LECTURE

Functional genetic dissection of nuclear receptor signalling in obesity, diabetes and liver regeneration using spatio-temporally controlled somatic mutagenesis in the mouse

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Abstract. The mouse is an excellent animal model for defining human diseases. The null allele mutations (knockouts, KO) have already provided valuable information about their functions, but have also revealed major complications and difficulties: (1) an early embryonic lethality, (2) temporal effect (developmental stage or adult stage), (3) functional redundancy and (4) spatio-effect (cell-autonomous or non-autonomous). To overcome these limitations, spatio-temporally controlled somatic mutagenesis, Cre-ER^T/LoxP system, was established. The nuclear receptors (NRs) play central roles in development, organogenesis, metabolism and energy homeostasis through their ability to transduce hormonal signals into modulation of gene activity. Obesity, excess energy storage in adipose tissue, has a strong link to diabetes. Among NRs, retinoid X receptor α (RXRα)-peroxisome proliferator-activated receptor γ (PPARγ) heterodimers can mediate adipocyte differentiation and obesity which has been demonstrated with *in vitro* cell culture systems and RXR- and PPARγ-specific ligand studies. Therefore an adipocyte-specific temporally controlled somatic mutagenesis system was established and analysed. Furthermore, the functional roles of NRs to control liver regeneration were also studied with similar system in hepatocytes. (Keio J Med 52 (3): 198–203, September 2003)

Key words: conditional somatic mutagenesis, nuclear receptor, obesity, diabetes, liver regeneration

Conditional Somatic Mutagenesis in the Mouse

The mouse, the genome of which is similar in size to the human genome, is an excellent animal model for defining the functions of mammalian genes as it exhibits genetic, immunological, reproductive, physiologic and pathologic similarities to man. The existence of techniques allowing the introduction of targeted mutations in the mouse genome should considerably help in the determination of the function of mammalian gene(s) and the characterisation of pathogenetic factors involved in numerous diseases, as well as produce good animal models for human pathologies.

The germ line mutations (knock-out, KO) in the mouse have yielded remarkable advances in understanding the roles played by specific gene products in mammalian development and adult physiopathology. However, this strategy has some inherent limitations, which are due to the introduction of the mutation in the germ line, as follows: (i) The lack of a protein that serves essential functions in embryogenesis can result in early lethality, thus precluding analysis of its possible functions at subsequent stages (lethality). (ii) A number of genes exert multiple functions in distinct cell types during ontogeny and postnatally (pleiotropy). (iii) Pleiotropic abnormality may result in complex phenotypes and in difficulties in distinguishing cell autonomous from more complex origins of abnormalities (cell autonomous or nonautonomous). (iv) The effect of germ line mutation may also be compensated during development, thus preventing the appearance of an abnormal phenotype in the adult animal. In the case of closely related genes belonging to multigene families, it may be necessary to mutate several members of the family which precludes identification of the function of a given member of the family. Defining the function of

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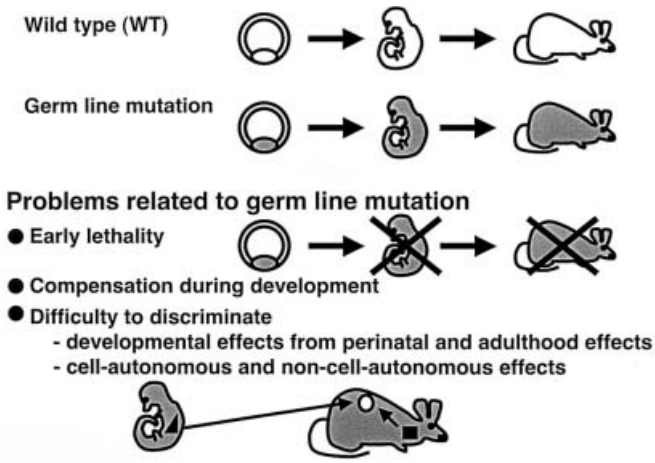

Fig. 1 Germ line mutation and its limitation. Schematic figures of wild type (WT) and germ line mutation in mice are shown. White and grey represent wild type and germ line mutation, respectively. Problems related to germ line mutation are also listed.

this member may become even more difficult when the family is involved in highly pleiotropic signalling pathways (functional redundancy). (v) Other potential effects confounding conventional KOs include the risk of impaired fertility and generalized, systemic disorders. In many instances, these limitations prevent the determination of the function of a given gene product in a defined subset of cells at any given time during life (Fig. 1). Thus, methods to achieve conditional gene inactivation are highly desired (Fig. 2).

