



Review

Multi-Functional Roles of Stat3 Revealed by Conditional Gene Targeting

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Abstract. Signal transducer and activator of transcription (STAT) is a family of transcription factors composed of seven members. Gene-targeted mice of each STAT family protein displayed defective responses to cytokines, demonstrating an important role in cytokine-mediated biological responses. However, unlike the mice lacking other STAT proteins, Stat3-deficient mice died during their early embryogenesis. Therefore, in an attempt to avoid the lethality and assess the role of Stat3 in cytokine-mediated functions in mouse adult tissues, conditional gene targeting utilizing a Cre-loxP system was achieved. By this method, Stat3 was disrupted in several types of tissue, including T cells, macrophages, skin, and mammary gland. Analyses of these Stat3-mutant mice revealed important roles of Stat3 in biological functions in each tissue.

Key words: Stat3; conditional gene targeting; cytokine; signal transduction.

Introduction

The signal transducer and activator of transcription (STAT) family of transcription factors is activated by cytokines. So far, 7 mammalian STAT families have been identified and shown to be involved in cytokine-mediated signaling pathways^{6, 8, 19}. The biological roles of STAT family proteins have been clearly demonstrated through the generation of gene-targeted mice. Knockout mice of each of the STAT family members displayed impaired responses to cytokines, demonstrating that STAT proteins have essential roles in cytokine-mediated biological responses³. Stat1-deficient mice were defective in interferon (IFN)-mediated functions and showed high susceptibility to viral infections^{7, 14}.

Stat4- and Stat6-deficient mice were impaired in interleukin (IL)-12- and IL-4-mediated functions, leading to defective Th1 and Th2 responses, respectively^{10, 11, 20, 24, 26}. Stat5a- and Stat5b-deficient mice showed defective functions mediated by several cytokines, such as prolactin, growth hormone, and IL-2^{9, 13, 15, 16, 25, 27}. However, in contrast to knockout mice of the other STAT family members, Stat3-deficient mice died during their early embryogenesis, displaying rapid degeneration between 6.5 days post coitum (dpc) and 7.5 dpc²³. Thus, in classical gene targeting, the role of Stat3 in cytokine-mediated functions was not elucidated due to the early embryonic lethality.

Stat3 is known to be activated in response to a variety of cytokines, such as IL-6 family cytokines (IL-6,

Abbreviations used: STAT – signal transducer and activator of transcription, ES – embryonic stem, IL-2R α – interleukin 2 receptor α chain, IFN – interferon, NO – nitric oxide, IGFBP5 – insulin-like growth factor binding protein 5.

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IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and cardiotrophin-1), growth factors (EGF, CSF-1, and PDGF), IFN- γ , IL-10, and IL-2². To address the roles of Stat3 in adult tissues, we utilized a Cre-loxP recombination system to delete Stat3 in a cell- or tissue-specific manner. Using this conditional gene targeting technique, we disrupted Stat3 in T cells, macrophages, skin, and mammary gland. Analyses of these Stat3-mutant mice revealed its essential role in cytokine-induced biological functions in mice.

Generation of Tissue-Specific Stat3-Deficient Mice

A classical approach of gene targeting sometimes resulted in embryonic lethality, in which case we could not examine the role of the gene in adult tissues. The Stat3-deficient mouse was such a case. To avoid lethality, conditional gene targeting has recently been achieved by applying a Cre-loxP recombination system to the classical gene targeting¹⁷. The Cre recombinase recognizes a palindromic sequence motif of 34 bp, termed the loxP site. When a DNA fragment is flanked by two loxP sites in the same orientation, Cre recombinase excises the fragment from the DNA. In conditional gene targeting, targeted embryonic stem (ES) cells were obtained by introducing a floxed-Stat3 construct with a neomycin-resistance gene. Floxed-Stat3 ES cell clones were then obtained from the targeted ES cells through excision of the neomycin resistance gene by transient expression of a Cre expression vector. Floxed-Stat3 mice were generated according to the strategy of classical gene targeting using floxed-Stat3 ES cell clones. In floxed-Stat3 mice, the *Stat3* gene was flanked by two loxP sites. Next, floxed-Stat3 mice were crossed with mice in which the Cre-transgene was expressed in a cell- or tissue-specific manner, leading to the generation of mutant mice lacking Stat3 in a cell- or tissue-specific manner.

