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# LOSS OF GPRC5A ENHANCES SURVIVAL IN NORMAL AND MALIGNANT LUNG EPITHELIAL CELLS BY ELICITING PERSISTENT STAT3 ACTIVATION INDUCED BY AUTOCRINE LIF

Yulong Chen

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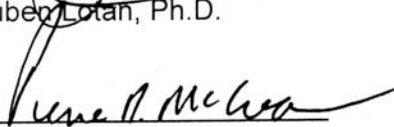
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By

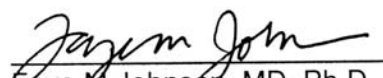
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MALIGNANT LUNG EPITHELIAL CELLS BY ELICITING PERSISTENT  
STAT3 ACTIVATION INDUCED BY AUTOCRINE LIF**

A  
DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment  
of the Requirements  
for the Degree of  
DOCTOR OF PHILOSOPHY

By

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Houston, Texas

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**LOSS OF GPRC5A ENHANCES SURVIVAL IN NORMAL AND  
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STAT3 ACTIVATION INDUCED BY AUTOCRINE LIF**

Publication No. \_\_\_\_\_

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Signal transduction and activator of transcription 3 (Stat3) is activated by cytokines and growth factors in many cancers. Persistent activation of Stat3 plays important role in cell growth, survival, and transformation through regulating its targeted genes.

Previously, we found that mice with a deletion of the G protein-coupled receptor, family C, group 5, member a (*Gprc5a*) gene develop lung tumors indicating that *Gprc5a* is a tumor suppressor. In the present study, we examined the mechanism of *Gprc5a*-mediated tumor suppression. We found that epithelial cells from *Gprc5a* knockout mouse lung (*Gprc5a*<sup>-/-</sup> cells) survive better in vitro in medium deprived of exogenous growth factors and form more colonies in semi-solid medium than their counterparts from wildtype mice (*Gprc5a*<sup>+/+</sup> cells). The phosphorylation of tyrosine 705 on Stat3 and the expression of Stat3-regulated anti-apoptotic genes Bcl-XL, Cryab, Hapa1a, and Mcl1 were higher in the *Gprc5a*<sup>-/-</sup> than in *Gprc5a*<sup>+/+</sup> cells. In addition, their responses to Lif were different; Stat3 activation was persistent by Lif treatment in the *Gprc5a*<sup>-/-</sup> cells, but was transient in the *Gprc5a*<sup>+/+</sup> cells. The persistent activation of Stat3 by Lif in

*Gprc5a*<sup>-/-</sup> cells is due to a decreased level of Socs3 protein, a negative inhibitor of the Lif-Stat3 signaling. Restoration of Socs3 inhibited the persistent Stat3 activation in *Gprc5a*<sup>-/-</sup> cells. Lung adenocarcinoma cells isolated from *Gprc5a*<sup>-/-</sup> mice also exhibited autocrine Lif-mediated Stat3 activation. Treatment of *Gprc5a*<sup>-/-</sup> cells isolated from normal and tumor tissue with AG490, a Stat3 signaling inhibitor, or with dominant negative Stat3(Y705F) increased starvation-induced apoptosis and inhibited anchorage-independent growth.

These results suggest that persistent Stat3 activation increased the survival and transformation of *Gprc5a*<sup>-/-</sup> lung cells. Thus, the tumor suppressive effects of Gprc5a are mediated, at least in part, by inhibition of Stat3 signaling through regulating the stability of the Socs3 protein.

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## **CHAPTER 1 INTRODUCTION**

### **1.1 Lung Cancer and Genetic Aberrations**

Lung cancer continues to be the leading cause of cancer death in both men and women in the United States and worldwide (Jemal et al., 2009). It is expected to account for 26% of all female cancer deaths and 30% of all male cancer deaths in United States in 2009 (Jemal et al., 2009). The fact that most lung cancer patients are diagnosed at advanced stages with metastasis when treatments are palliative causes the high mortality rate of this dreadful disease and high mortality happens even in early stage. The main types of lung cancer are non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). Lung cancer development is the consequence of multiple stepwise molecular and histopathological changes in the airway epithelial cells leading to invasive carcinoma and adenocarcinoma (Wistuba and Gazdar, 2006). The development of lung cancer is often attributed to many risk factors including cigarette smoking (Doll and Peto, 1976; Doll et al., 1994; Hecht, 1999), radon gas (Catelinois et al., 2006), asbestos (O'Reilly et al., 2007), air pollution (Chiu et al., 2006; Coyle et al., 2006; Kabir et al., 2007) and lung diseases such as chronic obstructive pulmonary disease (COPD) (Turner et al., 2007; Young and Hopkins, 2010). Tobacco smoke contains over 60 known carcinogens (Hecht, 2003) that have been implicated in lung carcinogenesis. Therefore, cigarette smoking is the major risk factor for lung cancer (Biesalski et al., 1998).



### Estimated New Cases\*

				Males	Females				
Prostate	192,280	25%				Breast	192,370	27%	
Lung & bronchus	116,090	15%				Lung & bronchus	103,350	14%	
Colon & rectum	75,590	10%				Colon & rectum	71,380	10%	
Urinary bladder	52,810	7%				Uterine corpus	42,160	6%	
Melanoma of the skin	39,080	5%				Non-Hodgkin lymphoma	29,990	4%	
Non-Hodgkin lymphoma	35,990	5%				Melanoma of the skin	29,640	4%	
Kidney & renal pelvis	35,430	5%				Thyroid	27,200	4%	
Leukemia	25,630	3%				Kidney & renal pelvis	22,330	3%	
Oral cavity & pharynx	25,240	3%				Ovary	21,550	3%	
Pancreas	21,050	3%				Pancreas	21,420	3%	
<b>All Sites</b>	<b>766,130</b>	<b>100%</b>				<b>All Sites</b>	<b>713,220</b>	<b>100%</b>	

### Estimated Deaths



				Males	Females				
Lung & bronchus	88,900	30%				Lung & bronchus	70,490	26%	
Prostate	27,360	9%				Breast	40,170	15%	
Colon & rectum	25,240	9%				Colon & rectum	24,680	9%	
Pancreas	18,030	6%				Pancreas	17,210	6%	
Leukemia	12,590	4%				Ovary	14,600	5%	
Liver & intrahepatic bile duct	12,090	4%				Non-Hodgkin lymphoma	9,670	4%	
Esophagus	11,490	4%				Leukemia	9,280	3%	
Urinary bladder	10,180	3%				Uterine Corpus	7,780	3%	
Non-Hodgkin lymphoma	9,830	3%				Liver & intrahepatic bile duct	6,070	2%	
Kidney & renal pelvis	8,160	3%				Brain & other nervous system	5,590	2%	
<b>All Sites</b>	<b>292,540</b>	<b>100%</b>				<b>All Sites</b>	<b>269,800</b>	<b>100%</b>	

Figure 1. Ten Leading Cancer Types for Estimated New Cancer Cases and Deaths, by Sex, United States, 2009. Adapted from (Jemal et al., 2009).

Cigarette smoking has been estimated to account for 87% of lung cancer cases (90% in men and 85% in women) in the United States (Samet et al., 1988).

However, about 10% of lung cancer patients are never smokers in the United States (Subramanian and Govindan, 2007; Subramanian and Govindan, 2010). These reports suggest that other factors, including different genetic background of individuals, and gene-environment interactions may play important roles in the development of lung cancer (Alberg and Samet, 2003; Gorlova et al., 2007; Hackshaw et al., 1997; Shields and Harris, 2000). Several genetic aberrations have been found in human non-small cell lung cancer (NSCLC) including mutations in epidermal growth factor receptor (EGFR) and RAS, Loss of Heterozygosity (LOH) of TP53 and RB, and promoter hypermethylation of RAR $\beta$  (Burbee et al., 2001; Meuwissen and Berns, 2005; Sato et al., 2007; Virmani et al., 2000; Zochbauer-Muller et al., 2001). Some of these genetic changes have been proved to induce lung carcinogenesis in transgenic mice including mutant K-ras<sup>G12D</sup> (Guerra et al., 2003; Jackson et al., 2001; Johnson et al., 2001; Tuveson et al., 2004) and mutant EGFR (Ohashi et al., 2008; Politi et al., 2006). Multiple signaling pathways including Akt, Erk and Stat3 are activated in mutant EGFR- and Kras<sup>G12D</sup>-induced lung tumors and deactivated during tumor regression suggesting these downstream signaling pathways are required to maintain tumor growth (Politi et al., 2006). Mutant EGFRs selectively promote cell survival by activating AKT and STAT signaling pathways but have no effect on proliferation (Sordella et al., 2004).

Treatment for lung cancer commonly includes surgery, chemotherapy, and radiation therapy. Non-small cell lung carcinoma is usually treated with cisplatin or carboplatin in combination with gemcitabine, paclitaxel, docetaxel, etoposide, or vinorelbine (Clegg et al., 2002). Cisplatin and etoposide are most commonly used to treat small cell lung carcinoma (Murray and Turrisi, 2006). Recently, the identification of molecular changes in lung tumors from never-smokers has generated new therapeutic strategies for this disease (Subramanian and Govindan, 2010). Most NSCLC patients show no response to the EGFR tyrosine kinase inhibitor gefitinib, whereas about 10 percent of patients have a rapid and dramatic clinical response (Lynch et al., 2004). Higher response rates and better outcomes are seen when never-smoker patients with advanced NSCLC are treated with EGFR tyrosine kinase inhibitors such as gefitinib or erlotinib compared to the rates and outcomes of patients with tobacco-associated lung cancer (Miller et al., 2004; Shepherd et al., 2005; Subramanian and Govindan, 2007). This can be explained by the finding that activated EGFR mutants are present more frequently in lung tumors from never smokers compared with tobacco-associated lung cancer (Subramanian and Govindan, 2007). Further, activating mutations in EGFR are correlated with clinical response of non-small cell lung cancer to tyrosine kinase inhibitor gefitinib (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). Although non-small cell lung cancer patients carrying EGFR-mutant have dramatic responses to EGFR inhibitors, patients may have a relapse, which is attribute to the presence of a second gefitinib resistance mutation or to the MET amplification

driving ERBB3-dependent activation of phosphoinositide 3-kinase (Engelman et al., 2007; Kobayashi et al., 2005). Although many cases of resistance are still undefined these data suggest that molecularly-targeted therapies using small molecules to inhibit the genetic aberrations, which tumor cells are addicted to will be an effective method to treat lung cancer patients (Linardou et al., 2009; Neal and Sequist, 2010). Although some genetic changes are found in lung tumors from patients, many lung cancer patients do not show changes in these potential targets. Therefore, there is a need to identify new biomarkers for early diagnosis and novel targets for lung cancer prevention and therapy, which may decrease the high mortality of lung cancer.

## **1.2 Signal Transducer and Activator of Transcription 3 (Stat3)**

Stat3 is a member of the Stat protein family. Stat3 is a cytoplasmic transcription factor that is rapidly activated in response to various cytokines [e.g., interleukine 6 (IL-6), oncostatin M, interleukin 5 (IL-5), interleukin 11 (IL-11), ciliary neurotrophic factor] and growth factors [e.g., epidermal growth factor (EGF)] that regulate cell proliferation, differentiation, survival, invasion, inflammation and immunity (Yu et al., 2009). For example, IL-6 and related cytokines bind to specific cell surface receptors that usually lack intrinsic tyrosine kinase activity but become associated through their cytoplasmic domain with tyrosine kinases such as the Janus kinase (JAK) family kinases (Yeh and Pellegrini, 1999). These kinases phosphorylate specific tyrosine residues in Stat proteins (e.g. Y705 in Stat3), leading to the formation of

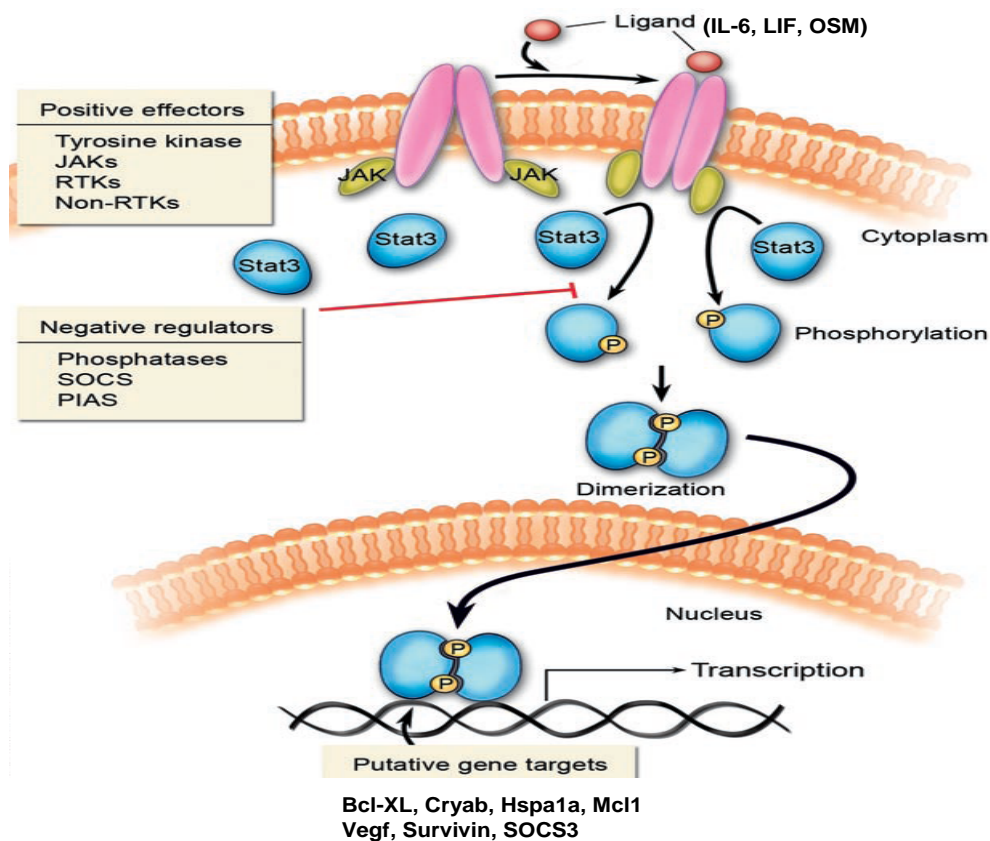


Figure 2. Model for Stat3 signaling. Stat3 is a transcription factor, which is activated in response to many cytokines and growth factors that bind to specific receptors. In the case of the nontyrosine containing cytokine receptors, the JAK kinases are recruited to the receptor complex and phosphorylate the receptor on tyrosine. This phosphorylation leads to recruitment of Stat3 protein to the receptor/kinase complex through the SH2 domain of Stat3. Stat3 is then tyrosine phosphorylated at a single residue (tyrosine 705) in its C-terminus. Tyrosine phosphorylation of the Stat3 leads to Stat3 dimerization and nuclear translocation. In the nucleus Stat3 binds through its DNA-binding domain (DBD) to consensus elements, resulting in gene transcription. Adapted from (Pedranzini et al., 2004).

homodimers or heterodimers with other Stat proteins by reciprocal interactions through phosphotyrosine–SRC homology 2 (SH2) domain then translocate into the nucleus and bind to consensus sequences in target genes to regulate gene transcription (Reich and Liu, 2006; Yu et al., 2009). In addition to specific tyrosine 705 phosphorylation, phosphorylation of Stat3 on serine 727 mediated by RSK2 is also required for maximal transcriptional activity of Stat3 (Decker and Kovarik, 2000; Wen et al., 1995; Zhang et al., 2003). Histone acetyltransferase p300-mediated Stat3 acetylation on Lys685 is important for Stat3 to form stable dimers, bind to DNA and regulate transcription of cell growth-related genes (Yuan et al., 2005). Activated Rac1 guanosine triphosphatase can form a complex with Stat3, which is critical for the Stat3 activation induced by growth factors (Simon et al., 2000). Substitution of 2 cysteine residues within the C-terminal loop of the SH2 domain of Stat3 produced a molecule that dimerized spontaneously, bound to DNA, and activated transcription (Bromberg et al., 1999).

Stat3 is an acute phase response transcription factor and Stat3 signaling is well-controlled by several mechanisms. Suppressor of cytokine signaling 3 (SOCS3), one of the Stat3 target genes, inhibits the activation of Stat3 through binding to the tyrosine kinase domain of JAK2 and serves as a negative feedback mediator of the Stat3 signaling (Masuhara et al., 1997). Protein inhibitor of activated Stat3 (PIAS3) blocks the DNA-binding activity of Stat3 and inhibits Stat3-mediated gene transcription by directly binding to Stat3 but did not interact with Stat1 nor affect its DNA-binding or transcriptional activity (Chung et

al., 1997). Stat3-beta is phosphorylated on tyrosine similar to Stat3 but co-expression of Stat3-beta inhibits the transactivation potential of Stat3 suggesting that Stat3-beta may function as a negative regulator of Stat3 (Caldenhoven et al., 1996).

Stat3 is a transcription factor critical for TH17 differentiation and required for the suppression of pathogenic TH17 responses by CD4<sup>+</sup> regulatory T cells [T (regs)] in mice (Chaudhry et al., 2009). Stat3 played a role in the cooperative signaling of BMP2 and LIF and the induction of astrocytes from neural progenitors by forming a complex with Smad1, which was bridged by p300 (Nakashima et al., 1999). Dominant-negative mutations in Stat3 were found in the hyper-IgE syndrome, an immunodeficiency syndrome showing increased innate immune response and recurrent infections (Holland et al., 2007; Minegishi et al., 2007). Specific deletion of Stat3 under the control of the Nes promoter-enhancer limited the migration of reactive astrocytes and resulted in markedly widespread infiltration of inflammatory cells, and neural disruption indicating that Stat3 is a crucial regulator of reactive astrocytes in the process of healing after spinal cord injury (SCI) (Okada et al., 2006). Stat3 is activated in airway epithelial cells and is required for repair of normal airway epithelial cell after naphthalene injury (Kida et al., 2008). Cell-selective deletion of Stat3 in respiratory epithelial cells did not alter prenatal lung morphogenesis or postnatal lung function but made the Stat3<sup>Δ/Δ</sup> mice more susceptible to oxygen-induced (exposure to 95% oxygen) injury than the wild type mice (Hokuto et al., 2004), whereas activation of the Stat3 pathway prevents hyperoxia-induced

inflammation and injury in the lung (Lian et al., 2005), suggesting that Stat3 plays important roles in preventing lung injury and in the repair process after lung injury.

It has also been shown that Stat3 can function as an oncogene and is frequently aberrantly activated in a variety of human solid tumor and blood malignancies including lung cancer, ovarian cancer, pancreatic cancer and lymphomas (Bromberg et al., 1999; Yu and Jove, 2004). Stat3 plays a critical role in both the initiation and the promotion stages of epithelial carcinogenesis. Mice with conditional knockout Stat3 in epidermal and follicular keratinocytes were completely resistant to DMBA-induced skin tumor development (Chan et al., 2004) indicating that Stat3 is required for the development of skin cancer induced by DMBA. Mice with specific Stat3 ablation in intestinal epithelial cells have reduced tumor growth and multiplicity indicating that Stat3 is required for the development of Colitis-Associated Cancer (Bollrath et al., 2009; Grivennikov et al., 2009). Inhibition of Stat3 by Stat3-beta, a dominant-negative variant, or Stat3 antisense in mouse tumor cell lines induced tumor-specific T-cell responses by upregulation of proinflammatory cytokines and chemokines that activate innate immunity and dendritic cells, which suggests that persistent activation of Stat3 suppressed the innate and adaptive antitumor immunity (Wang et al., 2004). Stat3 mediated constitutive NF-kappaB activity by enhancing NF-kappaB nuclear retention through acetyltransferase p300-mediated RelA acetylation, which interfered with NF-kappaB nuclear export, in both cancer cells and tumor-associated hematopoietic cells (Lee et al., 2009).



Together with C/EBP-beta, Stat3 functioned as synergistic initiator and regulator of mesenchymal transformation in malignant glioma (Carro et al., 2010). In addition to its nuclear transcriptional role, Stat3 was also located in the mitochondria and found to regulate metabolic function that supported Ras-dependent malignant transformation (Gough et al., 2009; Wegrzyn et al., 2009).

More than 60% of lung cancers have been reported to possess constitutively activated STAT3 (Song et al., 2003). One possible explanation for this activation is that the suppressors of cytokine signaling 3 (Socs3), which is an endogenous natural inhibitor of Stat3 signaling, is frequently silenced by hypermethylation in human lung cancer (He et al., 2003). Increased Stat3 activity regulates the expression of genes associated with wound healing and increased Stat3 activity is identified at the leading edge of lung tumors invading adjacent non-tumor stroma indicating that Stat3 is also important for tumor progression (Dauer et al., 2005). It has been reported that the Stat3 signaling pathway is activated in mutant EGFR and Kras<sup>G12D</sup>-induced lung tumors (Politi et al., 2006). A previous study has shown that mutations in the EGFR kinase domain mediate STAT3 activation by increasing IL-6 levels in primary human lung adenocarcinomas (Gao et al., 2007). Furthermore, inhibiting STAT3 function in fibroblasts abrogates transformation by mutant EGFR, indicating that STAT3 is required for the oncogenic effects of somatic EGFR mutations (Alvarez et al., 2006). Transgenic mice overexpressing constitutively activated Stat3 in alveolar type II epithelial cells exhibit severe pulmonary inflammation and develop spontaneous lung adenocarcinomas indicating that activating Stat3

in mouse lung is sufficient to induce these phenotypes (Li et al., 2007).

Since the JAK/STAT3 signaling pathway plays an important role in carcinogenesis, it has been targeted for cancer therapy (Costantino and Barlocco, 2008; Yu and Jove, 2004). For example, STAT3 activation can be suppressed by JAK inhibitors such as the tyrphostin AG 490 (Levitzki, 2002; Meydan et al., 1996) or AZD1480 (Hedvat et al., 2009), which induce apoptosis in cancer cells and inhibit tumor growth. Stat3 can also be targeted by inhibitors of its DNA-binding activity such as G-quartet oligonucleotides, which were found to promote apoptosis of non-small cell lung cancer (Weerasinghe et al., 2007).

