relationship between specific gene mutation and the phenotype may be important.

As reviewed above, developing a new drug is a complex and costly process (DiMasi *et al.*, 1991). If everything goes well according to plan, a new drug will be introduced to market ~10 years after the work first began. Pharmaceutical companies are constantly striving to shorten this time. Up to 10,000 compounds must be screened to get one potential drug. Even after released to market, only about

30% of drugs produce revenues that match average R&D costs. About one in four marketed drugs typically makes sufficient profits to recover the costs of its discovery and development. Thus, the best way to reduce the budgets and time of pharmaceutical discovery and development is reducing drug failures (Stewart *et al.*, 2001). Approximately 10% of the research budget is spent for preclinical studies today. It will be increased to 15% in the next 3~5 years. Target molecule screening, target validation, and preclinical studies are relatively inexpensive compared to the clinical phases, which may be good strategy for cost-effective drug development (Fig. 1).

Overall, preclinical studies serve a key role in the drug discovery and development processes. The main goal of preclinical studies is to discover potential drug molecules to provide better candidates to patients faster. Most candidates are failed due to poor pharmacokinetics, lack of efficacy and animal toxicities (Ulrich and Friend, 2002). Pharmacokinetics, bioavailability and efficacy had been the main reasons for the failures. Efficacy problem is still a big issue today. Another major problem that recently came up is the issue of toxicology or adverse effects. These factors can be detected in preclinical phases, implying that well-designed preclinical studies are crucial. These steps take less time and money, but are more important to increase the success rate of drug development. Preclinical data is also useful to choose the right compound, delivery method, and formulation for the overall strategic success.

### APPLICATION OF GENETICALLY MODIFIED TOOLS TO PRECLINICAL AND CLINICAL STUDIES

In the toxicological assessments during drug development, a big problem in preclinical studies is the lack of appropriate animal models for disease or toxicology of interest. No single model is likely to be suitable for all studies. Different animal models can help researchers better understand the pathogenesis of various disease and design reasonable therapeutics for patients. Since the first knockout mouse was created in 1989 (Thomas and Capecchi, 1987), transgenic and knockout animal models have become useful tools for the study of human diseases and their treatments.

As an example, Nrf2 is a transcription factor that plays a role in the defense mechanisms against xenobiotic-induced oxidative stress and toxicity. High sensitivity of Nrf2 knockout mice to toxicant-induced liver injury may result from the repression of detoxifying enzymes due to Nrf2 deficiency (Ramos-Gomez *et al.*, 2001; Chan and Kan, 1999; Enomoto *et al.*, 2001). Moreover, Nrf2-deficient mice exhibited decreased biliary excretion of sulfobromophthalein and decreased bile acid synthesis, which support the concept that the knockout animals may serve as a valuable tool to assess acute toxicity of drug candidate (Table 1). PPAR $\alpha$  is

**Table 1.** Frequently applied transgenic and knockout animals used in the field of toxicology

Knockout genes	Type of animals	Effects on toxicology	References
Nrf2	C57BL/6 mice	- increased hepatotoxicity to acetaminophen - decreased biliary excretion of sulfobromophthalein - decreased bile acid accumulation - decreased expression of detoxification enzymes and transporters	Reisman <i>et al.</i> , 2009a, b; Tanaka <i>et al.</i> , 2009; Reisman <i>et al.</i> , 2009c; Maher <i>et al.</i> , 2008
PPAR $\alpha$	C57BL/6 mice	- decreased expression of multidrug resistance-associated protein transporters - abrogated downregulation of Oatp1a1, 1a4, 1b2, and Ntcp and induction of Cyp4A14 by perfluorodecanoic acid - decreased PPAR- $\delta$ agonist-induced skeletal muscle damage and liver injury	Maher <i>et al.</i> , 2008; Cheng and Klaassen, 2008a, b; Faiola <i>et al.</i> , 2008
FXR	C57BL/6 mice	- enhanced susceptibility of mice to alpha-naphthyl isothiocyanate-induced liver injury	Cui <i>et al.</i> , 2009; Cheng and Klaassen, 2008a, b
CAR	C57BL/6 mice	- abrogated induction of Cyp2B10 by perfluorodecanoic acid	Phillips and Goodman, 2009; Cheng and Klaassen, 2008a, b
PXR	C57BL/6 mice	- no effect on alpha-naphthyl isothiocyanate- or perfluorocarboxylic acids-induced liver injury	Cui <i>et al.</i> , 2009; Cheng and Klaassen, 2008b