Stat3 is Indirectly Involved in T Cell Proliferation

By crossing floxed-Stat3 mice with transgenic mice expressing Cre recombinase under the control of the *Lck* promoter, Stat3 was knocked out in T cells²². In these mutant mice, T cell development was not significantly affected. However, Stat3-deficient T cells displayed severely impaired cell proliferation when stimulated with IL-6 plus anti-CD3 antibody or with IL-6

plus concanavalin A. Although IL-6 did not induce cell cycle progression by itself, it prevented apoptosis of normal T cells. In Stat3-deficient T cells, IL-6-induced prevention of apoptosis was not observed. Thus, the impairment of IL-6-mediated prevention of apoptosis resulted in the reduced T cell proliferation in Stat3-deficient T cells. Interestingly, IL-6 normally induced the expression of Bcl-2 in Stat3-deficient T cells. These findings indicate that IL-6-induced Stat3 activation in T cells is responsible for the prevention of apoptosis independently of Bcl-2 expression.

Stat3-deficient T cells also displayed partial impairment in IL-2-induced cell proliferation¹. When Stat3-deficient T cells were stimulated with a low concentration of IL-2, growth response was severely reduced compared with wild-type T cells. However, when Stat3-deficient T cells were cultured with a high concentration of IL-2, these cells showed almost the same growth response as did wild-type T cells. IL-2 is known to induce expression of the IL-2 receptor α chain (IL-2R α) to form the high-affinity IL-2 receptor complex. When Stat3-deficient T cells were stimulated with a low concentration of IL-2, induction of IL-2R α was severely impaired; however, a high concentration of IL-2 induced almost the same level of IL-2R α as in the case of wild-type T cells. Thus, IL-2-induced augmentation of IL-2R α expression was partially impaired in Stat3-deficient T cells. These might cause the partial reduction in IL-2-induced T cell proliferation in Stat3-deficient T cells.

Stat3 Activation in Macrophages and Neutrophils is Indispensable for Anti-Inflammatory Responses

Mice lacking Stat3 in macrophages and neutrophils were generated by crossing floxed-Stat3 mice with mice expressing Cre recombinase under the control of lysozyme M promoter²⁴. Lysozyme M is expressed in well-differentiated macrophages and neutrophils. The Stat3-mutant mice displayed high sensitivity to lipopolysaccharide (LPS)-induced endotoxin shock, showing a high mortality rate and increased serum concentration of inflammatory cytokines. Furthermore, the Stat3-mutant mice developed chronic enterocolitis with age. In addition, CD4⁺ T cells from the Stat3-mutant mice produced a significantly increased level of IFN- γ when compared with wild-type T cells. These demonstrate that the Stat3-mutant mice developed chronic inflammation with an enhanced Th1 cell activity.

When macrophage activity was analyzed, Stat3-deficient macrophages produced a significantly increased level of inflammatory mediators, such as tumor necrosis factor α (TNF- α), IL-6 and nitric oxide (NO), in response to LPS. Furthermore, Stat3-deficient macrophages showed an enhanced expression of MHC class II and B7-1, both of which are up-regulated in activated macrophages. These indicate that Stat3-deficient macrophages were highly activated. The finding that the Stat3-mutant mice displayed increased inflammatory responses indicates that Stat3 activation in macrophages and neutrophils is critical for anti-inflammatory responses in mice. Interestingly, the phenotypes of Stat3-mutant mice were quite similar to those of IL-10 knockout mice^{4, 12}. Therefore, IL-10-mediated functions in Stat3-deficient macrophages were analyzed. IL-10 pretreatment dramatically reduced LPS-induced production of several inflammatory mediators, such as TNF- α , IL-6 and NO, in wild-type macrophages and neutrophils. However, an IL-10-induced reduction in the production of inflammatory mediators was not observed in Stat3-deficient macrophages and neutrophils. Thus, the IL-10-mediated suppression of macrophage activity was abolished in the Stat3 mutant mice, indicating that the IL-10-induced Stat3 activation in macrophages and neutrophils is mandatory for anti-inflammatory responses.

Stat3 Is Responsible for Skin Remodeling

In addition to the immune system, Stat3 was disrupted in the skin using Keratin 5-Cre transgenic mice¹⁸. The Stat3-mutant mice showed normal development of epidermis and hair follicles when young. However, the Stat3-mutant mice displayed a severely compromised process of the second hair cycle, expressed sparse hair and developed spontaneously occurring ulcers with age. In addition, when the Stat3-mutant mice were wounded with a biopsy punch, wound healing was markedly delayed. The proliferation and differentiation of keratinocytes are regulated by several growth factors and cytokines. These include EGF, TGF- α , keratinocyte growth factor, hepatocyte growth factor, and IL-6, all of which can activate Stat3. In Stat3-deficient keratinocytes, the proliferative response was not impaired; however, *in vitro* migration in response to these factors was severely impaired. These findings indicate that Stat3 is essential for skin remodeling, such as hair cycle and wound healing, through the growth factor-dependent regulation of keratinocyte migration.

