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TITLE: **A Novel Strategy to Inhibit Hedgehog Signaling and Control Growth of Androgen-Independent Prostate Cancer Cells**

PRINCIPAL INVESTIGATOR: **James A. Cardelli, Ph.D.**

CONTRACTING ORGANIZATION: LOUISIANA STATE UNIVERSITY SYSTEM
Shreveport, LA 71103-4228

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6. AUTHOR(S)

James A. Cardelli, Ph.D.
Professor Microbiology

E-Mail: jcarde@lsuhsc.edu

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14. ABSTRACT

The original hypothesis was that a novel proprietary compound we identified in a screen named LS122, would be a potent inhibitor of the kinase STK36 which had been proposed to play a role in hedgehog signaling. This pathway was been hypothesized to contribute to progression of castrate resistant prostate cancer (CRPCa). The original proposal and statement of work proposed that inhibition of STK36 would prevent nuclear translocation of Gli, the transcription factor important in this signaling pathway. The proposal also described in Task 2 the use of a high content screen to identify additional STK36 inhibitors using an available kit. We initiated these proposed experiments and to our disappointment, LS122 did NOT affect the Hedgehog pathway at all and furthermore, no kit existed that would allow us to screen for additional STK36 inhibitors. Fortunately, we confirmed that LS122 was a potent NFKB inhibitor most likely by targeting the kinase RIP2K which is active upstream of NF-□B. Since NF-□B is an important signaling pathway implicated in CRPCa progression, our new SOW focusing on this pathways, is still is in line with the overarching mission of developing agents to treat CRPCa. Details of our findings will be presented in the final report. As a positive achievement, we were able to execute a patent of LS122 in the U.S. and Europe.

15. SUBJECT TERMS
Prostate cancer, RIP2K, proprietary drug, NF-kappa B, Castrate Resistance Prostate Cancer

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INTRODUCTION

This document represents the final report of grant W81XWH-12-1-0011 entitled "TARGETING OF RIP2K TO INHIBIT CRPCa PROGRESSION". The grant was activated in May of 2012 and following a 6 month no cost extension was terminated at the end of December 2013. An annual report was filed last month. We have discovered with the help of the PERD Institute a novel compound that appears to be anti-inflammatory and anti-cancer in part through targeting a kinase named RIP2K. This kinase is known to play a role in bacterial and viral infections but only recently has it become apparent that this kinase may play a role in cancer. In fact, a recent publication suggests it play an important role in triple negative breast cancer as well as prostate cancer. The compound we describe in this report, LS122, has been patented in the U.S. and in select countries in Europe and with the new information we supply in this report we hope to license this compound to a drug company to further advance development towards clinical trials in cancer patients.

KEYWORDS

Defined in the text

PROGRESS REPORT AND OVERALL SUMMARY

The following section contains the rationale for the modified SOW and our final progress report for this grant summarizing research data for each Task.

Rationale for a new SOW and No Cost Extension:

In the last few years we have seen an increase in new FDA-approved drugs for prostate cancer (CaP), more new molecular targets, and more hope for survival benefit than in the whole decade before. Despite this progress, advanced CaP therapy remains an underserved area of research and none of these newly approved drugs have shown long-term survival benefits. Death and suffering from prostate cancer is virtually all due to progressive, therapy-resistant disease and metastastic prostate cancer is the deadly form, particularly when it goes to the bone. Our ultimate goal is to provide: 1) a new drug for advanced, metastatic prostate cancer patients that has efficacy to prevent the progression of prostate cancer to hormone and chemotherapy resistance, 2) to limit dissemination of cells to visceral tissues and the bone, and 3) to treat those cells that reach the bone microenvironment.

It had been proposed in the original application, that LS122 would target the Hedgehog pathway which contributes to CRPCa progression. However, our preliminary experiments did not support this hypothesis. Therefore we investigated another pathway important in PCRPCa. In a set of preliminary pre-clinical studies presented below, we have found that the novel small molecule (LS122) to have low toxicity and high efficacy as an anti-cancer and anti-osteogenic molecule most likely by targeting an upstream component (RIP2K) of the NF-κB pathway. LS122 was discovered in collaboration with the PERD Institute and is classified as a thiophene. The position in the molecule of the substitutions and the substitutions themselves are novel and designed to reduce cardiotoxicity seen in similar drugs of this class, while increasing specificity and efficacy. LS122 was rationally designed as an anti-inflammatory and to protect against the cardiotoxicity associated with FDA-approved drugs in the same drug class. A patent has been filed and executed.

There are several reasons why LS122 is a desirable drug for clinical development: 1) it has potential as a sensitizer to standard of care chemotherapeutics, 2) its kinase inhibitory activity against a small number of important and unique kinases for prostate cancer, 3) the tissue distribution of its targets relevant to human prostate cancer progression, and 4) the low toxicity and high efficacy LS122 demonstrated in preliminary studies. In addition, LS122 is a quinazolinone, which as a class, has been described to re-sensitize resistant cells to chemotherapeutics, particularly taxanes [1]. RIPK2 is overexpressed in adaptive immunity, inflammation and cancer; particularly noteworthy is its overexpression in osteoblasts [2] and our observation of overexpression, as well as phosphorylation, in the PC-3 and LNCaP derivative C4-2B4 prostate cancer cells (unpublished data). RIPK2 is initiated by stimulation of the TNF-α receptor, a common occurrence in human CaP that is linked to progression to castrate-resistance as well as bone metastases; the clinical utility of TNF-α inhibitors for prostate cancer has been suggested for that reason [3-6].

NF-κB is a downstream target of RIPK2 that is also integral to both castrate-resistant progression and bone metastasis [7-12]. LS122 has shown both direct and indirect anti-NF-κB activities (see data below) and as such may be particularly useful in controlling functional changes downstream of NF-κB as well. LS122 has a limited number of kinase targets and therefore potentially fewer side-effects and off-target effects; problems associated with current FDA-

approved drugs of this class. We chose to characterize LS122 and prostate cancer because NF-κB is a key regulator of prostate cancer progression and metastasis, particularly in its role in bone metastasis. LS122 is a new chemical entity with novel substitutions and a novel mechanism of action. LS122 is in a drug class that has historically acted to sensitize chemo-resistant cells to standard-of-care chemotherapeutics. In particular, in our models LS122 has anti-angiogenic and anti-invasion activities, it limited dissemination of cancer cells to distant sites *in vivo*, and LS122 has also blocked osteoblastic and osteolytic activation and funtion. We therefore propose in this modified SOW that, like the first application, we will study the role of a understudied kinase, RIP2K, in prostate cancer progression and to further evaluated LS122 has a possible drug for clinical development.

FINAL STATEMENT OF WORK FOR:
W81XWH-12-1-0011
NEW TITLE: TARGETING OF RIP2K TO INHIBIT CRPCa PROGRESSION
This new SOW was approved by Joshua McKean

Task 1 Establish Cell 3D co-culture models for sensitive and taxane resistant prostate cancer cells along with tumor associated fibroblasts.

Task 2 Test LS122 as a NF-κB inhibitor via targeting RIP2K using 2D and 3D cell culture models.

A. Demonstrate using 2D culture that LS122 is a potent inhibitor of NF-κB and targets RIP2K. We propose to knockdown expression of RIP2K and MEKK3 to determine if they are the primary targets of LS122 responsible for regulating growth, motility and invasion of prostate tumor cells.
B. Demonstrate that LS122 acts in 3D culture to block prostate cancer motility and invasion via inhibition of RIP2K. We propose to knockdown expression of RIP2K and MEKK3 to determine if they are the primary targets of LS122 responsible for regulating growth, motility and invasion of prostate tumor cells.
C. Perform proliferation and apoptosis assays to determine if LS122 sensitizes PC3 and DU145 cells to taxanes in 2D and 3D culture

These proposed studies will be greatly aided using a high throughput real time imaging platform called the ZOOM incucyte. The P.I. directs the drug discovery and development core and has access to this imaging platform.

Task 3 Determine if RIP2K plays a role in osteoclasts/osteoblast function or differentiation . Preliminary results suggest LS122 alters the function and/or differentiation of bone localized cells critical in prostate cancer bone metastasis establishment and growth. These studies will be repeated and extended to determine effective doses of LS122 that work and the pathways it works on to target cells playing a key role in growth of PCa in the bone.

Task 4 Write up manuscript for submission. IN PROGRESS

RESEARCH PROGRESS

Task 1 ***Establish Cell 3D co-culture models for sensitive and taxane resistant prostate cancer cells along with tumor associated fibroblasts.***

Results and Discussion

FIGURE 1. STRUCTURE OF LS122, a modified theophene belonging to the quinazoline class of drugs. This is the compound we have selected from over 100 screened to date. We have an executed patent in the U.S. and select countries on this compound and related compound in this class.

FIGURE 2. DU145 tumor cells were grown on top of matrigel. After spheroids formed, HGF, the growth factor for the invasion and metastasis stimulating receptor Met, was added and cells were incubated for an additional 24 hours. HGF aloing with EGF (not shown) stimulate the formation of invasive cells into the matrigel. This was our first attempt at generating spheroids and it works reproducibly. The problems related to this approach is the slow image acquisition time and measurement of final spheroid size. Time lapse photography is difficult.The approach works for all the prostate cancer cell lines we have tried including PC3, PPC1 and LnCap in addition to DU145 shown here.

FIGURE 3. DU145 tumor cells were grown on top of matrigel containing or not containing WPMY prostate fibroblasts. Without fibroblasts the tumor cells grow into noninvasive spheroids while in the presence of the fibroblasts, invasive structures form. This also works with other cell lines. Unfortunately, this assay demonstrated that LS122 at concentrations approaching 20 micromolar did not block invasiveness.

FIGURE 4. We purchased the IncuCyte platform from Essen Bioscience which allows for real time image acquisition since the device is a camera and a microscope that remains in the tissue culture incubator. This experiment shows a time lapse sequence for PPC1 and a PPC1 cell line in which the Arl8 protein was knocked down by shRNA technology. It demonstrates the ability to measure changes in the same cell masses formed in matrigel over time and also demonstrates that a KD of Arl8b results in a dramatic reduction in growth.

FIGURE 5. We have also been successful at generating spheroids for any cell type in a 96 well plate format. Cells are seeded in Matrigel in round bottom low cell attachment plates. This results in the formation of a single spheroid in the center of each well of the identical size. Plates are placed in the IncuCyte system and photographs are automatically taken every 4 hours.

Figures 6 and 7. In these experiments, two different cell lines were seed in 96 well plates to form spheroids which were incubated with two versions of a natural product. Software associated with the IncuCyte can calculate the average volumes of each spheroid and this is presented in both figures. This is the approach we have taken to analyze the effects of LS122.

Task 2 ***Test LS122 as a NF-κB inhibitor via targeting RIP2K using 2D and 3D cell culture models.***

- ***A. Demonstrate using 2D culture that LS122 is a potent inhibitor of NF-κB and targets RIP2K. We propose to knockdown expression of RIP2K and MEKK3 to determine if they are the primary targets of LS122 responsible for regulating growth, motility and invasion of prostate tumor cells.***
- ***B. Demonstrate that LS122 acts in 3D culture to block prostate cancer motility and invasion via inhibition of RIP2K. We propose to knockdown expression of RIP2K and MEKK3 to determine if they are the primary targets of LS122 responsible for regulating growth, motility and invasion of prostate tumor cells.***
- ***C. Perform proliferation and apoptosis assays to determine if LS122 sensitizes PC3 and DU145 cells to taxanes in 2D and 3D culture***

Results and Discussion

Task 2A. Demonstrate using 2D culture that LS122 is a potent inhibitor of NF-κB and targets RIP2K. We propose to knockdown expression of RIP2K and MEKK3 to determine if they are the primary targets of LS122 responsible for regulating growth, motility and invasion of prostate tumor cells.

FIGURE 8 10 micromolar LS122 inhibits phosphorylation of the p65 subunit in the presence of TNF-α (left panel). In the right panel, LS122 and TNF alpha treated mice demonstrate lower levels of an NF-kB expression cassette (C) compared to control mice (A) or mice treated with TNF-α.

FIGURE 9 These studies were done in collaboration with the Agarwal laboratory, internationally recognized for studies on NF-kB. The cancer cell line SW480 was serum starved for 24 h, pretreated with 242-A, 242-NM and 242-bag (3 and 10 uM) for 2 h and then exposed to TNF-α (10 ng/ml) for 10 min. Each of these drugs are different synthesized lots.

Thereafter, nuclear and cytoplasmic fractions were prepared, and analyzed for NF-kB signaling molecules (p50, p65, IkBα) and by Western blotting and NF-kB activation by EMSA. Western blot analyses showed that in SW480 cells, TNF-α exposure induced the nuclear localization of p50 with a corresponding slight decrease in the cytoplasmic p50 level . 242-A, 242-NM and 242-bag (3 and 10 uM each) treatment strongly inhibited the p50 nuclear localization in SW480 cell with an increase in cytoplasmic p50 level. TNF-α exposure also increased the nuclear p65 level in SW480 cells along with a clear decrease in cytoplasmic p65 level. Even though an increase in cytoplasmic p65 was observed with 242-A (3 and 10 uM) a corresponding decrease in nuclear p65 level was not clearly evident. Both, 242-NM and 242-bag decreased the nuclear p65 expression but an increase in cytoplasmic p65 was observed only with 242-bag. IkBα is known to inhibit NF-kB activation, and our results showed that TNFα exposure resulted in a decrease in IkBα expression in SW480 cells. In general, all the three compounds increased the IkBα expression even though with different potency. Membranes were also stripped and re-probed for PARP as nuclear loading control and for tubulin as cytoplasmic loading control. Additionally experiments are being performed with DU145 and PC3 prostate cancer cells.

FIGURE 10 Western blot analyses showed the effects of 242-A, 242-NM and 242-bag (these are different lots of the same drug) on the expression and localization of NF-kB signaling molecules (p50, p65, and IkBα); therefore to clearly understand the effect of these three drugs on NF-kB activation we performed EMSA. In both SW480 and LoVo cells (not shown), TNFα exposure activated the NF-kB, measured in terms of its DNA binding. The treatment with 242-A, 242-NM and 242-bag (3 and 10 uM each) strongly inhibited the TNFα-induced NF-kB activation in SW480 cells at both the doses (Left Panel). The specificity of EMSA bands as well as constituents of NF-kB were determined by super-shift and competition assays, respectively. Competition assay confirmed the position and specificity of NF-kB bands (Middle Panel). In super-shift assay, nuclear extracts (TNFα exposed samples from SW480 and LoVo cells) were first incubated with either anti-p50 or anti-p65 antibody followed by EMSA. This assay showed a strong super shift to a higher molecular weight band in case of both anti-p50 and anti-p65 antibodies, suggesting that the observed NF-kB band consisted of these two subunits.

FIGURE 11 Quinazoline drugs are often kinase inhibitors. For example, erlotinib and gefitinib are 2 well-studied drugs of this class that have shown efficacy in some solid tumors, but have also been plagued with off-target effects against a wide variety of receptor tyrosine kinases and dose-limiting cardiotoxicities. LS122, along with all other hits from our initial reporter cell screen including all thiophene derivatives were analyzed [at DiscoveRx, previously KINOMEscan/Ambit Biosciences, San Diego, CA] for inhibition against a panel of 422 kinases (left panel). LS122 significantly inhibited only 4 kinases; most potently, RIPK2. Two of the other kinases, ZAK and TNNI3K, were in the same family, while STK36 (fused), the kinase responsible for nuclear translocation of Gli-1 downstream of sonic hedgehog activation, was the fourth kinase significantly inhibited by LS122 but further assays we performed indicated this kinase was not inhibited by LS122. We validated that in PC-3 cells; LS122 inhibited phospho-RIPK2 independent of TNF-α stimulation (bottom right panel), but had no effect on RIP2 total protein levels.

FIGURE 12 A model demonstrating the different pathways leading to activation of NF-□B. We propose that LS122 inhibits RIP2K which in turn acts on MEKK3 to initiate signaling to NF-kB.

FIGURE 13-14 <u>In vivo anti-cancer assays:</u> As a regulator of NF-κB and AP-1 signaling, LS122 may play an important role in many facets of prostate cancer progression and metastasis. We have found LS122 to be efficacious in a variety of standard anti-cancer assays. For example, as illustrated in Figure 15 and 16, LS122 significantly limited the tumor volume in 2 subcutaneous xenografts models; PC-3 and syngeneic RM-1. LS122 significantly decreased expression of VEGF and other angiogenic factors in cultured cells (data not shown) and potently reduced the number of new vessels in xenograft PC-3 and syngeneic RM-1 prostate tumors (Fig 16).

FIGURE 15 No significant organ toxicity has been seen with daily delivery of LS122 at 50 mg/kg oral or IP for as long as 90 days, nor did mice lose any body weight or show signs of systemic toxicity, making LS122 a potentially efficacious and low toxicity drug.

FIGURE 16 In the organ to organ metastasis model, tumor cells were directly injected into the spleen (n=5 per group) and allowed to migrate to the liver as an aggressive experimental metastasis model. Spleens from all mice were populated with numerous tumor nodules. Livers from untreated mice all had significant numbers of large tumor nodules; however, only one small nodule was found in one liver of LS122 treated mice.

FIGURE 17. We infected PC3 cells with Lenti Viruses expressing different shRNAs to RIP2K and we were successful in generating knockdowns. Clones 97-3 and 97-4 had a reduction in RIP2K of greater than 75% while the knock down levels were smaller for Clone 55-7. These cells and a non target selected control were used in the experiments described next.

Task 2B. Demonstrate that LS122 acts in 3D culture to block prostate cancer motility and invasion via inhibition of RIP2K. We propose to knockdown expression of RIP2K and MEKK3 to determine if they are the primary targets of LS122 responsible for regulating growth, motility and invasion of prostate tumor cells.

FIGURE 18 Using the technology described in figures 6 and 7, we analyzed the effects of LS122 on 3D growth of DU145 cells. HGF, EGF and TNF alpha all stimulated growth of spheroids compared to untreated cells. LS122 significantly inhibited the growth of spheroids treated with TNF alpha and had a lesser effect on HGF stimulated growth. EGF stimulated growth was not inhibited by LS122. We conclude that LS122 primarily targets TNF alpha signaling consistent with RIP2K being a primary target for this compound.

FIGURE 19 The Essen Bioscience ZOOM Incucyte real time imaging platform was used to measure motility of PC3 cells. The top panel represents PC3 cells in the presence or absence of the growth factor HGF and LS122 at 20 micromolar. At this concentration, LS122 blocks PC3 motility plus or minus HGF. The bottom panel shows comparable data for 10 micromolar LS122. LS122 also blocks invasion of PC3 cells through matrigel using the Incucyte system (not shown).

FIGURE 20-23. We first explored the role of RIP2K in proliferation in 2D cultures. Control cells or the three KD clones were left untreated, given solvent control, treated with LS122 (20 micromolar), or treated with TNF alpha with our without LS122. Figure 20 illustrates using the cell titer blue assay that none of these treatments effected proliferation of control cells. Figures 21-23 demonstrate that proliferation of clones 97-3 and 97-4 were comparable to control cells while clone 55-7 demonstated slower growth. None of the treatment conditions significantly altered proliferation. We conclude that RIP2K does not play a role in growth of prostate tumor cells in 2D growth environments.

FIGURE 24-27. We next determined with RIP2K and/or LS122 regulated apoptosis. For this assay, control cells or the KD clones were not treated or treated with either TNF alpha, LS122 or a combination of both. The incuCyte platform was used to measured increases in fluorescence of detected caspases over time. Figure 24 demonstrates that treatment of control cells with TNF alpha results in a relative 8 fold increase in apoptotic cells by day 3 while there is an observed 3 fold increase in untreated cells which is completely blocked by LS122. This compound by itself does not lead to an increase in apoptosis. This result is intriguing since one prediction would be that LS122 would block TNF alpha induction of a pro survival pathway thus leading to an increase in apoptosis, the opposite of what was observed. Also, the relative rate of increase in apoptotic cells in the two clones with the greatest KD of RIP2K, clone 87-3 and clone 97-4, was much greater in untreated and TNF alpha treated cells compared to control, consistent with the reduction in activity in the pro survival RIP2K active pathway. Surprisingly, LS122 greatly reduced apoptosis in the KD clones. One conclusion is that LS122 also targets a non RIP2K death pathway.

FIGURE 28-31. Next, we used the IncuCyte platform to measure motility in real time. Control cells and the 3 KD clones were grown to confluency in 96 well plates and then a wound was administered using a calibrated wounding device. Cells were left untreated or treated with HGF and TNF alpha plus and minus LS122. Cells were also treated with LS122 alone (Figure 28). HGF increased motility in control cells while TNF alpha decreased below untreated levels. Interesting, LS122 decreased motility by about 50 percent in control and TNF alpha treated cells. In the clone 97-3 KD, we observed essentially what we saw in control cells accept there was a smaller difference in the TNF alpha and TNF alpha LS122 treated cells, consistent with RIP2K being target for LS122. This was even more evident in clone 97-4 where the motility of TNF alpha treated cells was unchanged compared to the addition of this cytokine with LS122. Finally, clone 55-7

appeared almost identical to control cells as regards changes in motility. This clone also expressed higher levels of RIP2K as compared to the other two clones. These data are consistent with LS122 targeting RIP2K in response to TNF alpha.

