Elucidating functions of Tyrosine 705 and Serine 727 residues of *Stat3 in vivo*

Undergraduate Honors Thesis

Presented in partial fulfillment of the requirements for graduation with honors research distinction in Molecular Genetics in the College of Arts and Sciences at The Ohio State University

by

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BACKGROUND

Stat3 is a ubiquitously expressed transcription factor

Stat3 is a member of the Signal Transducer and Activator of Transcription family of proteins. As the name implies, it is a transcription factor that regulates gene expression in response to growth factors and cytokines as well as external factors such as stress, carcinogens, and infection. For example, carcinogens like diethylnitrosamine induces IL-6 expression, which stimulates the JAK-STAT pathway.[1] The multiple avenues of activation lead to the phosphorylation of STAT3. After phosphorylation, the STAT3 protein undergoes dimerization and translocates to the nucleus where it binds to the gene promoter to activate transcription (Figure 1).

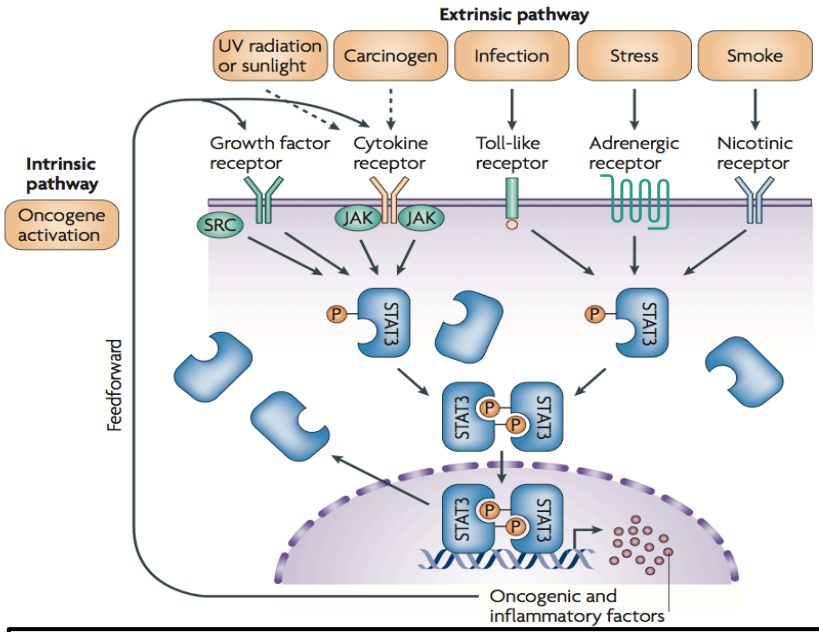


Fig. 1: A schematic representation *Stat3* activation pathways resulting in phosphorylation, dimerization, and translocation into the nucleus. *Nat Rev Cancer. STATs in cancer inflammation and immunity: A leading role for STAT3.* 2009 Nov; 9(11): 798-809

As a transcription factor, it regulates vital processes starting from maintenance of pluripotent stem cells to inflammatory responses, apoptosis, and proliferation.[2] Persistent activation of STAT3 has been observed in many cancers such as colorectal, liver, skin, and breast cancer, and attributes to increased carcinogenesis.[1] Activation of STAT3 was also found to have a significant role in acquired immune responses in colitis and inflammatory bowel disease.[3] Ubiquitous expression of *Stat3* and its role in inflammatory response promoting cancer makes it an important protein to characterize further, and findings will be potentially impactful in understanding human diseases that *Stat3* influences.

STAT3 protein and its key phosphorylation sites

STAT3 is a 90kDa multi domain protein.[4] The amino-terminus domain of STAT3 protein is involved in dimerization, the DNA-binding domain mediates binding of STAT3 to its consensus binding sites on the target gene promoter and is similar to that of other STAT family members. The carboxy-terminus of the protein harbors the transactivation domain (Figure 2).[1] There are two phosphorylation sites (Tyrosine 705 and Serine 727) in the transactivation domain that are involved in activation of STAT3 and has been studied extensively in the past.[5-7]

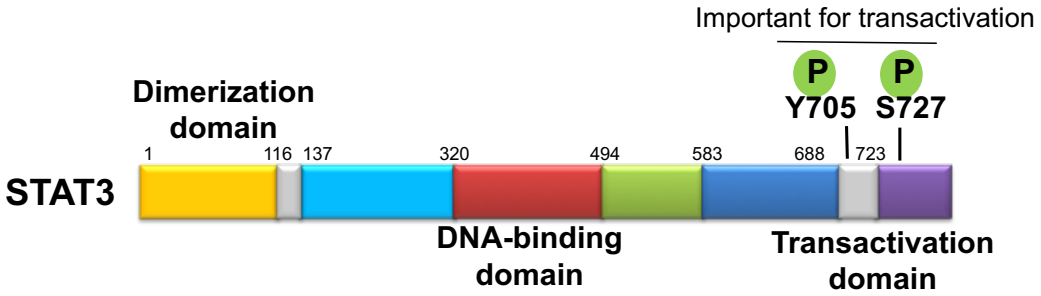


Figure 2: Schematic diagram of different domains of STAT3 protein

Canonically, Tyrosine 705 (Y705) residue was regarded as the primary and Serine 727 (S727) residue as the secondary phosphorylation site for STAT3 activation. Downstream targets of Y705 activation include apoptosis inhibitors (Mcl-1, HSP27, and Bcl-xL), cell cycle regulators (MEK5, c-Fox, and c-Myc), and inducer of tumor angiogenesis (VEGF, COX-2, MMP-1, MMP-2, and MMP-10), studied in breast cancer tissues. STAT3 is activated by cytokines such as IL-6, a common activator for the Janus kinase (JAK) and JAK-STAT pathway. Binding of IL-6 to its receptor activates JAK, which in turn phosphorylates STAT3 at Y705 residue leading to its activation and nuclear translocation.[1] As Y705 phosphorylation is downstream to cytokines mediated activation, it has an important role in inflammatory response, persistent activation of which is associated with the tumor microenvironment.[5,6] Functions of Y705 are not limited to inflammation. Phosphorylation of Y705 is necessary for mouse embryonic stem cell renewal during embryogenesis.[7]

The S727 residue on the other hand is phosphorylated by kinases, such as, ERK, JNK, p38, in response to mitogens.[8] The Ras-induced phosphorylation of S727 downstream of ERK kinase pathway plays a critical role in the mitochondrial activity of STAT3 which includes interactions with the electron transport chain complex.[8]

Question: Are functions of Y705 and S727 distinct or is there overlap?