Stat3 Is Required for Involution of Mammary Gland

Stat5a knockout mice showed defective mammary gland development and lactation during pregnancy, displaying the important role of Stat 5a in mammary gland development^{13, 25}. Indeed, Stat5a is activated during pregnancy and lactation. However, expression of Stat5a is rapidly reduced during involution. In contrast, Stat3 becomes activated as involution starts.

The role of Stat3 during mammary gland development was analyzed in the mice lacking Stat3 in the mammary gland⁵. These mutant mice were generated by crossing β -lactoglobulin (BLG)-Cre transgenic mice with floxed-Stat3 mice. In the Stat3-mutant mice, a decrease in the apoptosis of mammary gland epithelial cells and a significant delay of involution after weaning were observed. Thus, Stat3 is required for the involution of the mammary gland. Insulin-like growth factor binding protein 5 (IGFBP5) has been proposed as inducing the apoptosis of epithelial cells of the mammary gland, and its expression was dramatically enhanced during involution. In Stat3-deficient mammary tissue, induction of IGFBP5 expression was severely reduced, which might be a cause of the reduced apoptosis in Stat3-deficient mammary gland epithelial cells. This study clearly demonstrates the importance of Stat3 in the induction of apoptosis.

Stat3 Has Important Roles in Biological Functions Induced by a Variety of Cytokines

Classical gene targeting of Stat3 revealed its essential role in the embryonic development of mice. However, the role of Stat3 in cytokine-mediated functions was not delineated, due to early embryonic lethality. To avoid the lethality, we conducted conditional gene targeting, which led to the elucidation of important roles of Stat3 in several aspects of cytokine-mediated biological functions in mice (Table 1). First, Stat3 has an important role in the IL-6-induced anti-apoptotic activity in T cells; in contrast, Stat3 is responsible for the induction of apoptosis of epithelial cells during mammary gland involution. It is of note that one protein has opposing functions in different tissues. This outcome might be because Stat3 induces distinct target genes in different tissues, that is, Stat3 induces expression of anti-apoptotic gene in T cells, whereas genes responsible for apoptosis, such as IGFBP5, is induced in the mammary gland. Second, the activity of cells is dif-

Table1. Phenotypes of tissue-specific Stat3-deficient mice

Tissue	Phenotype
T cell	Impaired IL-6-dependent T cell growth due to lack of prevention of apoptosis Partial defect in IL-2-dependent T cell growth due to impaired IL-2R α expression
Macrophage, neutrophil	Development of chronic enterocolitis and enhanced Th1 activity due to lack of IL-10-mediated suppression of macrophage activity
Skin	Compromised hair-cycle and wound-healing processes due to impaired growth factor-dependent migration of keratinocytes
Mammary gland	Delayed involution due to decrease in apoptosis of mammary epithelial cells

ferentially regulated through the activation of distinct STAT proteins. In macrophages, IFN- γ -induced phosphorylation of Stat1 leads to activation of cells. In contrast, IL-10-induced Stat3 activation leads to suppression of macrophage activity. The activity of normal macrophages is finely regulated through the balance between Stat1 and Stat3 activation. The absence of Stat1 resulted in defective macrophage activity and a high susceptibility to viral infection in mice. The absence of Stat3 resulted in a high activity of macrophages and development of chronic inflammation in mice. A cell's fate is also regulated through the activation of distinct STAT family members. In the mammary gland, activation of Stat5a is responsible for the development of mammary gland and lactation, and Stat3 activation is indispensable for the involution of mammary epithelial cells after weaning.

Conclusion and Future Prospects

Conditional gene targeting of Stat3 revealed its important roles in a variety of aspects of biological function. Stat3 is a transcription factor involved in the signaling pathways of several cytokines. Therefore, it is expected that genes essential for the cytokine-mediated biological responses are absent or reduced in Stat3-deficient tissues. Identification of target genes of Stat3 in each tissue will lead to a clearer elucidation of the molecular mechanisms of cytokine-mediated responses in the future.

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