FIGURE 32-35. Next, we used the incuCyte system to measure invasion in real time. The experimental design is exactly that described in Figures 28-31, except that following wounding of the cultures, matrigel was added. Thus, cells need to invade through this artificial matrix to close the wound. As observed, HGF stimulates invasion and this appears to be independent of the levels of RIP2K. All the other treatments were significantly lower than HGF induced invasion and were not significantly different from each other.

FIGURE 36-40. These figures represent different pairings of the data presented in figures 32-35.

Task 3. ***Determine if RIP2K plays a role in osteoclasts/osteoblast function or differentiation . Preliminary results suggest LS122 alters the function and/or differentiation of bone localized cells critical in prostate cancer bone metastasis establishment and growth. These studies will be repeated and extended to determine effective doses of LS122 that work and the pathways it works on to target cells playing a key role in growth of PCa in the bone.***

Results and Discussion

FIGURE 41 The tumor microenvironment is altered during cancer progression and a drug with effects on this compartment in addition to the tumor cell could be particularly attractive. RIPK2's reported regulation of RANKL production from osteoblasts is highly significant to the present proposal. In addition to expression in PC-3 cells, RIPK2 is expressed in osteoblasts and controls a NOD1/2-modulated signaling cascade that results in the release of inflammatory cytokines and RANKL that stimulate the bone microenvironment to elicit the —vicious cycle of bone destruction. As illustrated in this figure, our preliminary in vitro studies show that LS122 blocks key steps in osteoblast and osteoclast activation. For example, osteoblast proliferation, alkaline phosphatase secretion and mineral deposition are inhibited. Also, in osteoclasts, LS122 inhibited RANKL-stimulated proliferation, as well as ruffling and fusion, expression of key osteogenic factors such as OPN, cathepsin K, and TRAP, and blocked destruction of mineralized bone. Because of technical issues we were not able to generate additional data for this task but additional experiments are being planned.

Task 4 Write up manuscript for submission. IN PROGRESS

KEY RESEARCH ACCOMPLISHMENTS

- Patent for LS122 has been executed in the U.S. and select countries in Europe, and we are now looking for licensing possibilities to move this drug closer to clinical trials for men with CRPCa.
- RIP2K was identified as a main target of LS122 and a new SOW was prepared along with a six month extension to further study this.
- LS122 worked to prevent metastasis in an animal model.
- Our data support but do not prove that the main target of LS122 is RIP2K.

REPORTABLE OUTCOMES

A patent for LS122 was executed in the U.S. and Europe and we are searching for companies to license this technology.

CONCLUSION

We developed a 3D cell co-culture system. We screened a 422 member kinase panel and found LS122 inhibited >90% of RIP2 kinase activity, but had no significant activity on other kinases tested. RIP2K is a TNF-α responsive, upstream

regulator of NF-κB, a "druggable target", and novel in prostate cancer. We verified that LS122 inhibits RIP2K phosphorylation at micromolar concentrations. In order to characterize the anti-cancer efficacy of LS122, we have conducted viability assays in PC-3, RM-1, LNCaP and its derivative C4-2, DU145 and other prostate cancer lines, as well as a set of non-tumor prostate cells including RWPE-1, WPMY-1, and 267B1. Additional non-prostate cells were also tested. In general, LS122 had minimal effect on viability of non-cancer and cancer cells. In TNF-α stimulated PC-3 cells, LS122 significantly blocked the NF kappa B signaling pathway. LS122 blocked TNF alpha induced apoptosis. In vivo, LS122 had a significant, dose-dependent effect on tumor doubling, final tumor volume and tumor angiogenesis in PC-3 human xenografts and in the highly aggressive RM-1 syngeneic model. LS122 blocked the dissemination to the liver of cancer cells injected into the spleen by >90%; nearly double that of another experimental NF-κB inhibitor of an entirely different drug class. LS122 inhibits the proliferation, ruffling, fusion and osteolytic activity of osteoclasts and inhibited the proliferation, alkaline phosphatase secretion and mineralization of osteoblasts. Our results support but do not prove that RIP2K is a primary target for the activity of LS122.

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SUPPORTING DATA – FIGURES

LS122: novel thiophene derivative dual NFκB/AP-1 inhibitor

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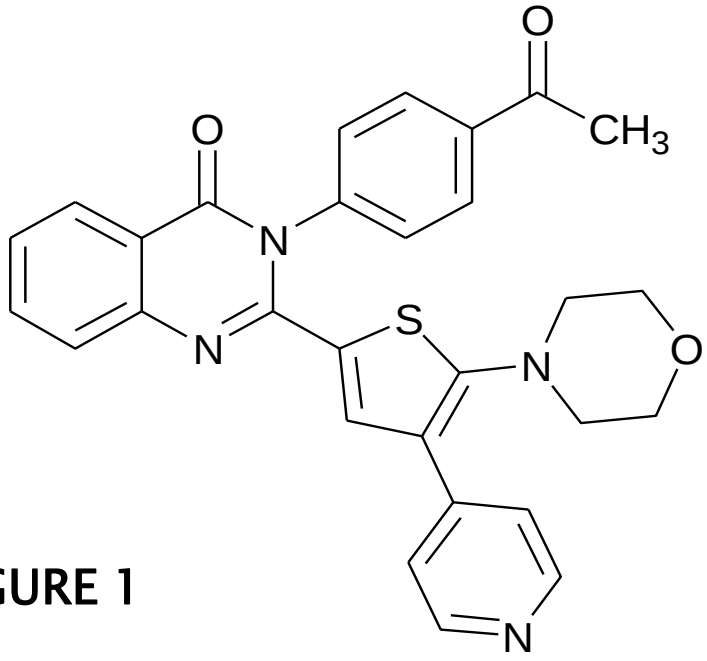
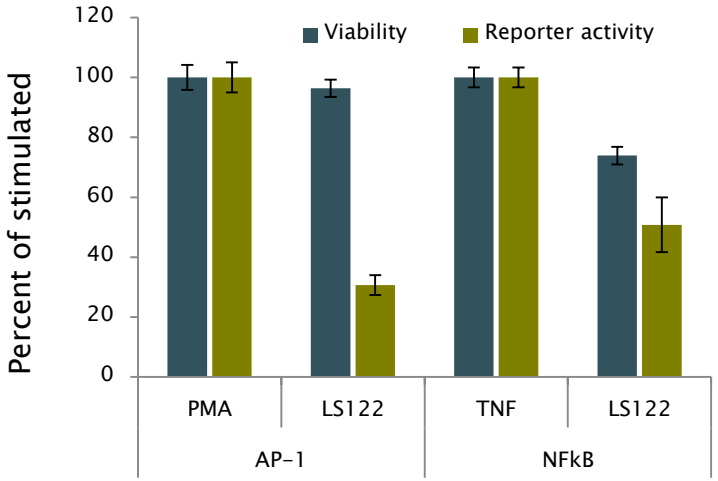


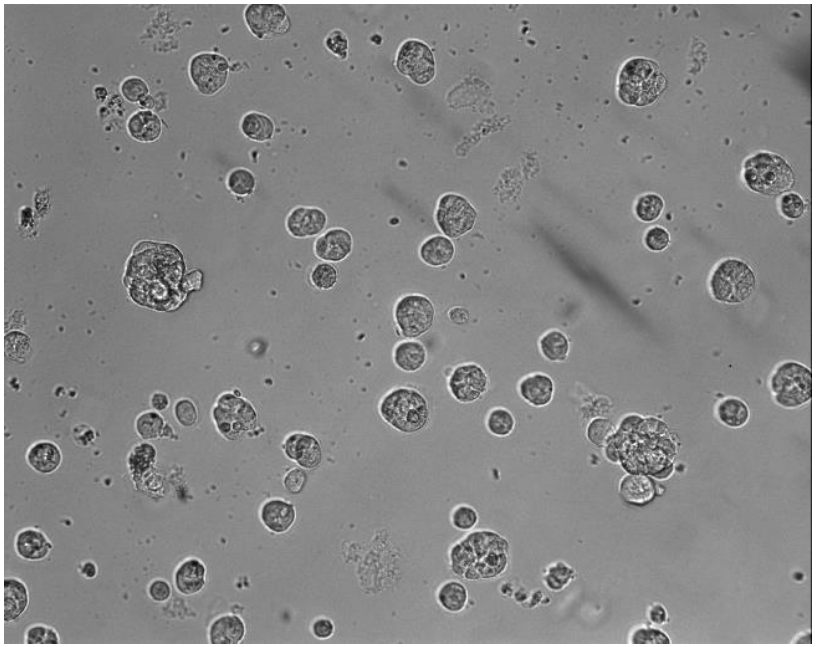
FIGURE 1



Initial screening at 10μM

FIGURE 2

CONTROL



HGF

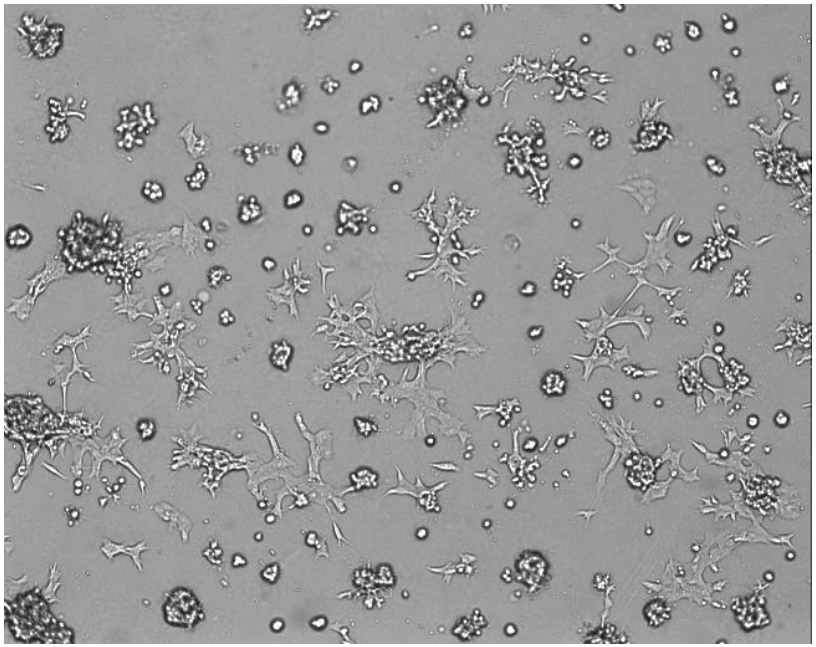
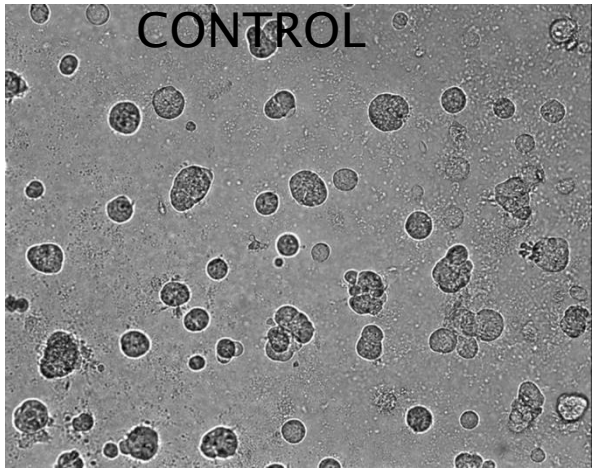
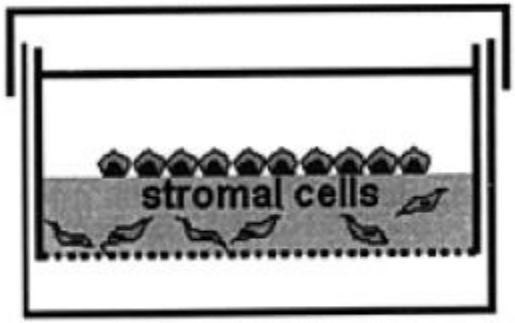


FIGURE 3

DU145

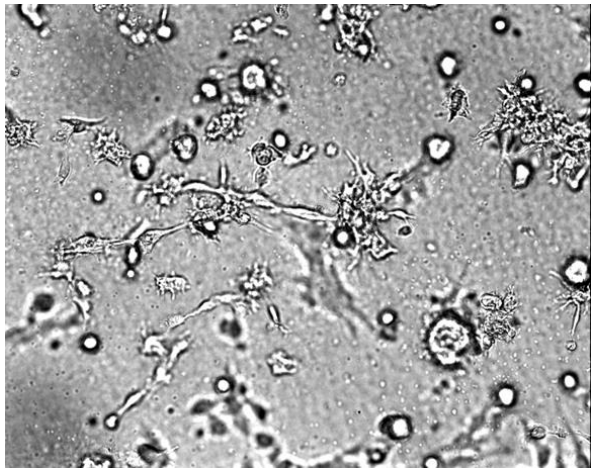


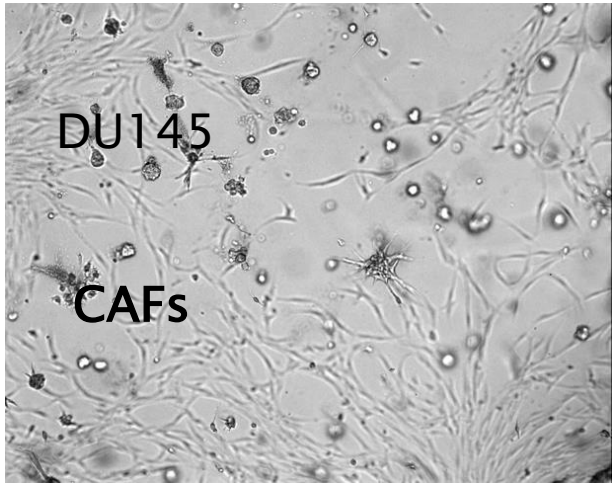


Co-culture with
Stromal Cells in BME

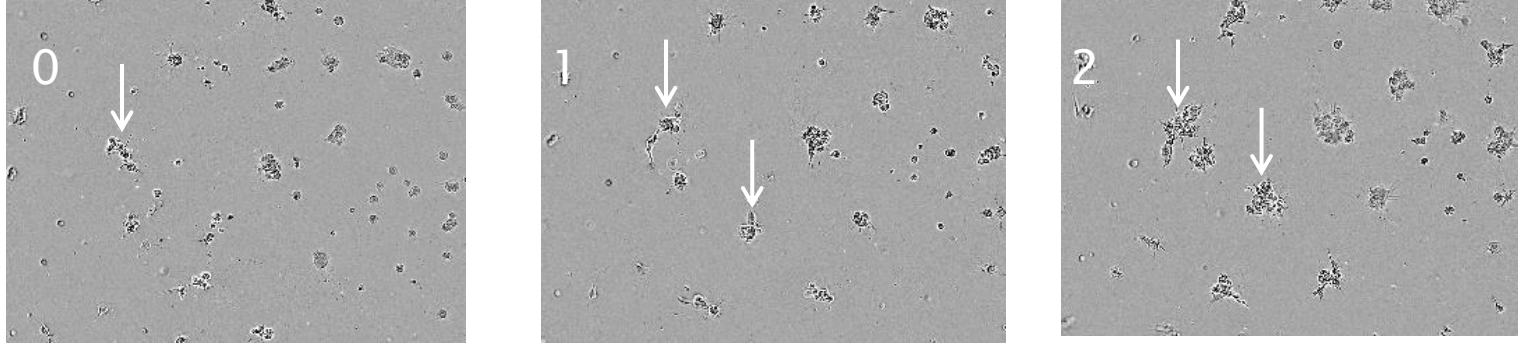
DU145

CAFs





OPTICAL FOCUS ON DU145 CELLS FOCUS ON FIBROBLASTTS



PPC1

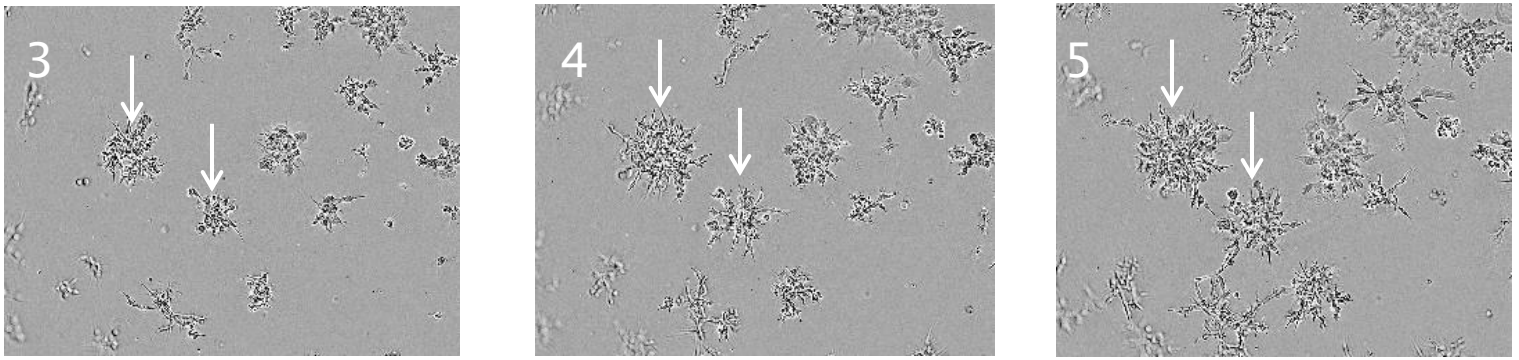
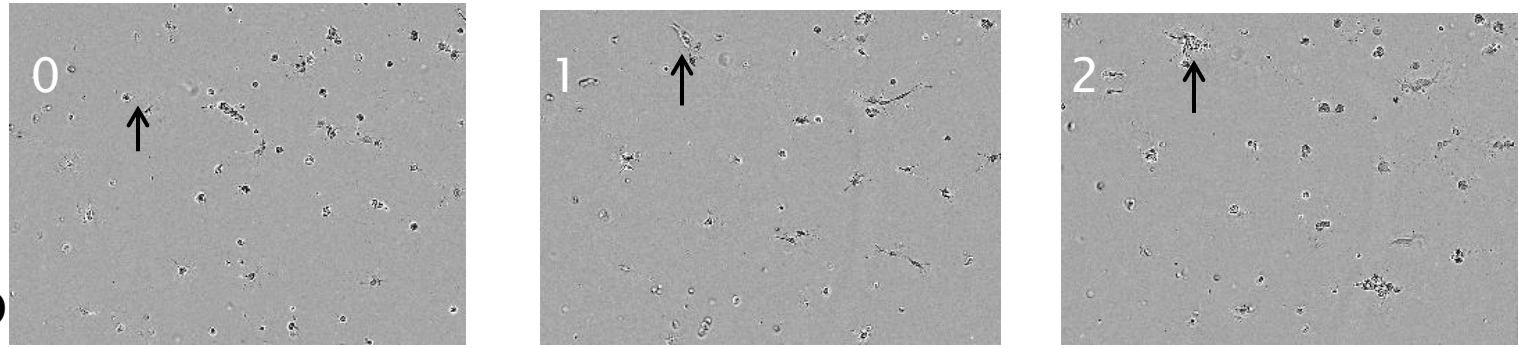
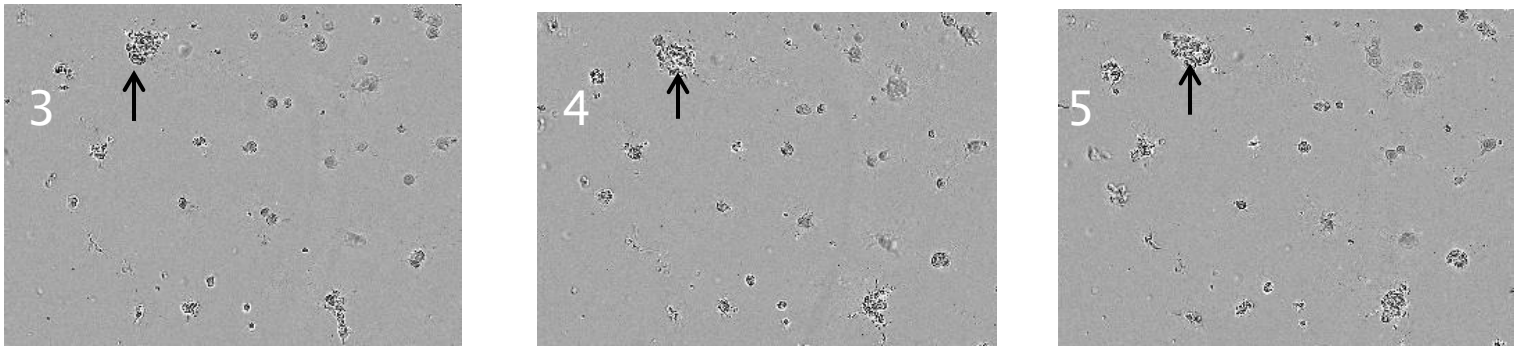