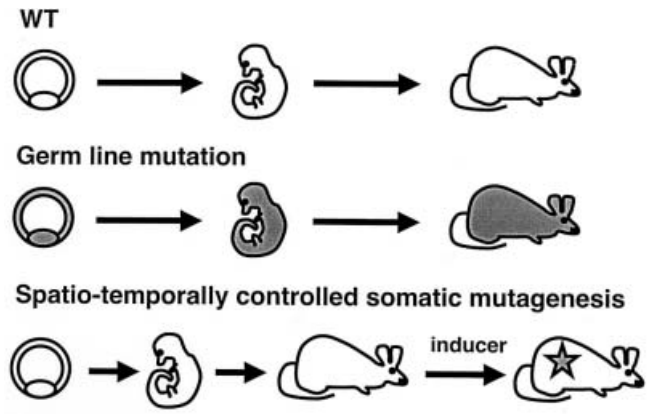

Fig. 2 Spatio-temporally controlled somatic mutagenesis. Schematic figures of WT, germ line mutation and spatio-temporally controlled somatic mutagenesis are shown. Before application of inducer, mice develop and grow as a wild type. By treatment of inducer (temporally controlled) somatic mutagenesis occur in specific cell types (spatio-controlled). White and grey represent wild type and genetic mutation, respectively.

The strategies of conditional gene targeting in mice is based on the Cre/LoxP system, and makes use of cell/tissue-specific expression of chimeric Cre recombinases (Cre-ER^{T}s), whose activity is induced by anti-estrogens such as Tamoxifen (Tam). It was shown that Tam administration to Cre-ER^T transgenic mice efficiently induced the excision of a chromosomally-integrated LoxP-flanked (floxed) DNA segment in most if not all the cells expressing Cre-ER^T, whereas no excision was detected in the absence of ligand treatment. Using the principle of this technology, conditional gene ablation in adipocytes and hepatocytes are established (Fig. 3).[1]

The Nuclear Receptor Superfamily

The last decade has seen an enormous rise in the interest in nuclear receptors (NRs) because of their central role in the coordination of development and metabolism. NRs can transduce hormonal signals into modulation of gene activity.[2] Thus, NRs are now seen as integrators of multiple signals and stimuli that participate in the control of all complex processes in living organisms. The transcriptional activity of these receptors is controlled by classical hormones, vitamin and dietary nutrient fatty acids derivatives, with the exception of the so-called orphan receptors for which no ligands have been identified.[3] The complexity and network nature of hormonal signalling via NRs require novel approaches and sophisticated tools in the integrated developing and adult organisms.

The Link Between Obesity and Diabetes

Obesity is a major health problem with more than one billion overweight people in the world. The number of obese among the Japanese is more than 23 million. Obesity most likely accompanies adult custom disease, such as hypertension, hyperlipidemia, and especially diabetes. In Japan, more than 2 million people are patients and 7 million people are thought to be afflicted without diagnoses. The disease is rapidly becoming more common among the Japanese. In patients of diabetes, the risk of heart disease and stroke are elevated 2–4 times. Moreover, diabetes is the leading cause of end stage nephropathy, neuropathy and retinopathy. While the human and economic costs of diabetes are difficult to calculate, the total medical costs incurred annually in the U.S.A. are more than $100 billion.[4]

Diabetes is defined as a situation in which carbohydrate and lipid metabolism are disordered by insulin. There are 2 major categories of the disease, types 1 and 2. The number of type 1 diabetes patients may account for 5–10% of all the diabetes cases. The cause of type 1 disease is thought to result from the destruction of the insulin-producing β cells of the islets of Langerhans,