### **1.3 LIF-LIFR/GP130-STAT3 Signaling**

Leukemia inhibitory factor (LIF) is one of the IL-6 family cytokines including ciliary neurotrophic factor (CNTF), oncostatin M, interleukin 11 (IL-11) and interleukin 6 (IL-6). The function of these cytokines is mediated by specific cell-surface receptors that have a unique  $\alpha$  chain and the shared signal transducer GP130 (Taga and Kishimoto, 1997). Activation of these receptors induces activation of the JAK family tyrosine kinases, which then stimulate multiple signaling pathways involving MAPKs, PI3Ks and STATs (Hong et al., 2007). LIF induces macrophage differentiation of the murine M1 myeloid leukaemia cell line indicating that it functions as a leukemia inhibitor (Gearing et al., 1987; Gough et al., 1988). LIF is also a human hemopoietic growth factor and maintains the proliferation of the murine interleukin-3-dependent leukemic

cell line suggesting it has both growth-promoting and differentiation-inducing activities depending on the target cell type (Moreau et al., 1988). LIF serves as a growth factor for the maintenance of the stem-cell phenotype of mouse embryonic stem (ES) cells *in vitro*, which have the potential to form chimaeric mice (Williams et al., 1988). The ureteric bud cells express and secrete LIF, and metanephric mesenchyme express its receptors, suggesting that LIF is a potential regulator of mesenchymal-to-epithelial transition during kidney development (Barasch et al., 1999). LIF is a pleiotropic cytokine present at the maternal-fetal interface and has been shown to play an essential role in the implantation of the embryo in mice (Escary et al., 1993; Stewart et al., 1992). Human leukocyte antigen type G (HLA-G) is a class I MHC molecule specifically expressed by human invasive cytotrophoblast cells and has been suggested to play a role in facilitating the immune tolerance of the conceptus (Aldrich et al., 2001; Hviid et al., 2003; Ober et al., 2003; Pfeiffer et al., 2001). LIF induced the expression of HLA-G mRNA and activated the HLA-G promoter indicating that LIF may play an important role in modulating HLA-G production and immune tolerance of conceptus at the maternal-fetal interface (Bamberger et al., 2000). LIF was also identified as a p53-regulated gene that functions as the downstream mediator of impaired implantation in female p53-null mice (Hu et al., 2007).

IL-6 family members were significantly increased in the lung and induced activation of Stat3 during *E. coli* pneumonia, which functions to promote neutrophil recruitment and to limit lung infection and injury (Quinton et al., 2008).

Transgenic mice with pulmonary expression of human LIF are more resistant to hyperoxia induced lung injury and LIF transgenic mice with IL-6 null mutation were more sensitive to the toxic effects of 100% O<sub>2</sub> than LIF-transgenic animals with a wild-type IL-6 indicating that LIF plays a protective role in hyperoxic acute lung injury partly through increasing the expression of IL-6 (Wang et al., 2003). The major source of LIF in human lung tissue is airway fibroblasts and airway smooth muscle cells, which indicate that LIF and other family cytokines play roles in the proliferative response of both epithelial and mesenchymal cells (Knight, 2001; Knight et al., 1999).

Smad-dependent induction of LIF in response to TGF-beta is required for the self-renewal capacity of Glioma-initiating cells (GICs) and prevents their differentiation which may be important for the initiation and recurrence of gliomas (Penuelas et al., 2009). IL-6 is an important activator of oncogenic STAT3 in lung adenocarcinomas and of Jagged-1/Notch signaling in breast tumor mammospheres (Grivennikov and Karin, 2008; Schafer and Brugge, 2007). LIF has been reported to act as an autocrine growth factor in human medulloblastomas, breast cancer, and pancreas carcinoma (Dhingra et al., 1998; Kamohara et al., 2007; Liu et al., 1999; Quaglino et al., 2007). LIF is a potential metastasis-promoting factor for melanoma and rhabdomyosarcomas (Maruta et al., 2009; Wysoczynski et al., 2007). It has been described that transformation of human bronchial epithelial cells increases the activation of STAT3 induced by LIF suggesting that altered cytokine responses may be an early event in primary premalignant cells and may play critical role in lung

tumorigenesis (Loewen et al., 2005). LIF mRNA was detected in some human lung cancer cell lines and LIF protein was found in the conditioned medium of human lung cancer cells (Kamohara et al., 1994) and human lung cancer xenografts (Kamoshida et al., 2006), suggesting that LIF may also be a growth factor in human lung cancer.

#### **1.4 GPRC5A as a New Lung Tumor Suppressor**

G protein-coupled receptor, family C, group 5, member A (GPRC5A) was cloned as a retinoic acid induced gene in UMSCC22B cells by differential display (Cheng and Lotan, 1998). GPRC5A is a protein with seven trans-membrane domains and is located mainly in the cell plasma membrane and perinuclear vesicles (Fig. 3). GPRC5A is an orphan receptor because the identity of its ligand is not known yet. In addition, it is not clear whether GPRC5A is activated by ligand binding or is constitutively activated by other mechanisms (Seifert and Wenzel-Seifert, 2002). The reason for this question is that the GPRC5A protein has a short (about 30 amino acids) extracellular amino terminal domain (ATD), whereas other members of the GPCR family C such as the glutamate receptor (mGluR1) and the calcium sensing receptor (CaR) have long ATDs (about 600 amino acids), which include their ligand-binding cleft (Brauner-Osborne et al., 2001). GPRC5A binds to frizzled receptors and may crosstalk and activate the non-canonical Wnt signaling pathway (Harada et al., 2007). GPRC5A was also involved in regulating the cAMP signaling and apoptosis (Hirano et al., 2006). GPRC5A was primarily and

abundantly expressed in human fetal and adult lung tissue and restricted to distal bronchiolar epithelial cells in postnatal lungs (Cheng and Lotan, 1998; Xu et al., 2005), which indicated that it might play important role in lung physiological and pathological function.

The expression of GPRC5A was suppressed in some human lung cancer cell lines and human non-small cell lung cancer when compared with the normal tissue from the same patient (Cheng and Lotan, 1998; Tao et al., 2007). In the absence of information on its activation mechanism, we attempted to gain some insight into the actions GPRC5A by restoration of its expression into tumor cells via transfection. Overexpression GPRC5A in H1792, and 293F cells decreased the anchorage-independent colony formation (Tao et al., 2007). The inhibition of anchorage-independent colony formation by expression GPRC5A in human lung cancer cells indicated that it may play a role in regulating cell survival and the transformed cell phenotype (Tao et al., 2007) and suggested that GPRC5A may function as a putative tumor suppressor in the lung. Controversially, other group reported that GPRC5A was a growth-promoting gene and a therapeutic target for breast cancer (Nagahata et al., 2005; Wu et al., 2005). To further confirm our hypothesis, we generated *Gprc5a* knockout mice; these mice do not have lung developmental defects (Tao et al., 2007). Another group has reported the similar result (Xu et al., 2005). Interestingly, we found that homozygous *Gprc5a* knockout (*Gprc5a*<sup>-/-</sup>) mice spontaneously developed many more lung tumors at an advanced age (incidence:

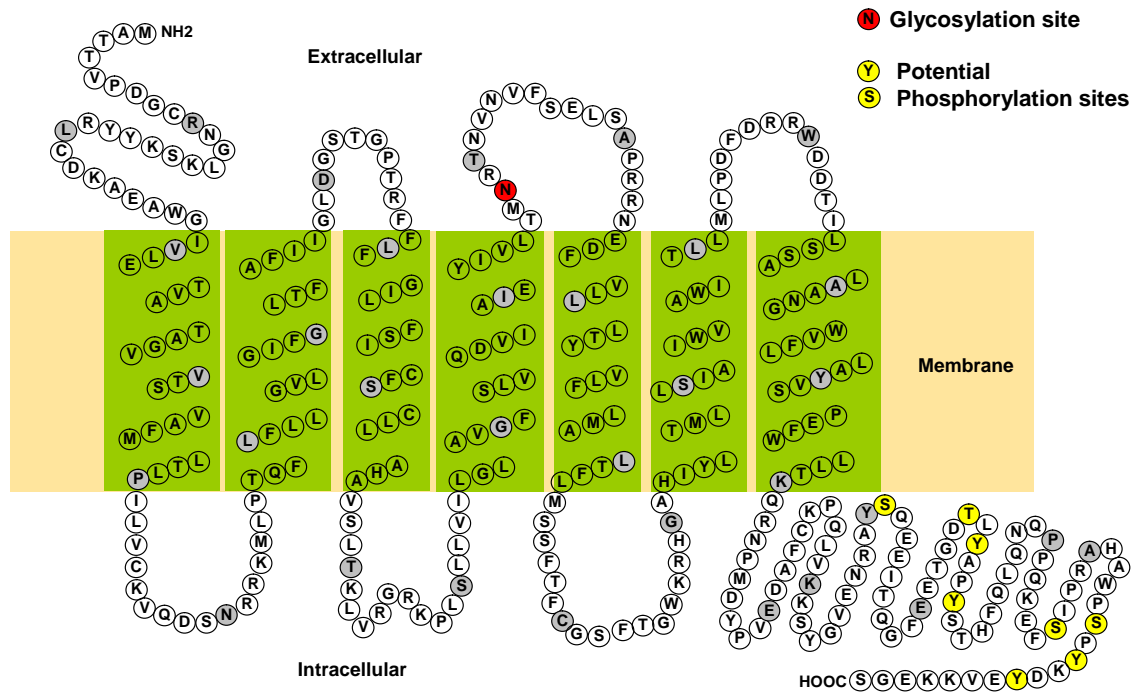


Figure 3. Schema of GPRC5A protein. GPRC5A is a protein with seven transmembrane domains. It has an N-glycosylation site at residue 158 and 8 potential phosphorylation sites predicted by using NetPhos 2.0 webtool (<http://www.cbs.dtu.dk/services/NetPhos/>).

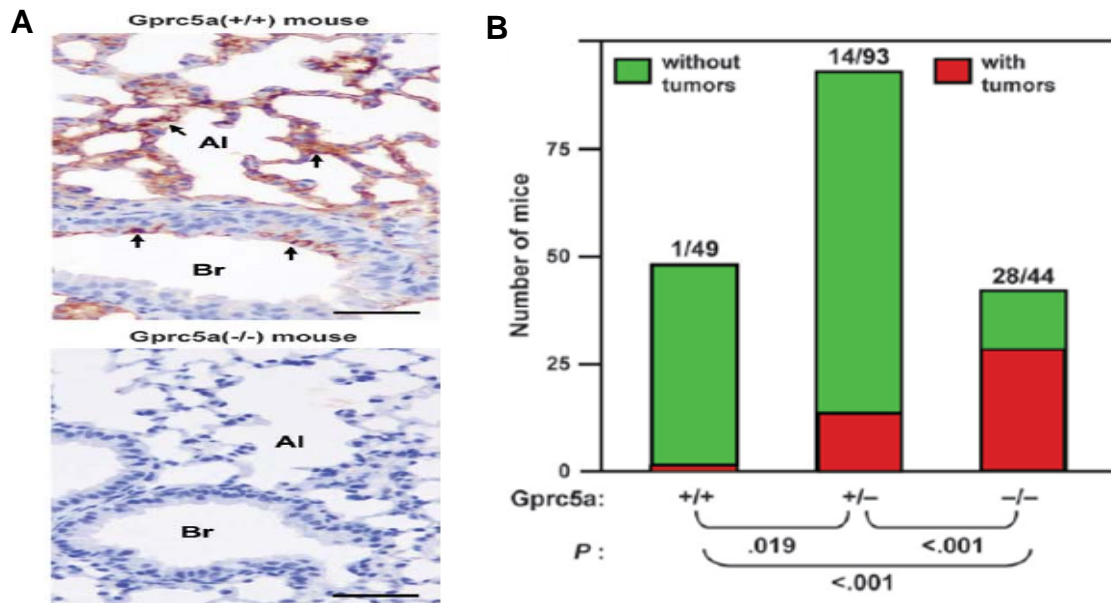


Figure 4. Gprc5a functions as a new lung tumor suppressor. A, The expression of Gprc5a protein was analyzed by immunohistochemical methods in sections of lung tissues from adult *Gprc5a*<sup>+/+</sup> or *Gprc5a*<sup>-/-</sup> mice. B, Mice were killed between the ages of 10 and 24 months, and tumors were detected by visual inspection of the lung exterior after necropsy. P values (calculated using a two-sided z test) at the bottom of the panel indicate the statistical significance of difference in tumor incidence in *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> mice compared with *Gprc5a*<sup>+/+</sup> mice (Tao et al., 2007).



76% adenomas and 17% adenocarcinomas) than wild type (*Gprc5a*<sup>+/+</sup>) (10% adenomas) (Fig. 4), which strongly indicated that *Gprc5a* functions as a tumor suppressor in mouse lung (Tao et al., 2007). 40% *Gprc5a* deficient mice developed pneumonia compared with 10% wild type group suggested that there are more inflammation in the *Gprc5a* knockout mice (Tao et al., 2007). Recently we reported that NF-kappaB activation was increased in *Gprc5a*<sup>-/-</sup> lung epithelial cells, which leads to increased autocrine and paracrine interactions, cell autonomy, and lung inflammation (Deng et al., 2010).

### **1.5 Hypothesis and project goals**

Previous studies have led to the conclusion that GPRC5A is a new lung specific tumor suppressor gene based on the following evidence: 1) *GPRC5A* is primarily and abundantly expressed in normal lung epithelial cells but its expression is decreased in human lung cancer cell lines and in human lung tumor tissues from patients. 2) *Gprc5a*<sup>-/-</sup> mice spontaneously developed many more lung adenomas and adenocarcinomas at advanced age than *Gprc5a*<sup>+/+</sup> mice. However, the mechanism(s) by which GPRC5A inhibits lung carcinogenesis are still not clear. It is worthy to determine these mechanisms because they may help us find new targets for lung cancer therapy and prevention to reduce the high mortality of lung cancer. Since Stat3 is an oncogene and the activation of Stat3 has been reported to occur in the majority of lung cancers, we hypothesize that loss of *Gprc5a* causes deregulated Stat3 signaling leading to gain of the transformed phenotypes, which contributes to

lung tumorigenesis in *Gprc5a*<sup>-/-</sup> mice. The objectives of my thesis research are to understand the importance of Stat3 signaling in lung carcinogenesis in *Gprc5a*<sup>-/-</sup> mice and the mechanisms by which loss of *Gprc5a* increases Stat3 activity.

First, we will find the differences between *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells isolated from tracheas of *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> mice. We will examine whether *Gprc5a*<sup>-/-</sup> normal airway epithelial cells present some of the transformed phenotypes like resistant to apoptosis and increased anchorage-independent growth. We will analyze the gene expression pattern using microarray and examine whether Stat3 signaling is deregulated and Stat3 targeted genes will differentially expressed between *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells, which may contribute to the transformed phenotype. Quantitative Realtime PCR and western blotting assays will be applied to verify the microarray data and deregulated signaling pathway. Stat3 activation will be determined by analyzing the tyrosine phosphorylation of Stat3 using western blotting in *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells.

Second, we will determine the mechanisms by which loss of *Gprc5a* increases Stat3 activity. We will examine whether Stat3 activation is induced by EGFR signaling since the culture medium contains EGF or by autocrine cytokine signaling. To examine whether *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells have different response to EGF leading to the different activation of Stat3, cells will be treated with EGF and analyzed for Stat3 activity. JAK inhibitors (AG490) will be used to examine whether Stat3 activity is induced by

the cytokines if Stat3 activation is not dependent on the EGFR signaling. *Gprc5a*<sup>-/-</sup> normal airway epithelial cells will be cultured in medium without any supplements to determine whether Stat3 activity is induced by autocrine Stat3 activator(s). We will determine whether different activation of Stat3 is due to different level of Stat3 activator(s) in the conditioned medium from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells or due to the different response to the Stat3 activator(s) in the conditioned medium. Neutralizing antibodies against different cytokines will be used to identify the Stat3 activator(s) in the conditioned medium of *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells. We will determine the mechanisms leading to the different level of Stat3 activator(s) in the conditioned medium from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells or the different response to the Stat3 activator(s). We will examine whether MDA959 tumor cells also have increased Stat3 activity and whether this activation is due to the same mechanism as *Gprc5a*<sup>-/-</sup> normal airway epithelial cells.

Finally, we will determine whether Stat3 activation is important for the transformed phenotypes of *Gprc5a*<sup>-/-</sup> normal airway epithelial cells and MDA959 tumor cells. *Gprc5a*<sup>-/-</sup> normal airway epithelial cells and MDA959 tumor cells will be transfected with dominant negative Stat3 construct (Y705F) to inhibit the activity of Stat3. The effect of inhibition Stat3 by the dominant negative Stat3 on the transformed phenotypes (apoptosis and anchorage independent growth) will be assayed. *Gprc5a*<sup>-/-</sup> normal airway epithelial cells and MDA959 tumor cells will also be treated with AG490, a JAK inhibitor, and effect of inhibition Stat3

AG490 on the transformed phenotypes (apoptosis and anchorage independent growth) will be assayed.

These studies will provide part of the mechanisms by which GPRC5A suppress lung tumorigenesis and Stat3 may play a critical role in the development of lung tumors in *Gprc5a*<sup>-/-</sup> mice. This mouse model may be a useful tool for chemoprevention study and for studies for Stat3 inhibitors *in vivo*.

## CHAPTER 2 MATERIALS AND METHODS

### 2.1 Cell lines and culture conditions

Epithelial cells were derived from normal tracheas of three week old *Gprc5a*<sup>-/-</sup> and *Gprc5a*<sup>+/+</sup> mice (C57Bl/6 x 129sv) F1 and from a spontaneous lung adenocarcinoma from a *Gprc5a*<sup>-/-</sup> mouse. Briefly, tracheas and tumor tissue were dissected from mice, cut into small fragments, and incubated in a tissue dissociating solution ACCUMAX (Innovative Cell Technologies, San Diego, CA). The dissociated cells and tissue fragments were then transferred to PRIMARIA tissue culture dishes (BD Biosciences, San Jose, CA) and incubated in AmnioMAX-C100 medium (Invitrogen, Carlsbad, CA). The epithelial cells that have grown in these dishes were detached by trypsinization, sub-cultured and grown in keratinocyte-serum-free medium (K-SFM) (GIBCO; Invitrogen, Grand Island, NY). The cell lines were karyotyped by G banding in the Institutional Molecular Cytogenetics Facility (MD Anderson Cancer center, Houston, TX) and found to be of mouse origin. The cells were expanded and numerous aliquots of low passage (5 to 8) were frozen in liquid nitrogen and thawed before use for up to three months. The normal epithelial cells were cultured in K-SFM supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE) (GIBCO, Invitrogen) and the tumor cells were cultured in Dulbecco's modification of Eagles medium (DMEM) and Ham's F12 medium (1:1) supplemented with 10% fetal bovine serum. 293T cells were cultured in Dulbecco's modification of Eagles medium (DMEM) and Ham's F12 medium

(1:1) supplemented with 10% fetal bovine serum.

## **2.2 Reagents and antibodies**

AG490 was purchased from EMD Chemicals (Gibbstown, NJ). Antibodies against pStat3(Y705), Stat3, pErk1/2, Erk1/2, Bcl-XL and Socs3 were purchased from Cell Signaling Technology (Danvers, MA). Mcl-1 antibody was purchased from Epitomics (Burlingame, CA). Hspa1a and Cryab antibodies were purchased from Assay Designs (Ann Arbor, MI). Flag antibody to detect expression of transfected flag-tagged dominant negative Stat3 was purchased from Sigma (St. Louis, MO). Neutralizing antibodies against mouse Lif, Il-6, Osm and normal goat IgG were purchased from R&D Systems (Minneapolis, MN). ESGRO® (LIF) was purchased from Millipore (Billerica, MA). EGF was purchased from Invitrogen.

## **2.3 Plasmid construction**

GPRC5A open reading frame was cloned by PCR from cDNA of 292G human lung carcinoma cell line using primers RAI3-F containing KpnI site (*Italic*) (GCAC *GGTACC* GCCACC ATG GCT ACA ACA GTC CCT GAT GGT TGC CGC AAT) and RAI3-R containing XbaI site (*Italic*) (GAGC *TCTAGA* CTA GCT GCC CTC TTT CTT TAC TTC ATA GTC TTT) and subcloned into pcDNA3.1(+) plasmid. To construct the pcDNA3.1(+)-GPRC5A-Myc plasmid, GPRC5A coding sequence with myc tag at its C-terminal was generated by PCR using primers RAI3-F and RAI3-Myc containing myc tag (underline) and

XbaI site (*Italic*) (GAGC *TCTAGA* CTA CAG ATC CTC TTC AGA GAT GAG TTT CTG CTC GCT GCC CTC TTT CTT TAC TTC ATA GTC TTT) and subcloned into pcDNA3.1(+) plasmid. To generate the pAcGFP1-Hyg-N1-GPRC5A plasmid, GPRC5A coding sequence was generated by PCR using primers RAI3-F and RAI3-R2 containing AgeI site (*Italic*) (GAGC *ACCGG* TGG GCT GCC CTC TTT CTT TAC TTC ATA GTC TTT) and subcloned in frame into pAcGFP1-Hyg-N1 plasmid (from Clontech). pCMV6-Entry-SOCS3 plasmid was purchased from Origene (Rockville, MD). To generate the pCMV-3Tag-8-SOCS3 plasmid expressing a flag tagged SOCS3 protein, the SOCS3 cDNA was released from pCMV6-Entry-SOCS3 plasmid by digested with BamHI and XhoI and subcloned into the pCMV-3Tag-8 plasmid. To generate the pDsred-Monomer-N1-SOCS3 plasmid, the SOCS3 coding sequence was generated by PCR using SOCS3 template released from pCMV-3Tag-8-SOCS3 plasmid and the primers hSOCS3-F containing XhoI site (*Italic*) (GCAC *CTCGAG* GCCACC ATG GTC ACC CAC AGC AAG TTT CCC GCC GCC) and hSOCS3-R2 containing EcoRI site (*Italic*) (GAGC *G AAT TCG* AAG CGG GGC ATC GTA CTG GTC CAG GAA CTC), then subcloned in frame into the pDsRed-Monomer-N1 plasmid (from Clontech). A plasmid containing mutant Stat3(Y705F) with flag tag generated in Dr. James E. Darnell, Jr's laboratory (The Rockefeller University, New York, NY) (Bromberg et al., 1998; Wen and Darnell, 1997) was purchased from Addgene (plasmid 8709). The plasmid pRc/CMV-Stat3(Y705F) was digested using SacI to release the Stat3(Y705F)-Flag cDNA, which was then subcloned into pIRES2-EGFP as an intermediate

step before releasing Stat3(Y705F)-Flag cDNA by digestion of pIRES2-EGFP-Stat3(Y705F)-Flag using BamHI and subcloning it into pIRESHyg3 to generate pIRESHyg3-Stat3(Y705F)-Flag expression vector.

## **2.4 Transfection reagent and stable transfected cells**

All transfections were done by using Eugene 6 transfection reagent purchased from Roche (Madison, WI). To generate stable cell line, *Gprc5a*<sup>-/-</sup> cells were transfected with pIRESHyg3 or pIRESHyg3-Stat3(Y705F)-Flag expression vector. After 48 hours, stably transfected cells were selected by adding hygromycin at 500 µg/ml (Clontech, Mountain View, CA) and cultured for 2 weeks.