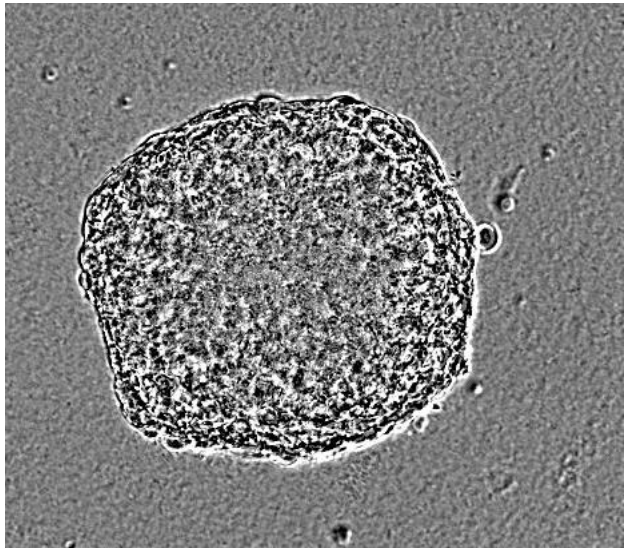
FIGURE 4



PPC1 KD



Spheroids in 96 well plates are all the same size



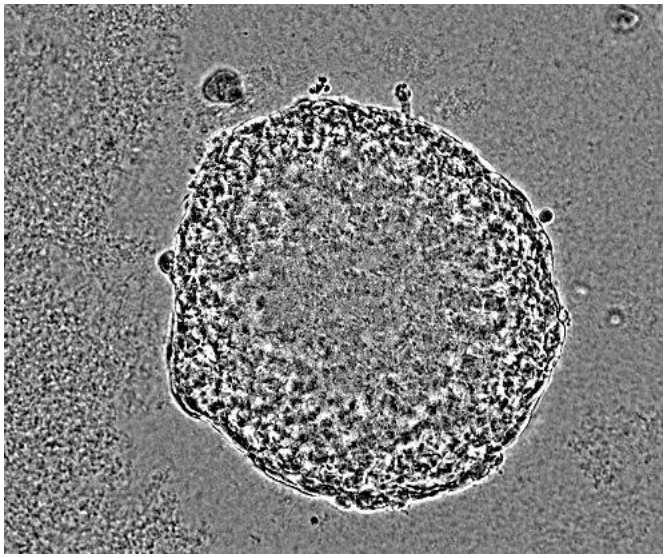
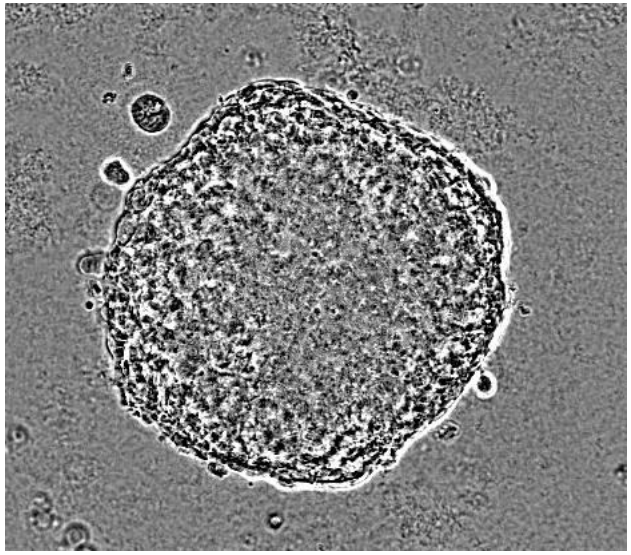
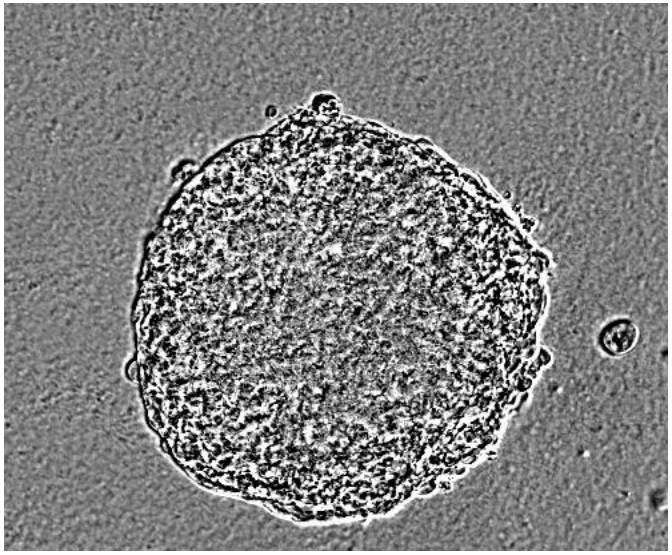
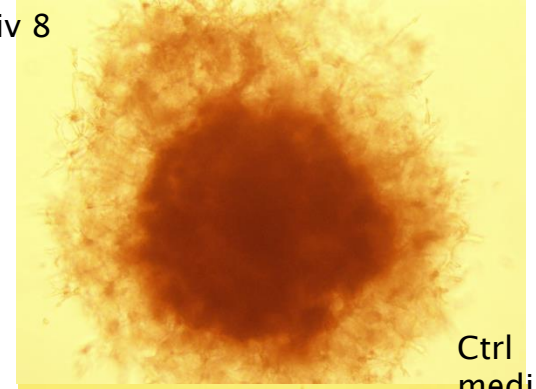


FIGURE 5

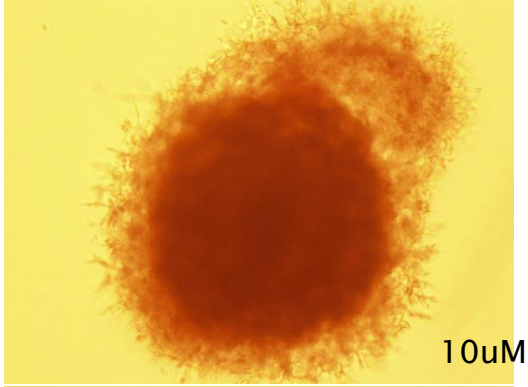




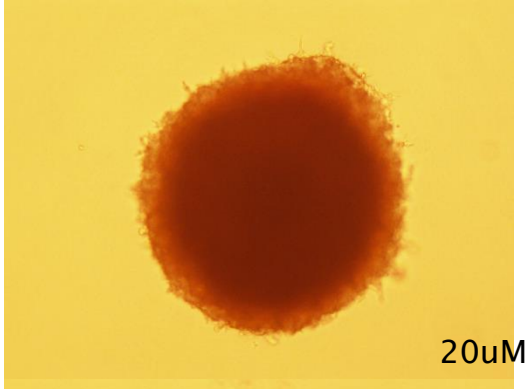
Div 8



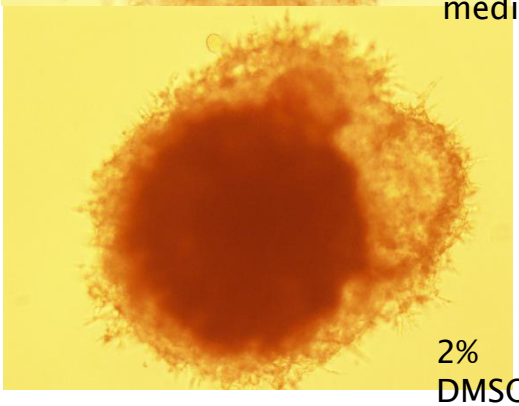
Ctrl media



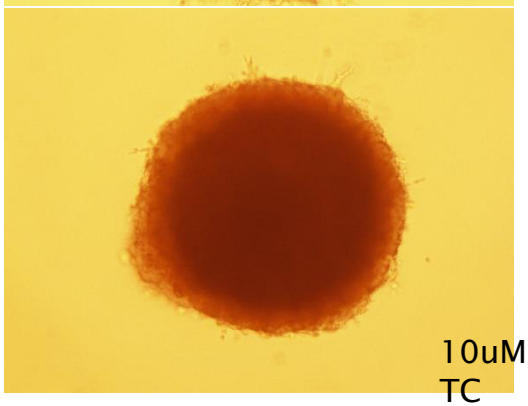
10uM FC



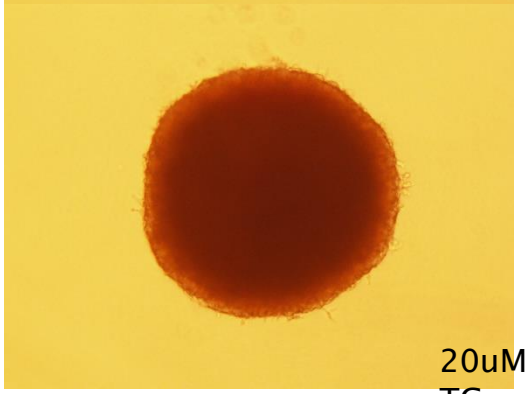
20uM FC



2% DMSO



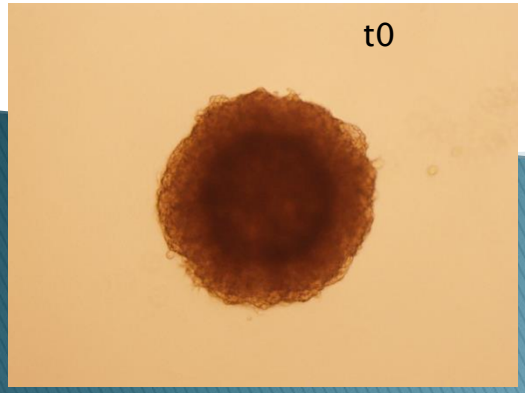
10uM TC

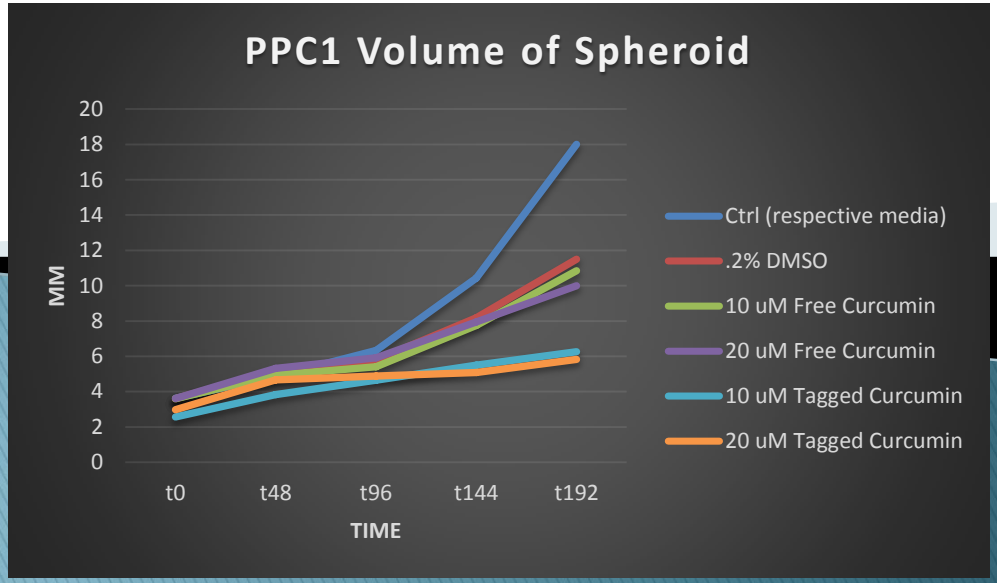


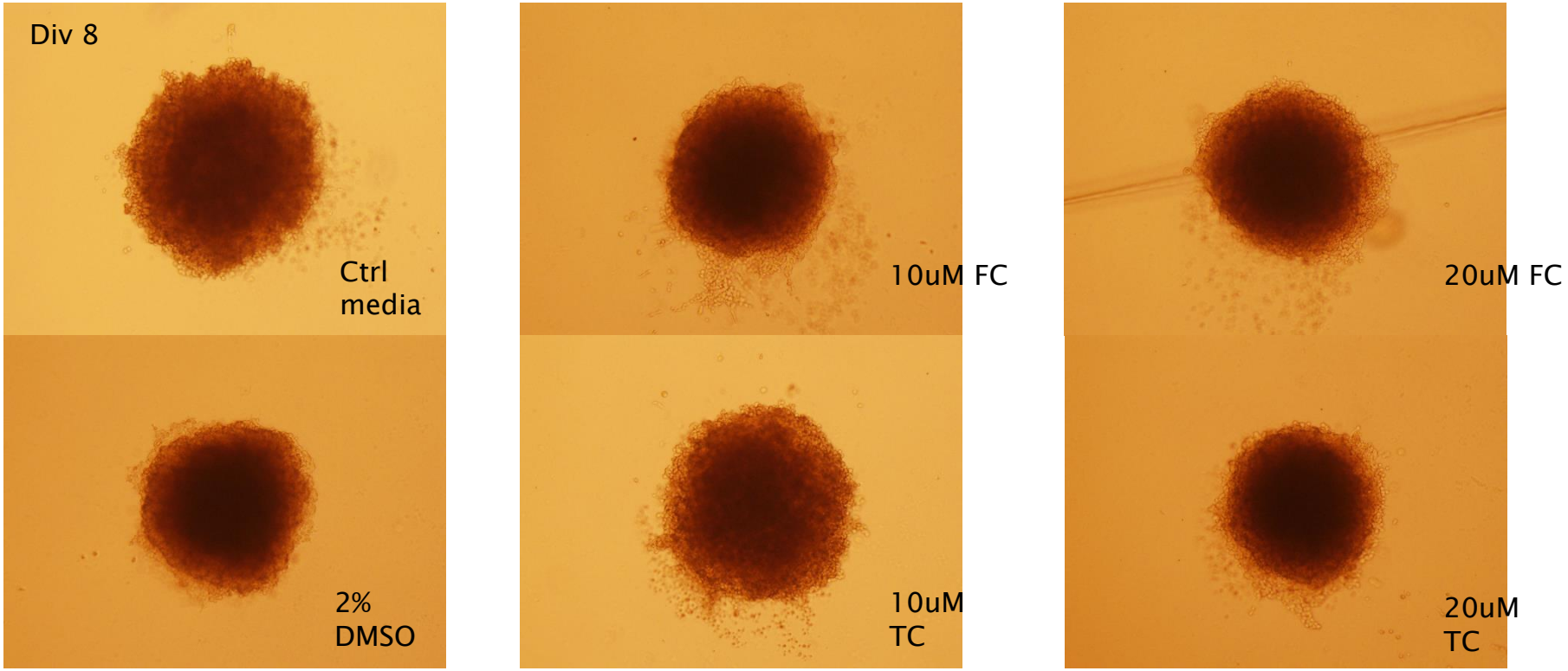
20uM TC

FIGURE 6

PPC1

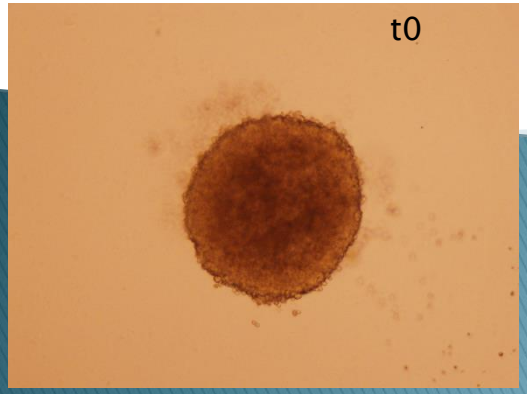


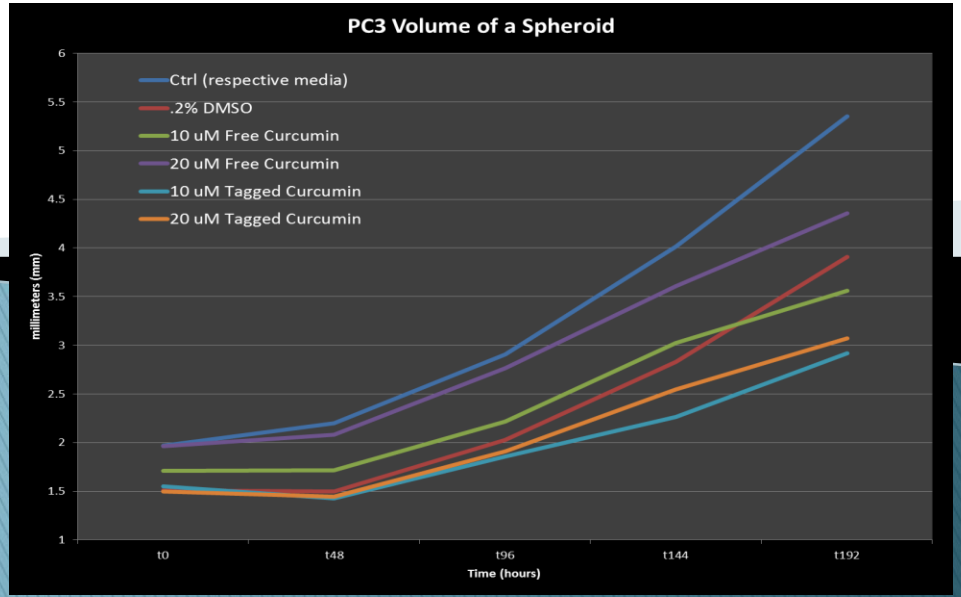



Div 8
Ctrl media
10uM FC
20uM FC
2% DMSO
10uM TC
20uM TC

PC3

FIGURE 7

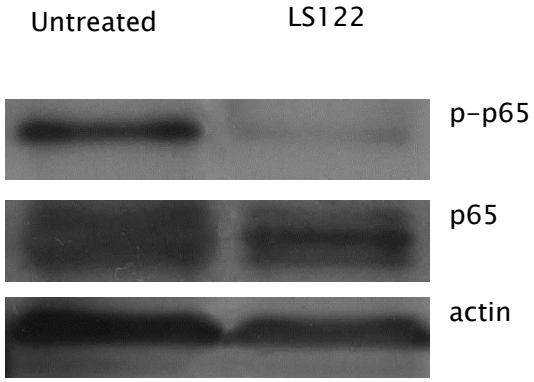

t0


PC3 Volume of a Spheroid
Ctrl (respective media)
.2% DMSO
10 uM Free Curcumin
20 uM Free Curcumin
10 uM Tagged Curcumin
20 uM Tagged Curcumin
millimeters (mm)
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
t0
t48
t96
t144
t192
Time (hours)

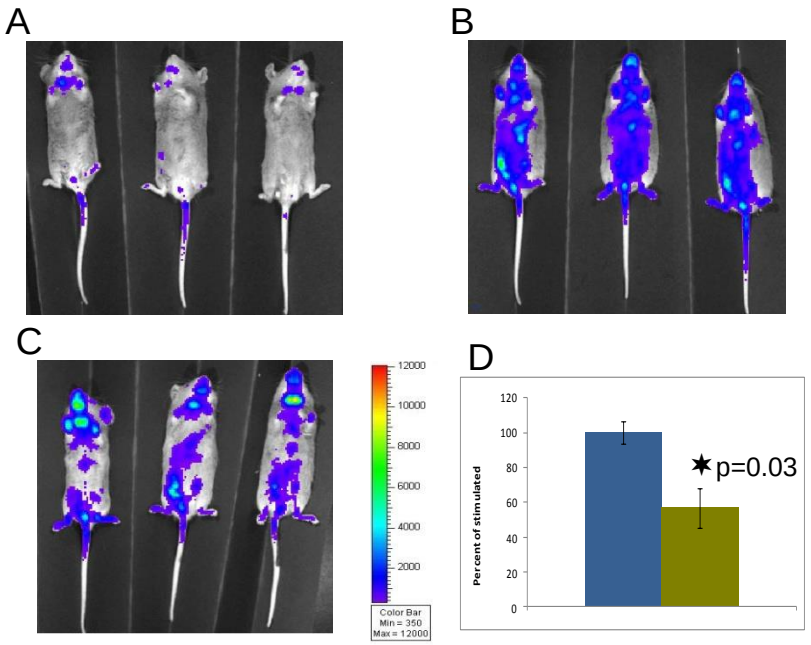
LS122 decreased *in vitro* and *in vivo* targets consistent with an NFκB inhibitor