Role of key phosphorylation sites of STAT3 in cancer cells

The downstream signaling events following phosphorylation of Y705 and S727 are distinct as well as there are some overlap and cooperation between the two sites. Qin *et al.* demonstrated the distinction between the two phosphorylation sites and proposed an alternative to canonical pathway which states that Y705 is the primary phosphorylation site. It has been

shown that human prostate cancer cells (LNCap) overexpressing non-phosphorylatable Y705 (inactive) and phosphomimetic S727 (constitutively active) grew faster than cells with non-phosphorylatable mutants of Y705 and S727 (inactive) under the same conditions. This finding was further confirmed when these cells were injected into mice, showing that their phenotypes remained the same *in vivo* and *in vitro*.[9] The data showed that constitutive phosphorylation at S727 site may be able to rescue the non-phosphorylatable Y705 mutation. As it has become evident now, many of *Stat3* studies regarding the transactivation domain artificially overexpress STAT3 *in vitro,* questioning the physiological relevance of these studies and demonstrating a need for an *in vivo* characterization of the transactivation domain of STAT3.

Question: Can *in vitro* studies and artificial overexpression of STAT3 realistically model the physiological functions of *Stat3*?

Stat3 in embryogenesis

Role of *Stat3* in embryogenesis has been studied using *Stat3* knock-out mice. *Stat3* null mice are embryonic lethal. Studies where mice heterozygous for *Stat3* allele were bred (*Stat3*$^{-/+}$ X *Stat3*$^{-/+}$) showed that homozygous embryos died around the seventh day of pregnancy.[10] This study demonstrated that STAT3 activity is critical during early embryogenesis. More detailed studies show that STAT3 activity fluctuates during embryogenesis. Initially, the embryo body seemed to have low STAT3 protein but around day 7 when differentiation is underway, STAT3 activity increased steadily until day 21, which is around partum.[11] Even though STAT3 is a member of a large family of proteins, other members of the STAT family is unable to rescue the embryonic lethality of *Stat3* null mutants. Studies conducted with *Stat3* null mice failed to

address if both Y705 and S727 residues of STAT3 are important for embryogenesis and if they have distinct function.

Question: Do Y705 and S727 have distinct roles during embryogenesis? Is phosphorylation of both sites, or just one of the two, critical for embryogenesis?

Stat3 in the mammary gland

Stat3 levels fluctuate when the mammary glands undergoes changes during pregnancy and involution. In an adult virgin female mouse, *Stat3* mRNA expression is high in the mammary epithelium and remains high until pregnancy. *Stat3* mRNA level declines during pregnancy and remains low during lactation.[12] After lactation, the mammary gland goes through involution, which is a process of gland remodeling to its pre-pregnancy stage. *Stat3* has been shown to be critical for initiation of involution. In a *Stat3* deleted mouse model, mammary gland involution following pregnancy and lactation is significantly delayed.[13] The cell death and tissue remodeling, which are characteristics of involution, occurred minimally in a mammary epithelium specific *Stat3* knock-out mouse model (*K14-Cre, Stat3*$^{FL/FL}$). In this mouse model, development and pregnancy were normal.[14] During involution, apoptotic functions of STAT3 facilitate massive cell death in the previously lactating glands, and this activity is autonomous regulating the non-classical lysosomal pathways of cell death during involution.[15] While it is clear that *Stat3* plays an important role in mammary gland involution, the role of the two key phosphorylation sites is still unclear.

Question: What specific roles do Y705 and S727 phosphorylation sites play in the mammary gland involution?

SIGNIFICANCE

Several *in vitro* studies have unraveled distinct and overlapping signaling pathways activated as a consequence of phosphorylation of Tyrosine705 and Serine727 residues of STAT3. All these studies were performed using cell lines overexpressing different mutants of *Stat3*. Very little is known about the physiological relevance of the *in vitro* studies. We therefore aimed to conduct a study for the first time to understand the role of the two key phosphorylation sites of STAT3 *in vivo*, using transgenic mouse models where *Stat3* mutants are knocked-in in the endogenous locus. By utilizing non-phosphorylatable and phosphomimetic mutations of Y705 and S727, functions of each phosphorylation site will be analyzed *in vivo*. Using the mouse embryonic fibroblasts derived from the knock-in mutant mice, we will have better understanding of the function of each of the residues in terms of their downstream signaling. We believe that utilizing physiologically relevant environments for *in vivo* studies and obtaining mouse embryonic fibroblasts (MEFs) from mice, contrary to overexpressing the mutant protein in cell lines, this will lead to a more accurate characterization of the transactivation domain of STAT3.

HYPOTHESIS

Previous studies have shown that loss of *Stat3* is embryonic lethal. One of the contributing factors is the role of *Stat3* in embryonic stem cell renewal. *Stat3* was shown to be indispensable for self-renewal of pluripotent stem cells.[16] Because both Y705 and S727 were shown to have significant roles in self-renewal of stem cells,[17] we hypothesize that the non-phosphorylatable mutations tyrosine705 to phenylalanine and serine727 to alanine (*Stat3* (Y705F) and *Stat3*(S727A)) will be embryonic lethal. On the contrary, we expect the

phosphomimemtic *Stat3*(S727E) knock-in mutants where S727 is mutated to glutamate with intact Y705 function to be viable.[10] Based on previous studies that showed that loss of Stat3 is lethal, we expect the *Stat3*(Y705F-S727A), where both sites are non-phosphorylatable, will be embryonic lethal as well. On the other hand, in *Stat3*(Y705F-S727E), the phosphomimetic *Stat3*(S727E) could rescue the loss of Y705 phosphorylation.

Mammary epithelial specific knock-out of Stat3 did not affect mammary gland development or lactation, it only delayed involution.[13] Given this, we hypothesized that while the *Stat3*(Y705F) and *Stat3*(S727A), the non-phosphorylatable mutants, will not have any effect on development of the mammary gland when compared to the wildtype *Stat3*, while *Stat3*(S727E), the constitutive phosphomimetic mutation, might. Activation of *Stat3* is important for involution, when massive cell death takes place.[13] We hypothesize *Stat3*(Y705F), *Stat3*(S727A), and *Stat3*(Y705F-S727A) will delay involution, as these are the key phosphorylation sites for activation of *Stat3*. On the contrary, the phosphomimetic *Stat3*(S727E) could accelerate the involution process.

AIMS

1. Elucidate the roles of STAT3 phosphorylation sites *in vivo*
    a. Generate transgenic mouse models expressing *Stat3* Tyrosine 705 (Y705) and Serine 727 (S727) mutants
    b. Determine the impact of the *Stat3* mutation on embryogenesis by studying the viability of homozygous mutants
    c. Study the effect of the *Stat3* mutants on mammary gland development
2. Characterize the Y705 and S727 mutants of *Stat3 in vitro*

STUDY DESIGN

1. Elucidate the roles of STAT3 phosphorylation sites *in vivo*.

1a. Generation of transgenic mouse models expressing *Stat3* Tyrosine 705 (Y705) and Serine 727 (S727) mutants.