## **2.5 Immunoblotting**

Cells were washed with PBS then lysed using the cell lysis buffer (Cell Signaling Technology) following the procedure described in manufacturer's protocol. Equal amounts of cell extract protein (20 µg) were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gels (10%) and transferred to a nitrocellulose membrane. After blocking with 5% (w/v) powdered non-fat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST), the membrane was incubated with first antibody diluted in 5% (w/v) Bovine Serum Albumin (BSA) solution in TBST at 4°C overnight. The membrane was washed three times (5 minutes each) with TBST and incubated with appropriate secondary antibody for 1 hour at room temperature. The membrane was washed three



times and detection of protein–antibody complexes was done by using SuperSignal West Pico Chemiluminescent Substrate from Thermo Fisher Scientific (Rockford, IL) and exposure to X-ray film. The signal strengths were quantitated by densitometric analysis using Quantity One software.

## **2.6 Immunoprecipitation (IP) assay**

Human embryonic kidney cell line 293T cells were transfected with the expression plasmids for GPRC5A and SOCS3. After 48 hours culture, the cells were washed with PBS and harvested using the cell lysis buffer (Cell Signaling Technology). The cell lysates were pre-cleared by incubating with protein G beads and mouse normal IgG control for 1 hour at 4°C. Then the lysates were immunoprecipitated by incubating them with anti-flag antibody or mouse normal IgG at 4°C overnight, then added protein G beads and incubated them at 4°C for one more hour. The beads were washed 5 times with PBS and protein complexes were released from the beads by adding SDS loading buffer and boiling for 10 minutes. The proteins were subjected to western blotting analysis.

## **2.7 Enzyme-linked immunosorbent assay**

Conditioned media from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells were harvested by centrifugation at 13000 rpm/min for 10 minutes and analyzed for the level of Lif by using Mouse LIF Quantikine ELISA Kit from R&D Systems following the manufacturer's protocol.

## **2.8 RNA extraction and real-time quantitative PCR**

Total RNA was extracted from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal tracheal epithelial cells using TRI Reagent from Molecular Research Center (Cincinnati, OH) according to the manufacturer's protocol. For reverse transcription (RT) reaction, 0.5 µg of total RNA of each sample was reverse transcribed into cDNA using RETROscript® Kit from A&B Applied Biosystems (Austin, TX) according to the manufacturer's instructions. Each cDNA was diluted 50 fold using UltraPure™ DNase/RNase-Free Distilled Water from Invitrogen for Quantitative real-time PCR reaction (QPCR). The primers for QPCR and TaqMan Gene Expression Master Mix were purchased from A&B Applied Biosystems. Mouse Actin was used as an internal control gene. In real time PCR, samples were denatured at 95°C for 10 minutes and 40 cycles were performed at 95°C for 15 seconds and at 60°C for 1 minute using Applied Biosystems 7500 Fast Real-Time PCR System. The expression data was analyzed and normalized to actin using the 7500 Fast System Software.

## **2.9 Anchorage-independent growth assay**

Cells were suspended in 0.2 ml of Matrigel (Collaborative Biomedical Products, Becton Dickinson Labware) diluted 1:1 (vol/vol) with growth medium. The mixture was then placed on top of 0.2 ml of semisolid 1% agarose in growth medium in each well of a 24-well cluster plate and incubated for 2 weeks. All the experiments were performed using triplicate wells. After 2 weeks, the total number of colonies (defined as >50 cells) in each well was counted under

an inverted microscope at X 40 magnification and photographed with a digital camera. The mean  $\pm$  SD of the number of colonies per well was calculated.

### **2.10 Apoptosis assay**

Apoptosis was measured by using FITC-Annexin V Apoptosis Detection Kit from BD Biosciences (San Jose, CA). Apoptosis was induced by starving cells in K-SFM medium without EGF and BPE for 48 hours. Cells were then washed twice with cold PBS and resuspended in 1X Binding buffer, then stained with FITC-Annexin V and PI (propidium iodide) for 15 minutes at room temperature in the dark and analyzed by flow cytometry. The assays were performed in triplicates and the mean  $\pm$  SD were calculated.

### **2.11 Statistical analyses**

All analyses were performed in triplicates and the significance of differences between groups was calculated using the student's t test. P value  $<0.05$  was considered to be statistically significant.

## CHAPTER 3 ISOLATION AND CHARACTERIZATION OF *GPRC5A*<sup>+/+</sup> AND *GPRC5A*<sup>-/-</sup> NORMAL AIRWAY EPITHELIAL CELLS

### 3.1 Isolation of the *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells

We have previously shown that *Gprc5a*<sup>-/-</sup> mice spontaneously develop lung adenoma and adenocarcinoma at an advanced age (Tao et al., 2007), which is similar to the process of human lung cancer development. However, the mechanisms by which GPRC5A suppresses lung tumor development are still unclear. To elucidate the mechanisms of GPRC5A lung tumor suppressor function, we established normal airway epithelial cell lines isolated from the tracheas of three week old *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> mice. These *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells grew well in vitro and showed epithelial morphology. To confirm that *Gprc5a* was deleted in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells, we harvested RNA and proteins from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells cultured in K-SFM supplemented with EGF and BPE for 48 hours, and analyzed them for the expression level of *Gprc5a* mRNA and protein. As shown in Figure. 5A and 5B, both mRNA and protein of *Gprc5a* were absent in *Gprc5a*<sup>-/-</sup> cells compared to *Gprc5a*<sup>+/+</sup> cells indicating that the cells exhibited the expected phenotypes.

### 3.2 *Gprc5a*<sup>-/-</sup> cells were resistant to starvation-induced apoptosis compared to *Gprc5a*<sup>+/+</sup> cells

Malignant transformation of cells usually involves increased survival

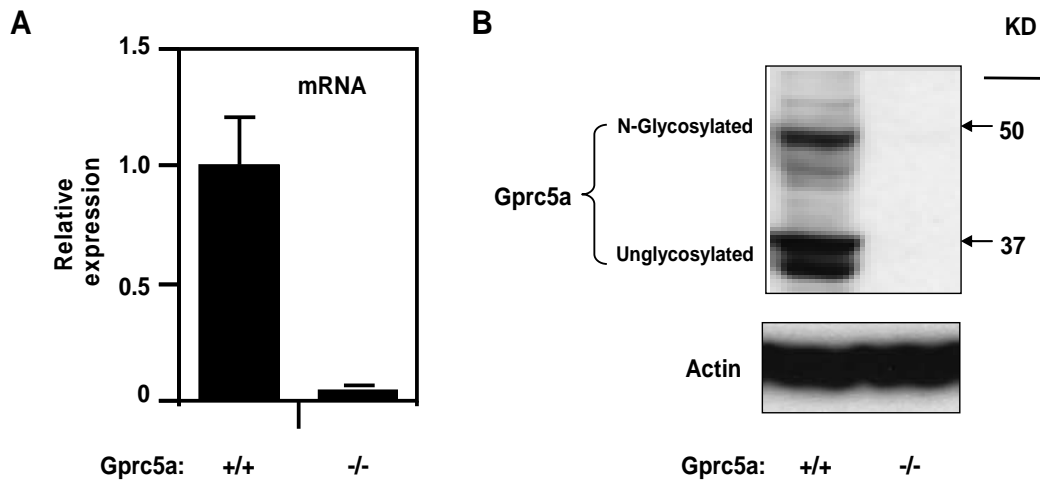


Figure 5. Characteristics of *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells. *A*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were cultured in K-SFM supplemented with EGF and BPE for 48 hours, then cells were extracted for RNA and analyzed for *Gprc5a* mRNA levels using QPCR. *B*, Cells cultured as in *A* were extracted for protein and analyzed for *Gprc5a* protein levels using western blotting.

signaling and resistance to apoptosis (Hanahan and Weinberg, 2000). Therefore, we compared the apoptosis of *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells under starvation. We observed that *Gprc5a*<sup>-/-</sup> cells had decreased apoptosis relative to *Gprc5a*<sup>+/+</sup> cells when starved for 48 hours in K-SFM without supplements (Fig. 6A). Specifically, starvation induced 22.9% cell death in *Gprc5a*<sup>+/+</sup> cells but only 9.1% in *Gprc5a*<sup>-/-</sup> cells (Fig. 6B) indicating that loss of *Gprc5a* resulted in increased cell survival.

### **3.3 *Gprc5a*<sup>-/-</sup> cells exhibited increased colony formation ability in Matrigel compared to *Gprc5a*<sup>+/+</sup> cells**

Next, we compared the ability of *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells to form colonies in semi-solid medium, an important property of transformed and tumor cells (Tucker et al., 1977). Since they were isolated from the normal tracheas, both *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells were not expected to form colonies in agar or agarose and indeed they failed to do so (data not shown). However, we found that *Gprc5a*<sup>-/-</sup> cells formed many more colonies than *Gprc5a*<sup>+/+</sup> cells when cultured in suspension in Matrigel (Fig. 7A). *Gprc5a*<sup>-/-</sup> cells formed about 40 colonies whereas *Gprc5a*<sup>+/+</sup> cells formed rare colonies (Fig. 7B). The increased anchorage-independent growth of *Gprc5a*<sup>-/-</sup> cells indicates that loss of *Gprc5a* tumor suppressor in normal airway epithelial cells caused acquisition of transformed phenotypes which are important for tumorigenesis.

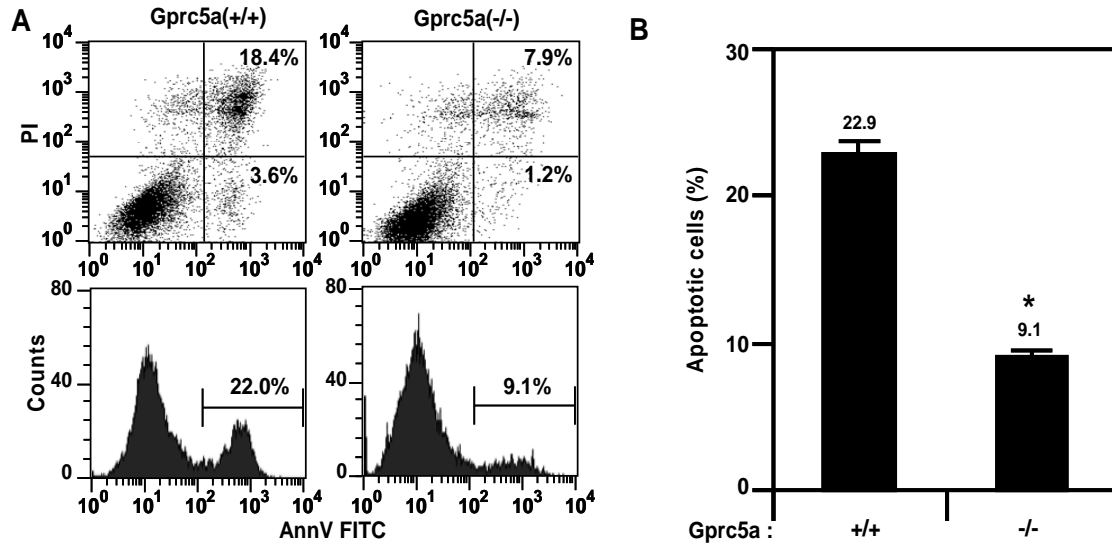


Figure 6. *Gprc5a*<sup>-/-</sup> normal airway epithelial cells are more resistant to starvation induced apoptosis compared to *Gprc5a*<sup>+/+</sup> cells. *A*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were cultured in K-SFM without supplements for 48 hours, then cells were double staining with PI and AnnV-FITC and then analyzed for apoptosis by flow cytometry. Results of one of the triplicates is presented. *B*, the mean  $\pm$  SD of three independent experiments in panel *A* was shown in bar graph. P value were calculated using Student T-test and \* means P < 0.05.

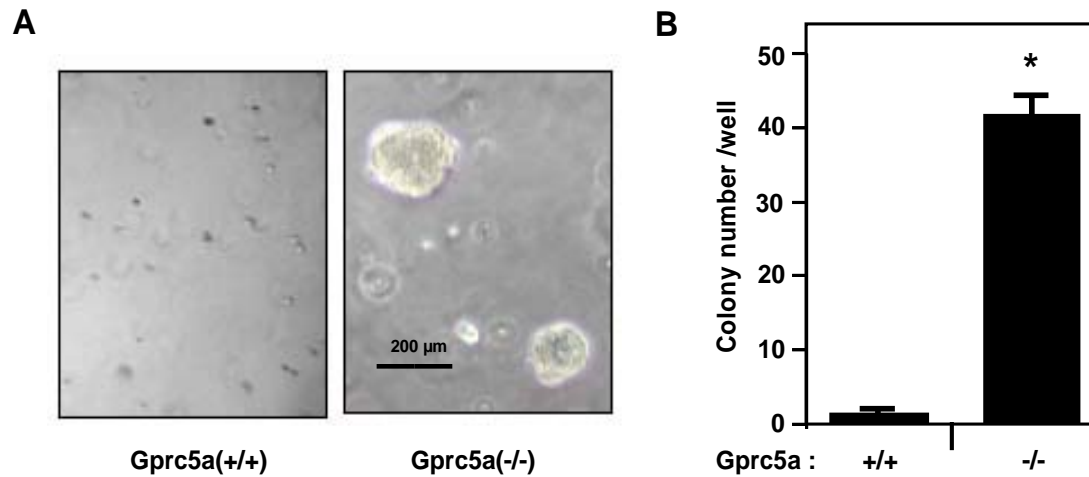


Figure 7. *Gprc5a*<sup>-/-</sup> normal airway epithelial cells exhibit anchorage independent growth ability. *A*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were suspended in Matrigel and cultured for two weeks to assay the colony formation. Photomicrographs of cultures at high magnification of one experiment were showed. *B*, colonies in three wells (each cell line) in panel *A* were counted and the data are presented in bar graph as mean  $\pm$  SD colonies/well. P value were calculated using Student T-test and \* means  $P < 0.05$ .



### **3.4 Stat3 signaling is deregulated in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells**

To investigate the difference in signal transduction pathways between *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells, we analyzed the transcription profiles of *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells using microarray and the signaling pathway using Ingenuity Pathway Analysis. We observed that many genes in the Stat3 pathway were differentially expressed between *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells (Fig. 8). Among these changed genes, we found that leukemia inhibitory factor (Lif), an IL-6 family member and upstream activator of Stat3 signaling, was increased in *Gprc5a*<sup>-/-</sup> cells compared to *Gprc5a*<sup>+/+</sup> cells (Fig. 8). Moreover, several anti-apoptotic genes which are Stat3 targets including Bcl-XL, Cryab, Hspa1a, and Mcl1 were upregulated in *Gprc5a*<sup>-/-</sup> cells relative to *Gprc5a*<sup>+/+</sup> cells (Fig. 8), which may explain the resistance to starvation induced apoptosis and increased colony formation of *Gprc5a*<sup>-/-</sup> cells.

### **3.5 Increased expression of anti-apoptotic genes in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells compared to *Gprc5a*<sup>+/+</sup> cells**

To further confirm that Stat3 regulated anti-apoptotic genes were upregulated in *Gprc5a*<sup>-/-</sup> cells compared to *Gprc5a*<sup>+/+</sup> cells, we analyzed the mRNA and protein levels of Bcl-XL, Cryab, Hspa1a and Mcl1 in *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells cultured in K-SFM medium supplemented with EGF and BPE for 48 hours. We found that the mRNA levels of these genes were significantly increased in *Gprc5a*<sup>-/-</sup> cells when compared to *Gprc5a*<sup>+/+</sup> cells (Fig. 9A). We

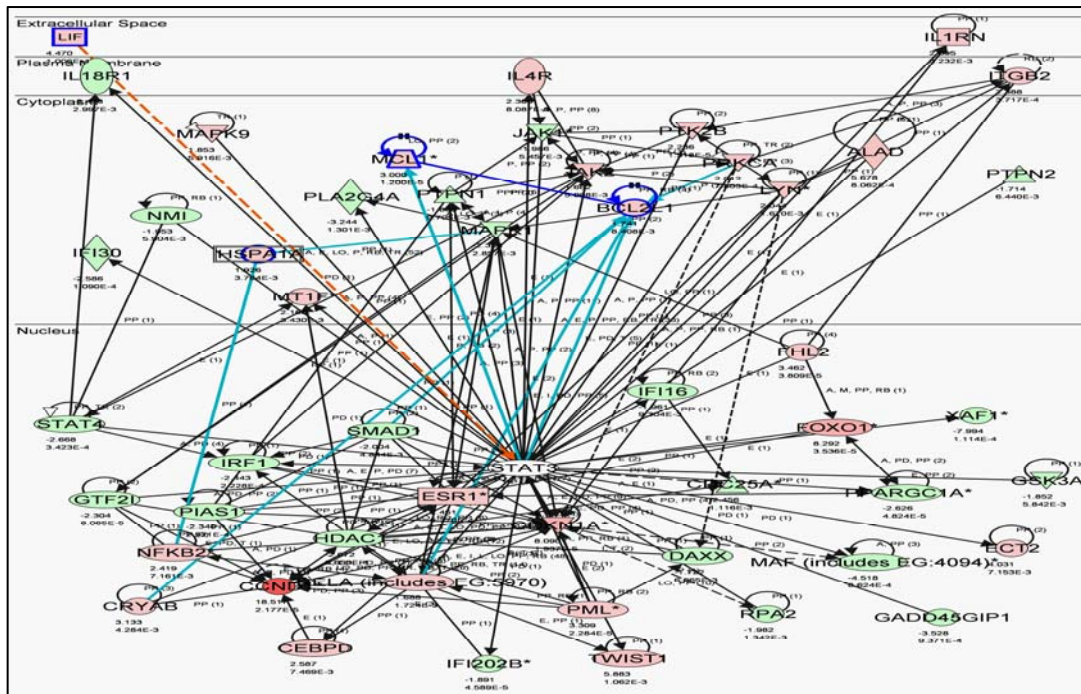


Figure 8. Deregulated Stat3 pathway in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells compared to *Gprc5a*<sup>+/+</sup> normal airway epithelial cells. *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were cultured in K-SFM supplemented with EGF and BPE for 48 hours, then cells were extracted for RNA and analyzed using Affymetrix microarray. The fold changes were calculated and Stat3 pathway analysis was done using Ingenuity Pathway Analysis (IPA).

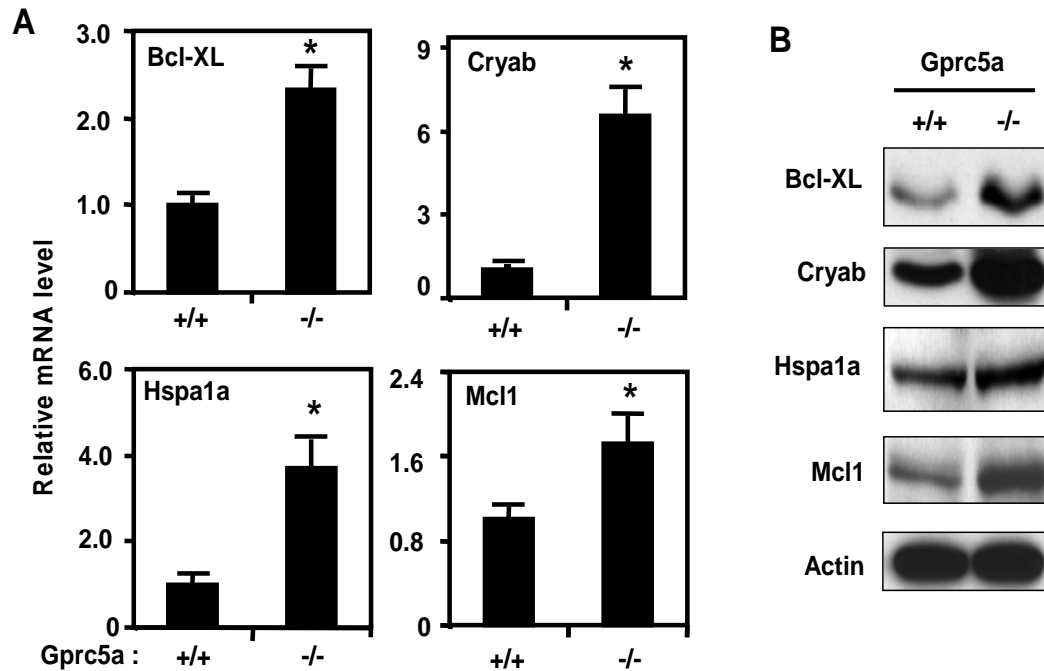


Figure 9. Increased expression of Stat3 regulated anti-apoptotic genes in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells relative to *Gprc5a*<sup>+/+</sup> cells. **A**, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were cultured in K-SFM without supplements for 48 hours, then RNA was extracted from the cells and analyzed for levels of Stat3 targeted genes by QPCR. \*, *P* < 0.05. **B**, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were cultured in K-SFM without supplements for 48 hours, then cells were extracted for protein and analyzed protein levels of Stat3 targeted genes by western blotting.

also observed that there *Gprc5a*<sup>-/-</sup> cells contained more Bcl-XL, Cryab, Hspa1a and Mcl1 proteins than *Gprc5a*<sup>+/+</sup> cells (Fig. 9B).

### 3.6 Discussion

In this thesis, we plan to explore the potential mechanisms by which the orphan G protein coupled receptor *Gprc5a* functions as a tumor suppressor in mouse lung in vivo. We isolated and established normal airway epithelial cell lines from tracheas of very young (3-week old) wild type mice (*Gprc5a*<sup>+/+</sup>) and knockout mice (*Gprc5a*<sup>-/-</sup>) after we found that mice with knockout of this gene develop lung adenomas and adenocarcinomas spontaneously (Tao et al., 2007). These cell lines will be very useful and suitable tools to study the lung-specific tumor suppressor function of *Gprc5a*. First, they were isolated from the airway epithelial cells where mouse lung tumors derived from so that they would be better than Mouse Embryonic Fibroblast (MEF) cells from these mice. Second, these cells were isolated from the normal airway of very young mice (only 3 weeks old) when no lung tumors developed, thus they may help us to find early biochemical and biological changes in the initiation stage of lung tumorigenesis in *Gprc5a*<sup>-/-</sup> mice.

The characterization of these cells as described here revealed that *Gprc5a*<sup>-/-</sup> normal airway epithelial cells isolated from normal trachea of a young mouse exhibited resistance to starvation-induced apoptosis and ability to form colonies in semi-solid medium, both of which are features associated with cell transformation (Hanahan and Weinberg, 2000). In contrast, *Gprc5a*<sup>+/+</sup> cells

were more sensitive to starvation-induced apoptosis than the *Gprc5a*<sup>-/-</sup> cells and failed to grow in semi-solid medium. However, the *Gprc5a*<sup>-/-</sup> cells are non-tumorigenic. Thus, our findings demonstrate that the *Gprc5a*<sup>-/-</sup> cells exhibit properties associated with the transformed cell phenotype but not the tumorigenic phenotype and therefore, they may be considered premalignant cells (Hanahan and Weinberg, 2000; Tucker et al., 1977). It is important to note that the acquisition of the malignant (tumorigenic) phenotype by cultured epithelial cells may require multiple genetic and epigenetic changes. For example human bronchial epithelial cells immortalized by overexpression of the human telomerase reverse transcriptase, transfection of CDK4 to bypass the p16/RB pathway, silencing the p53 pathway and introduction of mutant K-RASV12 or mutant EGFR, were able to form colonies in semi-solid medium but were still unable to form tumors in immunodeficient mice (Sato et al., 2006).