FIGURE 8

In vitro



In vivo



Phospho-p65 NFκB subunit expression decreased

NFκB reporter mice had 43% less expression

FIGURE 9

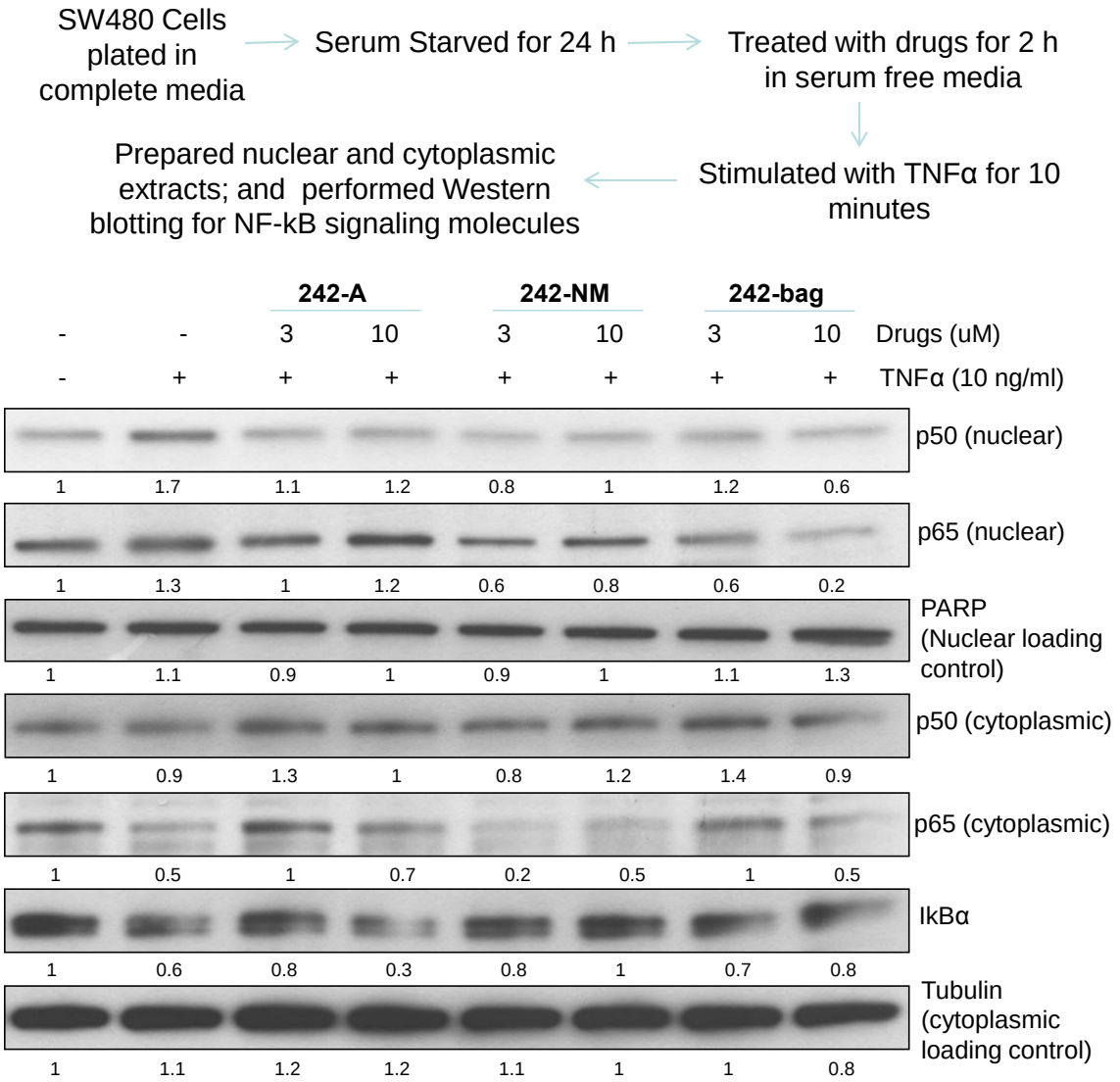


Figure 1

FIGURE 10

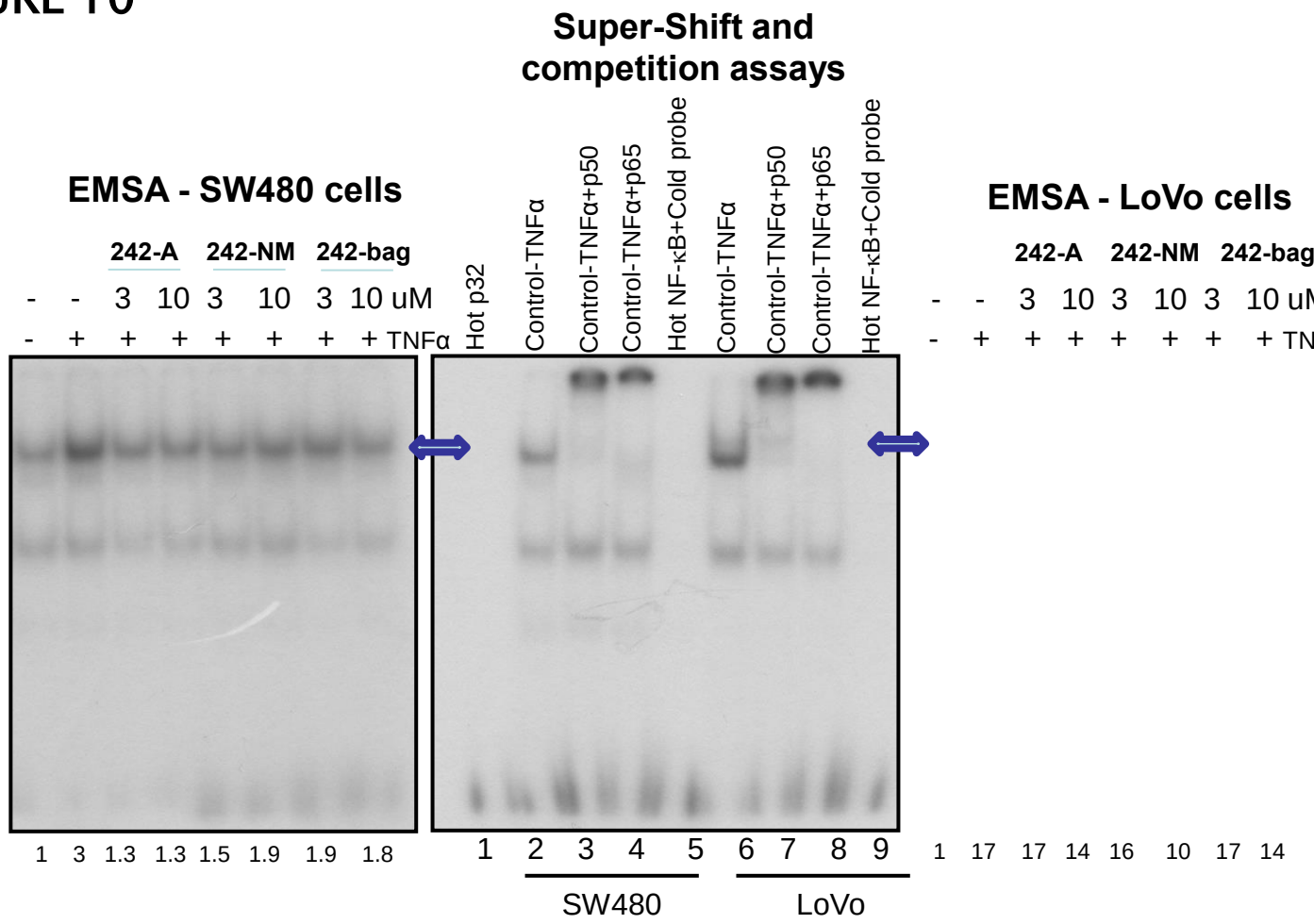
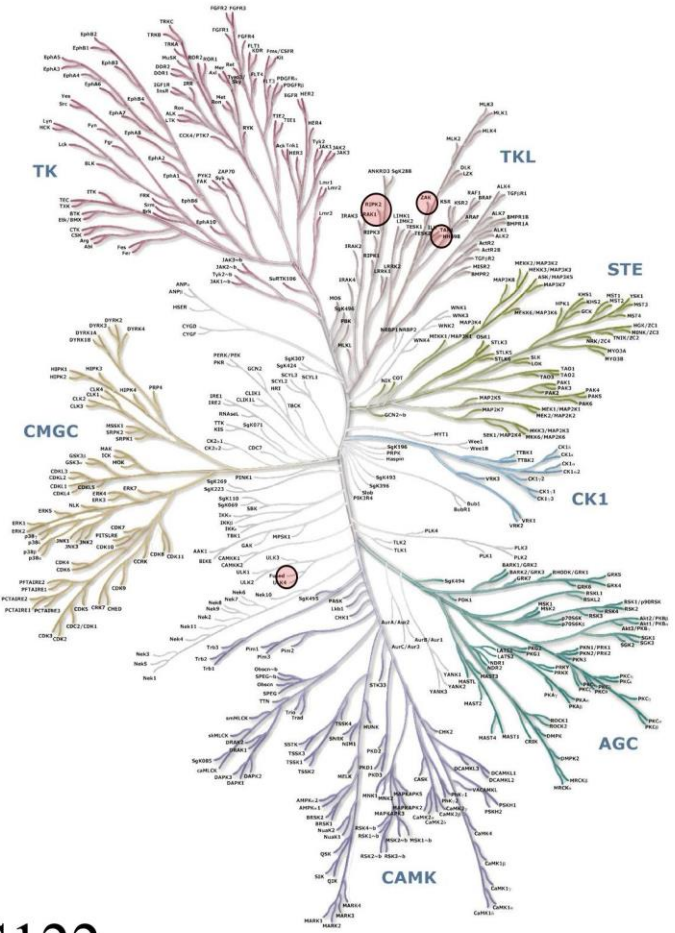


Figure 3

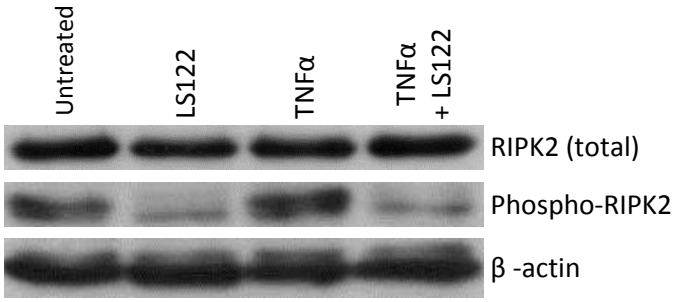
LS122 is most selective for RIPK2, a kinase relevant to cancer progression

FIGURE 11



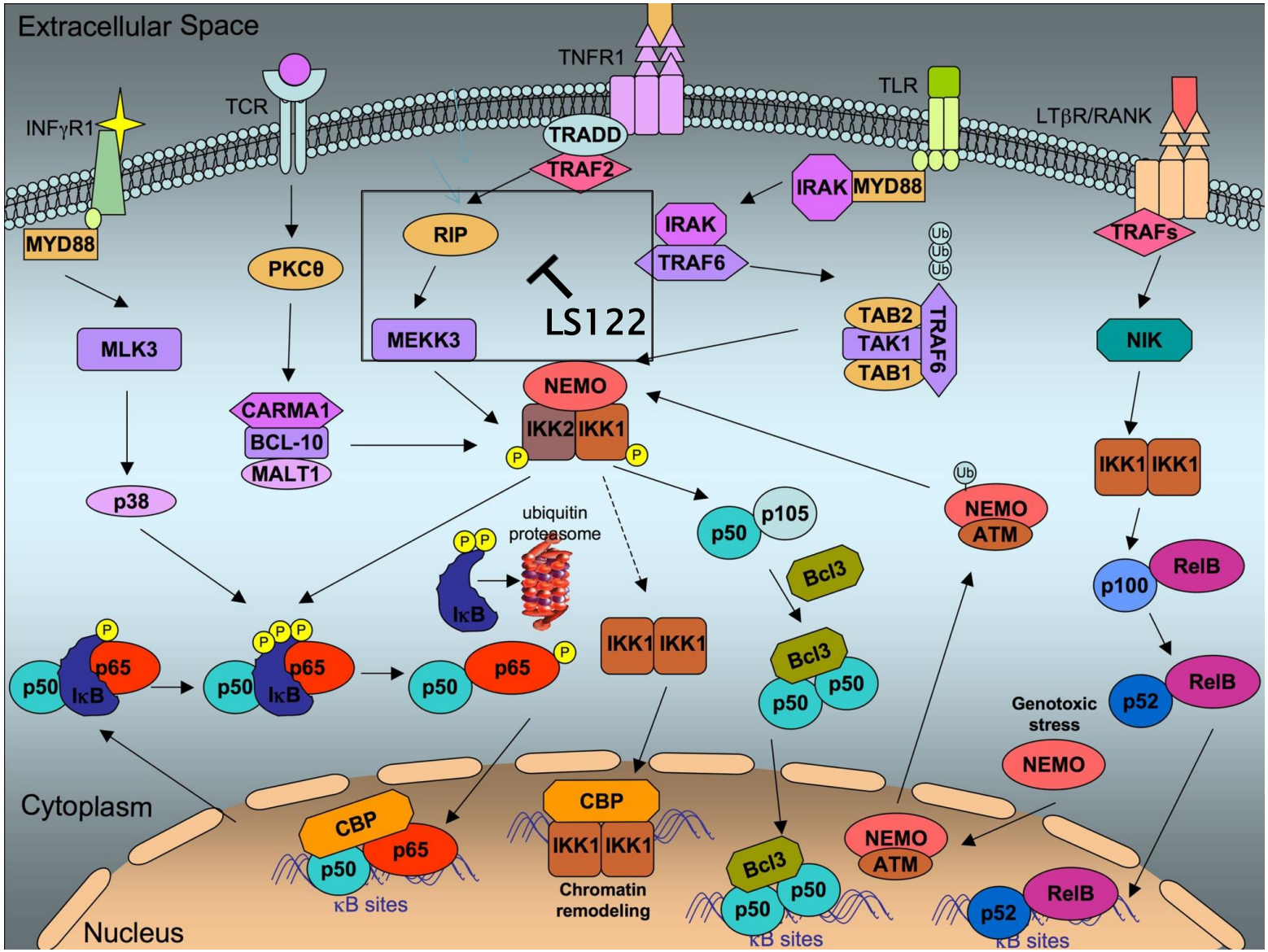
LS122
442 Total kinases tested
78 Kinases not mapped

Ambit Gene Symbol	Percent Inhibition
RIPK2	**91**
STK36	83
ZAK	73
TNNI3K	70



All other thiophene derivatives and all hits from the NFkB and AP-1 reporter screens were evaluated for RIPK2 and 5 relevant kinases (relevant biologically to us at the time) and no other compound was a RIPK2 inhibitor of ANY magnitude.

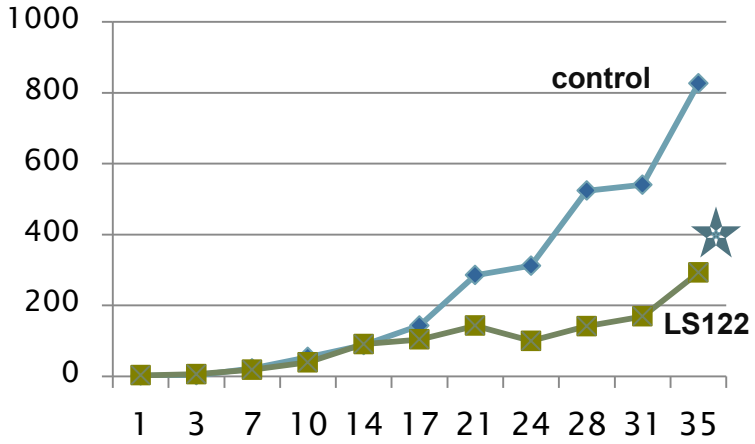
FIGURE 12 LS122 most likely targets RIP2K to inhibit NFKB activation



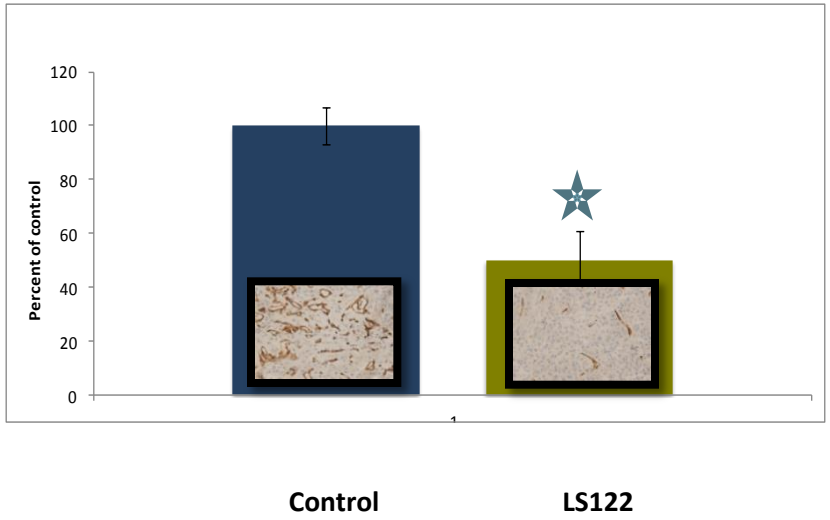
LS122 is a potent inhibitor of PC-3 human prostate cancer cell growth and angiogenesis *in vivo*

FIGURE 13

A.



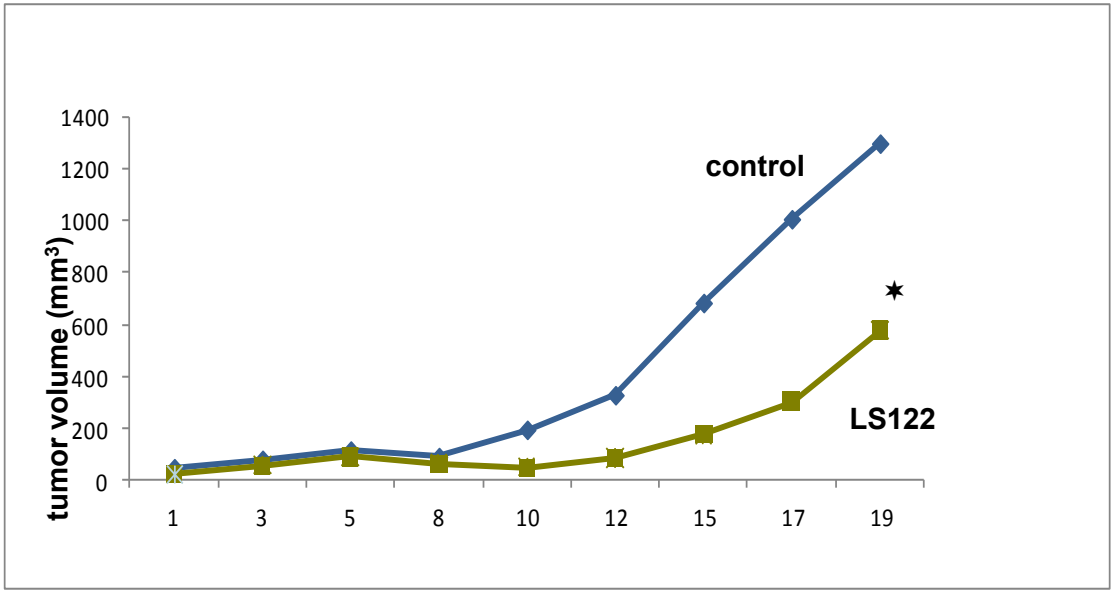
B.



A) PC-3 cells ($1x10^6$) were injected *sc* into SCID/bg mice. The mice were treated daily by oral gavage with 50mg/kg LS122 in 0.2% Noble agar or agar alone. Tumors were measured with calipers 3 times per week for 35 days. Tumor volumes were calculated using the standard formula [$vol=0.52 \times L \times W^2$], ✶$p = 0.02$. **B)** Tumors harvested from animals in Panel A were paraffin embedded, sectioned, and stained for CD34, a marker for angiogenesis. Mean microvessel density was calculated from 5 random images of each tumor (insets show representative images). Results shown are the average ± SEM, ✶$p < 0.001$. Staining was quantified using ImageJ.

LS122 decreased tumor growth in aggressive RM-1 syngeneic tumor model

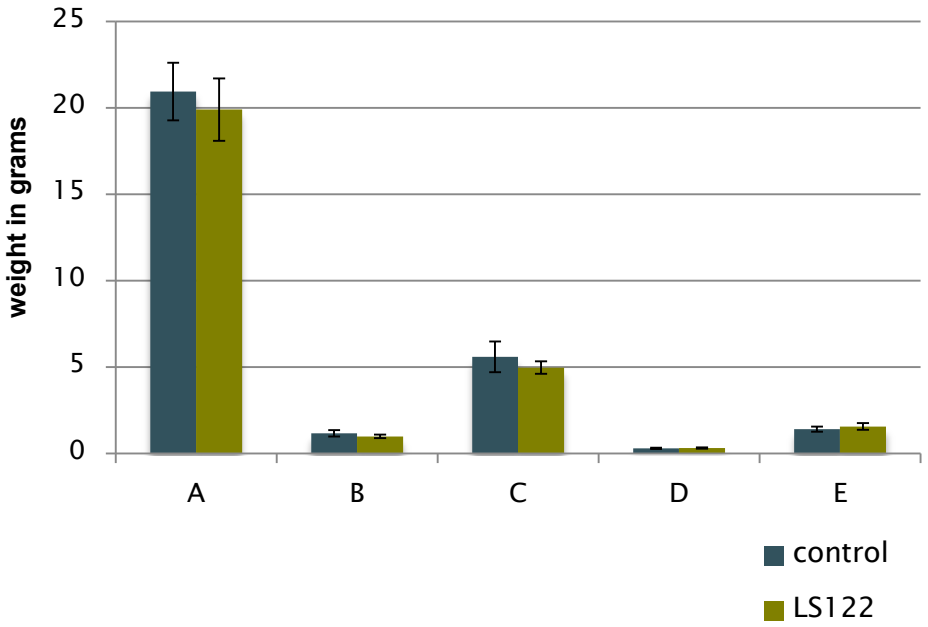
FIGURE 14



Male C57Bl/6 mice (8 weeks old) were injected in the right flank with $1x10^5$ RM-1 mouse prostate cancer cells. Animals were treated daily with 0.2% Noble agar alone (control) or 50 mg/kg LS122 in 0.2% Noble agar immediately after tumor cell inoculation. Mice were measured 3 times per week by caliper. The average tumor volume (n=10) is shown. LS122 dramatically restricted the tumor size compared to the control animals, ✶p = 0.02.