There are five mutants of *Stat3* targeting Y705 and S727, alone or in combination that we plan to study in this project (Figure 3A):

- Y705F (tyrosine to phenylalanine): non-phosphorylatable mutant
- S727A (serine to alanine): non-phosphorylatable mutant
- S727E (serine to glutamate): phosphomimetic
- Y705F-S727A: non-phosphorylatable at Y705 and S727
- Y705F-S727E: non-phosphorylatable at Y705 and phosphomimetic at S727

These *Stat3* mutations will be knocked-in in the endogenous *Stat3* locus in order to generate the transgenic model organism (Figure 3B). Generation of the transgenic mice, confirmation of the genotype, are described in the Methods section.

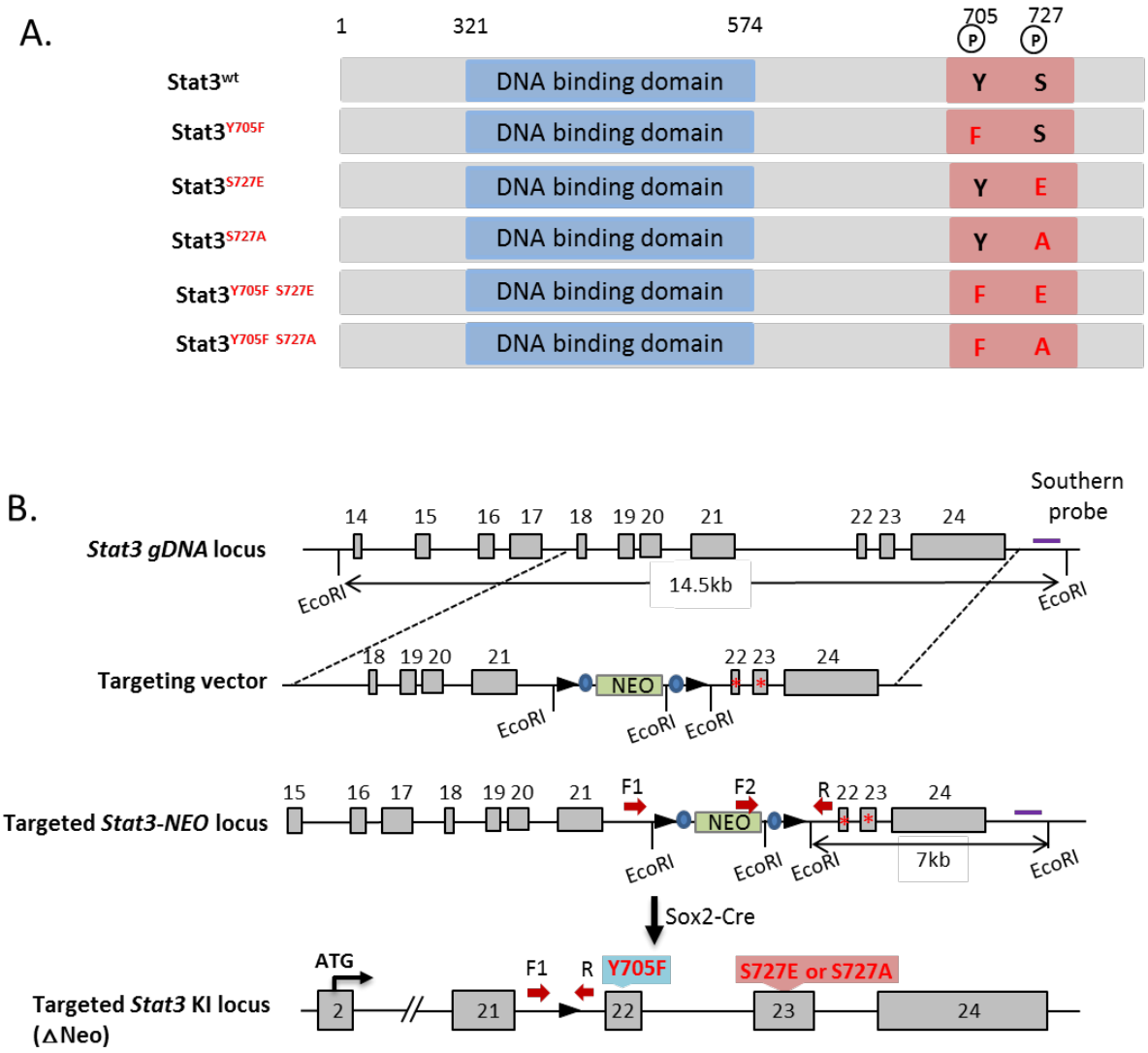


Fig. 3A: STAT3 activation and DNA binding domain.
Fig. 3B: Mutation constructs genetically recombined into transgenic mice. The Neo cassette is flanked by FRT sites (●) and LoxP sites (▶).