another important transcription factor in the liver which plays a role in fatty acid oxidation and inflammatory processes during hepatic peroxisome proliferation. In PPAR $\alpha$ - and Nrf2-null mice, perfluorodecanoic acid-mediated induction of Mrp3 and Mrp4 mRNA was attenuated (Maher *et al.*, 2008), indicating that compensatory hepatoprotective responses might be disrupted. Moreover, PPAR $\alpha$  was shown to be associated with down-regulation of liver uptake transporters such as Oatp1a1, 1a4, 1b2, and Ntcp (Cheng and Klaassen, 2008a). Knockouts of other nuclear receptors such as FXR, CAR and PXR may also be useful as toxicological tools as reported in the recent articles of Toxicological Sciences in the years of 2008~2009 (Table 1).

Advances in genomics and proteomics increased possible solutions for improved identification and validation of target molecules (Ohlstein *et al.*, 2000). Gene knockout or silencing, gene expression analysis using microarray technology, and genetics-based approaches of the study population are now commonly used across the industries. These mutants are the tools to directly understand the biological function and utility of the gene and genome. In the past, there were no technologies available to alter the genomic DNA sequences except random mutagenesis. The developed site-directed mutagenesis in the mouse is called “gene targeting” or “knockout mouse” since this technology specifically disrupts the gene of interest. In 1990, many fundamental technologies to elucidate the gene and genome function were set (Collins and McKusick, 2001). The development of site-directed mutagenesis to introduce a single base substitution in the mouse genome has been a dream in the mammalian genetics. The knock-in mouse has now become one of such technologies.

Human genome is 30 times larger in size and 3 times more in numbers than worm and fly. Since most proteins

and their functions are not identified yet, applications of genomics would provide potential drug targets. Currently, only ~800 targets have been identified and more than 7000 molecules are waiting to be explored (Birney *et al.*, 2001). The word ‘leads’ means compounds that have a binding affinity with the micro molar range or less. These are the starting materials for drug development in spite of difficulties presented by targeting protein, cytokine receptors and phosphatases. Thus, ‘drug-like compounds’ means materials that have sufficiently acceptable pharmacokinetic and toxicity properties to survive through the completion of phase I clinical trials. Drug efficacy study is necessary as a proof to ensure the effectiveness with regard to the therapeutic aims. Through drug efficacy study, researchers can evaluate medical effectiveness and narrow the range of “drug-like compounds”. In addition, drug toxicity study is required during drug development to exclude compounds that have unacceptable toxicity or side effects for volunteers in the clinical trials. These compounds have a common feature called ‘the rule of five’; molecular weight less than 500, hydrogen bonds less than 10, hydrogen bond donors less than 5, and calculated logP less than 5 (Lipinski *et al.*, 2001). After approved for marketing for human disease therapy by a regulatory agency (i.e., FDA), they are called ‘drugs’. Target validation by genomics can save the cost and time for drug development and help avoid unnecessary investments. Moreover, target validation is effective predictors of ‘leads’ generation success.

It is noteworthy that siRNA is a useful tool in drug discovery especially for target identification and validation (Wang *et al.*, 2004). A siRNA-based target identification may involve high throughput transfection of a large siRNA library into cultured cells and observation of the resulting phenotypes, most often by an established assay (Wang *et al.*

*al.*, 2004). Conditionally replicative adenoviruses also have emerged as a novel and promising approach for a range of advanced neoplasm (Kirn and McCormick, 1996). In this regard, direct translation of this approach from the laboratory to human clinical trials has proceeded at an unprecedented speed.