LS122 had no effect on body or organ weight and no significant gross or microscopic toxicity

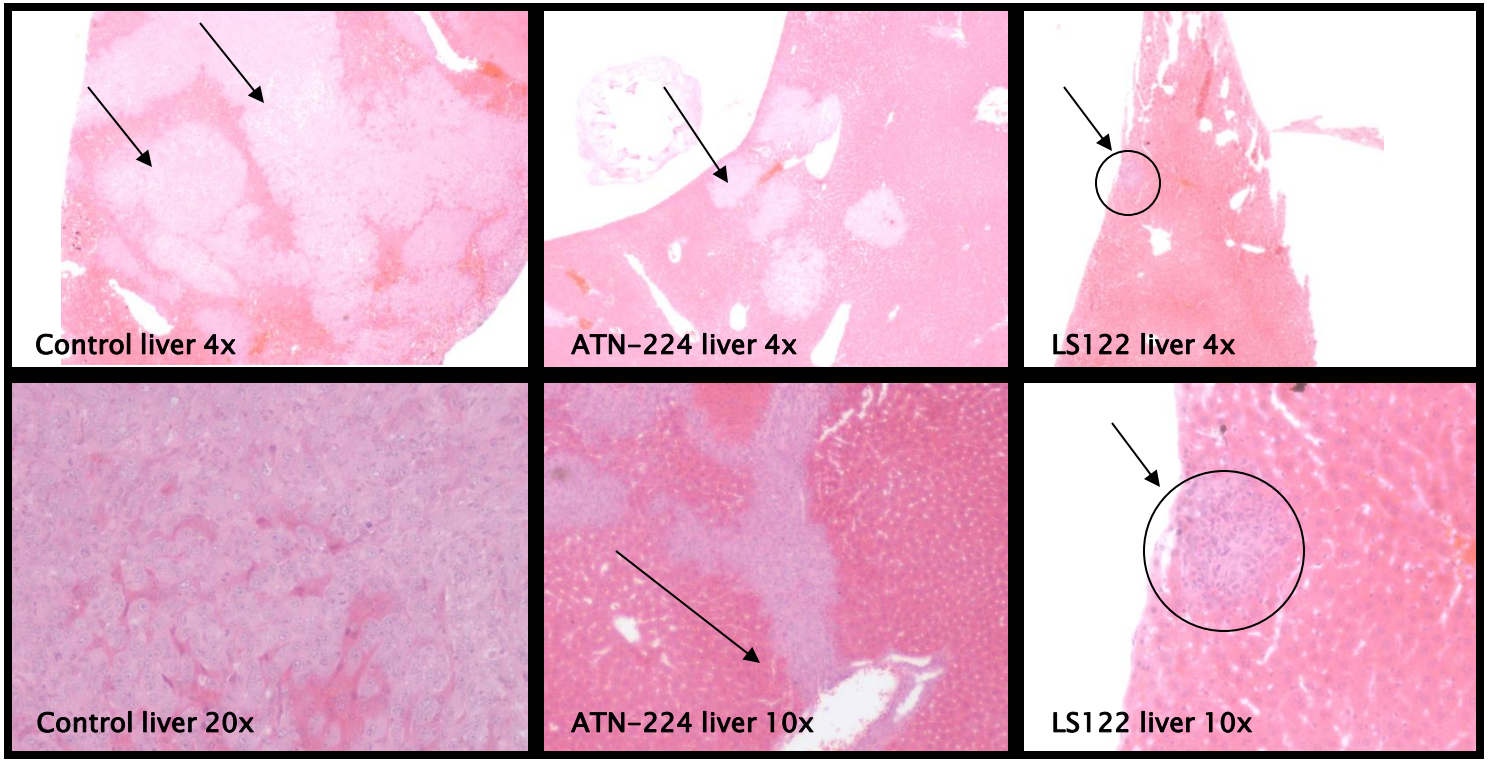
FIGURE 15



Male SCID/bg mice were given standard chow ± LS122. Mice were sacrificed after 30 days, at which time their **A)** body weight, **B)** liver, **C)** liver to body ratio, **D)** kidney weight and **E)** kidney to body ratio were calculated. The averages from the mice in each group are shown. LS122 has no gross adverse effects on the mice.

LS122 blocks organ-to-organ dissemination and invasion

FIGURE 16



RIP 2K Knock downs in PC-3

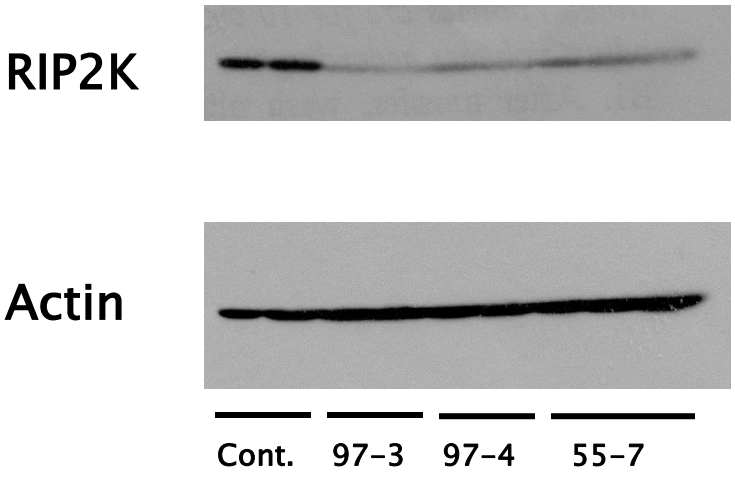


FIGURE 17

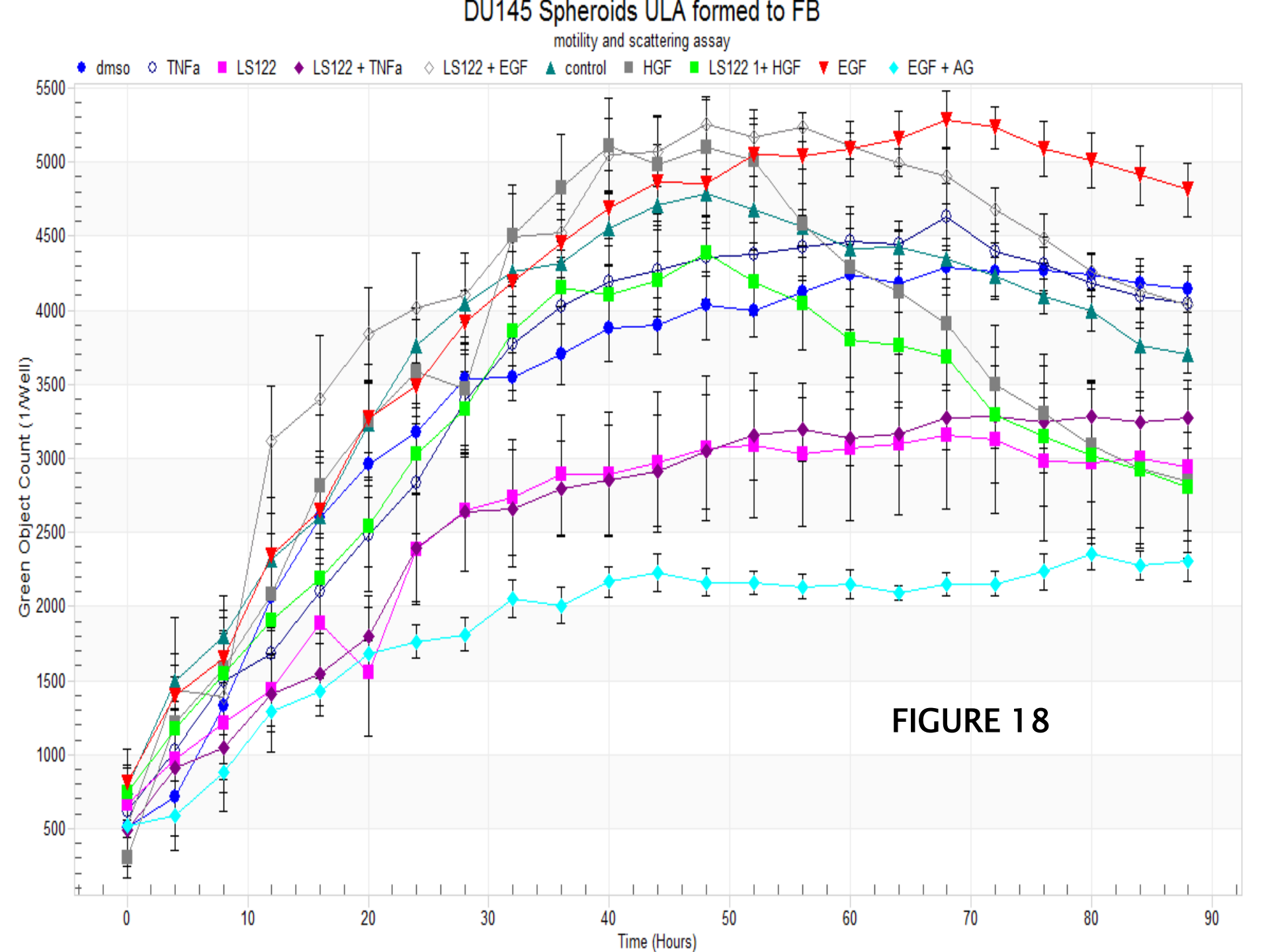
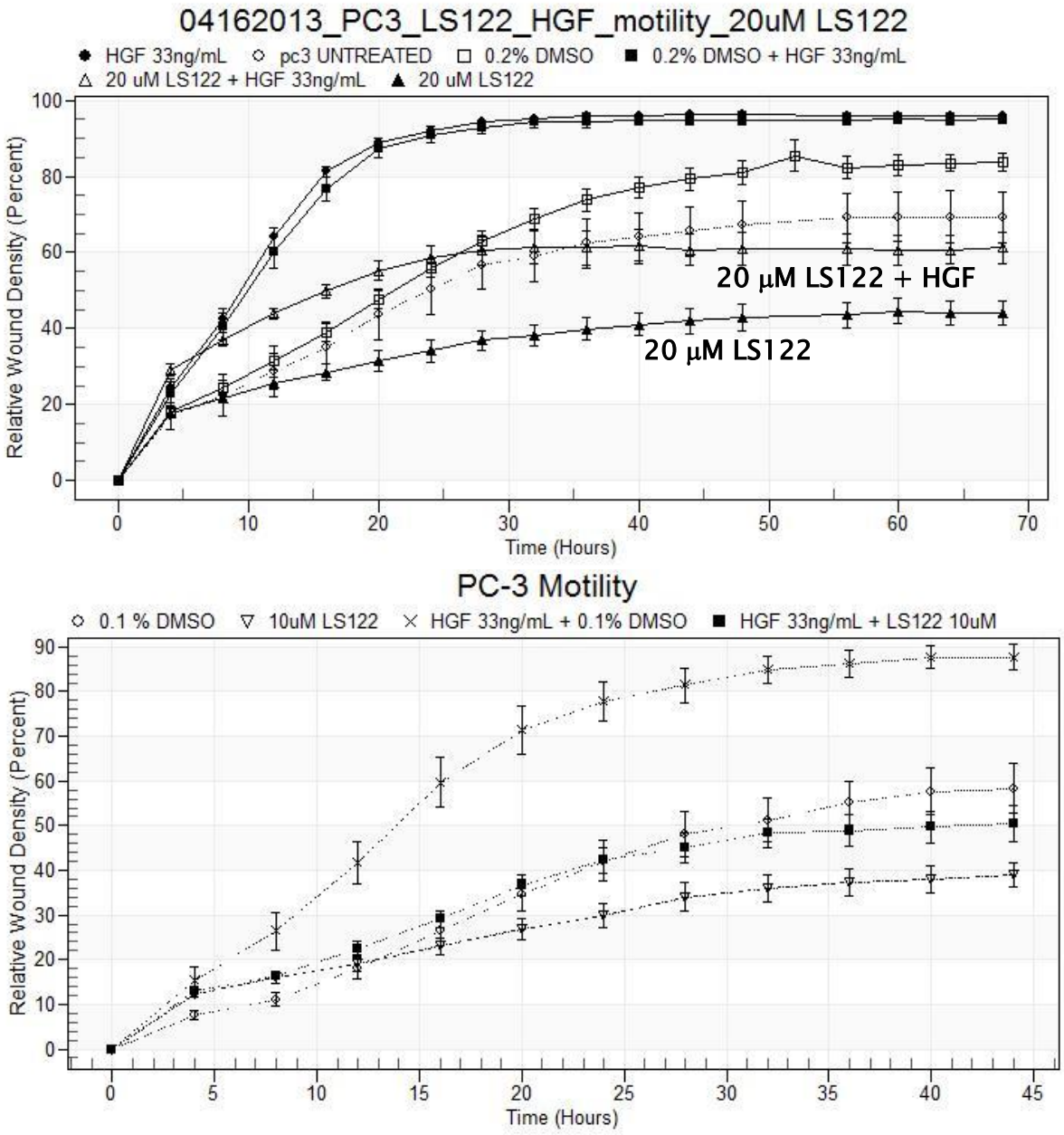
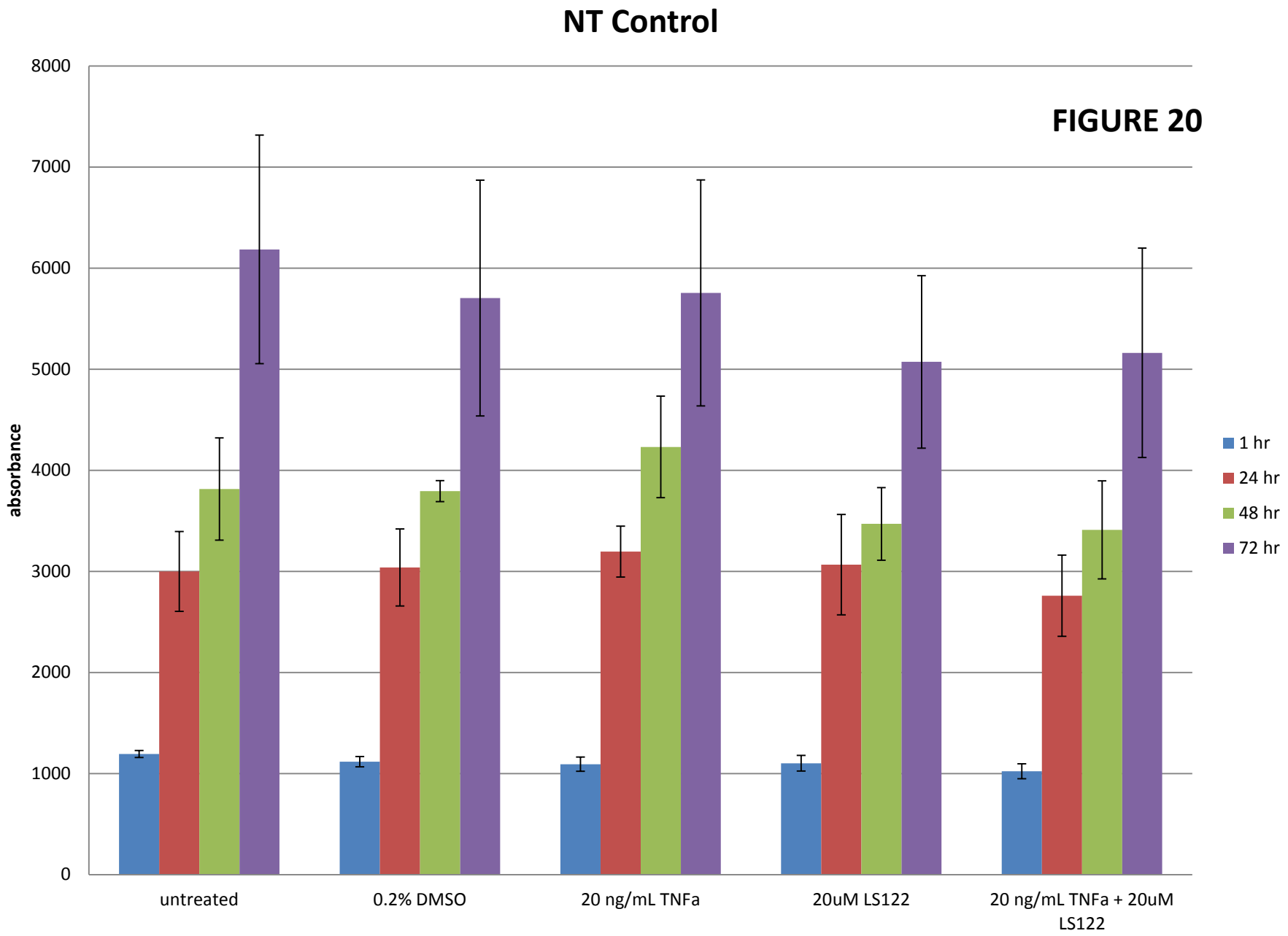

DU145 Spheroids ULA formed to FB
motility and scattering assay
dmso
TNFa
LS122
LS122 + TNFa
LS122 + EGF
control
HGF
LS122 1+ HGF
EGF
EGF + AG
Green Object Count (1/Well)
Time (Hours)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
10
20
30
40
50
60
70
80
90

FIGURE 18

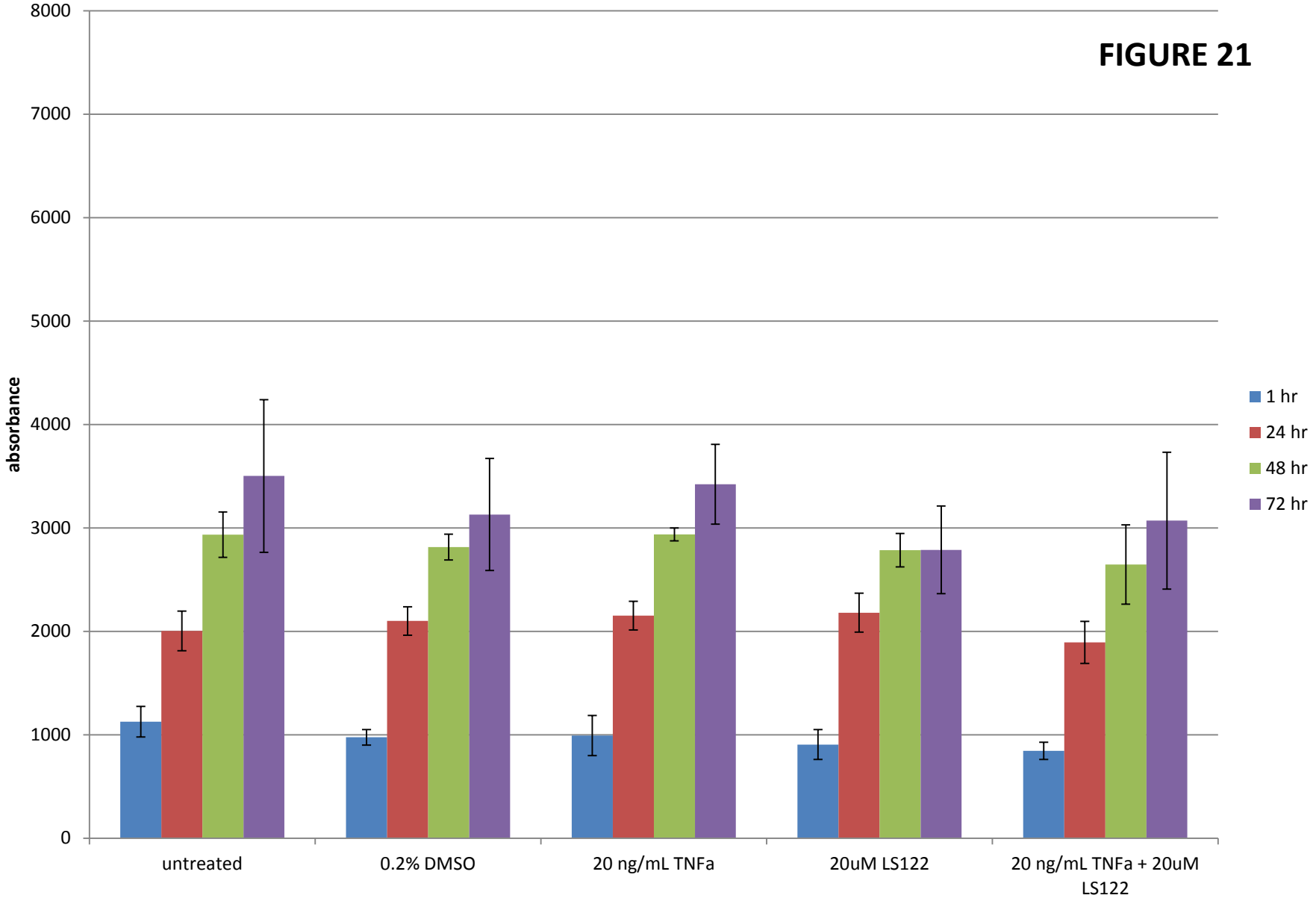
FIGURE 19


04162013_PC3_LS122_HGF_motility_20uM LS122
HGF 33ng/mL
pc3 UNTREATED
0.2% DMSO
0.2% DMSO + HGF 33ng/mL
20 uM LS122 + HGF 33ng/mL
20 uM LS122
Relative Wound Density (Percent)
Time (Hours)
20 µM LS122 + HGF
20 µM LS122
PC-3 Motility
0.1 % DMSO
10uM LS122
HGF 33ng/mL + 0.1% DMSO
HGF 33ng/mL + LS122 10uM
Relative Wound Density (Percent)
Time (Hours)


NT Control
FIGURE 20
absorbance
8000
7000
6000
5000
4000
3000
2000
1000
0
untreated
0.2% DMSO
20 ng/mL TNFa
20uM LS122
20 ng/mL TNFa + 20uM LS122
1 hr
24 hr
48 hr
72 hr