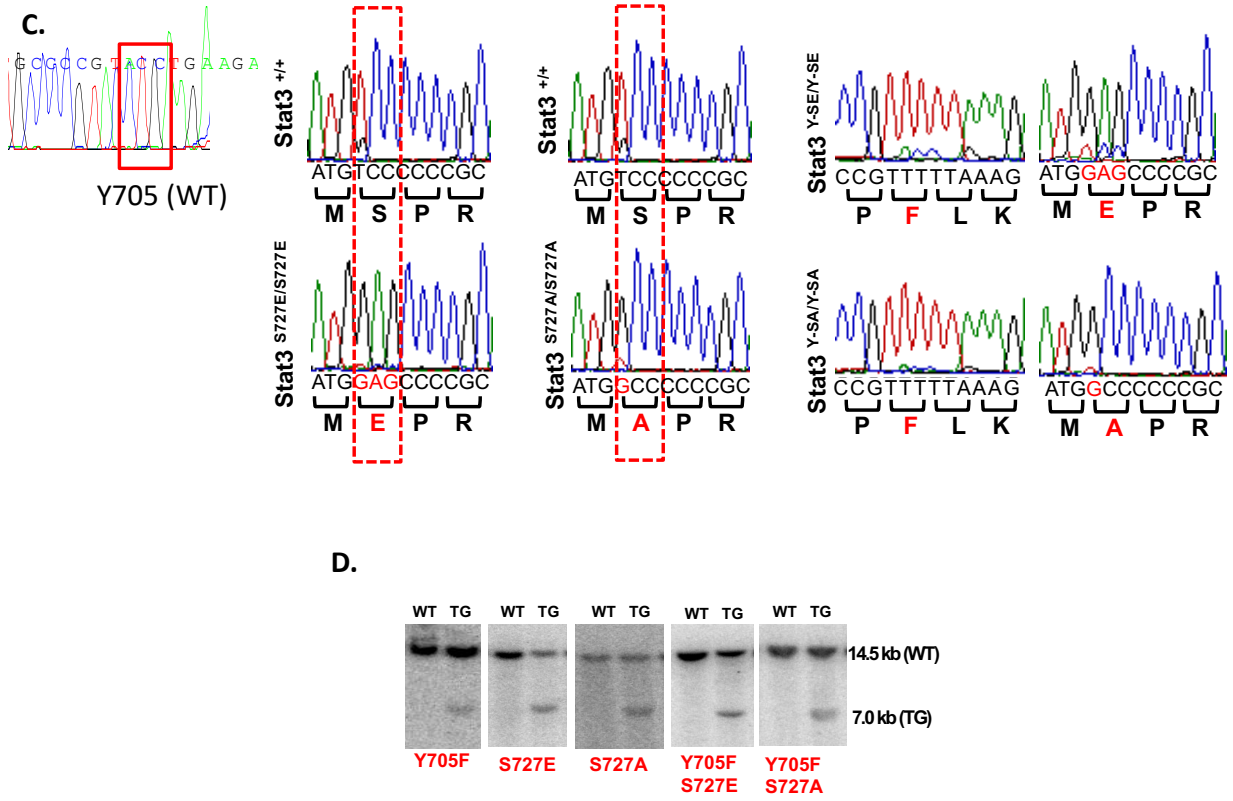


Fig. 3C: Nucleotide sequence of the mutated STAT3 clones used to generate the transgenic mutant mice, showing mutations at Y705 and S727 sites of STAT3.
Fig. 3D: Southern blot analysis of the DNA isolated from ES clones used to generate chimera of the transgenic mice. DNA was digested at restriction enzyme sites (EcoRI) as shown in Fig. 2B.

1b. Determine the impact of the *Stat3* mutation on embryogenesis by studying the viability of homozygous mutants.

Once germline transmission is successful and transgenic mice are obtained, the *Stat3* mutants will be knocked-in *in vivo* by breeding with Sox2Cre or ACTFlpe mice, as described in Methods. Next, mice heterozygous for knock-in mutants will be bred with each other to study embryonic viability. To assess the effect of the mutants on embryogenesis, we will compare the

observed ratio of the different genotypes with the expected Mendelian ratio from the heterozygous crosses.

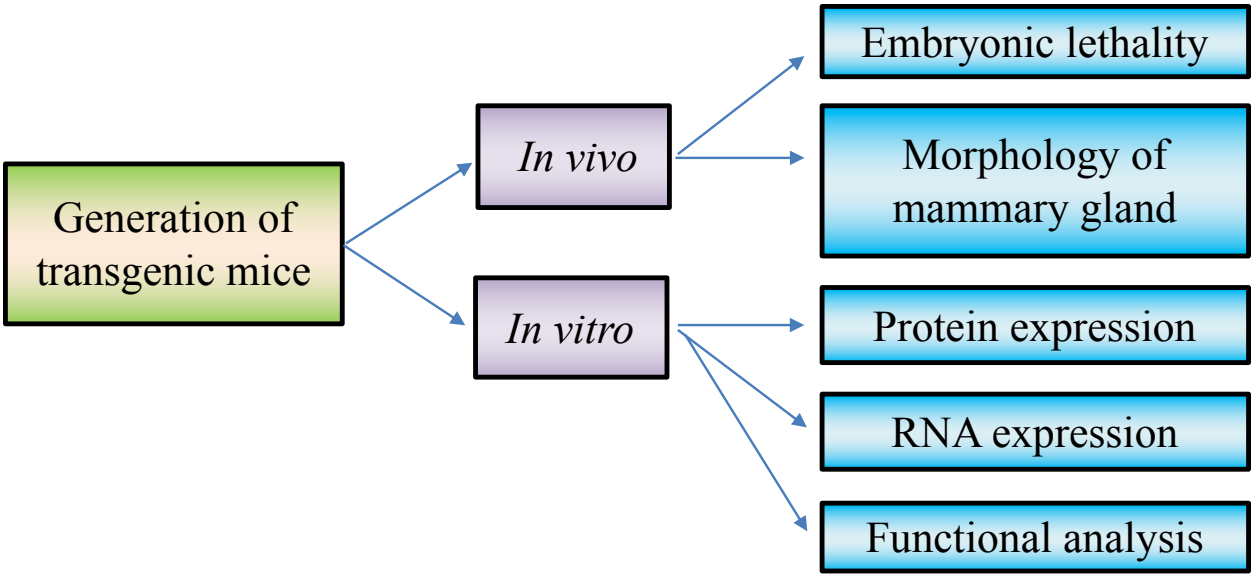
1c. Study the effect of the *Stat3* mutants on mammary gland development

Mice with constitutive activation of STAT3 will be harvested post-puberty (week 14). As our future plan is to study the role of the two phosphorylation sites of *Stat3* in involution, it is important to investigate how the mutant protein affects mammary gland development through puberty, pregnancy and lactation. The mammary glands will be collected from homozygous mutants, and wildtype mice to compare gross morphology of the glands from different mutants.

2. Characterize the Y705 and S727 mutants of *Stat3* in vitro

Embryonic fibroblasts will be harvested from mice harboring one mutant *Stat3* knock-in allele and one floxed *Stat3* allele. The floxed allele has LoxP sites spanning exons 18, 19, and 20. Deletion of which renders the allele functionally null after Cre-recombination. This will be done for each of the five genotypes stated above. After immortalization of the MEFs, they will be transduced with plasmids expressing Cre-recombinase, to delete the floxed allele, so that the only *Stat3* allele in the MEFs is the mutated allele. We will first analyze the mRNA and protein levels of each mutant *Stat3*, followed by analysis of downstream signaling events.

Synopsis of Study Design



METHODS

Transgenic mouse model

The transgenic mouse models were generated using the point mutations at the phosphorylation residues of STAT3. The mutations replace Tyrosine 705 with phenylalanine (Y705F), Serine727 with Alanine (S727A), Serine727 with glutamate (S727E). Additionally, there are two *Stat3* mutants that combine Y705F and S727A or Y705F and S727E (Figure 3A) that will be knocked-In in the mice. Y705F and S727A are non-phosphorylatable mutations, whereas S727E is a phosphomimetic mutation. A schematic of the mutations in the *Stat3* genomic DNA is shown in Figure 3B. A neomycin (neo) cassette was introduced between exons 21 and 22 of *Stat3*, harboring either Y705F mutation in exon22 or S727E/S727A mutation in exon 23. The neo cassette is flanked by LoxP sites as well as FRT sites at both ends to knock-in the mutant *Stat3* gene either in the whole body or in a tissue-specific manner (Figure 3B). Introduction of the neo cassette in the *Stat3* genetic locus blocks translation of the mRNA

transcribed from *Stat3* gene, because the neo cassette interferes with the reading frame of *Stat3*. Cre or Flpe-recombinase mediated removal of the neo cassette restores the reading frame while knocking-in the mutant. We also used *Stat3* floxed mice ($Stat3^{FL/FL}$), obtained from Jackson Laboratories (USA) where LoxP sites flanked exons 18, 19, and 20. Cre-mediated recombination of the LoxP sites renders the allele functionally null.