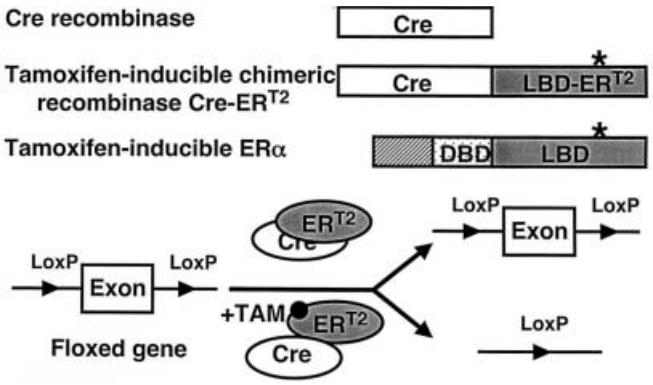

Fig. 3 The Cre-ER^{T2}/LoxP system. Chimeric recombinase (Cre-ER^{T2}) is a fusion protein between Cre recombinase (empty box) and mutated ligand binding domain of oestrogen receptor α (grey box). The loxP (filled triangle) flanked (floxed) gene is excised by Cre-ER^{T2} binding to tamoxifen (TAM). No recombination is observed in the absence of both factors. (DBD: DNA binding domain, dot box).

and type 1 diabetes patients are lead to insufficient of insulin production. Type 2 diabetes is far more common and caused from a combination of defects in insulin action and secretion.

The association of obesity with type 2 diabetes has been recognized for decades, especially in USA and Europe. The retinoid X receptors (RXRs) and the peroxisome proliferator-activated receptor γ (PPARγ) in adipocyte may play key roles in both obesity and diabetes. Therefore, the functional roles or link of these receptors to these diseases were analysed by adipocyte-specific conditional mutagenesis. PPARγ is a functional receptor for an interesting class of insulin-sensitizing drugs called thiazolinediones (TZDs), which are currently used in the treatment of type 2 diabetes mellitus.[5] RXR agonists (rexinoids) also have insulin-sensitizing activity *in vivo*. However, the target cells of these compounds are not known.

In vertebrates, the adipose tissue is critical for energy homeostasis.[6] Whereas the brown adipose tissue can dissipate energy through thermogenesis, the white adipose tissue (WAT) stores excess energy in the form of triglycerides, when caloric intake exceeds expenditure, and release free fatty acids when expenditure exceeds intake. The function of adipose tissue generally depends on their amount, and adipose tissue amount can be modulated by the formation of new adipocytes from precursor cells (adipocyte differentiation) and/or by increasing in adipocyte size (adipocyte hypertrophy). In the past adipose tissue was only largely regarded as a depot for fuel storage, however, adipocyte produce a number of enzymes and regulatory proteins involved in lipogenesis and lipolysis, as well as secreted paracrine and endocrine factors,[7] called adipokines.[8]

Adipocyte differentiation has mainly been studied using preadipocyte culture systems.[6] *In vitro* studies with rexinoid and TZD indicated that RXR-PPARγ heterodimers could play central roles in adipocyte, particularly in adipocyte differentiation and hypertrophy.[5] RXRα and PPARγ are highly expressed in adipocyte, as well as other tissues. The classic loss-of-function experiments for RXRα and PPARγ have proved difficult because homozygous null mice do not survive past the embryonic stage.[9,10] Therefore, mice in which RXRα and PPARγ are selectively ablated in adipocytes were established to study the link between obesity and diabetes.