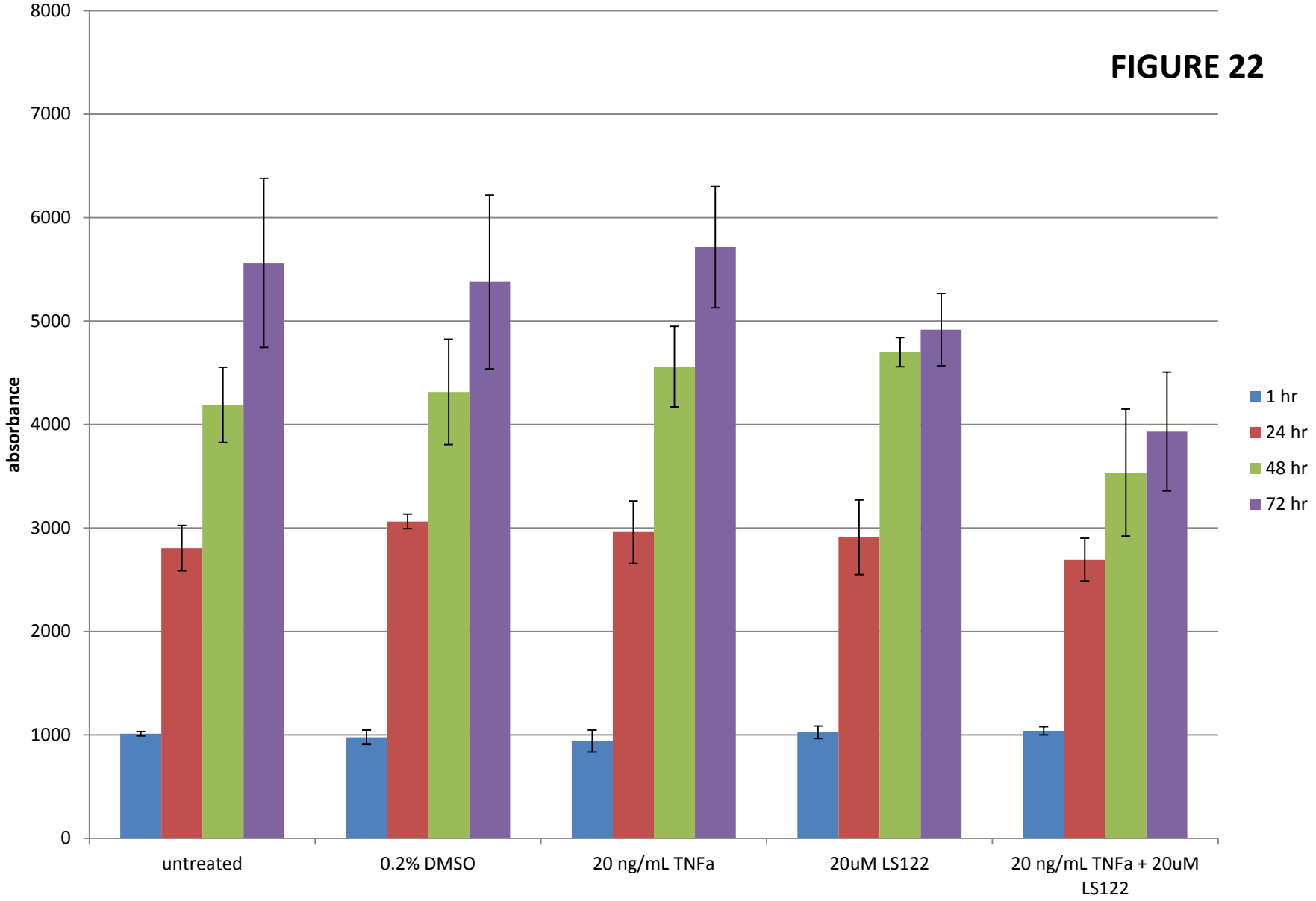
Clone 255-7

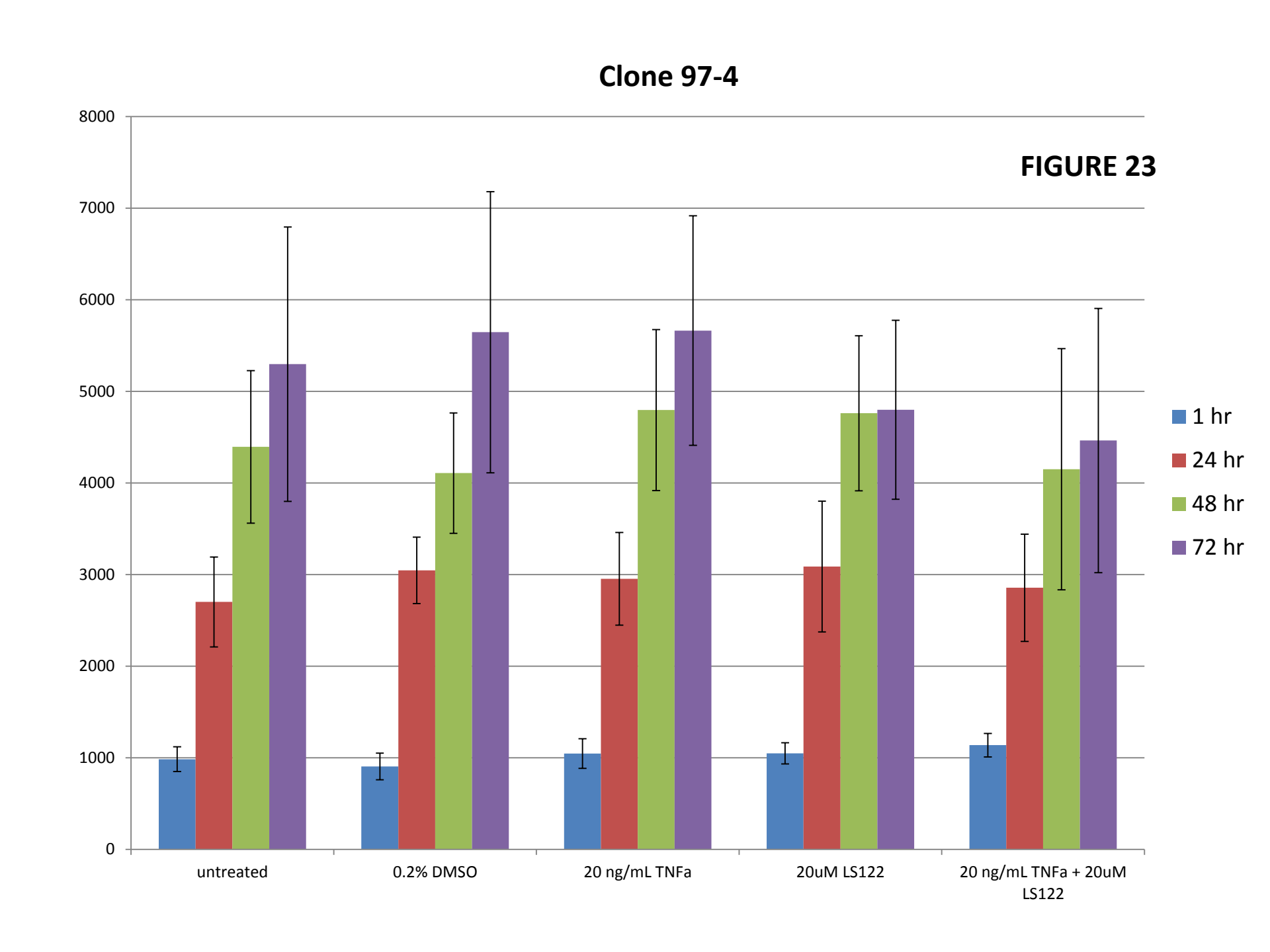
FIGURE 21



Clone 97-3

FIGURE 22




Clone 97-4
FIGURE 23
8000
7000
6000
5000
4000
3000
2000
1000
0
1 hr
24 hr
48 hr
72 hr
untreated
0.2% DMSO
20 ng/mL TNFa
20uM LS122
20 ng/mL TNFa + 20uM LS122

NT Control

FIGURE 24

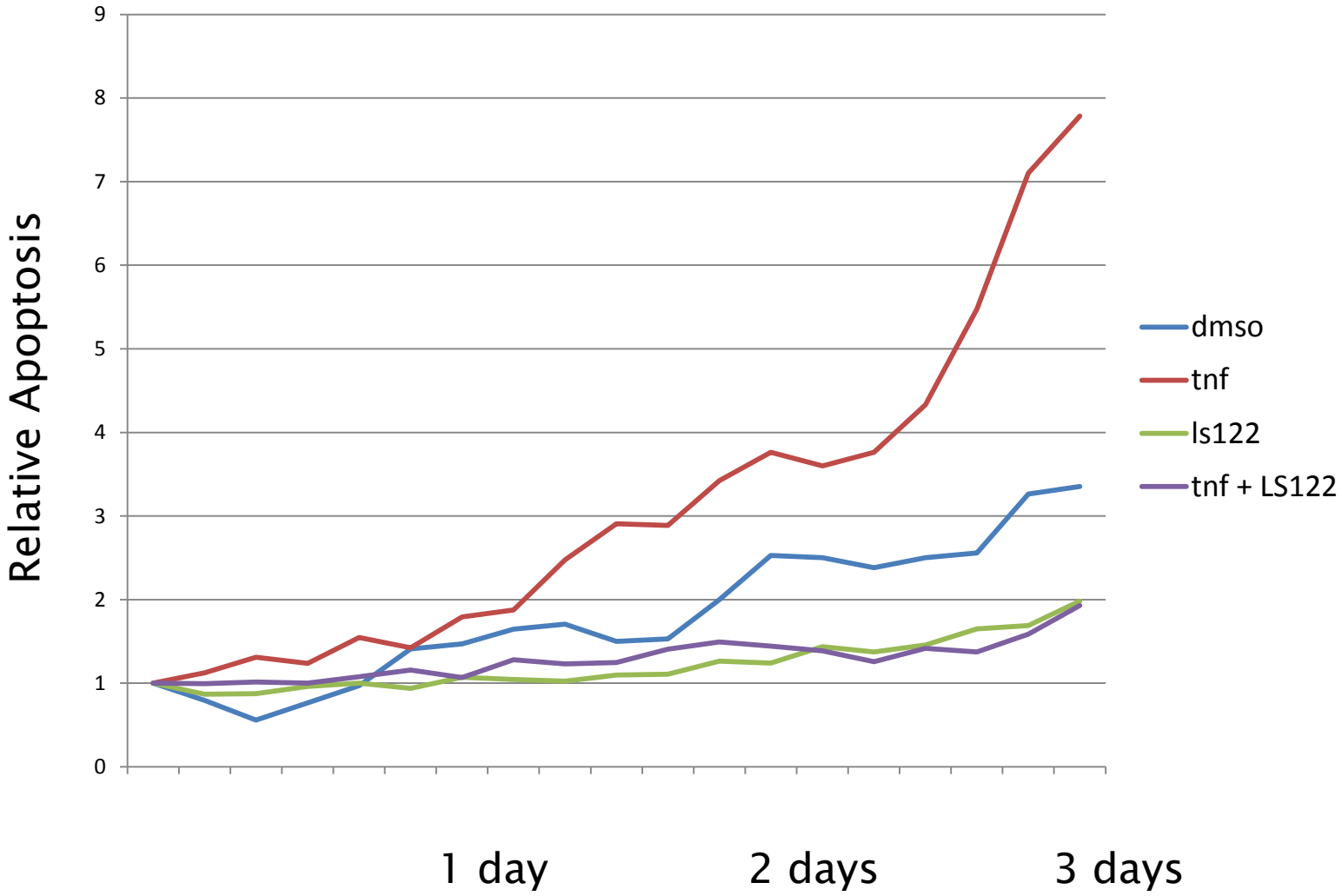
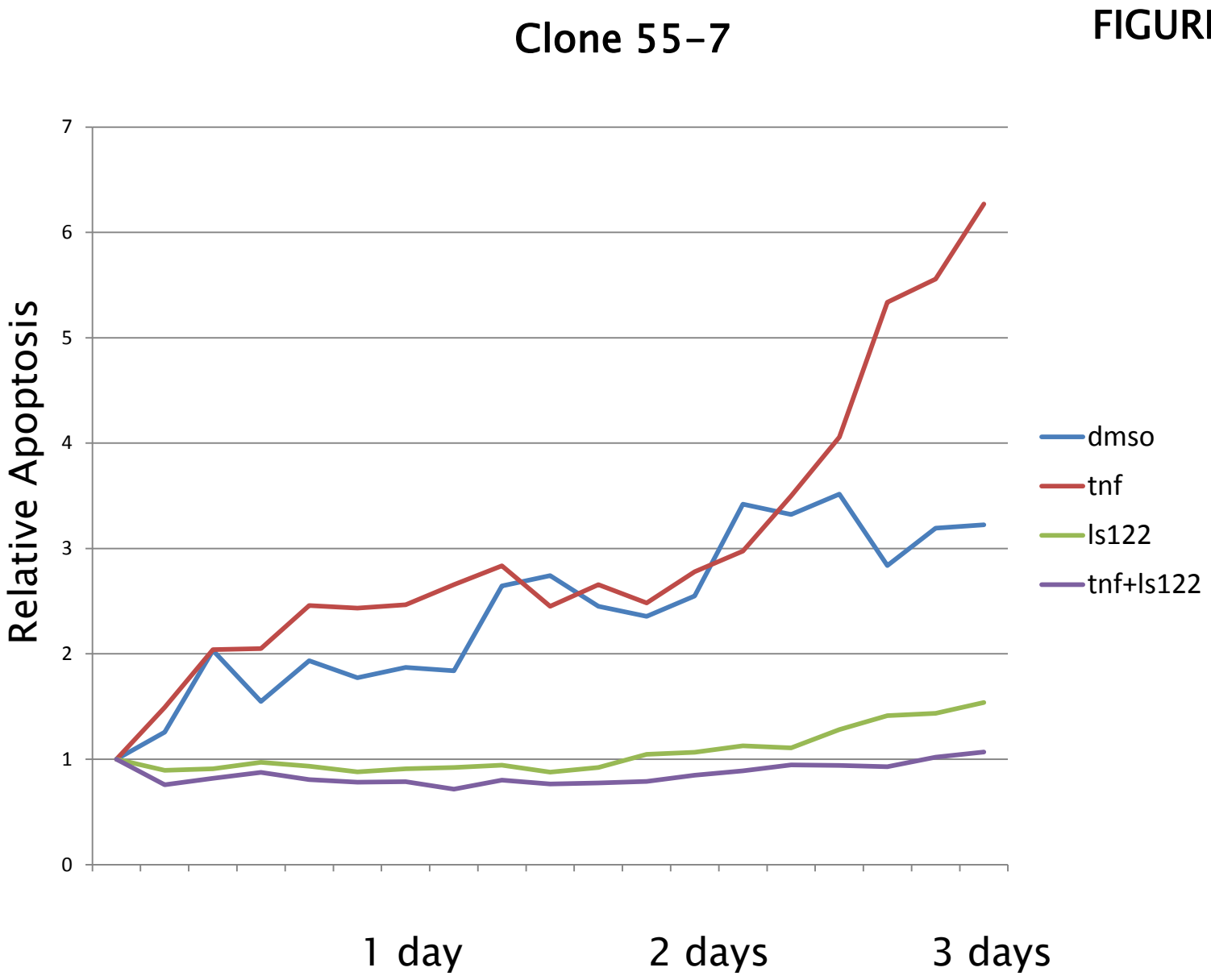
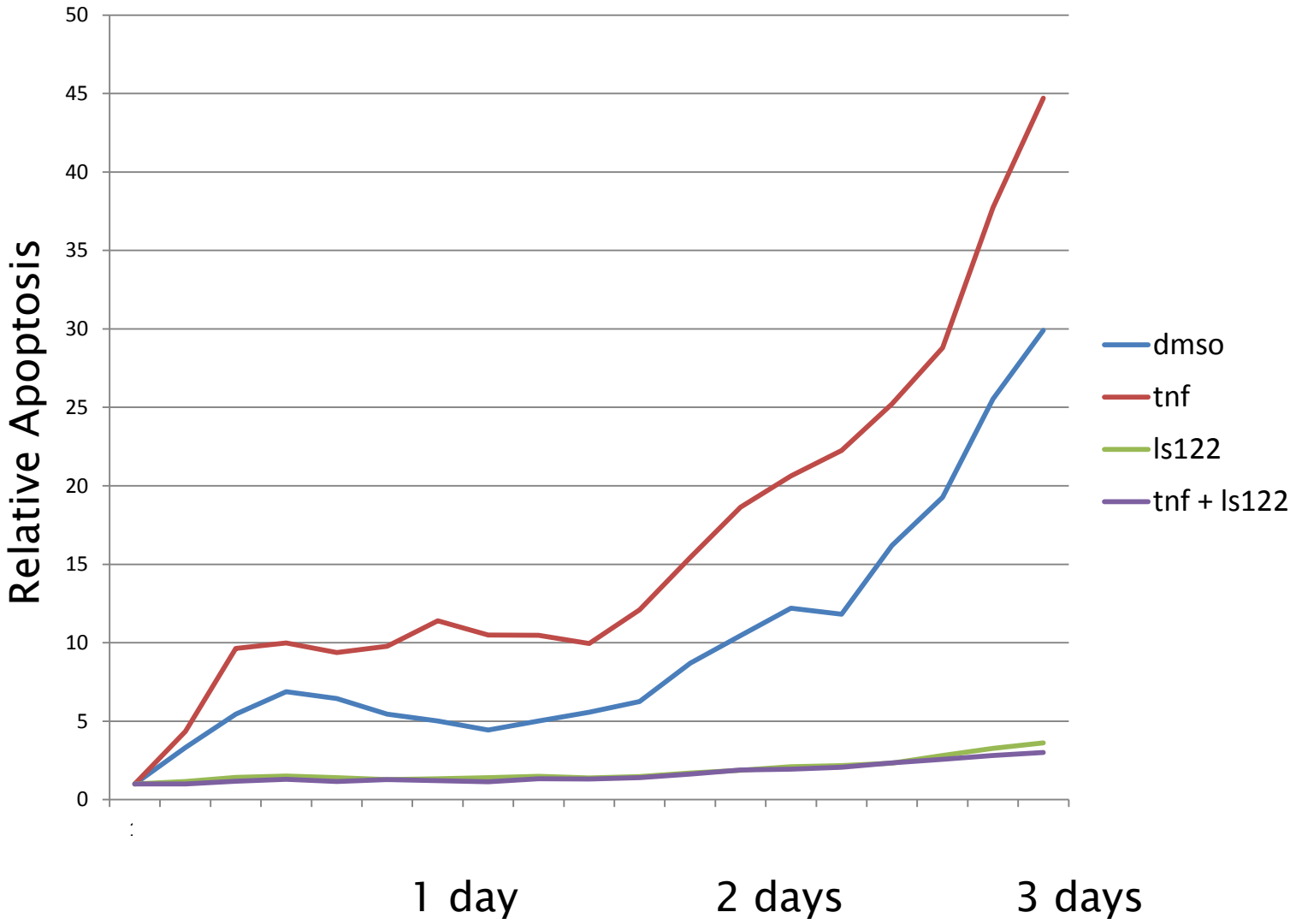


FIGURE 25


Clone 55-7
Relative Apoptosis
0
1
2
3
4
5
6
7
1 day
2 days
3 days
dmso
tnf
ls122
tnf+ls122