Plasmids carrying respective *Stat3* mutants were electrophoresed into mouse embryonic stem (ES) cells, which were subsequently used to generate *Stat3* mutant knock-in mice at Mouse Genomics core facility at The Ohio State University. The presence of the mutation in each mutant chimera (*Stat3* mutant mice with neo cassette) was confirmed by sequencing the tail DNA and Southern blot analysis (Figure 3C-D). Chimeras were bred with wildtype females to obtain germline transmission.

Knock-in mutation

The neo cassette from *Stat3*(S727A), *Stat3*(Y705F-S727E), and *Stat3*(Y705F/S727A) knock-in mutants was excised at the FRT sites by breeding mice harboring the transgene with ACTFlpe mice, harboring flipase. For *Stat3*(Y705F) and *Stat3*(S727E) mutants, the neo cassette was excised at the LoxP sites using Sox2Cre mice (Figure 3B). Both Sox2Cre and ACTFlpe breeder mice were from the Jackson Laboratory (USA). Sox2Cre and ACTFlpe are expressed throughout the whole body and as a result mutant *Stat3* was knocked-in in all the tissues.

Isolation of Mouse Embryonic Fibroblasts (MEFs)

In order to obtain MEFs with the knock-in and floxed alleles, mice were generated by breeding knock-in heterozygote with a flox/flox mutant mouse. MEFs were harvested on

embryonic day 13.5 (E13.5) for *in vitro* experiments. They were immortalized by repeated passaging in DMEM (Sigma Aldrich) with 10% fetal bovine serum (Sigma Aldrich), and antibiotic. Once immortalized, the floxed allele was excised using Cre-recombination.

Cre-mediated recombination

Cre-LoxP recombination was utilized to excise out the floxed allele in MEFs, using Lenti-virus harboring the Cre-recombinase. Exponentially growing HEK293T cells were transfected using jet-PEI reagent (Polyplus, USA) with pBabe-Hygromycin (empty vector) (ref) or pBabe-Cre plasmids, along with MuLV-Pac and MuLV-Env, coding for viral packaging and envelope proteins respectively. The viral supernatant from the HEK 293T was collected after 48 hours of transfection and filtered through 0.45μM filter. The viral supernatant was used to infect MEFs for 8 hours. The cells were infected twice. Upon Cre infection, the LoxP sites were recombined to delete the LoxP flanked exons 18, 19, and 20, rendering the floxed allele null. After the infection, the cells were selected for Cre-recombinase expressing cells only using the selectable drug hygromycin. Using this proceedure, we were able to obtain MEFs harboring only the mutant allele of *Stat3* for further analysis.

RNA isolation and analysis

RNA was isolated from exponentially growing MEFs using Trizol (Gibco). Total RNA was reverse transcribed using the High Capacity cDNA kit (Applied Biosystems). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) using SYBR green master mix (Bio-Rad) was used to measure the mRNA expression in the cells. Targets would include *Stat3* to first characterize the mutants and eventually, downstream targets of *Stat3*.

Whole cell lysate preparation and protein analysis

Whole cell lysates were prepared in RIPA buffer (150mM NaCl, 50mM Tris-HCl pH8.0, 1% Triton-X-100 or NP-40, 0.05% Na-DOC, 0.1%SDS) from cells in culture after washing twice with 1x PBS (phosphate-buffered saline, National Diagnostics). Whole cell lysates were separated on a 10% SDS-PAGE gel and transferred on to a PVDF membrane. The membrane was then blocked in Rockland buffer (Rockland Antibodies & Assays, USA), and incubated with anti-STAT3 mouse monoclonal antibody (Cell Signaling Technology) or anti-β-actin antibody (Cell Signaling Technology) over night at 4^0C. The membranes were next washed with 0.1% TBS-T (Tris-buffered saline containing 0.1% Tween-20) twice followed by incubation of the membranes in anti-mouse secondary antibody (Licor) for 1 hour at room temperature. The membranes were then washed in 1% TBS-T, 5 min each, and developed for fluorescent signals in Odyssey-CLX machine.

Mammary gland whole mount

Heterozygous *Stat3*(S727E) knock-in mice were bred to generate the homozygous mutant mice. Mammary glands harvested from *Stat3*(S727E) homozygous mutant mice were fixed in Carnoy's fixative (6:3:1 100 % EtOH : CHCl3 : glacial acetic acid) overnight. The fixed glands were stained in carmine aluminum solution (1g Carmine, 2.5g aluminum potassium sulfate in 500mL dH_2O, Sigma Aldrich) overnight and washed in increasing concentration of ethanol followed by mounting using xylene based mounting media.

RESULTS

Stat3 *Y705F mutation renders the protein unstable, acting as a functionally null allele* in vivo

Embryonic lethality

Stat3(Y705F) is an inactivating mutation of the Y705 phosphorylation site. Given the importance of Y705 residue as demonstrated by previous studies, specifically its role in embryonic stem cell renewal,[7] we hypothesized that mice harboring homozygous mutation for *Stat3*(Y705F) would be embryonic lethal. To study the effect of non-phosphorylatable Y705 amino acid on embryogenesis, we bred *Stat3*(Y705F) heterozygous knock-in mice and determined the genotype of the pups on postnatal day21 (P21). As shown in Table 4A, at P21, there was no homozygous *Stat3*(Y705F) mutant mice, when the expectation following Mendelian ratio was 25% (n=13) of the total number of animals (n=52). We next investigated viability of the embryos on embryonic day13.5, when embryos were harvested and genotyped. No homozygous mutant was observed, contrasting to the Mendelian expected ratio of 25% (n=6.25) out of total number of embryos (n=25). As *Stat3*(Y705F) was shown to be embryonic lethal on embryonic day13.5, we investigated at an earlier time point, embryonic day 8.5 to observe if homozygous mutant embryos could survive to this point. At embryonic day 8.5, there was no homozygous mutant, contrary to the expected Mendelian ratio of 25% (n=4.25) out of total number of embryos (n=17) (Figure 4A). The observed genotypes were significantly different than the expected ratios according to Mendelian genetics (p=0.005 E8.5, p=0.002 E13.5, p=9.1E-5 P21). In summary, *Stat3* lost its critical functions in embryogenesis with the non-phorphorylatable mutation of Y705 and no homozygous mutants were viable as early as embryonic day8.5.

Protein and RNA expression

As *Stat3*(Y705F) mimicked the *Stat3* null homozygous mice during embryogenesis, we next sought to determine whether protein and RNA expression were affected due to *Stat3*(Y705F). We analyzed STAT3 protein in the MEFs isolated from transgenic mice harboring $Stat3^{Y705F/FL}$ alleles.