Previously, drug discovery was usually started from empirical and unselected search for new candidate. These models are not predictable and they often show poor correlation of non-human with human pharmacology. The trend is now changing. Compounds that act through defined molecular mechanisms associated with disease will be developed according to the rationale. Gleevec, a Bcr/Abl kinase inhibitor, was developed using this strategy (Druker and Lydon, 2000). In this case, instead of compound screening, drug development starts with target screening. Target-dependent *in vivo* model studies using transgenic animals will be followed, and clinical trials include assessment of effect on

target. Whether the target of interest is associated with the disease, the frequency of association, mechanism of the link between target and disease, and whether pharmacologic modulation of the target can modify the disease might be important issues. Transgenic mice can help answer these questions on the efficacy and toxicology. For example, PPAR $\alpha$  knockout mice revealed the mechanism of hepatotoxic effects of amiodarone (McCarthy *et al.*, 2004). Amiodarone is a widely used antiarrhythmic agent but use of this drug is limited due to hepatotoxicity. Given the similar pathological consequences of PPAR $\alpha$  activation and amiodarone-induced hepatotoxicity, PPAR $\alpha$  was considered to be relevant to the toxicity associated with amiodarone. As expected, amiodarone did not induce hepatomegaly in PPAR $\alpha$  knockout mice, suggesting that the effects of amiodarone were PPAR $\alpha$ -dependent. As another example, P-glycoprotein-deficient mice provided valuable information as to the pharmacodynamic consequences of risperidone, a

**Table 2.** Recently approved new drugs and their targets identified using genetic applications (New Drug Applications Report, FDA, 2008-2009)

Drugs	Target molecule	Active components	Usages	Applications of genetic modulation
EXTAVIA	Unknown	Interferon $\beta$ -IB	multiple sclerosis	IFN- $\alpha/\beta$ receptor knockout mice (Müller <i>et al.</i> , 1994)
MULTAQ	Unknown	Dronedarone HCl	antiarrhythmic	PPAR $\alpha$ knockout mice (McCarthy <i>et al.</i> , 2004)
EFFIENT	P2Y12	Prasugrel HCl	thrombotic cardiovascular	P2Y12 knockout mice (Andre <i>et al.</i> , 2003)
INVEGA SUSTENNA	Unknown	Paliperidone palmitate	schizophrenia	P-glycoprotein (mdr1a/1b) knockout mice (Kirschbaum <i>et al.</i> , 2008)
ONGLYZA	DPP-4	Saxagliptin HCl	glycemic control in adults with type 2 diabetes mellitus	DPP-4 knockout mice and DPP-4 deficient Fischer rats (Fuchs <i>et al.</i> , 2009)
ILARIS	IL-1	Canakinumab	cryopyrin-associated periodic syndromes	IL-1 receptor knockout mice (Pineau <i>et al.</i> , 2009)
SAMSCA	vasopressin V2 receptor	Tolvaptan	hypervolemic and euvolemic hyponatremia	Only vasopressin V1 receptor (Avpr1a, b) knockout mice available
COARTEM	endoperoxide moiety, inhibits the formation of $\beta$ -hematin	Artemether; Lumefantrine	acute, uncomplicated malaria infections	Increased quinine uptake in mdr1a knockout mice (Pussard <i>et al.</i> , 2007)
AFINITOR	mTOR	Everolimus	advanced renal cell carcinoma	Pten knockout mice (Hernando <i>et al.</i> , 2007)
ULORIC	xanthine oxidase	Febuxostat	hyperuricemia in patients with gout	No transgenic animal model available
LUSEDRA	prodrug of propofol	Fospropofol disodium	monitored anesthesia care sedation	Glutamate decarboxylase 65 knockout mice (Kubo <i>et al.</i> , 2009)
FIRMAGON	GnRH receptor	Degarelix acetate	advanced prostate cancer	Only G protein-coupled receptor 54 knockout mice available
BANZEL	sodium channel	Rufinamide	seizures associated with Lennox-Gastaut syndrome	No transgenic animal model available
PROMACTA	TPO receptor	Eltrombopag olamine	thrombocytopenia	Mpl (thrombopoietin receptor) knockout mice (Jin <i>et al.</i> , 2006)
TOVIAZ	M receptor	Fesoterodine fumarate	overactive bladder	Muscarinic receptor subtype knockout mice (Ito <i>et al.</i> , 2009)

P-glycoprotein substrate (Kirschbaum *et al.*, 2008). These results are important because applications of these animals provide new mechanistic perspectives. Newly approved drugs and related transgenic knockout animal models are listed in Table 2.