The finding that Stat3 signaling pathway was deregulated in *Gprc5a*<sup>-/-</sup> cells by the transcription profile analysis using microarray data and Ingenuity Pathway Analysis was really interesting since Stat3 is a well known oncogene and related to tumorigenesis (Bromberg et al., 1999). Bcl-XL has been reported to regulate cell apoptosis and promote cell survival through controlling the electrical and osmotic homeostasis of mitochondria (Boise et al., 1993; Vander Heiden et al., 1997). Cryab is a member of the small heat-shock protein family and inhibited caspase-3 activation during myogenic differentiation leading to decreased apoptosis (Dubin et al., 1990; Kamradt et al., 2002). Hspa1a is a negative regulator of apoptosis through directly binding to ASK1 and inhibiting

ASK1-dependent apoptosis (Park et al., 2002). Mcl1 is a potent multidomain antiapoptotic protein of the BCL2 family that heterodimerizes with other BCL2 family members to protect against apoptosis (Mott et al., 2007). The upregulation of these Stat3-regulated anti-apoptotic genes at both the mRNA and protein levels in the *Gprc5a*<sup>-/-</sup> cells compared to *Gprc5a*<sup>+/+</sup> cells suggested that Stat3 may be more activated in the *Gprc5a*<sup>-/-</sup> cells than in the *Gprc5a*<sup>+/+</sup> cells, which may contribute to the resistant to apoptosis and increased colony formation of *Gprc5a*<sup>-/-</sup> cells and the lung tumorigenesis in *Gprc5a*<sup>-/-</sup> mice. These observations are based on isolated *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells cultured *in vitro* and it will be worthy to look *in vivo* data by analyzing the mRNA and protein levels of these Stat3 regulated anti-apoptotic genes in lung tissues from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> mice.

## **CHAPTER 4 AUTOCRINE LIF INDUCED STAT3 ACTIVATION LEADS TO THE TRANSFORMED PHENOTYPES IN *GPRC5A*<sup>-/-</sup> CELLS AND MDA959 LUNG TUMOR CELLS**

### **4.1 Higher Stat3 activation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells compared to *Gprc5a*<sup>+/+</sup> cells**

Since we found that Stat3 signaling pathway was abnormal and Stat3 targeted genes were increased in *Gprc5a*<sup>-/-</sup> cells (CHAPTER 3), we investigated the status of Stat3 activation in *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells by analyzing the level of tyrosine (residue 705) phosphorylation of Stat3, which is an activated form of Stat3. As shown in Fig. 10, we found that Stat3 was more tyrosine phosphorylated in *Gprc5a*<sup>-/-</sup> cells than in *Gprc5a*<sup>+/+</sup> cells when cultured in K-SFM medium supplemented with EGF and BPE for 24 hours, indicating that Stat3 activation was increased in *Gprc5a*<sup>-/-</sup> cells.

### **4.2 EGF-independent activation of Stat3 in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells**

Stat3 can be activated by EGFR signaling (Gao et al., 2007; Politi et al., 2006). We next investigated whether Stat3 activation in *Gprc5a*<sup>-/-</sup> cells was mediated by EGF because the serum-free culture medium for the *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells contains EGF as a supplement. We found that EGF signaling was activated similarly in these cells as indicated by the transient increase in pErk1/2 level when treated with exogenous EGF (Fig. 11A). In contrast, the

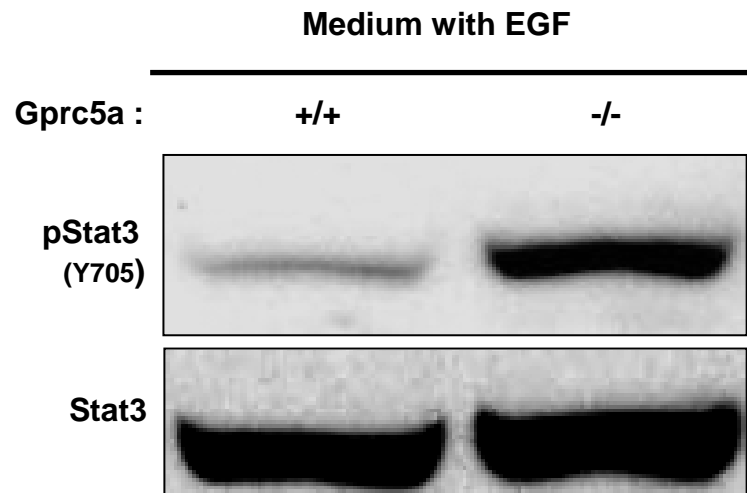


Figure 10. Stat3 is more activated in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells than in *Gprc5a*<sup>+/+</sup> cells. *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were cultured in K-SFM supplemented with EGF and BPE for 24 hours, then cells were extracted for protein and analyzed for the levels of phosphorylated and total Stat3 protein by western blotting.



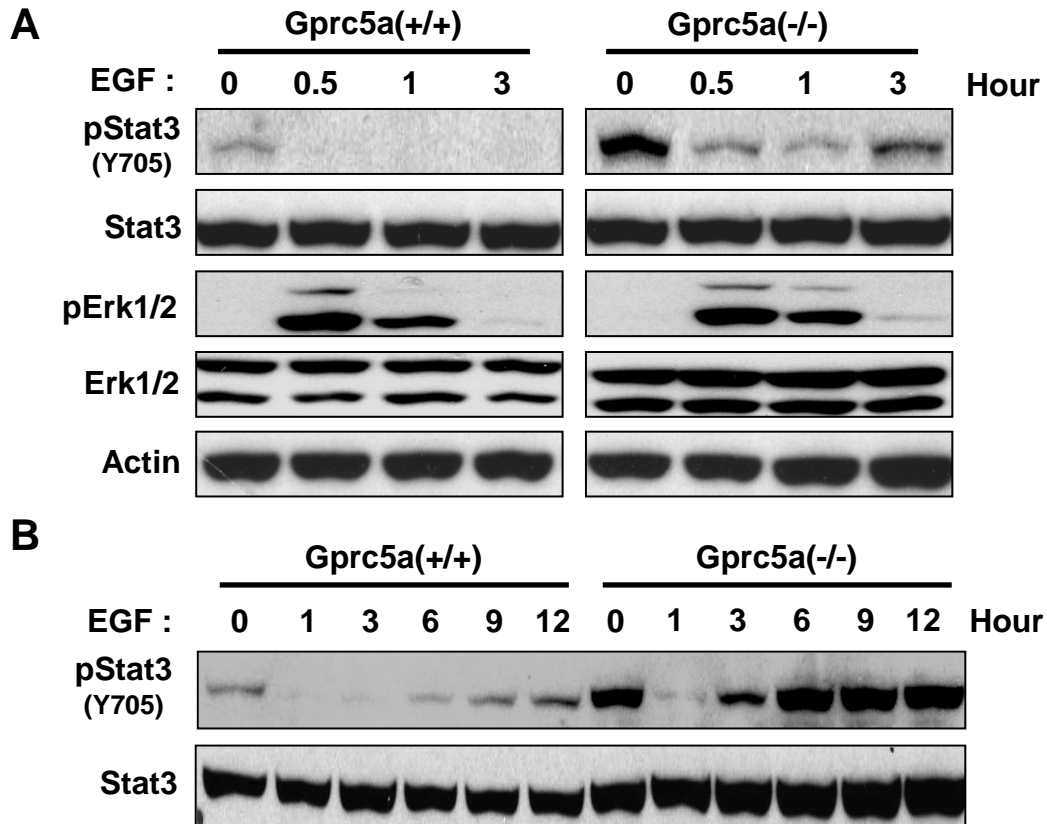


Figure 11. EGF does not stimulate Stat3 activation in *Gprc5a*<sup>-/-</sup> and *Gprc5a*<sup>+/-</sup> normal airway epithelial cells. *A*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were starved in K-SFM overnight and then treated with EGF at 10 ng/ml for up to 3 hours. Cells were extracted for protein and analyzed for the levels of phosphorylated and total Stat3, phosphorylated and total Erk1/2, and actin by western blotting. *B*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were starved in K-SFM overnight and then treated with EGF at 10 ng/ml for up to 12 hours. Cells were extracted for protein and analyzed for the levels of phosphorylated and total Stat3 by western blotting.

level of tyrosine phosphorylated Stat3, which was higher in *Gprc5a*<sup>-/-</sup> cells than in *Gprc5a*<sup>+/+</sup> cells before EGF treatment, decreased 30 min after EGF addition and began to increase slightly after 3 hours (Fig. 11A) suggesting that EGFR activation does not directly mediate Stat3 phosphorylation in either of these cells. The surprising decrease in tyrosine phosphorylation of Stat3 early after EGF treatment of the *Gprc5a*<sup>-/-</sup> cells was reversed after longer incubation (> 3 hours) and remained high up to 12 hours (Fig. 11B). A similar response pattern but with a substantially lower level of tyrosine phosphorylation of Stat3 was also observed in *Gprc5a*<sup>+/+</sup> cells when treated with EGF. Because in this experiment, we replaced the “old ” EGF-free cell growth medium with fresh EGF-supplemented medium, we surmised that the transient decrease in the level of tyrosine phosphorylated Stat3 is due to removal of autocrine factor(s) from the conditioned medium (“old”). Indeed, we found that Stat3 was activated in *Gprc5a*<sup>-/-</sup> cells cultured in medium without EGF for 24 hours but the level of tyrosine phosphorylated Stat3 decreased sharply in parallel cultures one hour after replacing culture medium conditioned for the preceding 23 hours with fresh medium containing no EGF (Fig. 12A and 12B). These results indicate that the higher Stat3 activation in *Gprc5a*<sup>-/-</sup> cells is EGF-independent but autocrine-dependent.

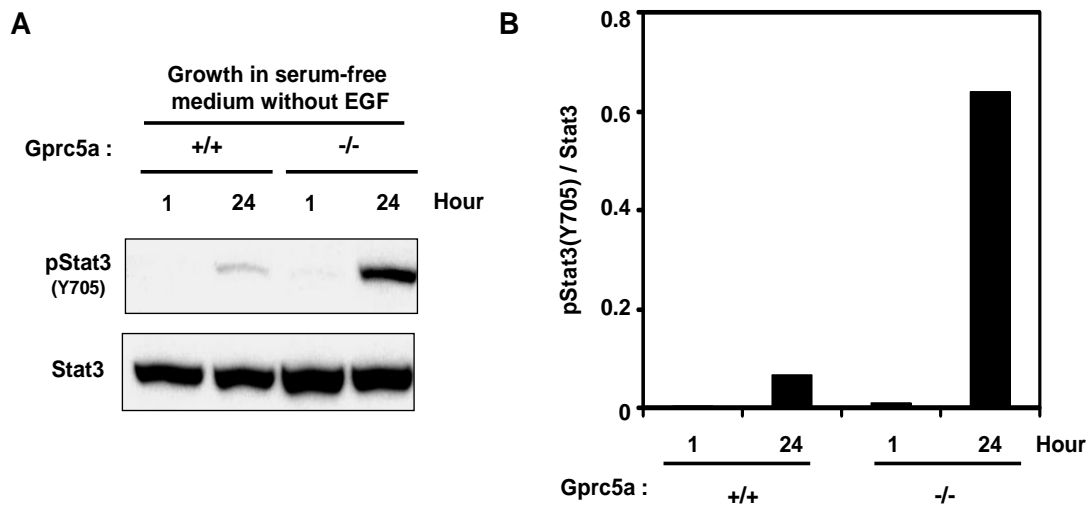


Figure 12. Stat3 is activated in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells independent of EGF. *A*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells were cultured in K-SFM and harvested after 24 hours. In parallel cultures, the cells were cultured for 23 hours and then their “old” medium was replaced with fresh medium and the cells were incubated in this medium for one hour. The cells were harvested and analyzed for phosphorylated and total Stat3 by western blotting. *B*, the X-ray films (panel *A*) were scanned and the bands were quantified using Quantity One software. The ratios of phosphorylated Stat3 to total Stat3 were calculated and shown in bar graph.

#### **4.3 Differential response to the conditioned media mediated the hyper activation of Stat3 in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells**

We have shown that the Stat3 activation in *Gprc5a*<sup>-/-</sup> cells is dependent on autocrine signaling but not on EGF signaling. We then investigated why Stat3 is more activated in *Gprc5a*<sup>-/-</sup> cells compared to *Gprc5a*<sup>+/+</sup> cells. There are two possibilities for the differential activation of Stat3 between these cells. One is that the *Gprc5a*<sup>-/-</sup> cells secrete a Stat3 activator(s) into their conditioned medium whereas the *Gprc5a*<sup>+/+</sup> cells did not. The other is that both cells secrete a Stat3 activator(s) into their conditioned medium but the responses to the Stat3 activator(s) of *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells were different. We treated both cell lines with conditioned media from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells and observed that both conditioned media activated Stat3 to a very high level in *Gprc5a*<sup>-/-</sup> cells but only to an extremely low level in *Gprc5a*<sup>+/+</sup> cells (Fig. 13A and 13B). These data strongly suggest that the difference of Stat3 activation between the *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells is due to different response to the Stat3 activator(s) in these conditioned media.

#### **4.4 JAK is required for the activation of Stat3 in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells**

We next examined whether Stat3 is activated by JAK-mediated cytokine signaling. First, we treated *Gprc5a*<sup>-/-</sup> cells with AG490, an inhibitor of JAK kinase, which is the important mediator of Stat3 activation induced by cytokines.

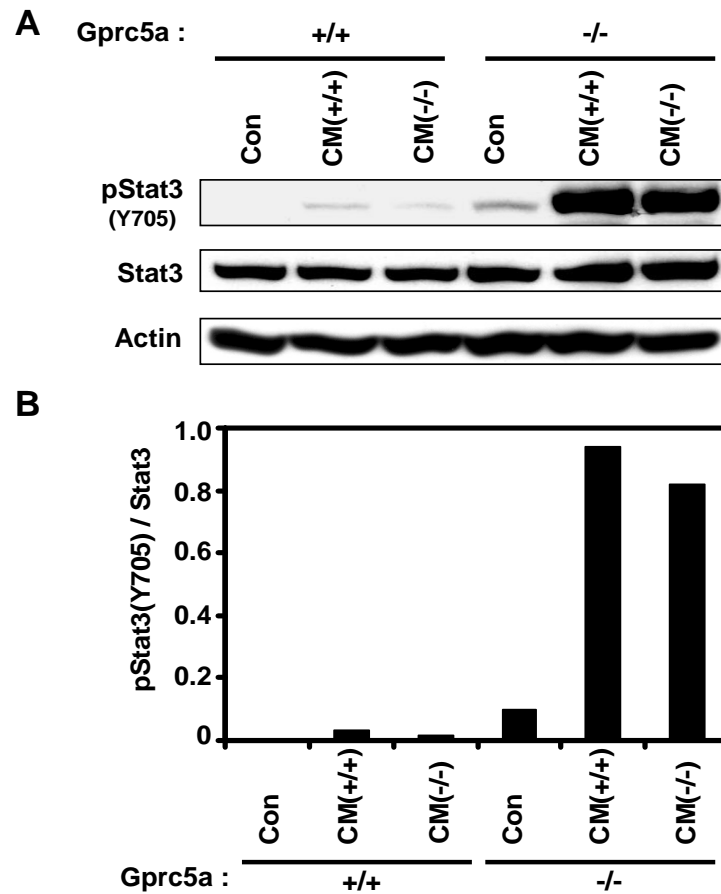


Figure 13. Stat3 is highly activated in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells induced by conditioned media from *Gprc5a*<sup>+/+</sup> or *Gprc5a*<sup>-/-</sup> cells. *A*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were incubated in medium conditioned for 24 hours by other cultures of either *Gprc5a*<sup>+/+</sup> or *Gprc5a*<sup>-/-</sup> cells as indicated. The cells were harvested after one hour and analyzed for phosphorylated and total Stat3 by western blotting. *B*, the X-ray films (panel *A*) were scanned and the bands were quantified using Quantity One software. The ratios of phosphorylated Stat3 to total Stat3 were calculated and shown in bar graph.

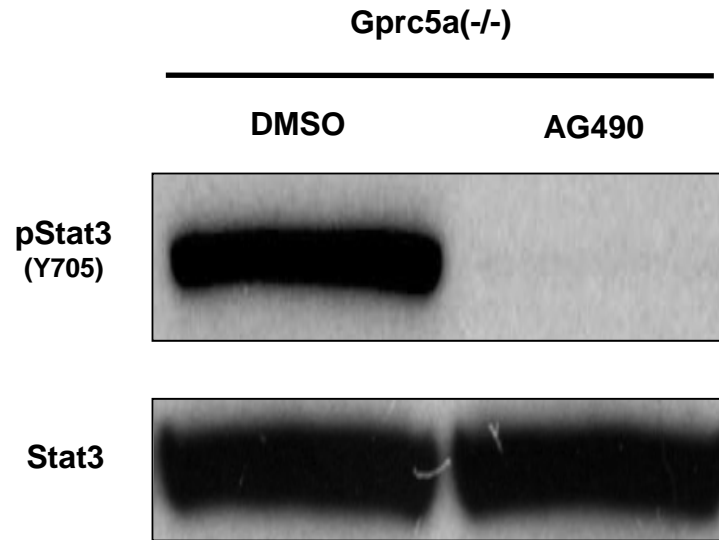


Figure 14. Stat3 activation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells is inhibited by JAK inhibitor AG490. *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were treated with AG490 at 30  $\mu$ M or DMSO (0.1% V:V) in K-SFM without supplements for 24 hours. The cells were harvested and analyzed for phosphorylated and total Stat3 by western blotting.

We found that treatment of *Gprc5a*<sup>-/-</sup> cells with AG490 for 24 hours completely blocked the activation of Stat3 in these cells (Fig. 14). This result indicates that Stat3 activation in *Gprc5a*<sup>-/-</sup> cells is dependent on JAK and suggests that Stat3 may be activated by autocrine cytokines.

#### **4.5 Inhibition of Stat3 activation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells by neutralizing antibody against Lif**

Cytokines of the IL-6 family members like IL-6, LIF and OSM are well known activators of STAT3 (Yu et al., 2009). To identify the cytokine(s) which activated Stat3 in the *Gprc5a*<sup>-/-</sup> cells, we treated the cells with neutralizing antibodies against Il-6, Lif and Osm for 24 hours. As can be seen in Fig. 15A and 15B, neutralizing antibody against mouse Lif decreased the tyrosine phosphorylation of Stat3 in *Gprc5a*<sup>-/-</sup> cells compared to the normal goat IgG treatment whereas the neutralizing antibodies against Il-6 and Osm did not. These data indicate that Lif may be the main autocrine activator of Stat3 in the *Gprc5a*<sup>-/-</sup> cells.

#### **4.6 Neutralizing antibody against Lif inhibited the Stat3 activation in *Gprc5a*<sup>-/-</sup> cells induced by both conditioned media from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells**

We have shown that conditioned media from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells activated Stat3 to a similar level (Fig. 13A and 13B) and Lif may be the autocrine Stat3 activator in the *Gprc5a*<sup>-/-</sup> cells (Fig. 15). We next examined

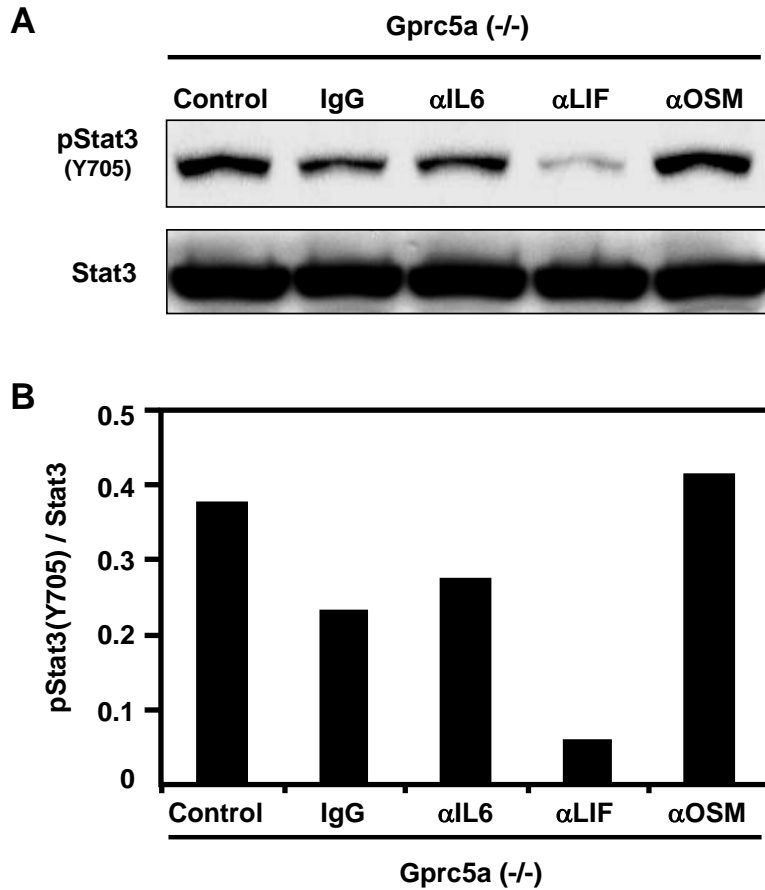


Figure 15. Stat3 activation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells is decreased by neutralizing antibody against mouse Lif. *A*, *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were cultured in K-SFM with neutralizing antibodies against mouse Il-6, Lif, and Osm or with normal goat IgG (all at 30  $\mu$ g/ml) for 24 hours. The cells were harvested and analyzed for phosphorylated and total Stat3 by western blotting. *B*, the X-ray films (panel *A*) were scanned and the bands were quantified using Quantity One software. The ratios of phosphorylated Stat3 to total Stat3 were calculated and shown in bar graph.



