Background:
STAT3 signaling has been shown to play a role in almost all aspects of cancer biology including anti-apoptosis, cell transformation, growth and proliferation, angiogenesis, metastasis and cancer stem cell maintenance. A large amount of clinical and preclinical data in solid and hematological cancers supporting STAT3 as a therapeutic target. As a result, there are a number of STAT3 inhibitors in clinical trials and many more in active development. TTI-101 currently in phase 1 clinical trials is a STAT3 inhibitor that competitively inhibits STAT3 activation by directly targeting the pY-peptide binding site within STAT3’s SH2 domain, thus blocking key steps in its activation process, starting with the recruitment to activated cytokine and peptide hormone receptors and consequently; cytokine dependent homodimerization, nuclear translocation and DNA binding. In this work we generated and characterized breast cancer cell lines MDA-MB-468 for validation of mechanism of STAT3 inhibitor TTI-101.

Materials and Methods:
15 MDA MB breast cancer cells were cultured in DMEM, 10% FBS at 37°C with an atmosphere of 5% CO2. Protein expression was analyzed with western blots as well as Lumix Assay. STAT3 activation was also examined using the TransAM® STAT3 Activation Assay. Immunofluorescence microscopy was used to visualize nuclear translocation. qPCR was utilized to measure the effect of TTI-101 exposure on the expression of STAT3 dependent genes: CCL2, IL8, MUC1, SOC3, and Cyclin D1.

Results:
CRISPR-Cas9 approach was used to assess the genetic effects of STAT3 blockade. Three different types of MDA MB 468 cell lines were generated; STAT3 knockout (KO), STAT3 Y705H mutants and STAT3 WT controls. The cells were sequenced to confirm changes at the DNA level. Absence of STAT3 protein expression of knockout clones and response to IL6 was confirmed using western blot analysis. Optimal conditions for IL6 stimulation and TTI-101 treatment were determined using western blot analysis, real time PCR and immunofluorescence. The results show that maximum STAT3 phosphorylation was achieved after 15min of 100ng/ml IL6 treatment. TTI-101 at a concentration of 33µM was shown reduce IL6 dependent phosphorylation by more than 90% after 4Hrs of pretreated. Importantly, levels of iSTAT3 and beta actin remained unchanged at this concentration. Immunofluorescence data showed that Y705H mutated cells prevented STAT3 from translocating to the nucleus in response to IL6 stimulation, showing that nuclear translocation is dependent on phosphorylation at Y705.

Conclusions
Optimal experimental treatment conditions of IL6 exposure length and TTI-101 concentration were identified. These conditions will guide future experiments to further investigate the impact of TTI-101 on MDA MB 468 cells. The KO Clone identities were confirmed and can be used in larger experiments. Y705H mutants, STAT3 does not translocate to the nucleus, as depicted in green, when exposed to IL6. The control clone shows STAT3 once translocated to the nucleus.

References