Functional Role of the RXRα in Adipocytes

To perform conditional somatic mutagenesis in adipocytes, we have generated aP2-Cre-ER^{T2} transgenic mice expressing Cre-ER^{T2} selectively in adipocytes. We have shown that Tam efficiently and selectively induces floxed DNA excision in adipocytes. We have therefore performed conditional mutagenesis of RXRα in adipocytes using aP2-Cre-ER^{T2} line. The analysis of mice in which RXRα was selectively ablated in adipocytes of adult mice (RXRα$^{ad-/-}$ mice) allowed us to demonstrate that RXRα plays a key role in adipocyte differentiation, in adipogenesis and in lipolysis. Indeed RXRα$^{ad-/-}$ mice were resistant to dietary- and chemically-induced obesity, and were impaired in fasting-induced lipolysis.[11] Moreover, RXRα$^{ad-/-}$ mice showed hyperglycemia with insulin resistance under regular diet with normal adiposity. Interestingly among adipokines, TNFα, leptin and adiponectin expression, which is disregulated in diabetic animal models and humans, was also impaired in RXRα$^{ad-/-}$ mice. Using micro array analysis comparing the adipose transcriptome of RXRα$^{ad-/-}$ and control mice, a gene expression profile was obtained and analysed. Three kind of gene clusters were identified, they are insulin signalling, secreted proteins and lipolysis genes. Apparently WAT of mutated mice impaired insulin-stimulated glucose uptake. Some genes that activate glucose uptake in adipocyte were down regulated in RXRα$^{ad-/-}$ mice, such as GLUT4, CAP, glycogen synthase and so on. It suggests that RXRα regulates insulin signalling in adipocytes through the regulated expression of some genes which stimulate insulin signalling. Moreover, insulin resistance in muscle and liver are also observed in the mice with ablated RXRα in their adipocytes. These abnormalities are due to adipokines. In addition to the result of impaired expression of adipokines described above, some new candidates of possible adipokines which are up or down regulated in RXRα$^{ad-/-}$ mice have been obtained from gene profiling analysis. Detailed analyses of their expression patterns are being performed, and the function of the promising ones will be analysed in transgenic animals either by overex-

pression or conditional mutagenesis. These new adipokines will be the new targets for medical application of type 2 diabetes patients.

Taken together, RXRα plays a key role in adipocyte differentiation, adipocyte hypertrophy and lipolysis, and regulates key genes for glucose homeostasis in adipocytes. Moreover, RXRα$^{ad-/-}$ mice are a diabetic mouse model with normal adiposity, suggesting that RXRα is one of the molecules which dissociate obesity from diabetes.

Functional Role of the RXRs and the PPARγ in Adipocytes

As RXRγ is upregulated in adipocytes in RXRα$^{ad-/-}$ mice,[11] and might therefore partially compensate for the loss of RXRα function, we disrupted both RXRα and RXRγ in adipocytes. Surprisingly RXRα and RXRγ ablated adipocytes (RXRαγ$^{ad-/-}$ mice) induced lipodystrophy, thus indicating that RXRs are essential for the maintenance/survival of adipocytes.

To analyse PPARγ function in adipocytes, we selectively ablated PPARγ in adipocytes of adult mice using a similar strategy (PPARγ$^{ad-/-}$ mice). As described above in RXRα and RXRγ ablated adipocytes, PPARγ ablation induced lipodystrophy, indicating that PPARγ is not only involved in adipocyte differentiation, but also in adipocyte maintenance/survival, and that RXRα and RXRγ are its heterodimeric partners.

New Signalling Pathway in Lipodystrophy

Conditional genetic mutation of PPARγ or RXRs alleles in adipocytes induces lipodystrophies. Lipodystrophies are a heterogenous group of adipose tissue disorders characterized by selective loss of fat from various parts of the body, and associated with insulin resistance, hypertriglycemia, hepatic steatosis and early onset of diabetes in humans and mice. Genetic mutation of Seipin, AGPAT2, Lipin and Lamin A/C loci lead to lack or reduced number of adipocytes in humans and mice.[12–17] Genetic mutation of Seipin or AGPAT2 lead to one type of human lipodystrophy, congenital generalized lipodystrophy, and Lamin A/C mutation leads to another type of lipodystrophy, called Dunnigan type familial partial lipodystrophy, thus several signalling pathways may lead to a variety of lipodystrophies.

To identify the cascade from PPARγ/RXRs function leading to lipodystrtophy in mice, expression of Seipin, AGPAT2, Lipin and Lamin A/C were analyzed in PPARγ$^{ad-/-}$ and RXRαγ$^{ad-/-}$ mice. Surprisingly, the expression of all the genes are impaired in both mice, thus one signalling pathway (PPARγ/RXRs) regulates various types of lipodystrophies. This master regulator of lipodystrophies is PPARγ and RXRs heterodimers. It may suggest linkages between different types of lipodystrophies with PPARγ/RXRs signalling.