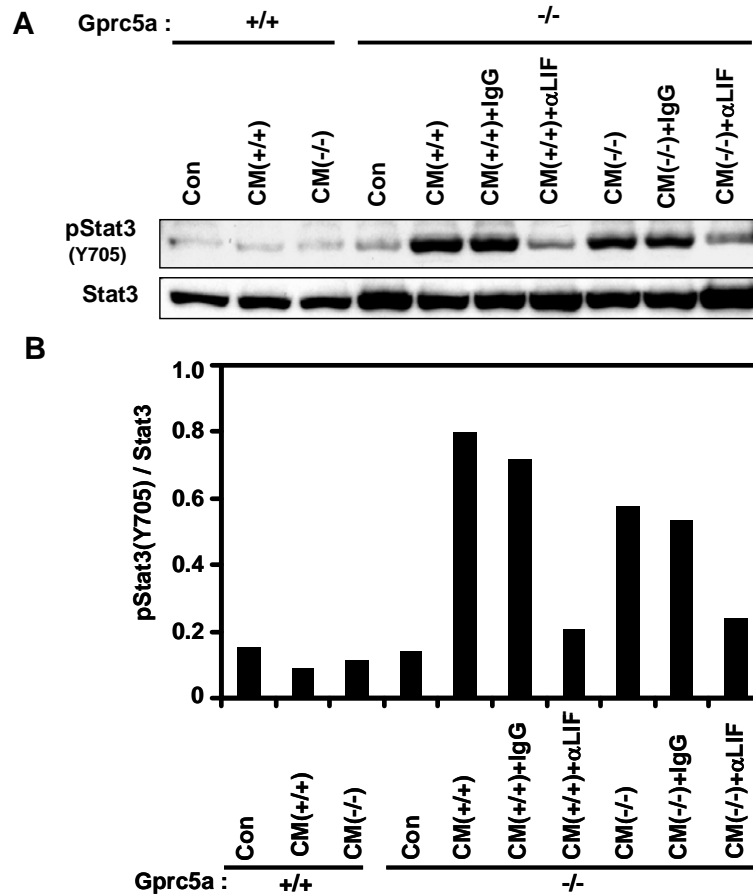


Figure 16. Neutralizing antibody against mouse Lif inhibited conditioned medium-induced Stat3 activation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells. *A*, 24-hour conditioned media from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cell cultures were treated for 1 hour with Lif neutralizing antibody or with normal goat IgG (both at 30  $\mu$ g/ml). These media were used to treat *Gprc5a*<sup>-/-</sup> cells that had been starved for one hour by incubation in fresh K-SFM. After 1 hour of incubation with the treated media and the cells were analyzed for phosphorylated and total Stat3 by western blotting. *B*, the X-ray films (panel *A*) were scanned and the bands were quantified using Quantity One software. The ratios of phosphorylated Stat3 to total Stat3 were calculated and shown in bar graph.

whether Lif was also the autocrine Stat3 activator in the conditioned medium from *Gprc5a*<sup>+/+</sup> cells. As shown in Fig. 16A and 16B, we observed a similar result as in Fig. 13 in that both conditioned media from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells activated Stat3 in the *Gprc5a*<sup>-/-</sup> cells but did not in the *Gprc5a*<sup>+/+</sup> cells indicating that these results were reproducible. Pre-incubating the conditioned medium from *Gprc5a*<sup>-/-</sup> cells with a neutralizing antibody against Lif for one hour inhibited the conditioned media-induced Stat3 activation in *Gprc5a*<sup>-/-</sup> cells while pre-incubating with the goat normal IgG did not (Fig. 16A and 16B), supporting our hypothesis that Lif is the autocrine Stat3 activator in the *Gprc5a*<sup>-/-</sup> cells. Notably, we found that pre-incubating the conditioned medium from *Gprc5a*<sup>+/+</sup> cells with neutralizing antibody against Lif for one hour also inhibited this conditioned medium induced Stat3 activation in *Gprc5a*<sup>-/-</sup> cells while pre-incubating with the goat normal IgG did not (Fig. 16A and 16B), indicating that Lif is also the autocrine Stat3 activator in the conditioned medium from *Gprc5a*<sup>+/+</sup> cells. These data also suggest that the *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells have different response to the autocrine Lif in the conditioned media leading to the difference in Stat3 activation between these cells.

#### **4.7 *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells secrete Lif**

We have shown that Lif-neutralizing antibodies suppressed the autocrine Stat3 activation in both *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells. To obtain more direct evidence that both cells secreted Lif into their conditioned media, we collected

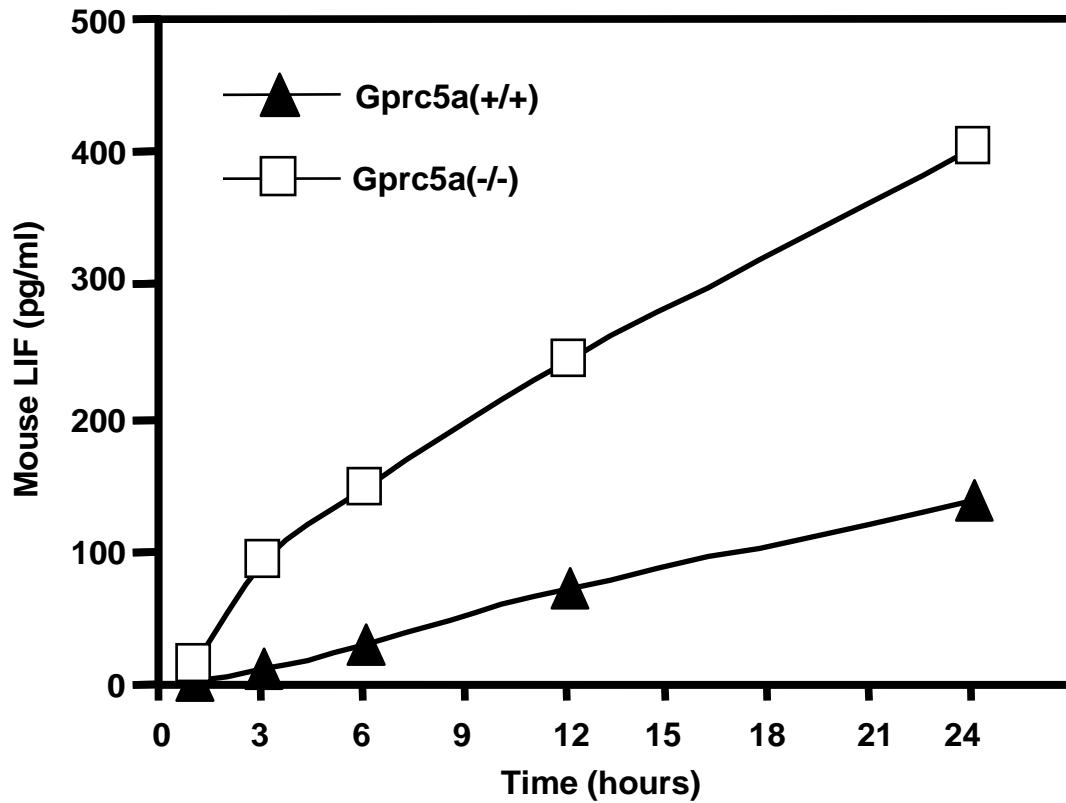


Figure 17. *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells secrete LIF. *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells were cultured in fresh K-SFM at 2 x 10<sup>5</sup> per 35-mm diameter well and their conditioned media were collected after 0, 1, 3, 6, 12, and 24 hours and analyzed by ELISA for secreted LIF.

conditioned media conditioned for different time from both cells and analyzed the Lif levels using ELISA. As seen in Fig 17, Lif existed in both the conditioned media from *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells indicating that Lif could be the Stat3 activator in these conditioned media. The level of Lif in the conditioned media increased following the increasing conditioned time indicating the Lif was produced and secreted by these cells than acted as an autocrine factor and was not derived from the culture medium (Fig. 17). We also observed that *Gprc5a*<sup>-/-</sup> cells secreted more Lif into their conditioned medium than *Gprc5a*<sup>+/+</sup> cells (Fig. 17).

#### **4.8 Differential response to Lif underlies the persistent Stat3 activation in *Gprc5a*<sup>-/-</sup> compared to *Gprc5a*<sup>+/+</sup> normal airway epithelial cells**

We have shown that the *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells responded differently to autocrine Lif. To further confirm our finding, we treated both cells with exogenous Lif for one hour and found that Lif activated Stat3 in the *Gprc5a*<sup>-/-</sup> cells whereas no Stat3 activation was observed in the *Gprc5a*<sup>+/+</sup> cells (Fig. 18). The Stat3 activation increased when increasing the dose of Lif from 250 to 10000 unit/ml suggesting Stat3 activation induced by the exogenous Lif was dose-dependent between these concentrations (Fig. 18). We then treated both cells with exogenous Lif at 1000 units/ml, which is the concentration for culturing mouse stem cells *in vitro*, for different times to monitor the time course response of these cells to Lif. We found that Stat3 activation was transient in the *Gprc5a*<sup>+/+</sup> cells, peaking around 30 min and declining rapidly by 60 min

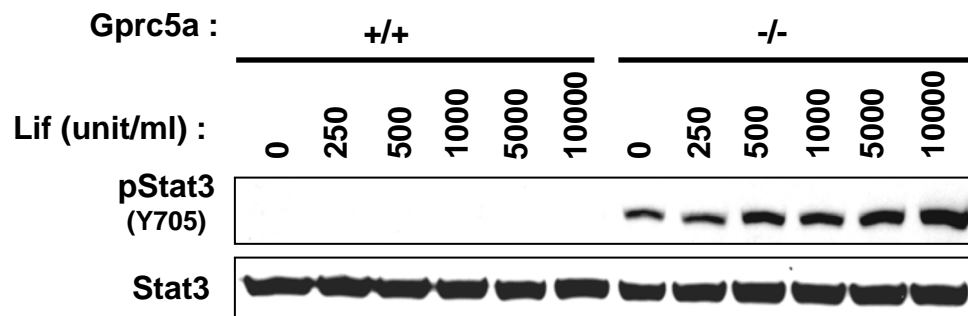


Figure 18. Dose response of *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells to exogenous Lif. *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells were starved in fresh K-SFM for one hour, then treated with different doses of exogenous Lif for one additional hour. The cells were then analyzed for phosphorylated and total Stat3 by western blotting.

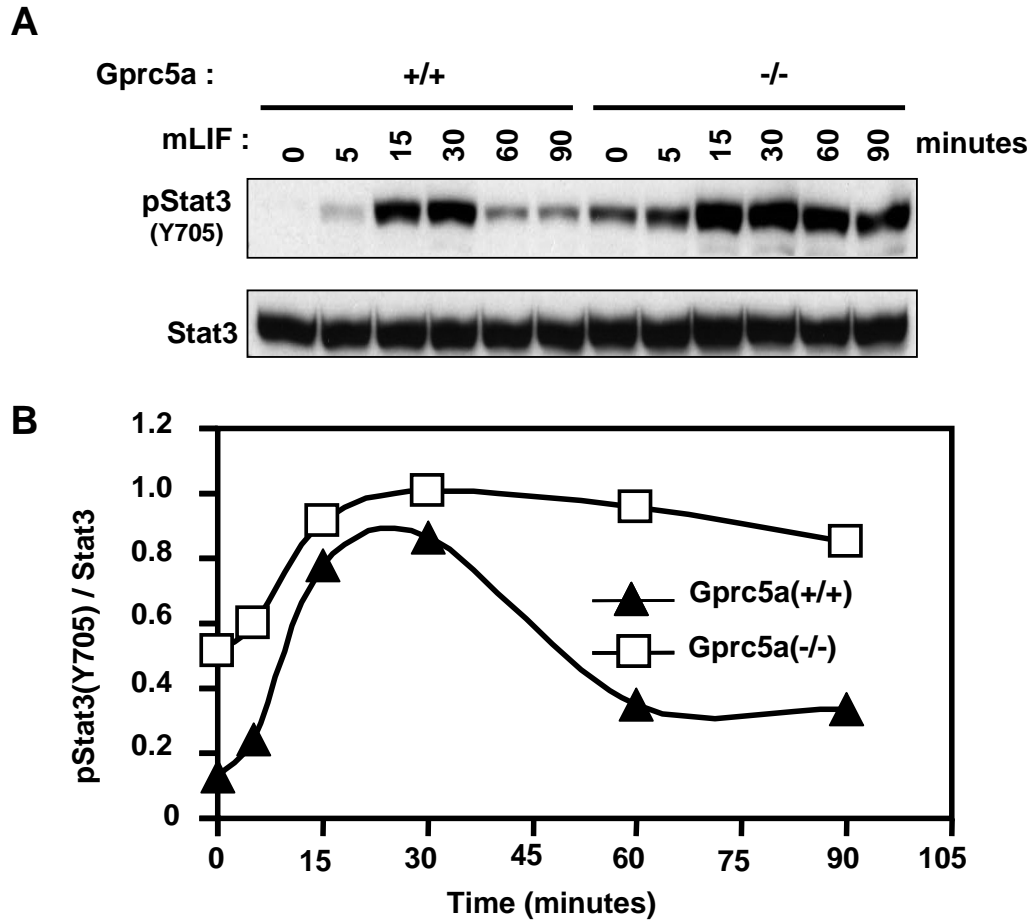


Figure 19. *Gprc5a*<sup>-/-</sup> normal airway epithelial cells have prolonged response to exogenous Lif compared with *Gprc5a*<sup>+/+</sup> cells. *A*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells were starved in fresh K-SFM for one hour, then treated with exogenous Lif (at 1000 unit/ml) for the indicated times. The cells were analyzed for phosphorylated and total Stat3 by western blotting. *B*, the X-ray films (panel *A*) were scanned and the bands were quantified using Quantity One software. The ratios of phosphorylated Stat3 to total Stat3 were calculated and shown.

(Fig. 19A and 19B). In contrast, Stat3 activation in *Gprc5a*<sup>-/-</sup> cells was persistent; after peaking at 30 min it remained activated for at least 90 min (Fig. 19A and 19B). These results suggest that *Gprc5a* loss leads to a prolonged response to Lif as indicated by persistent Stat3 activation that was also reported to occur in various tumor cells (Lee et al., 2009).

#### **4.9 Autocrine Lif mediates Stat3 activation in MDA959 lung tumor cells**

We have shown that Stat3 was activated in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells induced by autocrine Lif. Next we investigated whether Stat3 was also activated in tumors from *Gprc5a*<sup>-/-</sup> mice. We established a mouse lung tumor cell line (MDA959) from a spontaneously-developed mouse lung adenocarcinoma from a *Gprc5a*<sup>-/-</sup> mouse. We found that MDA959 tumor cells had a constitutively active Stat3, which was completely inhibited by AG490 (Fig. 20A), implicating the Jak/Stat3 signaling pathway in this activation. To examine whether the Stat3 activation in MDA959 tumor cells was also mediated by autocrine Lif, we pre-incubating the conditioned medium from MDA959 tumor cells with neutralizing antibody against Lif and found that the antibody decreased the tyrosine phosphorylation of Stat3 induced by conditioned medium from MDA959 tumor cells (Fig. 20B). To validate that MDA959 tumor cells also secreted Lif, we analyzed the level of Lif in the conditioned medium from MDA959 tumor cells using ELISA and found that the MDA959 tumor cells also released Lif into their conditioned medium as shown in Fig. 21.

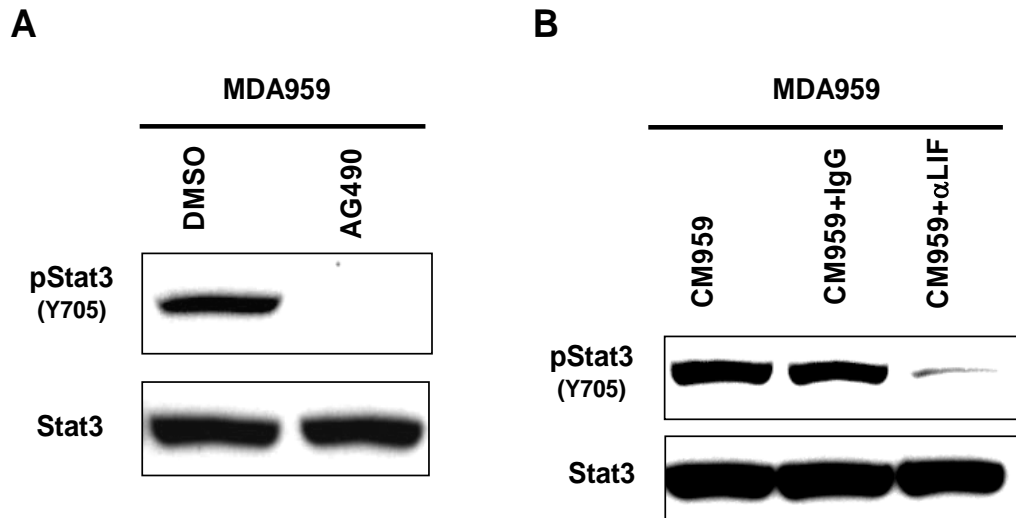


Figure 20. LIF mediates Stat3 activation in MDA959 mouse lung tumor cells. *A*, MDA959 cells were treated with 30  $\mu$ M AG490 or DMSO for 24 hours. The cells were analyzed for phosphorylated and total Stat3 by western blotting. *B*, 24 hours conditioned medium from MDA959 cell cultures was treated for 1 hour with LIF neutralizing antibody or with normal goat IgG (both at 30  $\mu$ g/ml). These media were used to treat MDA959 cells that had been starved for one hour by incubation in DMEM/F12 medium without serum. After 1 hour of incubation with the treated media the cells were analyzed for phosphorylated and total Stat3 by western blotting.



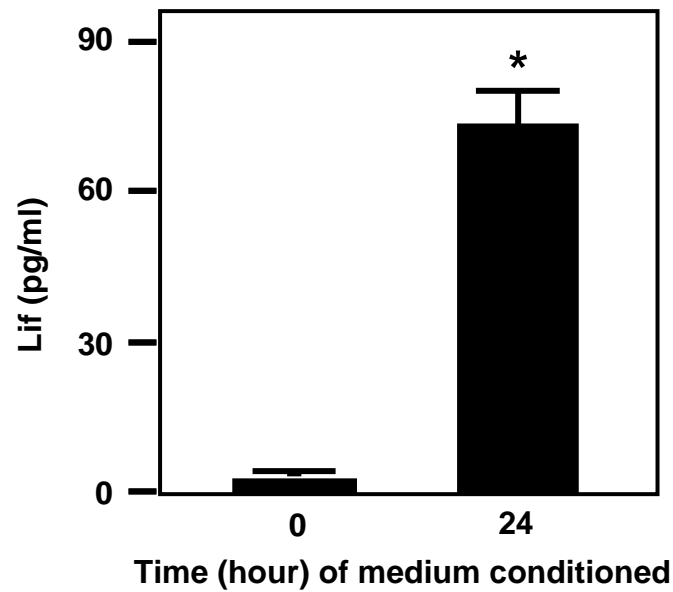


Figure 21. MDA959 mouse lung tumor cells secreted Lif. MDA959 cells were cultured in DMEM/F12 for 24 hours and then the medium was collected and analyzed for secreted Lif by ELISA. DMEM/F12 medium without conditioning by cells was used as a control. \* means  $P < 0.05$ .

#### **4.10 Stat3 activation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells was inhibited by dominant negative Stat3**

We have shown that Stat3 was activated by autocrine Lif in *Gprc5a*<sup>-/-</sup> cells, and then we investigated whether dominant negative Stat3 can block the activation of Stat3 in the *Gprc5a*<sup>-/-</sup> cells. *Gprc5a*<sup>-/-</sup> cells expressing the dominant negative Stat3 (Y705F) were established by transfecting the cells with the Stat3 (Y705F) expression vector and selecting with hygromycin. Meanwhile, a control cell line was also established by transfecting the cells with the empty vector. The expression of the dominant negative Stat3 (Y705F) was confirmed by western blotting (Fig. 22A). We observed that the mutated Stat3 (Y705F) inhibited the activation of the endogenous Stat3 in *Gprc5a*<sup>-/-</sup> cells as indicated by the decreased level of tyrosine phosphorylated Stat3 compared to the vector control (Fig. 22A). The increased total Stat3 protein in the Stat3 (Y705F) transfected cells was caused by the fact that the antibody for total Stat3 also recognized the dominant negative form of Stat3 (Y705F). Moreover, we found that the expression of Stat3 (Y705F) suppressed the expression of the Stat3-regulated anti-apoptotic proteins (Fig. 22A). Since Stat3 regulated these anti-apoptotic genes at transcriptional level, we analyzed the mRNA levels of these genes and found the mRNA of these genes also decreased significantly in *Gprc5a*<sup>-/-</sup> cells expressing the dominant negative Stat3 (Y705F) compared to the vector control (Fig. 22B). These data indicate that the upregulation of these anti-apoptotic genes in *Gprc5a*<sup>-/-</sup> cell compared to the *Gprc5a*<sup>+/+</sup> cells resulted from the increased activation of Stat3 in *Gprc5a*<sup>-/-</sup> cells.

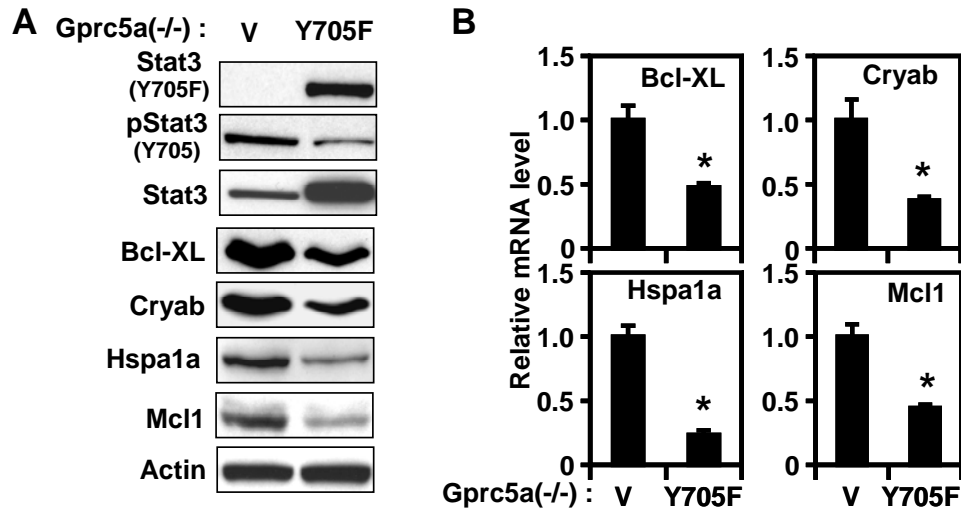


Figure 22. Dominant negative Stat3 inhibits Stat3 activation in *Gprc5a*<sup>(-/-)</sup> normal airway epithelial cells. *A*, *Gprc5a*<sup>(-/-)</sup> cells transfected with vector or Stat3(Y705F) were starved for 48 hours then extracted and analyzed by western blotting for the indicated proteins. *B*, *Gprc5a*<sup>(-/-)</sup> cells transfected with vector or Stat3(Y705F) were starved for 48 hours then extracted for mRNA analysis by QPCR for Stat3 regulated genes. The experiments were done by triplicates and \* means  $P < 0.05$ .