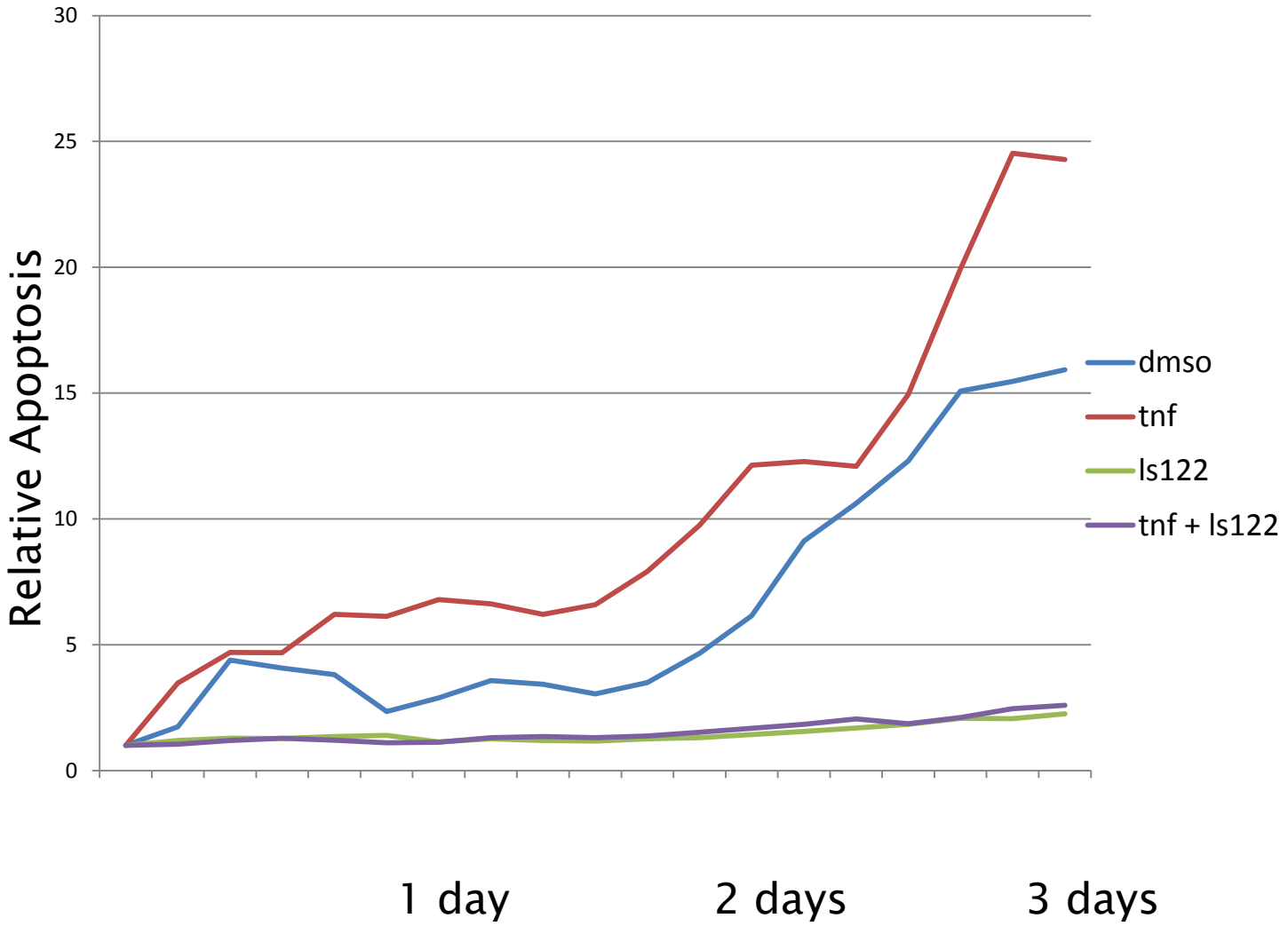
Clone 97-3

FIGURE 26



Clone 97-4

FIGURE 27



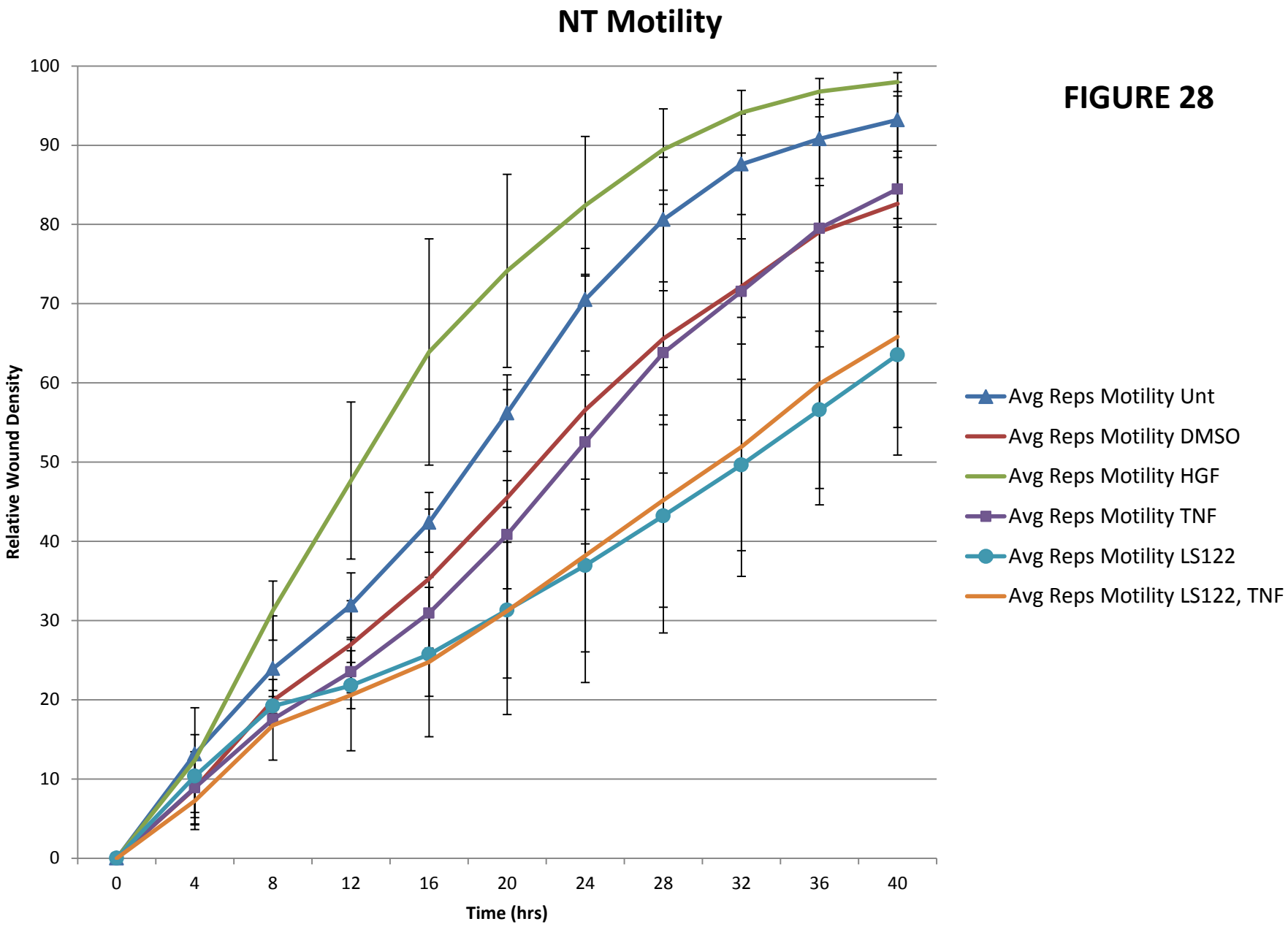

NT Motility
Relative Wound Density
Time (hrs)
0
10
20
30
40
50
60
70
80
90
100
0
4
8
12
16
20
24
28
32
36
40
Avg Reps Motility Unt
Avg Reps Motility DMSO
Avg Reps Motility HGF
Avg Reps Motility TNF
Avg Reps Motility LS122
Avg Reps Motility LS122, TNF

FIGURE 28

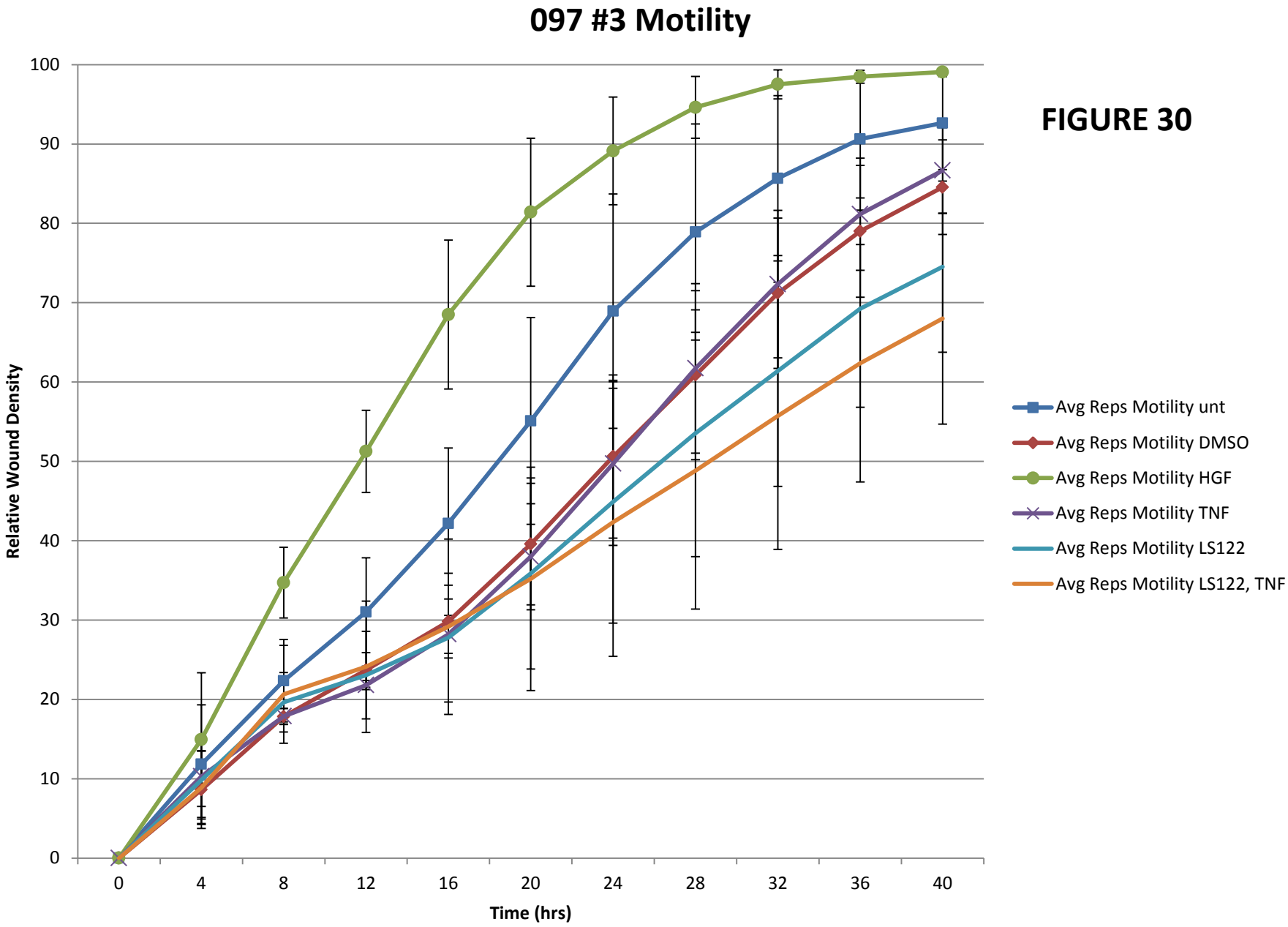

097 #3 Motility
Relative Wound Density
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
32
36
40
Time (hrs)
Avg Reps Motility unt
Avg Reps Motility DMSO
Avg Reps Motility HGF
Avg Reps Motility TNF
Avg Reps Motility LS122
Avg Reps Motility LS122, TNF

FIGURE 30

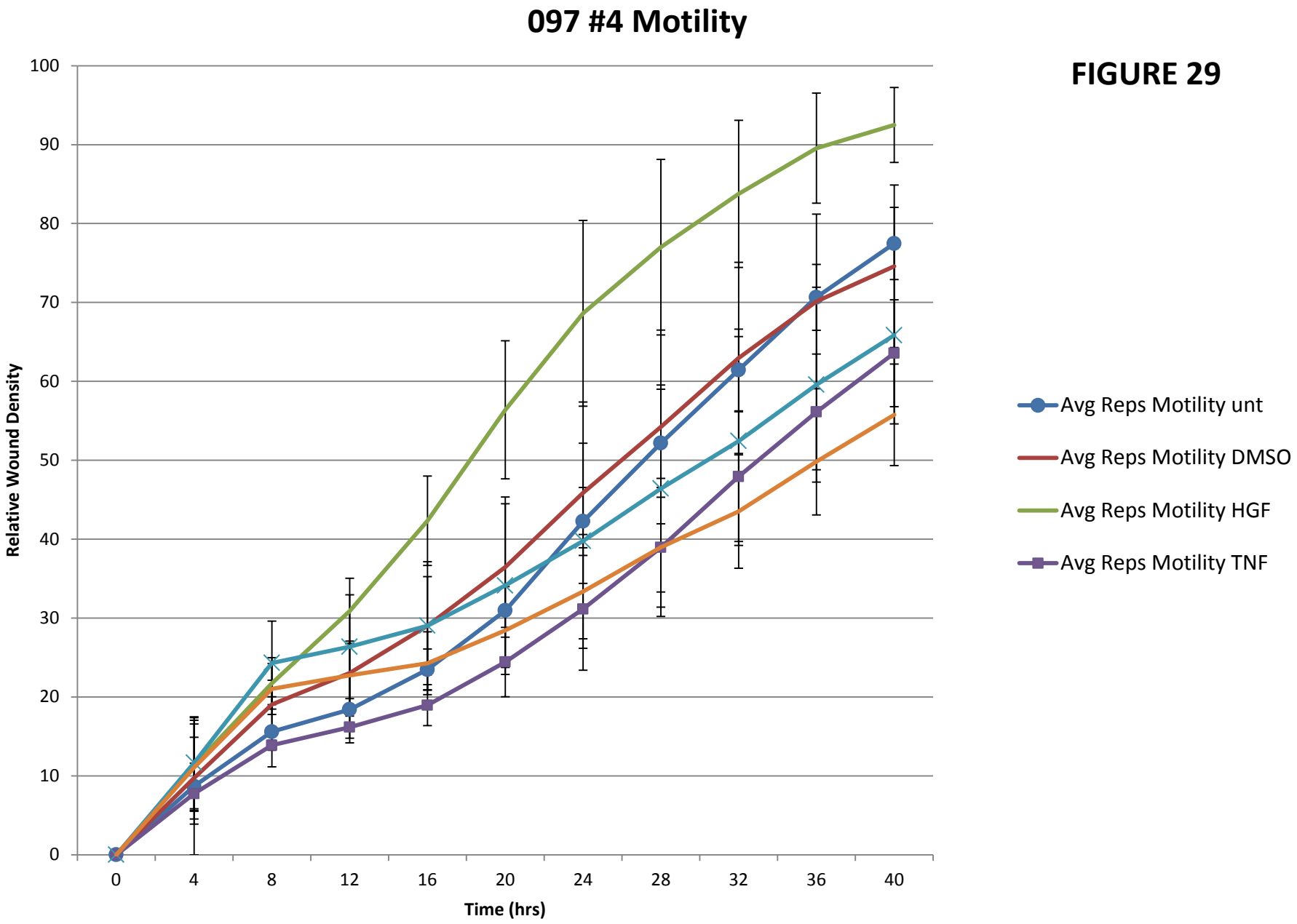

097 #4 Motility
Relative Wound Density
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
32
36
40
Time (hrs)
Avg Reps Motility unt
Avg Reps Motility DMSO
Avg Reps Motility HGF
Avg Reps Motility TNF

FIGURE 29

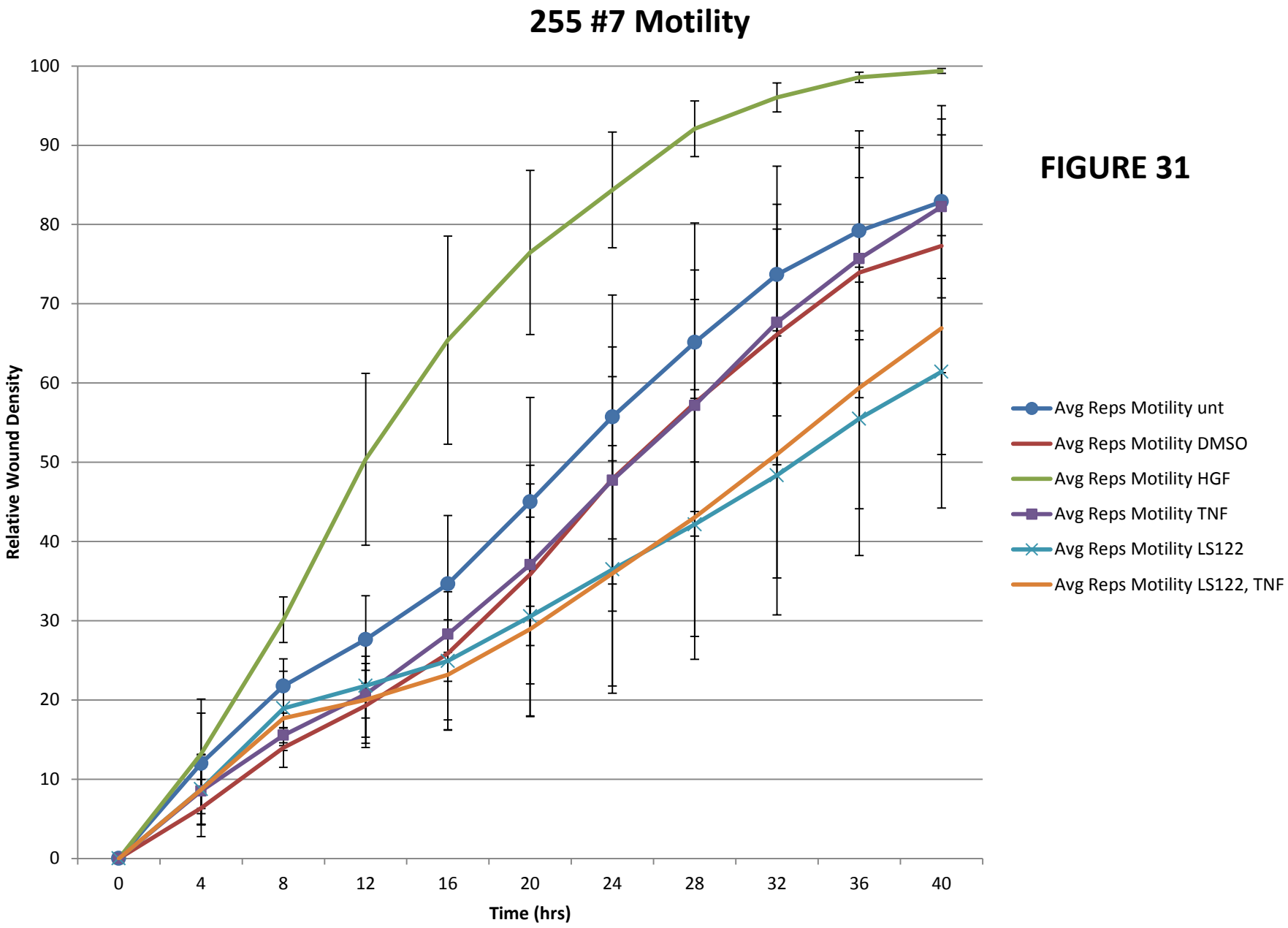

255 #7 Motility
Relative Wound Density
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
32
36
40
Time (hrs)
Avg Reps Motility unt
Avg Reps Motility DMSO
Avg Reps Motility HGF
Avg Reps Motility TNF
Avg Reps Motility LS122
Avg Reps Motility LS122, TNF

FIGURE 31

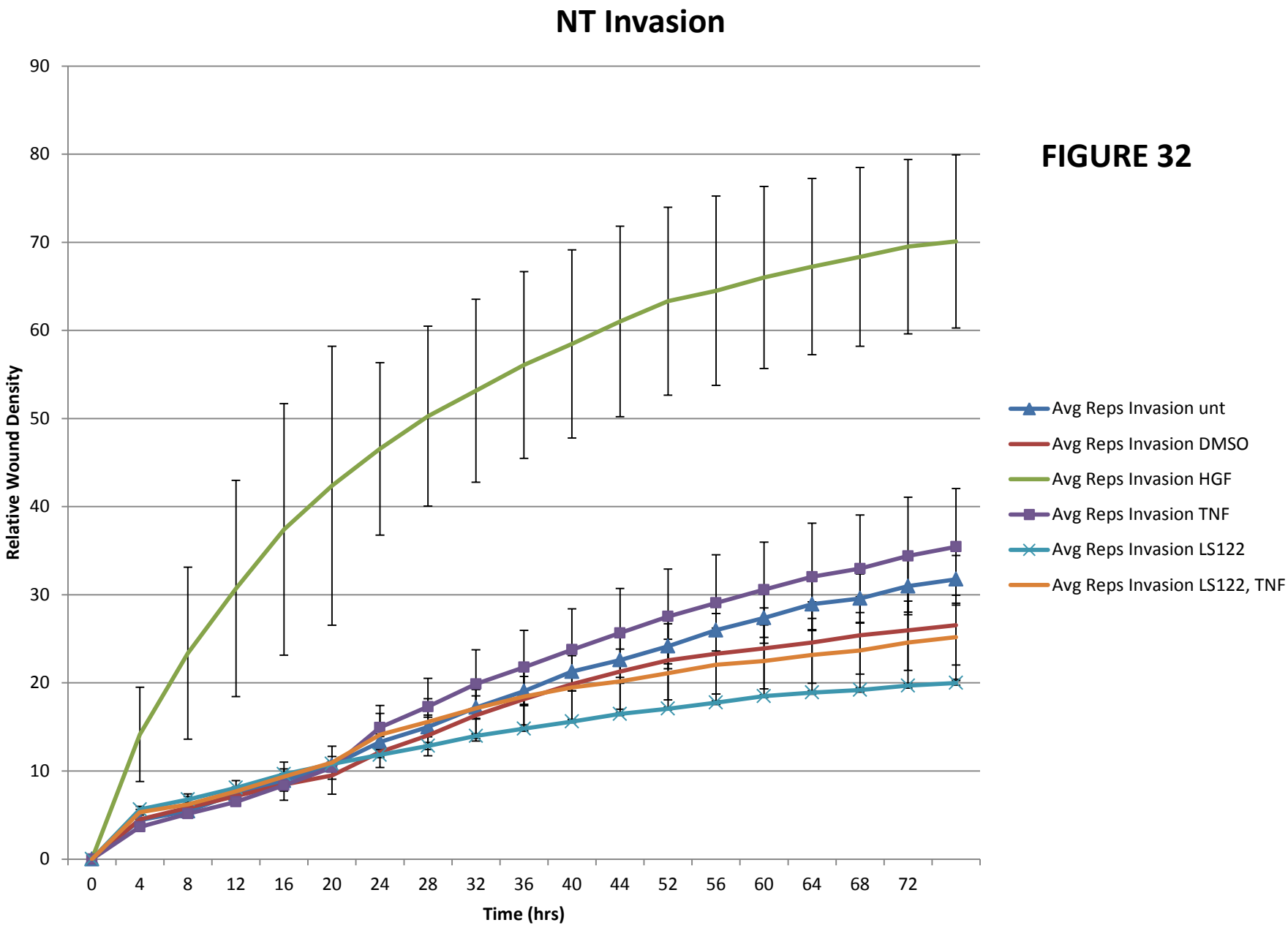

NT Invasion
Relative Wound Density
Time (hrs)
0
10
20
30
40
50
60
70
80
90
0
4
8
12
16
20
24
28
32
36
40
44
52
56
60
64
68
72
Avg Reps Invasion unt
Avg Reps Invasion DMSO
Avg Reps Invasion HGF
Avg Reps Invasion TNF
Avg Reps Invasion LS122
Avg Reps Invasion LS122, TNF