NR Signalling in Obesity, Diabetes and Lipodystrophy

NR signalling pathways regulate a number of adipocyte functions. RXRα is a key player in the link between obesity and diabetes. Using conditional ablation of RXRα in adult adipocyte leads to diabetes without obesity, thus diabetes and obesity are distinguished by RXRα function in adipocytes. Moreover, PPARγ/RXRs are master regulators for a variety of lipodystrophies.

Functional Roles of NRs in Liver Regeneration

The liver is a vital organ which performs critical functions including energy homeostasis, protein synthesis, bile secretion and detoxification. It has an optimal functional mass that is set in relationship to the body size. The highly differentiated adult hepatocytes have a half-life of several months. However, they can very rapidly begin to replicate in response to conditions that induce cell loss by physical, infectious or toxic injury, until the liver regains its functional mass, and returns to a non-proliferative state. The exact signals that control the functions and the size of the liver are not clearly defined.

To perform conditional somatic mutagenesis in hepatocytes, we generated transgenic mice that express Cre-ER^T under the control of the α-anti-trypsin promoter (αAT-Cre-ER^T). We selected one line which expresses selectively the tamoxifen-inducible Cre-ER^T recombinase in more than 50% of the hepatocytes, and efficiently induced recombination of floxed DNA segments after Tam treatment.[18]

Functional Role of RXRα in Hepatocytes

Many members of the NR superfamily including RXRs are expressed in the liver, as well as a wealth of genes that contain response elements for RXR-NR heterodimers. As RXRα is the most abundant of the three RXR isotypes in the liver, it may play a prominent role in hepatic growth, regeneration and homeostatic functions. The possible postnatal role of RXRα could however not be analysed in knock-out mice lacking RXRα (RXRαKO), as RXRαKO fetuses die at E13.5–E16.5.[9]

Using αAT-Cre-ER^T mice, as well as transgenic mice expressing Cre under the control of the albumin promoter selectively in hepatocytes (Alb-Cre; gift from A.

Magnusson), RXRα was efficiently and selectively ablated in hepatocytes either after Tam treatment, or in a constitutive manner during development, respectively. We have demonstrated that RXRα plays important cell-autonomous functions in the mechanism(s) involved in the physiological lifespan of hepatocytes and during liver regeneration after partial hepatectomy (PH). Our results show that the lifespan of adult hepatocytes lacking RXRα is shorter than that of their wild-type counterparts. Moreover, proliferative hepatocytes of a regenerating liver lacking RXRα exhibited an even shorter lifespan. This shortening of the lifespan was accompanied by increased polyploidy and multinuclearity.[19]

Estrogen Signalling Triggers Liver Regeneration

Several lines of evidence indicate that the liver is an oestrogen target tissue in vertebrates. Indeed, the level of a number of liver proteins, including coagulation factors and fibrolytic proteins, sex hormone binding globulin are regulated by oestrogens in mammals.[20] Moreover, *in vitro* studies suggest a supportive role for estrogens in hepatocyte proliferation, and estrogens have been shown to be involved in the control of liver growth in mammalian neonate, and to stimulate hepatocyte proliferation and liver weight recovery after PH or liver transplantation.[21] Most of the actions of oestrogens are mediated by two NRs, the oestrogen receptor α (ERα) and β (ERβ). However, in the liver, only ERα has been shown to be expressed.

To investigate the oestradiol signalling pathway in hepatocytes, we analysed ovariectomized and E2-treated WT and ERα-null mice,[22] as well as mice in which ERα is selectively ablated in hepatocytes ($ER\alpha^{hep-/-}$ mice) obtained with Alb-Cre mice. The study demonstrated that E2 is an early signal, upstream of TNFα, IL-1α, IL-1β and NF-κB that is released after PH and stimulates hepatocyte proliferation, and that hepatocyte ERα plays a central role for transducing the E2 proliferation signal in hepatocyte as well as Kupffer cells before and after PH, in both males and females. Moreover short oestrogen treatments might possibly be used in the clinic to improve liver regeneration.

Conclusion

The results demonstrate the feasibility of targeted spatio-temporally-controlled somatic mutagenesis in hepatocytes and adipocytes. Importantly, the established systems are not only valuable to study NR function, but also to investigate the function of any gene in these cell types. The studies should provide animal models for a number of human diseases, in addition to that we have already established.

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