Western blot analysis of the whole cell lysate prepared from MEFs with only *Stat3*(Y705F) mutated showed that there was no mutant protein suggesting that STAT3 protein was destabilized upon this mutation (Figure 4C). Another important finding in the protein expression was that before Cre-recombination, MEFs from $Stat3^{Y705F/FL}$ had comparable amount of protein with respect to MEFs either one or both *Stat3* alleles were floxed (Figure 4C). Quantitative RT-PCR analysis showed 50% reduction of *Stat3* mRNA, as the floxed allele was lost upon Cre-mediated recombination (Figure 4D). The lack of detectable STAT3 protein suggests that a) phosphorylation of Y705 residue of STAT3 is critical for its stability and b) that loss of STAT3 protein in the *Stat3*(Y705F) homozygous mutant mice, leads to embryonic lethality, mimicking the *Stat3* null mice. Although our plan was to analyze the functional consequences of this mutation but it was not possible to study the downstream signaling events of Y705 residue because of the loss of STAT3 upon mutation.

Fig. 4: Characterization of Stat3^{Y705F} in vivo and in vitro.

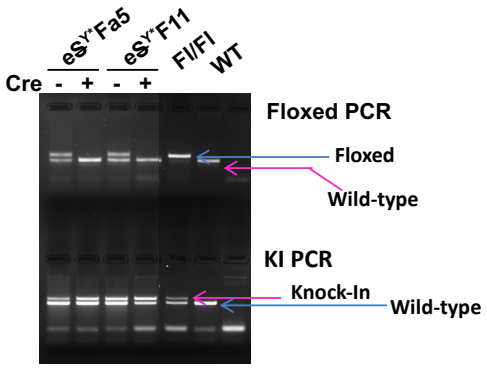
A. Mouse viability

P21	Observed	Expected	pValue
+/+	14	13	9.1E-5
Y705F/+	38	26	
Y705F/Y705F	0	13	
Total	52	52	

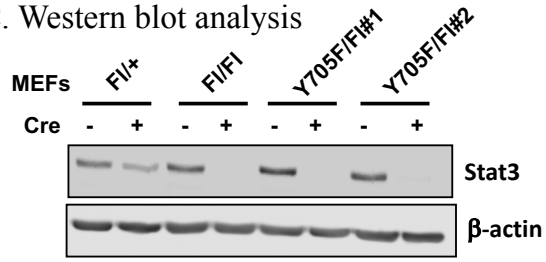
E13.5	Observed	Expected	pValue
+/+	4	6.25	0.002
Y705F/+	21	12.5	
Y705F/Y705F	0	6.25	
Total	25	25	

E8.5	Observed	Expected	pValue
+/+	2	4.25	0.005
Y705F/+	15	8.5	
Y705F/Y705F	0	4.25	
Total	17	17	

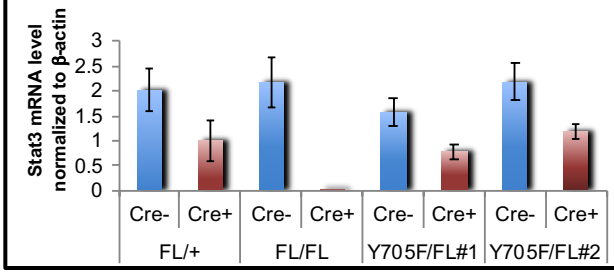
B. Genotyping PCR



C. Western blot analysis



D. qRT-PCR



Quantification

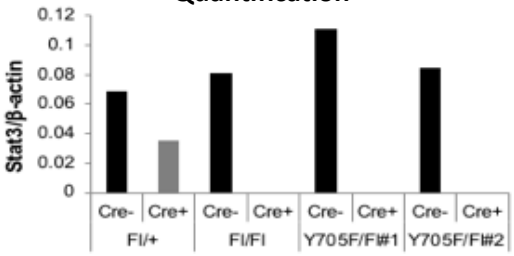


Fig. 4: Characterization of *Stat3*(Y705F) *in vivo* and *in vitro*

A. Comparison of the genotypic ratio of the offsprings obtained from a heterozygous mutant breeding to the expected Mendelian ratio. P21: postnatal day. E13.5: embryonic day 13.5. E8.5: embryonic day 8.5

B. Genotyping PCR of the Floxed and KI alleles using genomic DNA isolated from the MEFs, before and after Cre-recombination. Arrows indicate the bands corresponding to the floxed, knock-in, and wildtype alleles.

C. Western blot analysis of Stat3 using whole lysates prepared from MEFS as indicated. Amount of Stat3 protein normalized to the housekeeping gene β-actin is quantified in the bar diagram. MEFs with *Stat3*$^{Fl/+}$ and Stat3$^{Fl/Fl}$ alleles were included as controls , showing the result of deletion of one floxed allele as well as both floxed alleles.

D. Real-time PCR analysis of total RNA isolated from MEFs as indicated.

Phosphomimetic* Stat3 *Serine727 does not affect its stability or embryogenesis.

Embryonic lethality

To determine the effect of *Stat3*(S727E) mutation on mouse embryogenesis, we analyzed the embryonic viability of the homozygous mutants. We bred heterozygous knock-in *Stat3*(S727E) mice to obtain homozygous, heterozygous and wild type pups. The number of homozygous mutant (Stait3$^{S727E/S727E}$) mice born was comparable to the expected number calculated based on Mendelian ratio (p=0.4531, P21) (Figure 5A). Thus, we concluded that constitutive activation of *Stat3* at S727 does not affect mouse embryogenesis.

Protein and RNA expression

We next analyzed the stability of the *Stat3*(S727E) mutant STAT3 protein, using MEFs isolated from *Stat3*$^{S727E/FL}$ mice and deleted the floxed allele using Cre recombinase (as described in the Methods). Western blotting of the Cre positive MEFs (Cre+, *Stat3*$^{S727E/FL}$) showed half the amount of STAT3 protein compared to the Cre negative counterparts (Cre-, *Stat3*$^{S727E/FL}$). *Stat3*(S727E) mutation did not have any apparent effect on the stability of the protein (Figure 5C). As expected, the mRNA levels in Cre positive MEFs (Cre+, *Stat3*$^{S727E/FL}$) were ~ 50% of that in the Cre negative counterparts (Cre-, *Stat3*$^{S727E/FL}$) (Figure 5D). As the protein and mRNA levels were as expected, these studies will be followed by functional analyses of *Stat3*(S727E) in the future.