#### **4.11 Dominant negative Stat3 increases starvation induced apoptosis and decreases colony formation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells**

Next, we investigated whether inhibition of Stat3 activation by dominant negative Stat3 (Y705F) would reverse the transformed phenotypes in *Gprc5a*<sup>-/-</sup> cells. As shown in Fig. 23A and 23B, *Gprc5a*<sup>-/-</sup> cells expressing the dominant negative Stat3 (Y705F) were significantly more sensitive to starvation induced apoptosis compared to cells transfected with the control vector. The starvation induced apoptosis rate increased from 9.3% to 22.4% in *Gprc5a*<sup>-/-</sup> cells after transfected with the dominant negative Stat3 (Y705F). These data indicate that persistent Stat3 activation is required for the resistance to starvation induced apoptosis in *Gprc5a*<sup>-/-</sup> cells. Moreover, we found that *Gprc5a*<sup>-/-</sup> cells expressing the dominant negative Stat3 (Y705F) formed less colonies in Matrigel than cells expressed the control vector (Fig. 24A). The decrease was statistically significant and more than 3 folds (Fig. 24B). These data indicate that persistent Stat3 activation is also required for the increased colony formation in *Gprc5a*<sup>-/-</sup> cells.

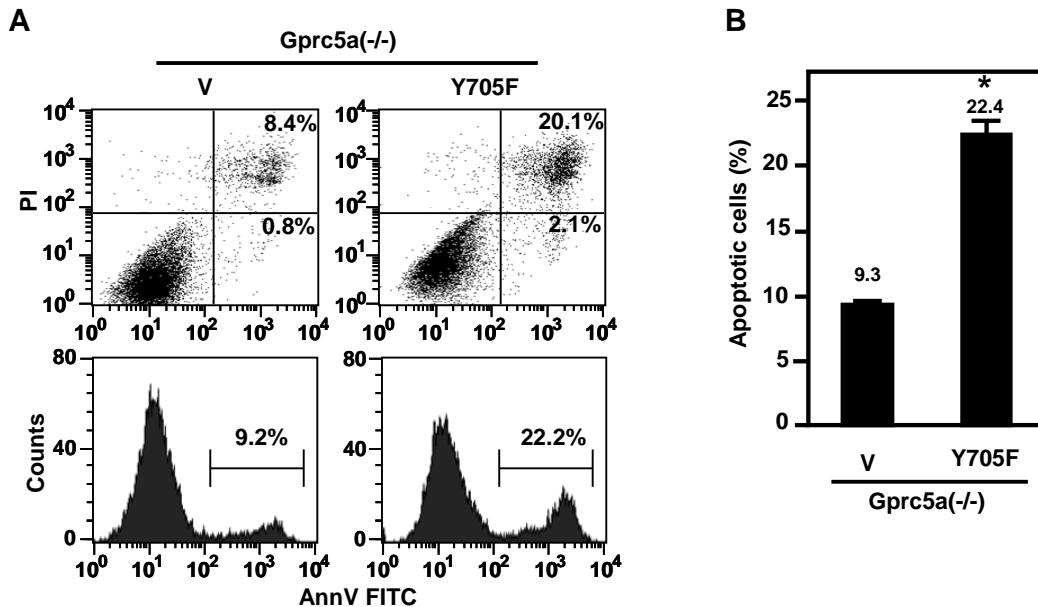


Figure 23. Dominant negative Stat3 increases starvation-induced apoptosis in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells. *A*, *Gprc5a*<sup>-/-</sup> cells transfected with vector or Stat3(Y705F) were cultured in K-SFM without supplements for 48 hours, then cells were double staining with PI and AnnV-FITC and then analyzed for apoptosis by flow cytometry. Results of one of the triplicates is presented. *B*, the mean  $\pm$  SD of three independent experiments in panel *A* was shown in bar graph. P value were calculated using Student T-test and \* means  $P < 0.05$ .

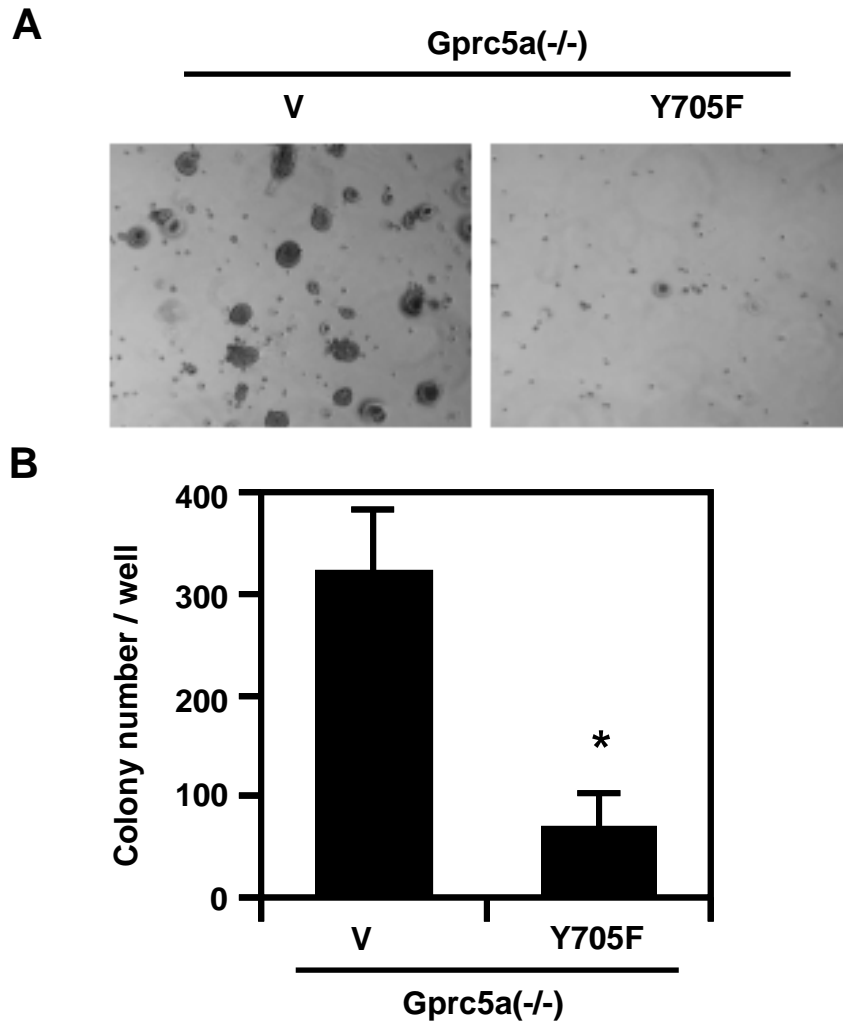


Figure 24. Dominant negative Stat3 inhibits colony formation of *Gprc5a*<sup>-/-</sup> normal airway epithelial cells. *A*, *Gprc5a*<sup>-/-</sup> cells transfected with vector or Stat3(Y705F) were suspended in Matrigel and cultured for two weeks to assay their colony formation ability. Photomicrographs of cultures at high magnification of one experiment were showed. *B*, colonies in three wells (each cell line) in panel *A* were counted and the data are presented in bar graph as mean ± SD colonies/well. P value were calculated using Student T-test and \* means P < 0.05.

#### **4.12 Dominant negative Stat3 blocks Stat3 activation; increases starvation induced apoptosis and decreases colony formation in MDA959 lung tumor cells**

We have shown that autocrine Lif mediated persistent Stat3 activation also existed in MDA959 tumor cells, and then we investigated whether dominant negative Stat3 can block the activation of Stat3 in the MDA959 tumor cells. MDA959 tumor cells expressing the dominant negative Stat3 (Y705F) were established by transfecting the cells with the Stat3 (Y705F) expression vector and selecting with hygromycin. Meanwhile, a control cell line was also established by transfecting the cells with the empty vector. The expression of the dominant negative Stat3 (Y705F) was confirmed by western blotting (Fig. 25A). We observed that mutant Stat3 (Y705F) inhibited the activation of the endogenous wildtype Stat3 in MDA959 cells as indicated by the decreased level of tyrosine phosphorylated Stat3 compared to the vector control (Fig. 25). The increased total Stat3 protein in the Stat3 (Y705F) transfected cells was caused by the fact that the antibody for total Stat3 also recognized the dominant negative form of Stat3 (Y705F).

Next, we investigated whether inhibition of Stat3 activation by dominant negative Stat3 (Y705F) would reverse the transformed phenotypes in MDA959 tumor cells. As shown in Fig. 26A and 26B, MDA959 tumor cells expressing the dominant negative Stat3 (Y705F) were significantly more sensitive to starvation induced apoptosis compared to cells transfected with the empty vector. The starvation induced apoptosis rate increased from 11.0% to 24.2% in MDA959

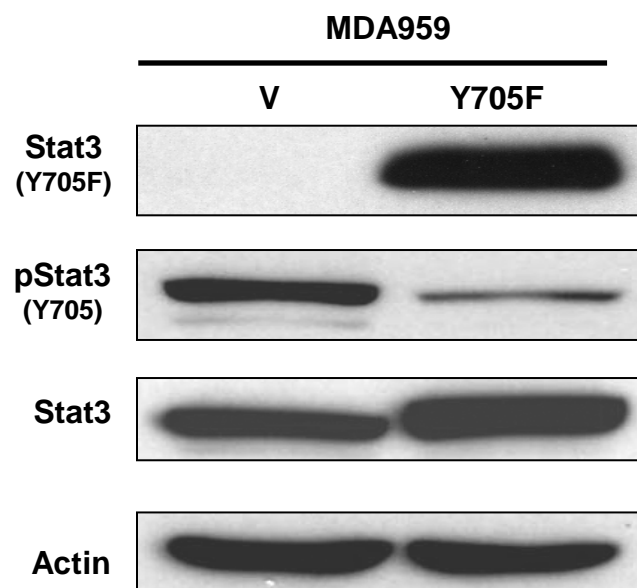


Figure 25. Dominant negative Stat3 inhibits Stat3 activation in MDA959 mouse lung tumor cells. MDA959 tumor cells transfected with vector or Stat3(Y705F) were starved for 48 hours then extracted and analyzed by western blotting for the indicated proteins. Flag antibody was used to detect the dominant negative Stat3(Y705F).



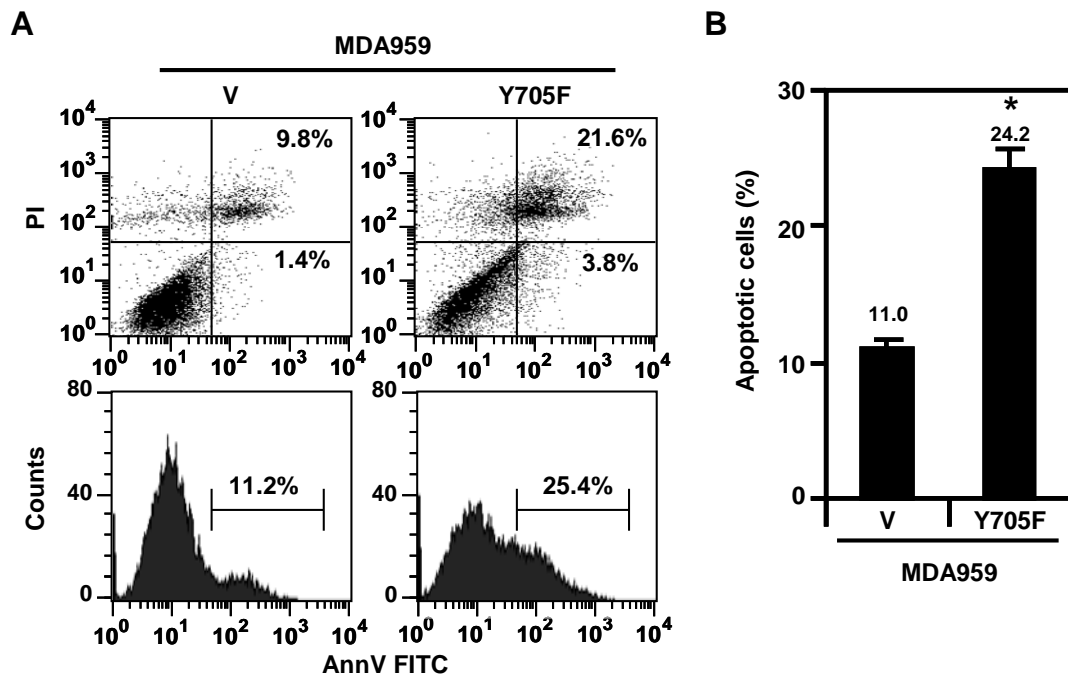


Figure 26. Dominant negative Stat3 increases starvation-induced apoptosis in MDA959 mouse lung tumor cells. *A*, MDA959 cells transfected with vector or Stat3(Y705F) were cultured in MDEM/F12 medium without FBS for 48 hours, then cells were double stained with PI and AnnV-FITC and analyzed for apoptosis by flow cytometry. Results of one of the triplicates is presented. *B*, the mean  $\pm$  SD of three independent experiments in panel *A* was shown in bar graph. P value were calculated using Student T-test and \* means  $P < 0.05$ .

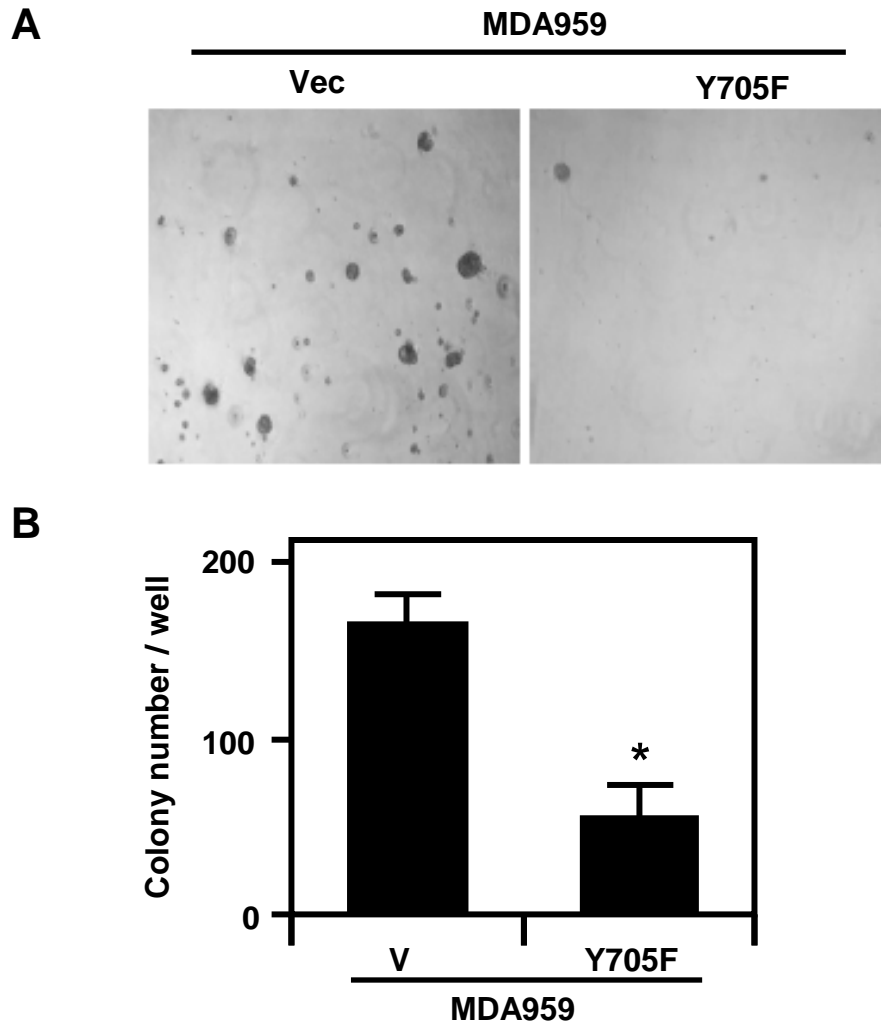


Figure 27. Dominant negative Stat3 inhibited colony formation of MDA959 mouse lung tumor cells. *A*, MDA959 tumor cells transfected with vector or Stat3(Y705F) were suspended in Matrigel and cultured for two weeks to assay for colony formation ability. Photomicrographs of cultures at high magnification of one experiment were showed. *B*, colonies in three wells (each cell line) in panel *A* were counted and the data are presented in bar graph as mean  $\pm$  SD colonies/well. P value were calculated using Student T-test and \* means  $P < 0.05$ .

tumor cells after transfected with the dominant negative Stat3 (Y705F). These data indicate that persistent Stat3 activation is required for the resistance to starvation-induced apoptosis in MDA959 tumor cells. Moreover, we found that MDA959 tumors cells expressed the dominant negative Stat3 (Y705F) formed less colonies in Matrigel than cells expressed the control vector (Fig. 27A). The decrease was statistically significant and more than 3 folds (Fig. 27B). These data indicate that persistent Stat3 activation is also required for the increased colony formation in MDA959 tumor cells.

#### **4.13 AG490 increases starvation induced apoptosis and decreased colony formation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells**

We have shown that AG490, an inhibitor of JAK, completely inhibited Stat3 activation in *Gprc5a*<sup>-/-</sup> cells (Fig. 14). Thus, we also examined whether this small molecular inhibitor can reverse the transformed phenotypes in *Gprc5a*<sup>-/-</sup> cells. As seen in Fig. 28A and 28B, AG490 treatment dramatically increased the starvation induced apoptosis in *Gprc5a*<sup>-/-</sup> cells. The starvation induced apoptosis rate increased from 10.6% to 38.1% in *Gprc5a*<sup>-/-</sup> cells after treatment with AG490. Meanwhile, we also found AG490 treatment significantly inhibited the colony formation ability of *Gprc5a*<sup>-/-</sup> cells in Matrigel (Fig. 29A and 29B). These data indicate that using small molecular inhibitor of Stat3 signaling also can reverse the transformed phenotypes in *Gprc5a*<sup>-/-</sup> cells.

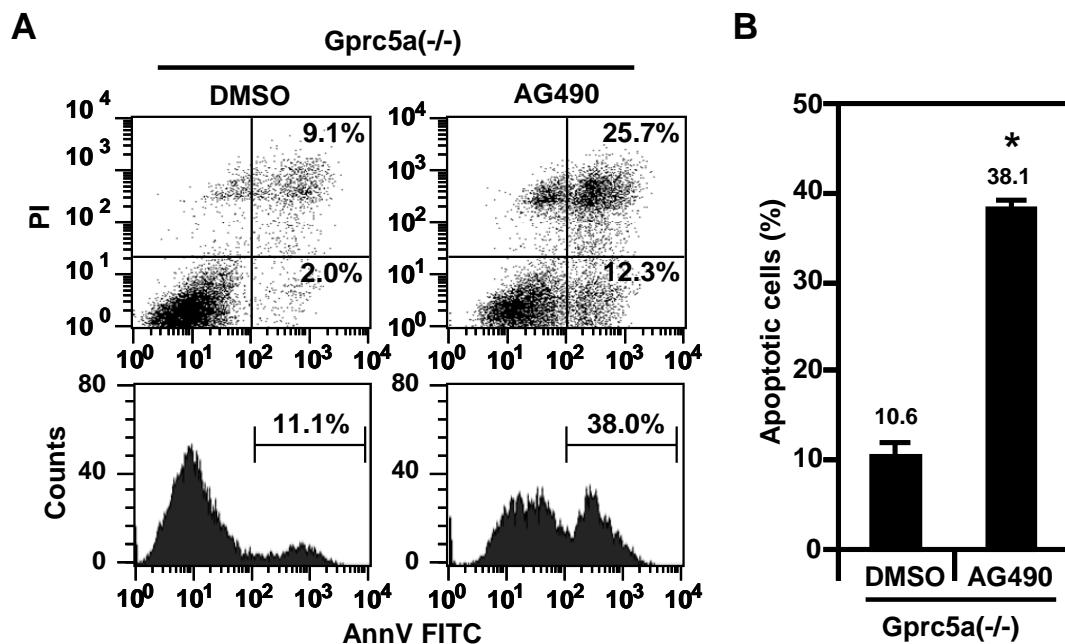


Figure 28. Inhibition of Stat3 activation using AG490 increases starvation induced apoptosis in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells. *A*, *Gprc5a*<sup>-/-</sup> cells were treated with AG490 (30  $\mu$ M) or DMSO in K-SFM for 48 hours, then cells were double stained with PI and AnnV-FITC and then analyzed for apoptosis by flow cytometry. Results of one of the triplicates is presented. *B*, the mean  $\pm$  SD of three independent experiments in panel *A* was shown in bar graph. P value were calculated using Student T-test and \* means  $P < 0.05$ .

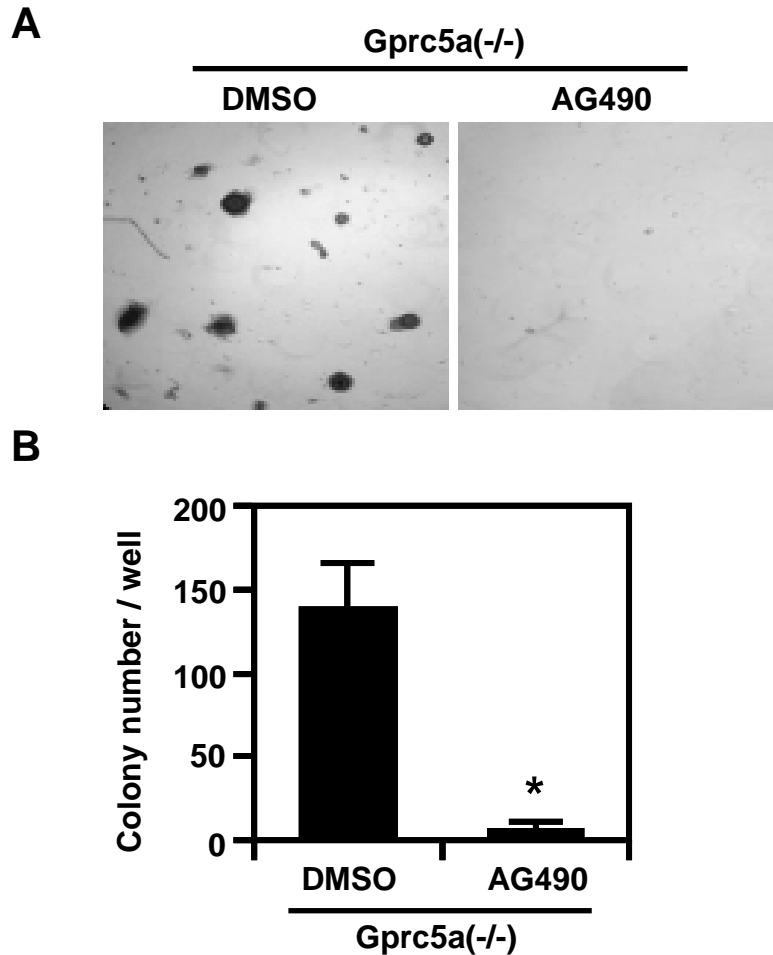


Figure 29. Inhibition of Stat3 activation using AG490 decreases colony formation of *Gprc5a*<sup>-/-</sup> normal airway epithelial cells. *A*, *Gprc5a*<sup>-/-</sup> cells were suspended in Matrigel with AG490 (30μM) or DMSO and analyzed for colony formation over two weeks. Photomicrographs of cultures at high magnification of one experiment were showed. *B*, colonies in three wells (each cell line) in panel *A* were counted and the data are presented in bar graph as mean ± SD colonies/well. P value were calculated using Student T-test and \* means P < 0.05.