Nowadays, compounds entering development are increasing every year (Feher and Schmidt, 2003). Along with it, identified innovative targets also increase, which carry increased risk of failure due to problems associated with toxicity and adverse effects (Nebeker *et al.*, 2004). Limited range of efficient decision making tools to pursue late phase drug development might be another barrier. Increasing pressure to shorten development cycle times and decreasing R&D budgets are the reasons for the necessity of efficient research on target validation and evaluation of preclinical efficacy and toxicity. More efficient decision making tools, higher rates of early attrition and lower rates of late phase attrition in drug development, and shorter early development cycle times would be needed. To discard those compounds which will most likely fail as early as possible and concentrate on the resources which will most likely succeed, introduction of well-designed models becomes more important. It will speed up drugs to the market and produce additional income to companies.

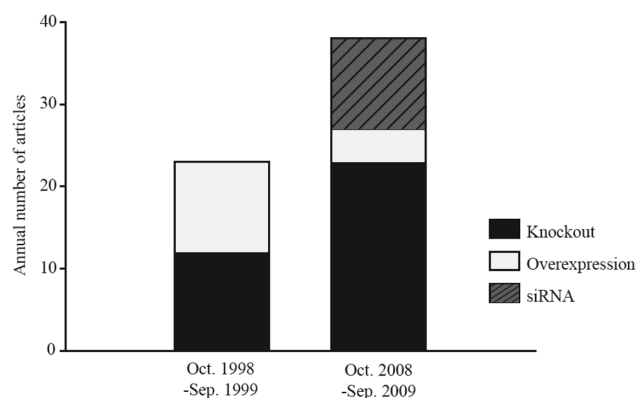
Nonetheless, well-designed animal studies still have some problems because of the differences in etiology, natural history, and/or molecular pathogenetic events between animal model and human disease. Moreover, animals and humans have different pharmacokinetics/pharmacodynamics in the way drugs interact with each other (Graham and Lake, 2008). These limits make it difficult to predict outcomes in human. To overcome these problems, transgenic mice engineered to express human homologs called 'humanized' mice had been developed. These transgenic mice can be used in drug development for assessment of efficacy and toxicity (Yoshizato and Tateno, 2009). Knockout mice of CYP450

genes or phase II enzymes might be useful to study pharmacokinetics and pharmacodynamics properties (McKinnon and Nebert, 1998). Moreover, using the Cre recombinase, conditional gene deletion would also be possible. Gene knockout modulation is now increasingly used; the usage of knockout technique has been increased ~4-fold in the past 10 years. Certainly, it will grow faster in the next decade. Nowadays, siRNA technologies are also used in preclinical study to evaluate the mechanism of actions and specificity of drug candidates, and to identify better candidates earlier in the drug development process. The number of articles describing the applications of genetic modulations including siRNA technique to new drug development is depicted in Fig. 2.

## CONCLUSIONS AND FUTURE DIRECTIONS

Pharmaceutical industries are facing productivity crisis over the last decade, despite advances in technologies for developing new candidate molecules and steady increase in R&D investment. The principal problem is the difficulty of developing efficient and safe drug with no adverse events; adverse event is the main reason for drug withdrawal and high rate of drop-out in clinical trials. Simplified and operationally efficient preclinical testing processes can dramatically reduce the high attrition rate and cost in new drug development steps. Today, multi-disciplinary approaches involving novel technologies are available; these technologies may improve in designing a pre-clinical study, and supply an effective framework to test alternative assumptions and facilitate the collection and interpretation of relevant information. Moreover, as all genes are potentially drug targets, strategies must be developed to gain insight that allows rate-limited R&D resources to be focused on genes with the greatest therapeutic potential.

Gene modulation is one of the most important tools of modern biology, and is commonly employed to examine the function of genes of interest (Debouck and Metcalf, 2000). Advances in new drug development have been achieved by exploding information on target selection and validation using transgenic knockout mice. These days, information obtained from gene knockout is accepted as a viable cost-effective alternative for mutagenicity and carcinogenicity testing. In addition, gene knockout can be used to define the biological mode of action by helping to discriminate between the closely related members of a gene family. The examples shown in this article illustrate the potential power of transgenic knockout animal model as a source of functional information and tools that can be used in studies at various other stages of the drug discovery process. New models by which we can evaluate *in vivo* toxicity will allow us to extrapolate therapeutic efficacy and toxicity to translational purposes that are used as therapeutic indexes for human trials.



**Fig. 2.** A trend of applications of genetic modulations to toxicological studies published in Toxicological Sciences. 1998; 45 (2)-1999; 51 (1), 2008; 105 (2)-2009; 111 (1).

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