Mammary gland study

As the homozygous mutants were viable, we first sought to monitor the effect of *Stat3*(S727E) mutation on mammary gland development *in vivo*, as this is a constitutively active mutant at the S727 site, unlike wildtype *Stat3*. While the *Stat3* null mice showed normal

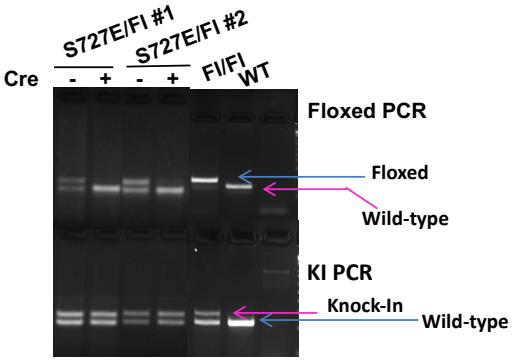
mammary gland development[9], the effect of constitutively active *Stat3* at S727 on mammary gland development has not been studied before. Specifically, gross morphology of the mammary gland was compared between homozygous mutants and wildtype animals using mammary gland whole mounts (Figure 5E). Our data revealed that post-pubertal mammary glands of $Stat3^{S727E/S727E}$ mice had more proliferative branching than the wildtype. As the estrous cycle and associated hormonal changes affect mammary gland proliferation, we stage-match all the mice of different genotypes. Both mice shown in Figure 5E were at the estrous stage of their estrous cycle. The data suggests that *Stat3*(S727E) mutation could stimulate proliferation of the mammary gland. We are continuing to study the effect of the *Stat3*(S727E) mutation on mammary gland development. In future, we will explore the effects of the *Stat3*(S727E) mutation on mammary gland involution, as any abnormal development would affect pregnancy, lactation, and involution.

Fig. 5: Characterization of Stat3^{S727E} *in vivo* and *in vitro*.

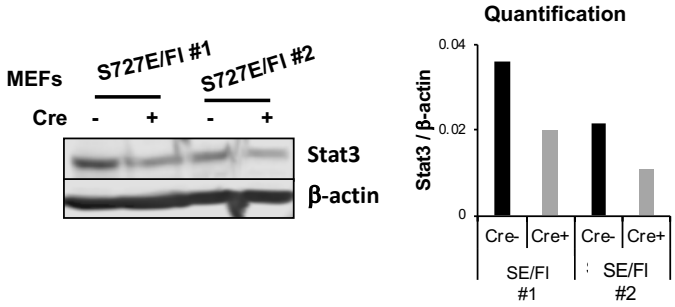
A. Mouse viability

P21	Observed	Expected	pValue
+/+	5	6	0.4531
S727E/+	15	12	
S727E/S727E	4	6	
Total	24	24	

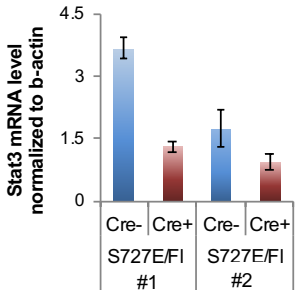
B. Genotyping PCR



C. Western Blot and Quantification



D. qRT-PCR



E. Mammary glands

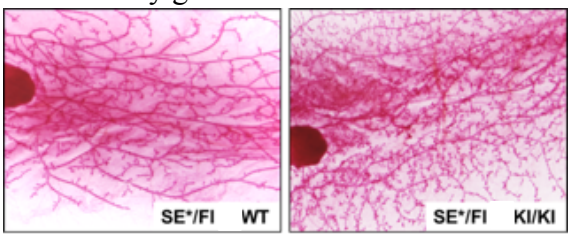


Fig. 5: Characterization of *Stat3*(S727E) *in vivo* and *in vitro*

A Comparison of the genotypic ratio of the offsprings obtained from a heterozygous mutant breeding to the expected Mendelian ratio. P21: postnatal day 21

B. Genotyping PCR of the Floxed and KI alleles using genomic DNA isolated from the MEFs, before and after Cre-recombination. Arrows indicate the bands corresponding to the floxed, knock-in, and wildtype alleles.

C. Western blot analysis of STAT3 using whole lysates prepared from MEFS as indicated. Amount of STAT3 protein normalized to the housekeeping gene β-actin is quantified in the bar diagram.

D. Real-time PCR analysis of total RNA isolated from MEFs as indicated.

E. Whole mounts of mammary glands harvested from post-pubertal mice harboring *Stat3*(S727E) knock-ins (*Stat3*$^{S727E/S727E}$) and wildtype alleles.

Stat3 Y705F-S727E is embryonic lethal and the mutant protein is unstable.

Embryonic lethality

Stat3(Y705F-S727E) is a non-phosphorylatable mutant at Y705 and phosphomimetic mutant at S727. Our studies showed that *Stat3*(Y705F) is embryonic lethal while *Stat3*(S727E) supports embryogenesis. We sought to study if *Stat3*(S727E) can rescue the lethality of *Stat3*(Y705F) mutation of *Stat3*. We bred mice heterozygous for *Stat3*(Y705F-S727E), $Stat3^{Y705F\text{-}S727E/+}$, to study the viability of the mutation. We have not obtained any homozygous mutants on postnatal day 21, when the expected Mendelian ratio was 25% (n=9.75) out of the total number of mice (n=39). Like *Stat3*(Y705F), *Stat3*(Y705F-S727E) knock-in homozygous mice were embryonic lethal (p=0.0014) (Figure 6A).

Protein and RNA expression

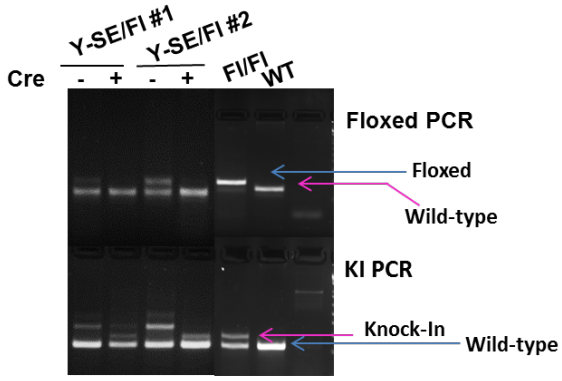
Using MEFs isolated from embryos harboring *Stat3*(Y705F-S727)E and floxed alleles, we further analyzed the effect of *Stat3*(Y705F-S727E) *in vitro.* As the floxed allele has LoxP sites spanning three exons, after Cre-recombination we obtained MEFs that only expressed the *Stat3*(Y705F-S727E) mutation(Cre+, $Stat3^{Y705F\text{-}S727E/Fl}$) . We found this mutation to be another functionally null mutation. Following Cre recombination of $Stat3^{Y705F\text{-}S727E/FL}$, no STAT3 protein was observed and RNA expression was significantly decreased (Figure 6C-D). The results showed that with respect to protein stability and embryogenesis, phosphomimietic of the S727 residue, was unable to rescue the dominant negative effects of inactivating the *Stat3*(Y705F) mutation. In terms of protein stability, the phosphorylation of Y705 is critical for STAT3 protein stability and phosphomimetic mutant at S727 was not able to rescue the phenotype.

Fig. 6: Characterization of Stat3$^{Y705F-S727E}$ *in vivo* and *in vitro*.