#### **4.14 AG490 increases starvation induced apoptosis and decreases colony formation in MDA959 lung tumor cells**

We have shown that AG490, an inhibitor of JAK, completely inhibited Stat3 activation in MDA959 tumor cells (Fig. 20A). Thus we examined whether inhibition of Stat3 by this small molecular inhibitor can reverse the transformed phenotype in MDA959 tumor cells. As seen in Figs. 30A and 30B, AG490 treatment significantly increased the starvation induced apoptosis in MDA959 tumor cells. The starvation induced apoptosis rate increased from 14.3% to 43.9% in MDA959 tumor cells after treated with AG490. Meanwhile, we also found AG490 treatment dramatically inhibited the colony formation of MDA959 tumor cells in Matrigel (Fig. 31A and 31B). These data indicate that using small molecular inhibitor of Stat3 signaling also can reverse the transformed phenotypes in MDA959 tumor cells.

#### **4.15 Discussion**

The relative resistance of *Gprc5a*<sup>-/-</sup> normal airway epithelial cells to starvation-induced apoptosis compared to *Gprc5a*<sup>+/+</sup> normal airway epithelial cells suggests increased self-sufficiency in growth signals, enhanced ability to evade apoptosis or both (Hanahan and Weinberg, 2000). The increased expression of anti-apoptotic Stat3 target genes including Bcl-XL, Cryab, Hspa1a, and Mcl1 in the *Gprc5a*<sup>-/-</sup> cells is a likely explanation for their relative resistance to apoptosis. Because Stat3 activation has been reported to play important

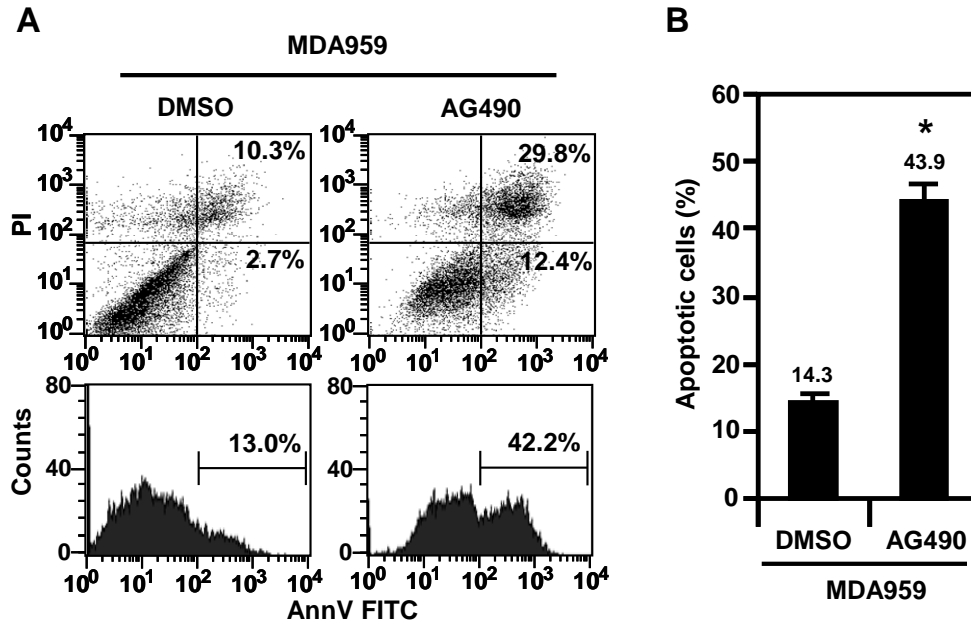


Figure 30. Inhibition of Stat3 activation using AG490 increases starvation induced apoptosis in MDA959 mouse lung tumor cells. *A*, MDA959 cells were treated with AG490 (30  $\mu$ M) or DMSO in DMEM/F12 medium without FBS for 48 hours, then cells were double staining with PI and AnnV-FITC and then analyzed for apoptosis by flow cytometry. Results of one of the triplicates is presented. *B*, the mean  $\pm$  SD of three independent experiments in panel *A* was shown in bar graph. P value were calculated using Student T-test and \* means  $P < 0.05$ .

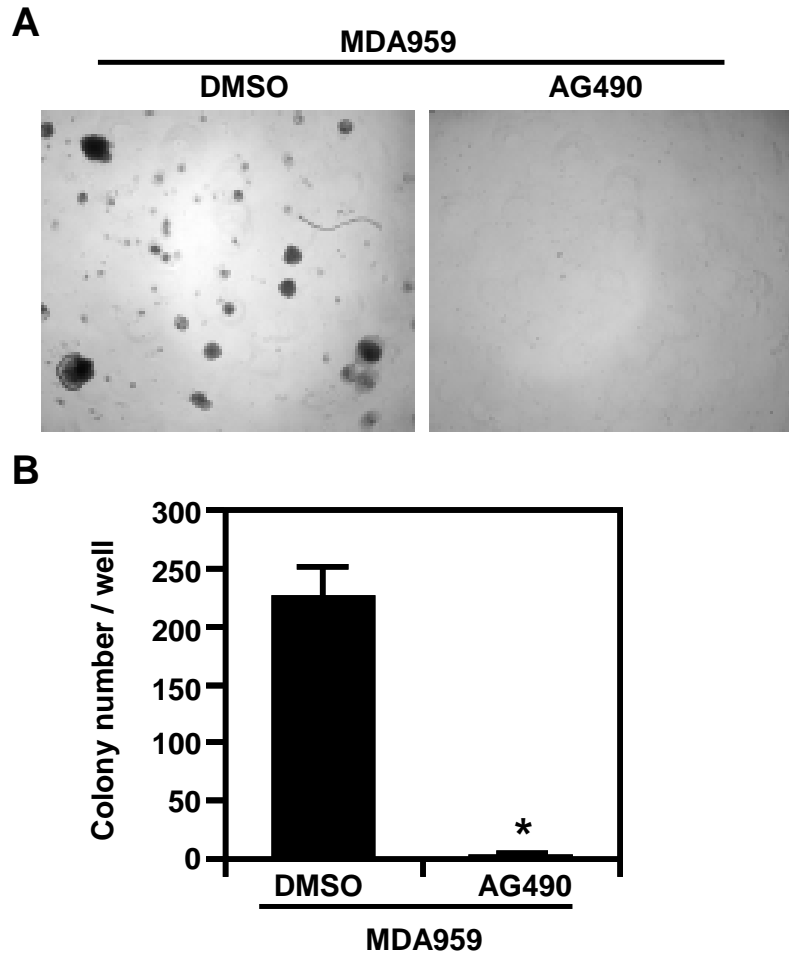


Figure 31. Inhibition of Stat3 activation using AG490 decreases colony formation of MDA959 mouse lung tumor cells. *A*, MDA959 cells were suspended in Matrigel with AG490 (30 $\mu$ M) or DMSO and analyzed for colony formation over two weeks. Photomicrographs of cultures at high magnification of one experiment are showed. *B*, colonies in three wells (each cell line) in panel *A* were counted and the data are presented in bar graph as mean  $\pm$  SD colonies/well. P value were calculated using Student T-test and \* means  $P < 0.05$ .



roles in both mouse and human lung cancer development (Gao et al., 2007; Li et al., 2007) and these increased anti-apoptotic genes in *Gprc5a*<sup>-/-</sup> cells were Stat3 targeted genes, we examined the status of Stat3 activation in our cells and found that Stat3 signaling pathway was activated in *Gprc5a*<sup>-/-</sup> cells to a much higher level than in *Gprc5a*<sup>+/+</sup> cells. Previous reports have shown that the EGFR signaling activated Stat3 and mediated growth in tumor cells (Akca et al., 2006; Colomiere et al., 2009; Gao et al., 2007; Vigneron et al., 2008). However, the Stat3 activation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells was independent of exogenous EGF and EGFR signaling but was dependent on autocrine Stat3 activator(s) released by both *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells.

Further studies using the Jak/Stat3 inhibitor AG490 demonstrated that Jak activity is required for Stat3 activation in *Gprc5a*<sup>-/-</sup> cells, and identified Lif, a member of the Il-6 family cytokines, as the autocrine mediator of Stat3 activation in the *Gprc5a*<sup>-/-</sup> cells. Previous reports have shown that human carcinoma cell lines including lung cancer produce Lif (Kamohara et al., 1994) and that Lif functions as an autocrine or paracrine growth factor in breast, pancreas and glioblastoma tumor cells (Kamohara et al., 2007; Kellokumpu-Lehtinen et al., 1996; Penuelas et al., 2009; Quaglino et al., 2007). Lif also plays important roles in tumor metastasis (Maruta et al., 2009; Wysoczynski et al., 2007). Our studies have shown that while both *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells produce and release Lif, their response to Lif was different insofar as Lif induced a persistent Stat3 activation in the *Gprc5a*<sup>-/-</sup> cells but only a transient

activation of Stat3 in the *Gprc5a*<sup>+/+</sup> cells. The data is consistent with the previous report that transformed bronchia epithelial cells have increased stat3 activation induced by LIF (Loewen et al., 2005). This difference in Stat3 activation may explain the higher levels of the anti-apoptotic genes and proteins and the partial resistance of the cells to starvation. Moreover, we also found that Stat3 was constitutively activated by autocrine Lif in MDA959 lung tumor cells suggesting that Lif mediated Stat3 activation happened in both premalignant and malignant cells.

The importance of Stat3 activation for the expression of the transformed phenotype in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells and in MDA959 tumor cells was demonstrated by the finding that blocking Stat3 activation by dominant negative Stat3(Y705F) or by AG490 increased the sensitivity of both cell lines to starvation-induced apoptosis and decreased their colony forming potential. Therefore, we propose that persistent Stat3 activation induced by autocrine Lif in *Gprc5a*<sup>-/-</sup> cells and MDA959 tumor cells may play important roles for the development of lung cancer in the *Gprc5a*<sup>-/-</sup> mice. This possibility is also supported by the report that transgenic mice overexpressing constitutively activated Stat3 in alveolar type II epithelial cells develop spontaneous lung adenocarcinomas (Li et al., 2007), which suggests that Stat3 activation alone can lead to lung carcinogenesis. The relevance of the findings with mouse cells to human lung cancer is indicated by the findings that STAT3 is persistently activated by chronic stimulation of JAK by cytokines in a variety of human tumors including lung tumors (Bromberg et al., 1999; Hedvat et al., 2009).

Further, STAT3 target genes have been proposed as biomarkers for human chronic obstructive pulmonary disease (COPD) and lung adenocarcinoma diagnosis and prognosis (Qu et al., 2009).

We have shown that autocrine LIF is the Stat3 activator in *Gprc5a*<sup>-/-</sup> cells and MDA959 tumor cells. These data were mostly based on studies of cells cultured *in vitro*. To further investigate the important roles of autocrine LIF in lung carcinogenesis of *Gprc5a*<sup>-/-</sup> mice *in vivo*, it would be worthwhile to generate *Gprc5a*<sup>-/-</sup>*Lif*<sup>-/-</sup> double knockout mice by crossing the *Gprc5a*<sup>-/-</sup> mice with *Lif*<sup>-/-</sup> mice (Escary et al., 1993; Stewart et al., 1992) and examine whether the *Gprc5a*<sup>-/-</sup>*Lif*<sup>-/-</sup> mice have decreased lung tumors compared with the *Gprc5a*<sup>-/-</sup>*Lif*<sup>+/+</sup> mice. To examine the important roles of persistent Stat3 activation in lung carcinogenesis of *Gprc5a*<sup>-/-</sup> mice *in vivo*, we suggest to generate *Gprc5a*<sup>-/-</sup>*Stat3*<sup>-/-</sup> double knockout mice by crossing the *Gprc5a*<sup>-/-</sup> mice with lung specific *Stat3*<sup>-/-</sup> mice (Hokuto et al., 2004; Kida et al., 2008) and investigate whether the *Gprc5a*<sup>-/-</sup>*Stat3*<sup>-/-</sup> mice have decreased lung tumors compared with the *Gprc5a*<sup>-/-</sup>*Stat3*<sup>+/+</sup> mice.

## CHAPTER 5 THE PERSISTENT STAT3 ACTIVATION IN *GPRC5A*<sup>-/-</sup> CELLS IS THE RESULT OF REDUCED SOCS3 PROTEIN

### 5.1 Socs3 protein level decreased in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells compared with *Gprc5a*<sup>+/+</sup> cells

Stat3 activation induced by Lif was transient in *Gprc5a*<sup>+/+</sup> cells but persistent in *Gprc5a*<sup>-/-</sup> cells suggesting that *Gprc5a*<sup>-/-</sup> cells have defects in controlling the Lif/Stat3 signaling. To explore this further, we examined the level of Socs3, a Stat3 induced protein that functions as a negative feedback inhibitor of the Stat3 activation induced by various cytokines (Crocker et al., 2003; Lang et al., 2003; Nicola and Greenhalgh, 2000; Yasukawa et al., 2003). Although we did not observe a large difference in the mRNA level of Socs3 between *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells (Fig. 32A), we found that the level of Socs3 protein was greatly reduced in *Gprc5a*<sup>-/-</sup> cells compared to *Gprc5a*<sup>+/+</sup> cells (Fig. 32B). These data suggest that the level of Socs3 is regulated at the post transcriptional level. Previous reports have shown that Socs3 protein can be degraded through the proteasome pathway (Haan et al., 2003; Sasaki et al., 2003; Zhang et al., 1999). To determine whether the decreased Socs3 protein in *Gprc5a*<sup>-/-</sup> cells was caused by increased proteasome mediated degradation, we treated cells with the proteasome inhibitor MG132. We found that MG132 treatment did not increase the protein level of Socs3 in *Gprc5a*<sup>-/-</sup> cells and also did not change the protein level of Socs3 in *Gprc5a*<sup>+/+</sup> cells (Fig. 33), suggesting the Socs3

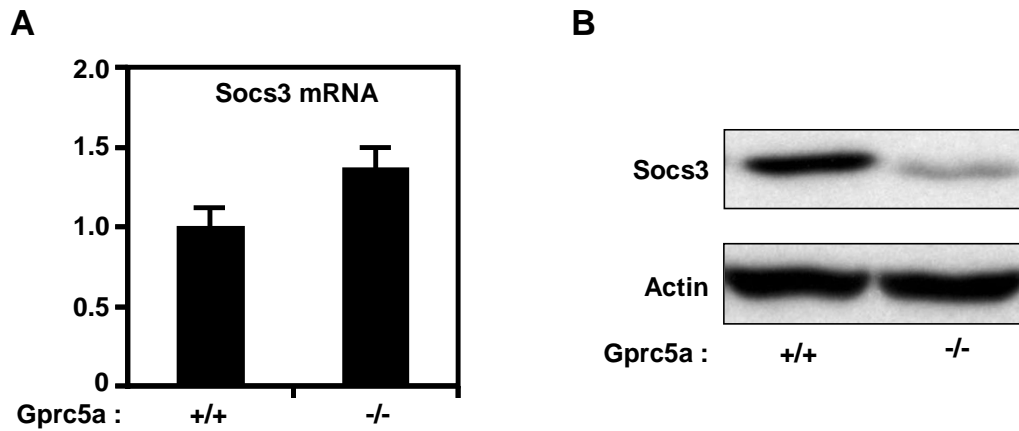


Figure 32. Decreased Socs3 protein level in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells compared with *Gprc5a*<sup>+/+</sup> cells. *A*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were cultured in K-SFM medium supplemented with EGF and BPE for 24 hours, and then cells were harvested and analyzed for Socs3 mRNA level using QPCR. *B*, *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were cultured in K-SFM medium supplemented with EGF and BPE for 24 hours, and then cells were harvested and analyzed for protein level of Socs3 by western blotting.

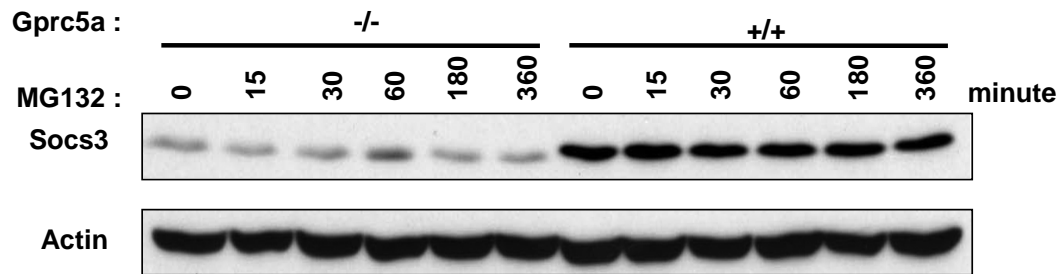


Figure 33. Decreased Socs3 protein level in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells is not caused by increasing proteasome dependent degradation. *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> normal airway epithelial cells were treated with 10  $\mu$ M MG132 for the indicated times, and then the cells were harvested and analyzed for protein level of Socs3 by western blotting.

protein is not regulated by proteasome mediated degradation in *Gprc5a*<sup>+/+</sup> and *Gprc5a*<sup>-/-</sup> cells.

## **5.2 Restoration of SOCS3 expression in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells inhibited Stat3 activation**

To confirm that the persistent Stat3 activation of Stat3 in *Gprc5a*<sup>-/-</sup> cells was due to a decrease of Socs3, we restored the expression of SOCS3 into *Gprc5a*<sup>-/-</sup> cells by transfection with a SOCS3-HA expression vector. As can be seen in Fig. 34A, the expression of SOCS3-HA was confirmed by immunoblotting using HA antibody. We found that the over-expression of SOCS3 decreased the persistent activation of Stat3 in *Gprc5a*<sup>-/-</sup> cells as indicated by the reduced level of tyrosine phosphorylated Stat3 (Fig. 34A). To investigate whether over-expression of SOCS3 will change the response to Lif stimulation, we treated the cells with exogenous Lif. As shown in Fig. 34B, *Gprc5a*<sup>-/-</sup> cells expressing SOCS3 exhibited reduced response to exogenous Lif stimulation, which is similar to *Gprc5a*<sup>+/+</sup> cells. These data strongly indicate that decreased Socs3 protein due to loss of Gprc5a tumor suppressor leads to the prolonged response to Lif and persistent activation of Stat3 in *Gprc5a*<sup>-/-</sup> cells.

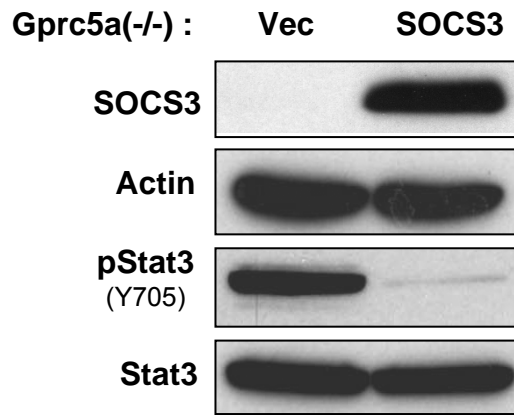
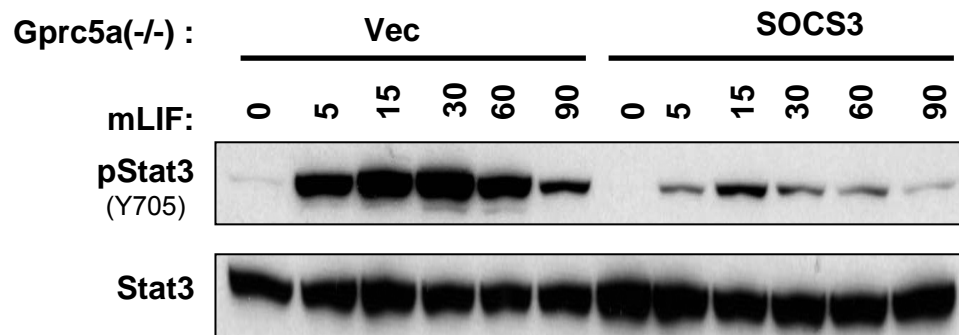
**A****B**

Figure 34. SOCS3 inhibits Stat3 activation in *Gprc5a*<sup>-/-</sup> normal airway epithelial cells. *A*, *Gprc5a*<sup>-/-</sup> cells transfected with vector or SOCS3-HA were starved for 48 hours then extracted for western blotting analysis of the indicated proteins. *B*, *Gprc5a*<sup>-/-</sup> cells transfected with vector or SOCS3-HA were starved in fresh K-SFM for one hour, then treated with exogenous Lif (1000 unit/ml) for the indicated times. The cells were analyzed for phosphorylated and total Stat3 by western blotting.



### **5.3 GPRC5A increases SOCS3 protein in co-transfected 293T cells**

The above data suggested that Gprc5a increased Socs3 protein by post-transcriptional regulation. To determine whether human GPRC5A also increase SOCS3 protein level, we co-transfected SOCS3 expression vector with GPRC5A expression vector in 293T cells. We found that SOCS3 protein level increased in cells co-transfected with GPRC5A compared to control vector, strongly indicating that GPRC5A may increase SOCS3 protein through post-transcriptional regulation (Fig. 35).

### **5.4 GPRC5A stabilizes SOCS3 protein**

We have shown that SOCS3 protein level was higher in GPRC5A expressing cells. We propose that it may be through translational regulation or through regulating the stability of the SOCS3 protein. To determine whether GPRC5A stabilized SOCS3 protein, we co-expressed SOCS3 with GPRC5A and treated the cells with cycloheximide, an inhibitor of protein synthesis. As shown in Fig. 36A and 36B, SOCS3 protein degraded more slowly in cells co-expressing GPRC5A than in cells co-transfected with control vector. The half life of SOCS3 increased when co-expressed with GPRC5A relative to co-transfected with control vector (Fig. 36B). These data indicate that GPRC5A increases SOCS3 protein level through regulating the SOCS3 protein stability and half-life.

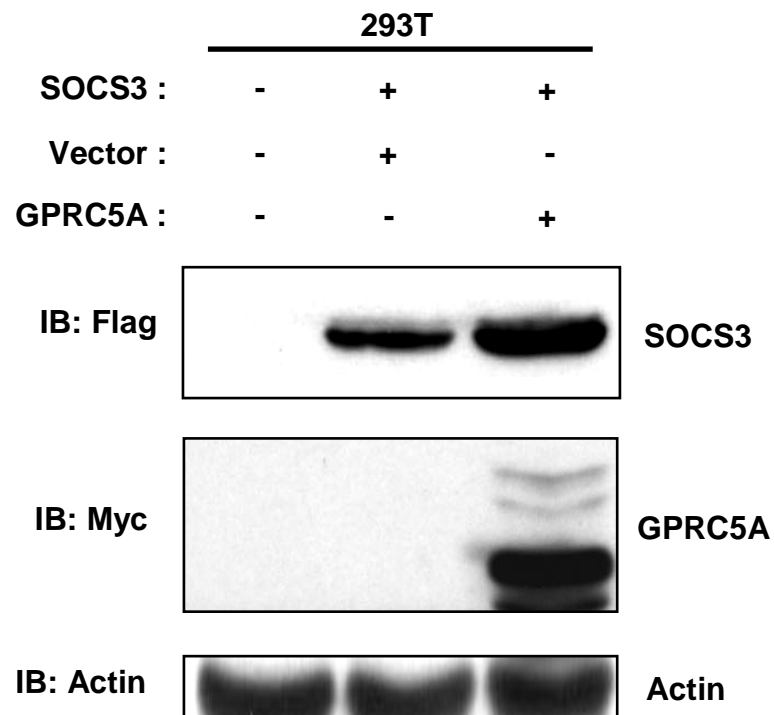


Figure 35. GPRC5A increased SOCS3 protein level in 293T cells. 293T cells were transfected with expression vectors of SOCS3 tagged with Flag, GPRC5A tagged with Myc as indicated and cultured for 48 hours. The cells were then harvested and analyzed for protein levels of SOCS3, GPRC5A and Actin by western blotting.

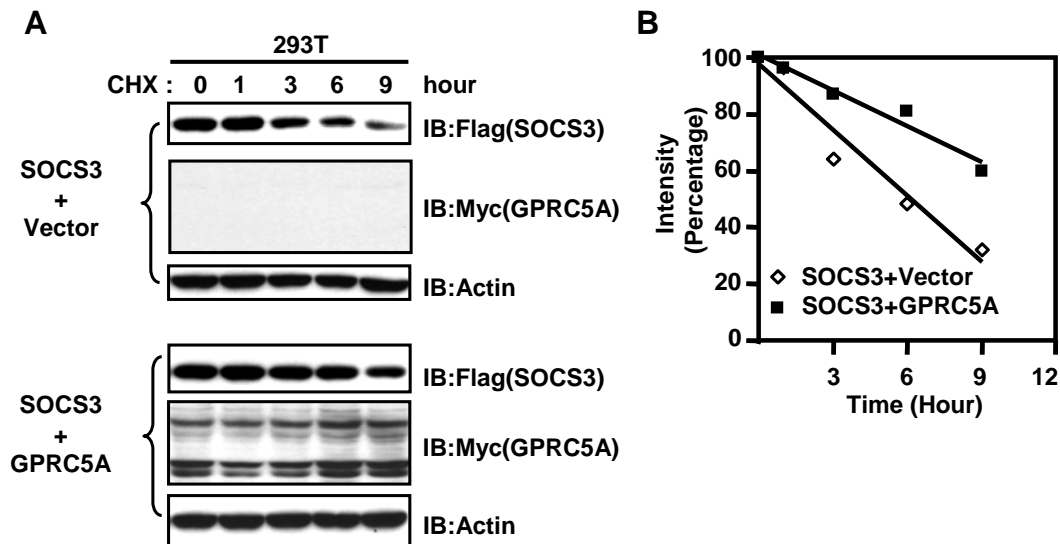


Figure 36. GPRC5A stabilizes SOCS3 protein in 293T cells. *A*, 293T cells were transfected with expression vectors of SOCS3 with Flag tag and vector control, or SOCS3 with Flag tag and GPRC5A with Myc tag and cultured for 48 hours. Cells were then treated with cycloheximide (CHX) at 20  $\mu$ M for the indicated times then harvested and analyzed for protein levels of SOCS3, GPRC5A and Actin by western blotting. *B*, the X-ray films (panel *A*) were scanned and the bands were quantified using Quantity One software. The intensities (percentage) were calculated by comparing to the 0 hour point. The 0 hour point is 100%.

## 5.5 SOCS3 co-localizes and interacts with GPRC5A in 293T cells

Next, we investigated the locations of SOCS3 and GPRC5A when co-expressed in 293T cells. We fused SOCS3 with red fluorescence protein DsRed and GPRC5A with green fluorescence protein AcGFP1 and co-expressed them in 293T cells. As seen in Fig. 37, SOCS3 co-localized with GPRC5A-AcGFP1 fusion protein but not with AcGFP1 protein, indicating that SOCS3 co-localized with GPRC5A protein. The co-localization of SOCS3 and GPRC5A suggested that SOCS3 may interact with GPRC5A, which may regulate SOCS3 stabilization. To determine whether SOCS3 interacts with GPRC5A, we co-expressed SOCS3 with flag tag and GPRC5A with myc tag in 293T cells and performed immunoprecipitation. We found that GPRC5A was associated in the SOCS3 protein complex immunoprecipitated using anti-flag antibody, whereas the normal IgG did not pull down either SOCS3 or GPRC5A (Fig. 38).

## 5.6 Discussion

The Stat3 activation induced by autocrine Lif was transient in *Gprc5a*<sup>+/+</sup> normal airway epithelial but was persistent in *Gprc5a*<sup>-/-</sup> cells, suggesting that *Gprc5a*<sup>-/-</sup> cells may lose some negative feedback inhibitors of the Lif/Stat3 signaling. Following that, we found that Socs3 protein, a well known inhibitor of Stat3 signaling, was decreased in the *Gprc5a*<sup>-/-</sup> cells compared to *Gprc5a*<sup>+/+</sup> cells which may explain the persistent Stat3 activation induced by autocrine Lif.

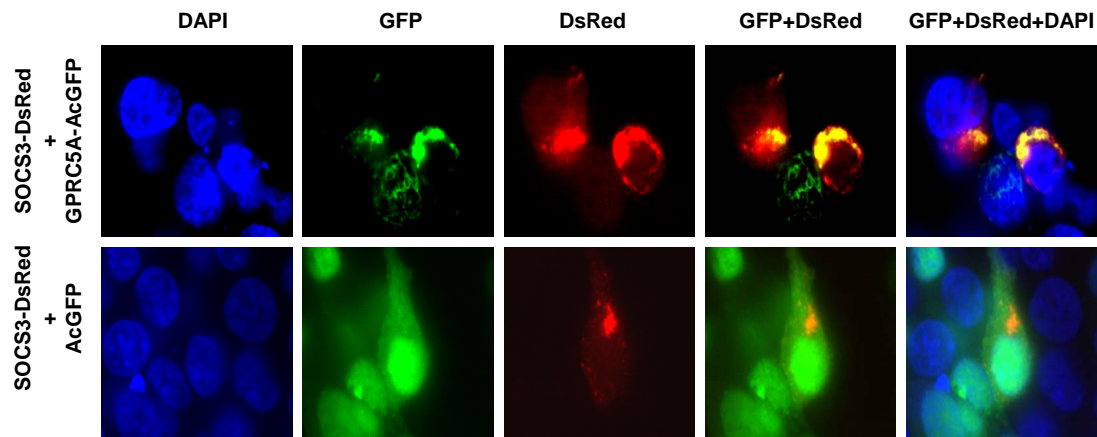


Figure 37. GPRC5A colocalizes with SOCS3 protein in 293T cells. 293T cells were transfected with expression vectors of SOCS3 with DsRed tag and GPRC5A with AcGFP tag, or SOCS3 with DsRed tag and AcGFP alone. After culturing for 48 hours, cells were fixed, stained for nuclei using DAPI and analyzed by using Olympus confocal microscope.

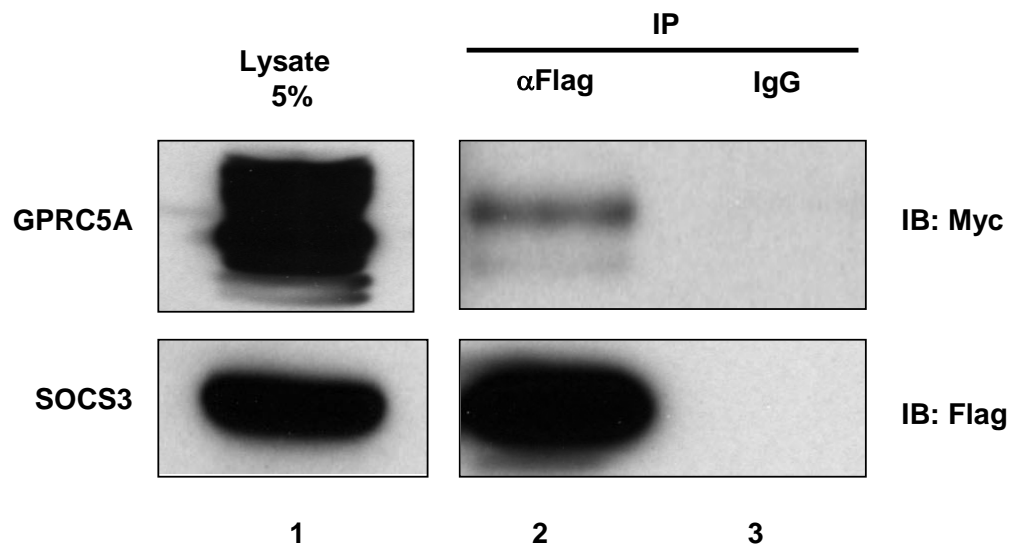


Figure 38. GPRC5A interacts with SOCS3 protein in 293T cells. The cells were transfected with expression vectors of SOCS3 with Flag tag and GPRC5A with Myc tag. After culturing for 48 hours, cells were harvested and cell lysates were subjected to immunoprecipitation using Flag antibody or normal IgG. The protein complexes were analyzed by western blotting.

Previous study showed that SOCS3 was silenced by promoter hypermethylation in human head and neck cancer cells and lung cancer (He et al., 2003; Weber et al., 2005). However, we found that the reduction of Socs3 protein in *Gprc5a*<sup>-/-</sup> cells was regulated through post-transcriptional level since the mRNA level of Socs3 were even somewhat higher in *Gprc5a*<sup>-/-</sup> cells compared with *Gprc5a*<sup>+/+</sup> cells, which may be explained by the fact that Socs3 is also a Stat3 targeted gene. Thus, Socs3 level may be down-regulated by different pathways including transcriptional or posttranscriptional regulation in lung cancer cells. It has been reported that SOCS3 protein may be degraded through the proteasome pathway (Haan et al., 2003; Sasaki et al., 2003). However, the proteasome inhibitor MG132 did not alter the Socs3 protein level in *Gprc5a*<sup>-/-</sup> cells and *Gprc5a*<sup>+/+</sup> cells, consistent with a previous report that wild type SOCS3 was degraded through a proteasome-independent pathway (Babon et al., 2006). The degradation pathway of wild type SOCS3 and the mechanism by which GPRC5A stabilized SOCS3 require further investigation, which is out of the scope of this thesis.

In summary, we demonstrated a potential mechanism involving Stat3 activation by which *Gprc5a* functions as a lung-specific tumor suppressor. In *Gprc5a*<sup>+/+</sup> airway epithelial cells (Fig. 39), Socs3 protein is stabilized by *Gprc5a* and negatively regulates the autocrine Lif-induced Stat3 activation leading to a transient Stat3 activation in response to the autocrine Lif. The Stat3 regulated anti-apoptotic genes are then expressed at a low level and the *Gprc5a*<sup>+/+</sup> airway epithelial cells are

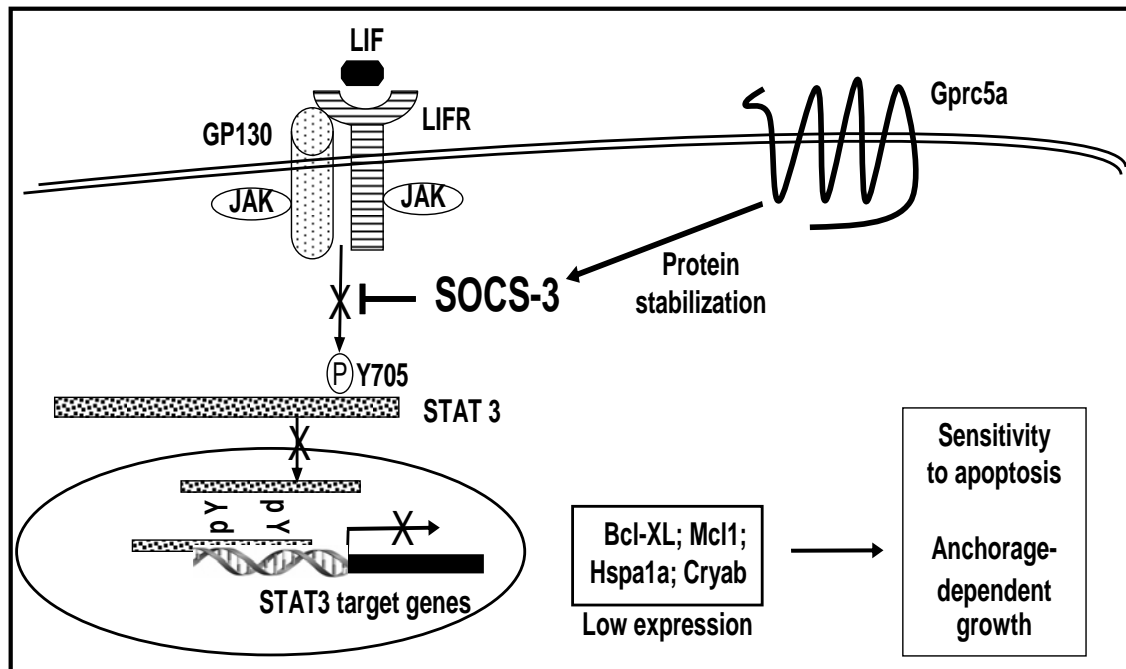


Figure 39. Stat3 signaling in *Gprc5a*<sup>+/+</sup> cells. In *Gprc5a*<sup>+/+</sup> cells, Socs3 protein was high through Gprc5a-mediated stabilization. The autocrine Lif induced Stat3 activation was transient and inhibited by Socs3, leading to low expression of anti-apoptotic proteins like Bcl-XL, Cryab, Hspa1a and Mcl1. Thus, the *Gprc5a*<sup>+/+</sup> cells were more sensitive to starvation induced apoptosis and grew dependent on anchorage.



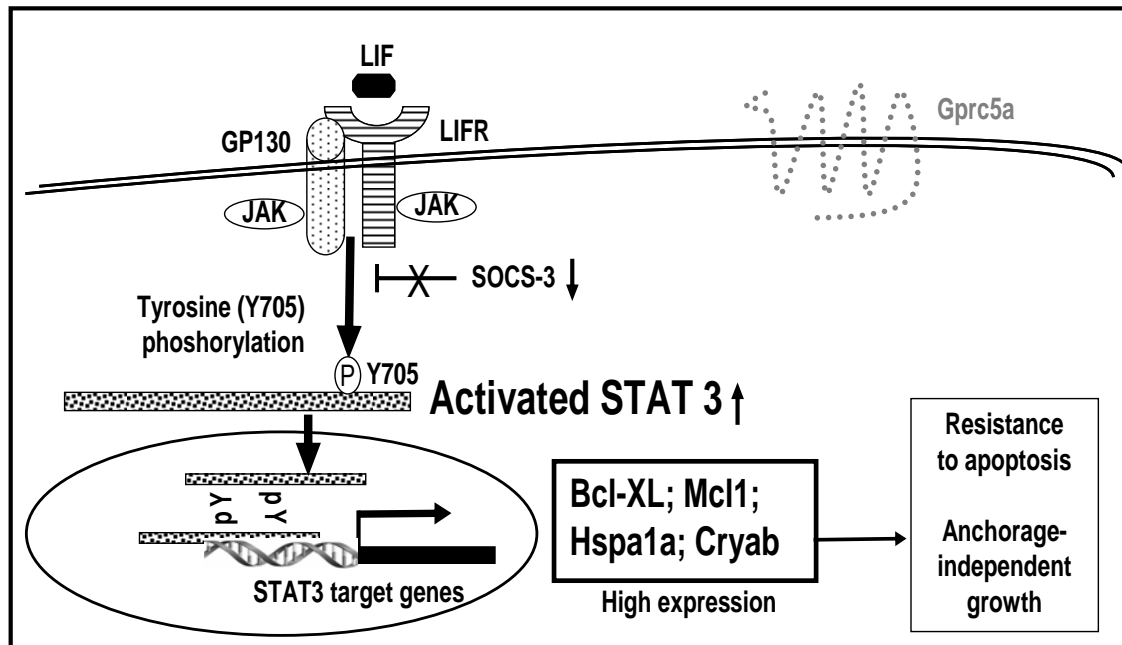


Figure 40. Stat3 signaling in *Gprc5a*<sup>-/-</sup> cells. In *Gprc5a*<sup>-/-</sup> cells, Socs3 protein level is much lower than in *Gprc5a*<sup>+/+</sup> cells due to loss of stability. The autocrine Lif induced a persistent activation of Stat3 because of the low level of Socs3, the negative feedback inhibitor of the Lif/Stat3 signaling. The persistent Stat3 activation increased the expression of anti-apoptotic genes including Bcl-XL, Cryab, Hspa1a and Mcl1, leading to increased survival, acquisition of anchorage independent growth in *Gprc5a*<sup>-/-</sup> cells, and lung tumorigenesis in *Gprc5a*<sup>-/-</sup> mice.

more sensitive to starvation induced apoptosis and their growth is anchorage-dependent. In *Gprc5a*<sup>-/-</sup> airway epithelial cells (Fig. 40), Socs3 protein is lower than in *Gprc5a*<sup>+/+</sup> cells due to the loss of Gprc5a and the autocrine Lif/Stat3 pathway is hyper-activated. The *Gprc5a*<sup>-/-</sup> airway epithelial cells have a prolonged response to autocrine Lif which causes a persistent activation of Stat3, leading to the high expressing these Stat3 targeted anti-apoptotic genes including Bcl-XL, Cryab, Hspa1a and Mcl1. The *Gprc5a*<sup>-/-</sup> airway epithelial cells exhibit increased survival and anchorage-independent growth and behavior like transformed cells which contributes to the development of spontaneous lung tumors in *Gprc5a*<sup>-/-</sup> mice.

## CHAPTER 6 DISCUSSION

### 6.1 New and unique mouse model of lung cancer

Human lung cancer is a dreadful disease causing ~30% of cancer death every year. The development of mouse models bearing lung tumors will likely bring in additional insights into the pathophysiologic perturbations of lung cancer; provide more targets for developing therapy drugs and serve as a good *in vivo* screen system for compounds against lung cancer. Several mouse models of lung cancer have been generated using genetic methods by knocking in oncogenes like mutant EGFR, mutant Kras and constitutive activated Stat3 or by knocking out tumor suppressors like p53. However, p53 tumor suppressor knockout mutant animals succumb to other tumors like lymphomas and sarcomas in early stage, precluding the development of lung cancer, although knockout mutant animals are not embryonic lethal like other tumor suppressor Rb-1 and WT-1. Our *Gprc5a* knockout mutant mouse model is a unique lung cancer model since that deletion of *Gprc5a*, a single tumor suppressor gene, is sufficient to mimic the human lung cancer phenotype in the mouse. Moreover, *Gprc5a* functions as a lung-specific tumor suppressor because it is primarily expressed in mouse lung tissue and no other organs developed tumors in *Gprc5a*<sup>-/-</sup> mice.

Human lung cancer patients are usually diagnosed at advanced stage with metastasis diseases, which are important reason for lung cancer death. *Gprc5a* is not a metastasis inhibitor since *Gprc5a*<sup>-/-</sup> mice do not show

metastasis at later stage so that we can not work on lung cancer metastasis just on the *Gprc5a*<sup>-/-</sup> mice. However, we can investigate the mechanisms of other factors which have effect on lung cancer metastasis using the combination of *Gprc5a*<sup>-/-</sup> mutant mice with other transgenic mice. It has been reported that combination of mutant p53 with Kras mutant or combination of knockout LKB with Kras mutant increased lung cancer metastasis. It is unknown that whether mutant p53 or knockout LKB affecting lung cancer metastasis needs the specific background on Kras mutant. To address this question, we can generate *Gprc5a* and p53 double mutant mice or *Gprc5a* and LKB double mutant mice to investigate whether mutant p53 or knockout LKB will increase the lung cancer metastasis on the *Gprc5a*<sup>-/-</sup> background mice. We believe that *Gprc5a* knock out model is better than the Kras model because *Gprc5a* is primary expressed in lung while Kras is expressed in variety tissues so that it will have less none specific effects.

## **6.2 Translational use of *Gprc5a*<sup>-/-</sup> lung cancer mouse model**

*Gprc5a*<sup>-/-</sup> mice spontaneously develop lung inflammation and lung adenocarcinoma and tobacco-specific carcinogen NNK not only accelerated the tumorigenesis but also increased the multiplicity of lung tumors, thus this model will be useful for screening and testing drugs for the prevention and therapy of lung cancer. *Gprc5a* function as a protector of tobacco carcinogen induced lung cancer and may also serve as a predict marker for lung tumor development of smoking people since hundred percentage of *Gprc5a*<sup>-/-</sup> mice developed lung

tumors when treated with NNK compared with none of *Gprc5a*<sup>+/+</sup> mice developed lung cancer. Smoking people with low or none expression of GPRC5A will be at higher risk of development of lung cancer relative to smoking people with normal expression of GPRC5A.

Our finding that Stat3 is persistently activated in *Gprc5a*<sup>-/-</sup> normal and malignant airway epithelial cells suggests Stat3 inhibitors like Stat3 decoy composing of a double-stranded oligonucleotide which corresponded closely to the Stat3 response element may be used to treat lung cancer patient with low expression of GPRC5A. We can also target the upstream of Stat3 activation using JAK inhibitors, dominant negative LIF protein and cell permeable SOCS3 recombinant protein. *Gprc5a* suppress Stat3 activation by stabilization of Socs3 protein so that we can identify the domain or small peptides from *Gprc5a* which stabilize Socs3 and will also be good drug for lung cancer treatment.

In summary, *Gprc5a* knockout mice are new and unique mouse model for lung cancer providing new targets like LIF and stratagem for lung cancer treatment. This model will be a useful tool to study the mechanism of lung cancer development and lung cancer metastasis.

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