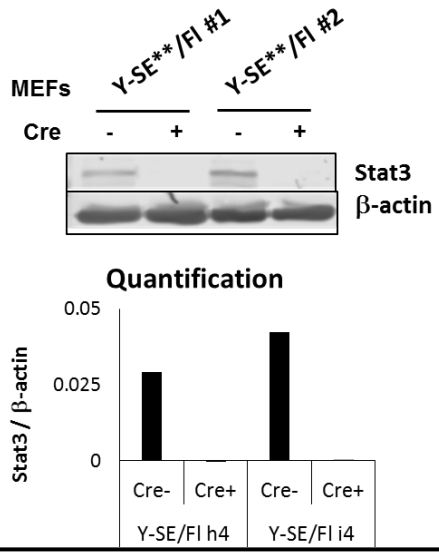
A. Mouse viability

P21	Observed	Expected	pValue
+/+	14	9.75	0.0014
Y705F-S727E/+	25	19.5	
Y705F-S727E/Y705F-S727E	0	9.75	
Total	39	39	

B. Genotyping PCR



C. Western blot analysis



D. Real-time PCR

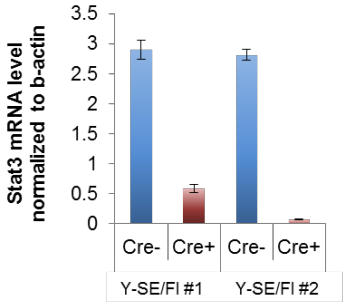


Fig. 6: Characterization of *Stat3*(Y705F-S727E) *in vivo* and *in vitro*
A. Comparison of the genotypic ratio of the offsprings obtained from a heterozygous mutant breeding to the expected Mendelian ratio. P21: postnatal day 21
B. Genotyping PCR of the floxed and KI alleles using genomic DNA isolated from the MEFs, before and after Cre-recombination. Arrows indicate the bands corresponding to the floxed, knock-in, and wildtype alleles.
C. Western blot analysis of STAT3 using whole lysates prepared from MEFS as indicated. Amount of STAT3 protein normalized to the housekeeping gene β-actin is quantified in the bar diagram.
D. Real-time PCR analysis of total RNA isolated from MEFs as indicated.

DISCUSSION

Previous studies have shown that *Stat3* null mutants are embryonic lethal, revealing critical role of *Stat3* during embryogenesis[11]. We have analyzed the importance of Y705 and S727, the two key phosphorylation sites of *Stat3,* in embryogenesis using transgenic knock-in mice. Our studies revealed that the mutation of Y705 to its non-phosphorylatable form *Stat3*(Y705F) rendered the homozygous mutants embryonic lethal. Our study also revealed that activation mutation of S727, *Stat3*(S727E), had no adverse effect on embryogenesis. In the *Stat3*(S727E) homozygous mutants the Y705 residue is intact and that could be the key to the function of *Stat3* in embryogenesis. Mice homozygous for double mutant of *Stat3*(Y705F-S727E) were also found to be embryonic lethal suggesting that loss of function due to *Stat3*(Y705F) mutation is not rescued by *Stat3*(S727E) mutation and that phosphorylation of these two key residues must have some distinct function during embryogenesis. Whether or not S727 residue is also critical for embryogenesis will be answered by our ongoing studies using S727A, a non-phophorylatable mutant of *Stat3*. These studies for the first time revealed critical and distinct role of Y705 residue of *Stat3* during embryogenesis.

Our in vitro studies using MEFs revealed that STAT3(Y705F) protein is unstable, which explains the embryonic lethality that was observed with $Stat3^{Y705F/Y705F}$ mice (Figure 4A). We have not observed any effect of the mutation on gene expression. However, it is important to note that the MEFs with one *Stat3*(Y705F) knock-in allele and one floxed allele ($Stat3^{Y705F/Fl}$) had same amount of protein as the MEFs that had two floxed alleles ($Stat3^{Fl/Fl}$) or are heterozygous for the floxed allele ($Stat3^{Fl/+}$). It is possible that in the $Stat3^{Y705F/Fl}$ MEFs, the wildtype STAT3 protein might be dimerizing with the STAT3(Y705F) and protecting it from degradation. In fact, previous studies have demonstrated dimerization of wild type STAT3 with

STAT3(Y705F) *in vitro*[18,19]. Alternatively, it is also known that *Stat3* regulates its own transcription by binding to its promoter[20]. Loss of the mutant protein might be compensated by increased transcription and translation of the stable wild-type *Stat3* as observed in Figure 4C. Because of the loss of STAT3(Y705F) protein we were unable to further characterize the downstream signaling events regulated by Y705 phosphorylation during embryogenesis.

Our data also demonstrated that constitutive activation of S727 site has no effect on protein stability, suggesting that loss of Y705 phosphorylation might be facilitating ubiquitination and degradation of STAT3. Previous studies have shown that STAT3 can undergo Ubiquitin –Proteasome mediated degradation, specifically in neonatal hippocampal neurons [21]. Similar to STAT3(Y705F) mutant, the double mutant STAT3(Y705F/S727E) protein is also unstable. This again shows the dominance of Y705 over S727 in terms of protein stability. We were not able to pursue further functional analysis as these mutant proteins were undetectable. It is also intriguing to note that the double mutation rendered mRNA level of *Stat3* to be significantly less than the single mutants. Whether or not rate of transcription or mRNA stability is affected when both mutations are present will be an interesting area for further studies.

As the mice homozygous for *Stat3*(S727E) mutants (*Stat3*$^{S727E/S727E}$)are viable, we studied its effect on mammary gland development and observed increased secondary and tertiary branching. This was somewhat surprising, as *Stat3* has previously shown to control lysosomal-mediated cell death during involution by upregulating cathepsin B and L[15]. *Stat3* may play a different role during development, as our data showed that constitutive phosphomimetic activity led to further branching, and not necessarily more cell death. We plan to further study the effect of *Stat3*(S727E) and *Stat3*(S727A) in mammary gland development and involution, provided the STAT3(S727A) mutant protein is stable.

Now that we know that non-phosphorylatable mutation at Y705 site of STAT3 renders the protein unstable, we are interested in the effect of the non-phosphorylatable *Stat3*(S727A) mutation on embryogenesis and protein stability. Studies along these lines are in progress. If this mutant protein is unstable, we expect to see embryonic lethality of the *Stat3*(S727A) homozygous mutants. This will unravel the importance of both the phosphorylation sites in stability of STAT3. If the protein is stable but the mutants are embryonic lethal, it will suggest that downstream signaling events regulated by S727 is critical for embryogenesis.

Stat3 is an important gene for various processes in the cell. For precisely this reason, understanding the nuances and intricacies of the two phosphorylation sites in the activation domain can lead us to new information and further our understanding of how *Stat3* fundamentally functions.

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