FIGURE 32

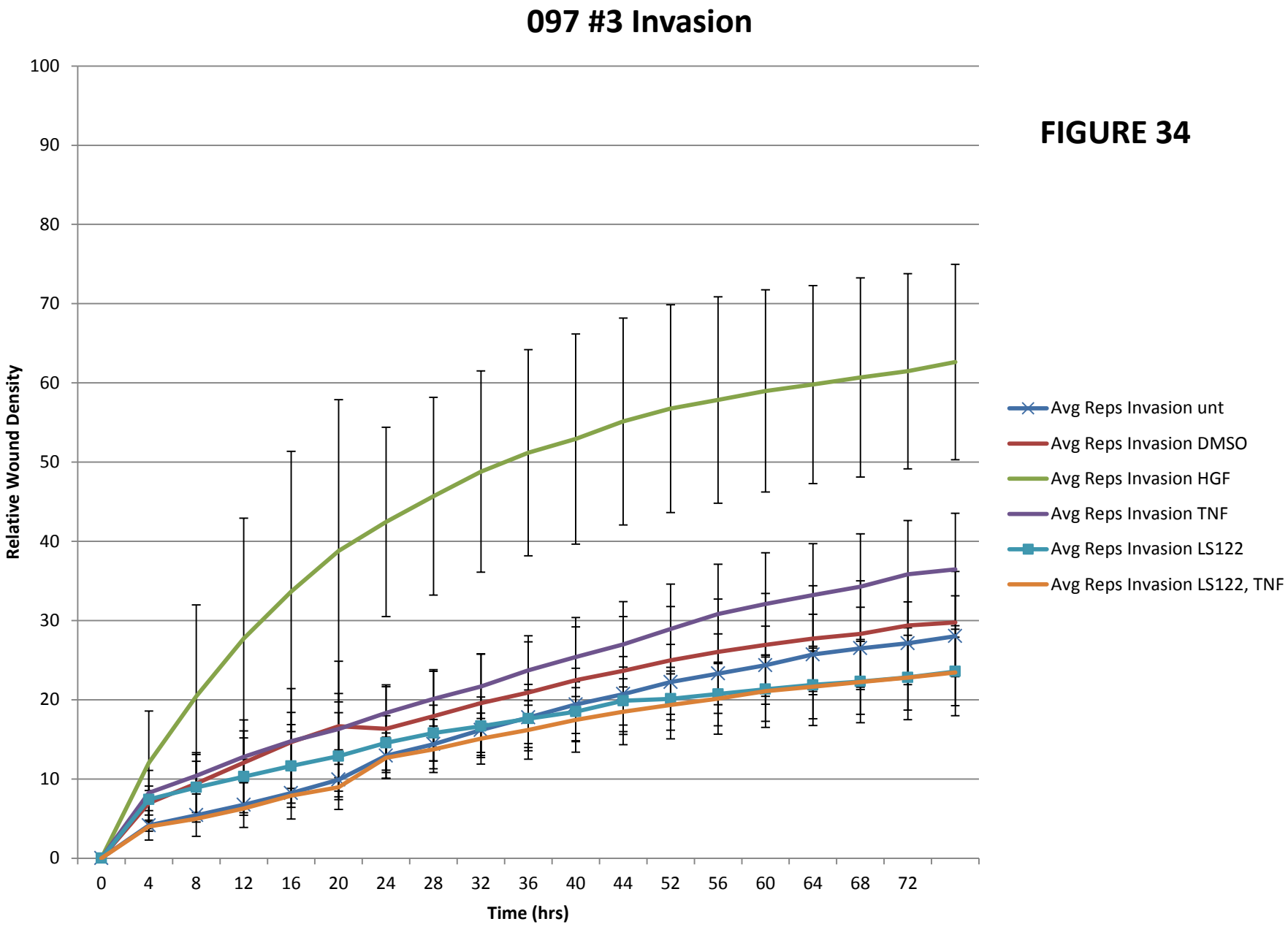

097 #3 Invasion
Relative Wound Density
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
32
36
40
44
52
56
60
64
68
72
Time (hrs)
Avg Reps Invasion unt
Avg Reps Invasion DMSO
Avg Reps Invasion HGF
Avg Reps Invasion TNF
Avg Reps Invasion LS122
Avg Reps Invasion LS122, TNF

FIGURE 34

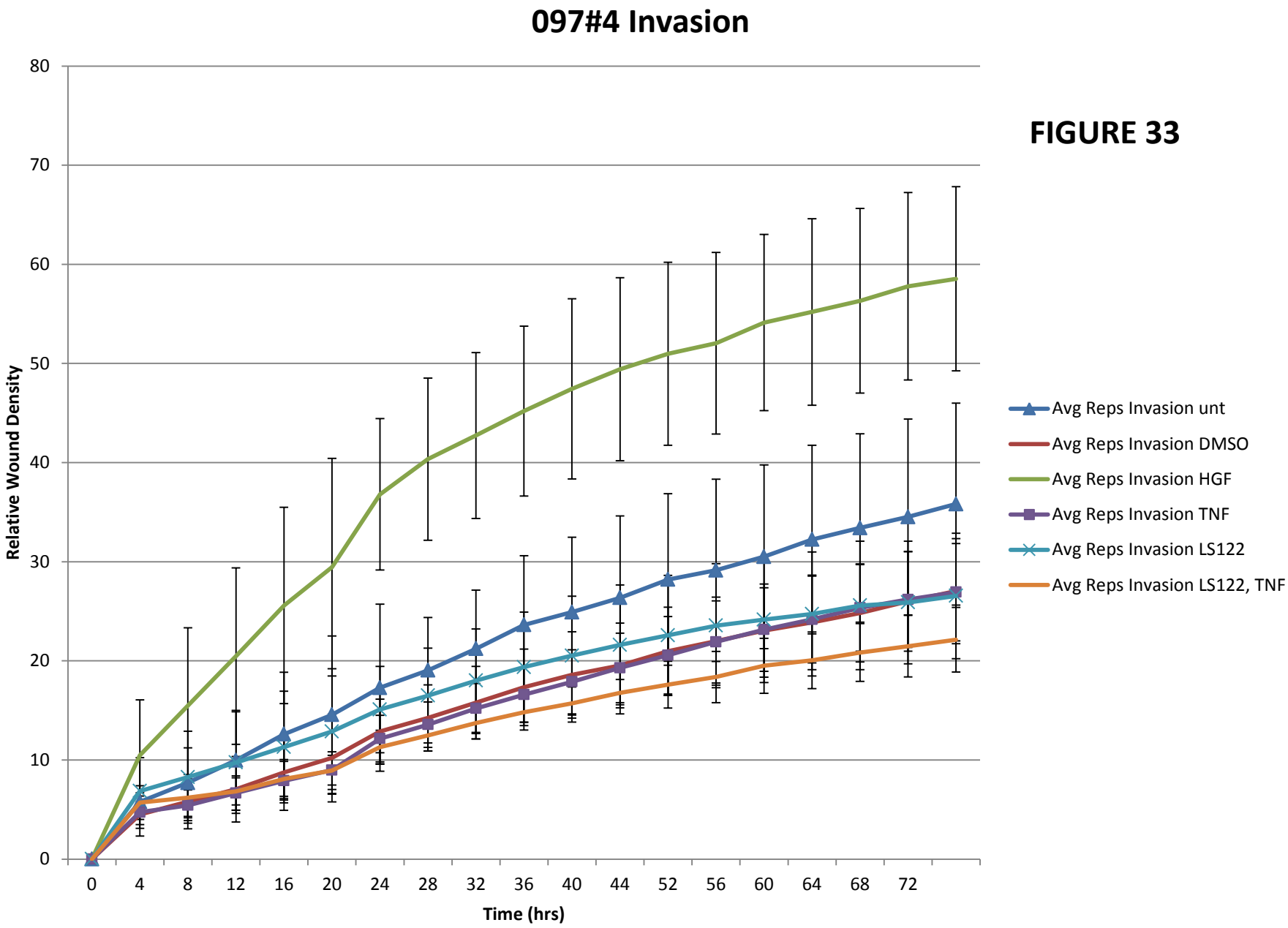

097#4 Invasion
Relative Wound Density
Time (hrs)
0
10
20
30
40
50
60
70
80
0
4
8
12
16
20
24
28
32
36
40
44
52
56
60
64
68
72
Avg Reps Invasion unt
Avg Reps Invasion DMSO
Avg Reps Invasion HGF
Avg Reps Invasion TNF
Avg Reps Invasion LS122
Avg Reps Invasion LS122, TNF

FIGURE 33

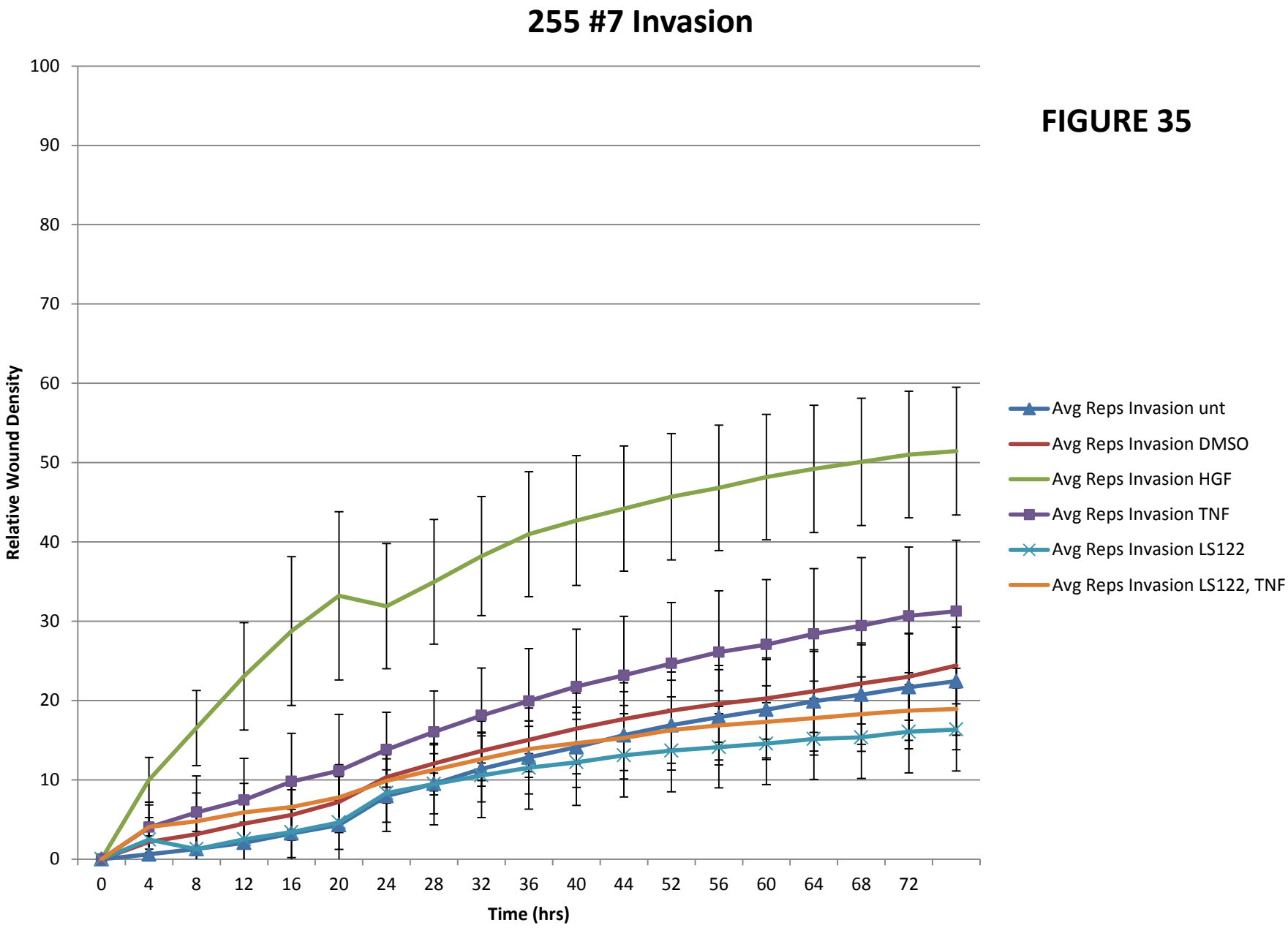

255 #7 Invasion
Relative Wound Density
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
32
36
40
44
52
56
60
64
68
72
Time (hrs)
Avg Reps Invasion unt
Avg Reps Invasion DMSO
Avg Reps Invasion HGF
Avg Reps Invasion TNF
Avg Reps Invasion LS122
Avg Reps Invasion LS122, TNF

FIGURE 35

LS122 + TNF and DMSO vs Time, motility

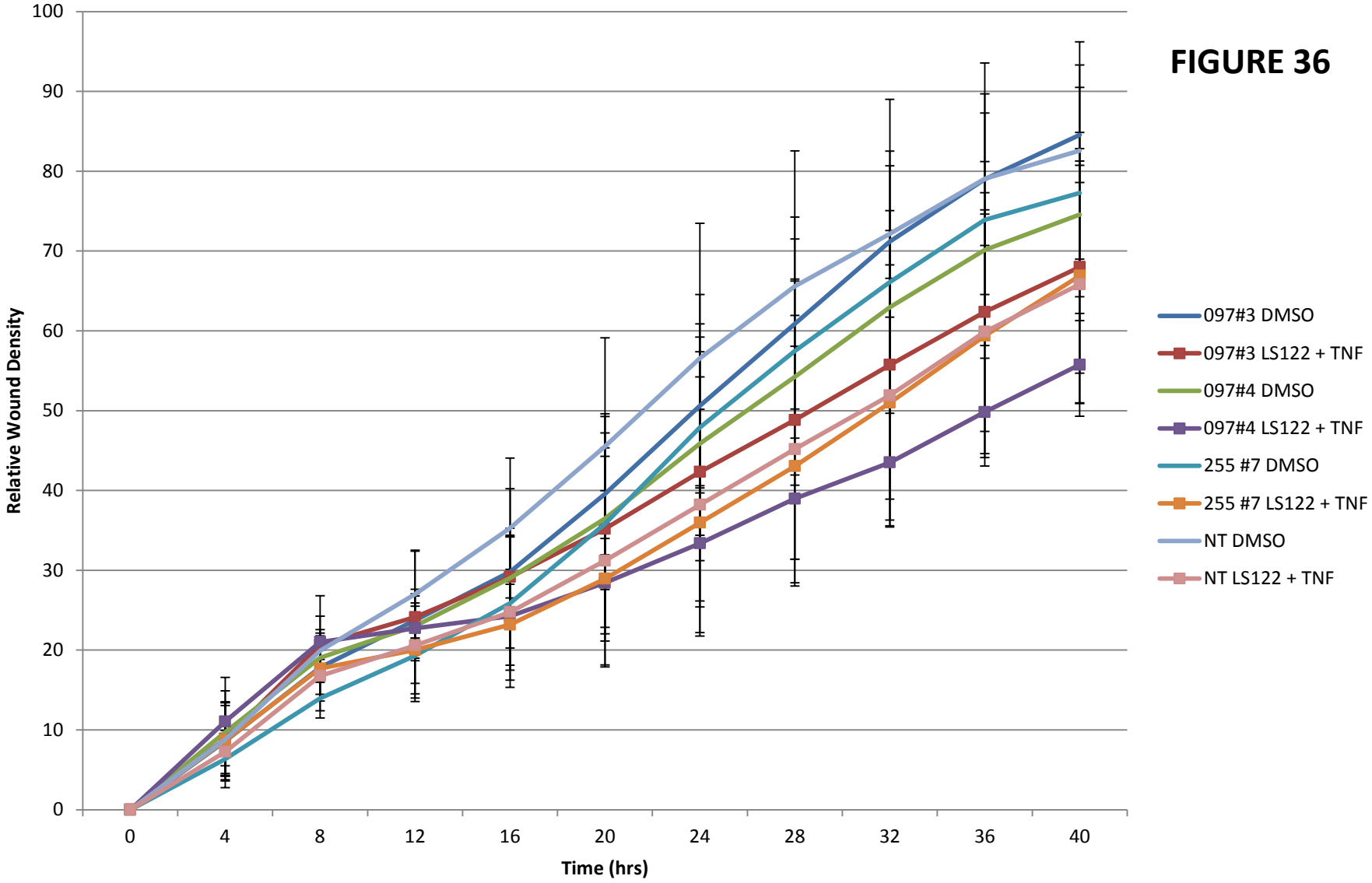


FIGURE 36

LS122 + TNF and DMSO vs Time, motility

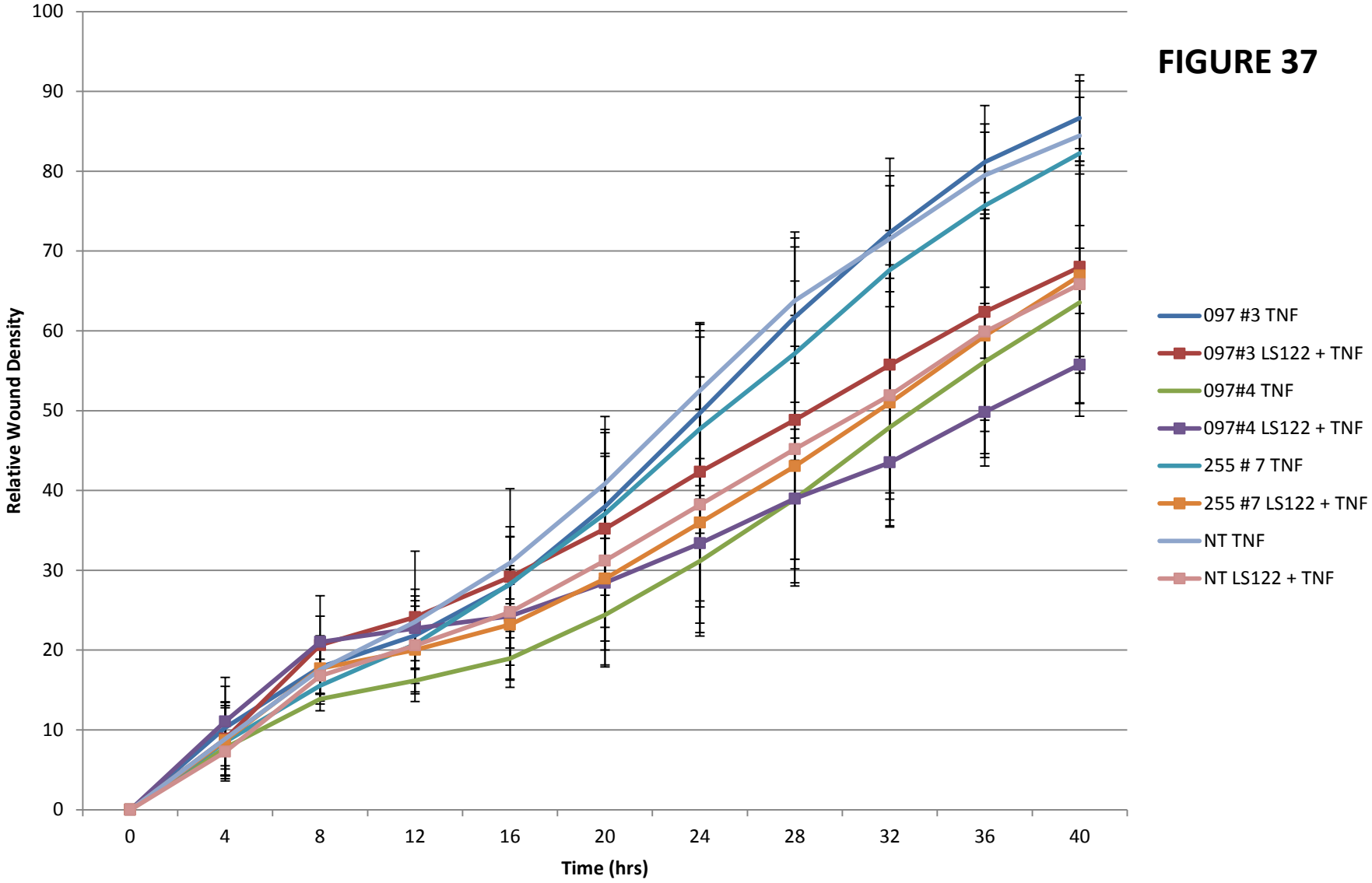
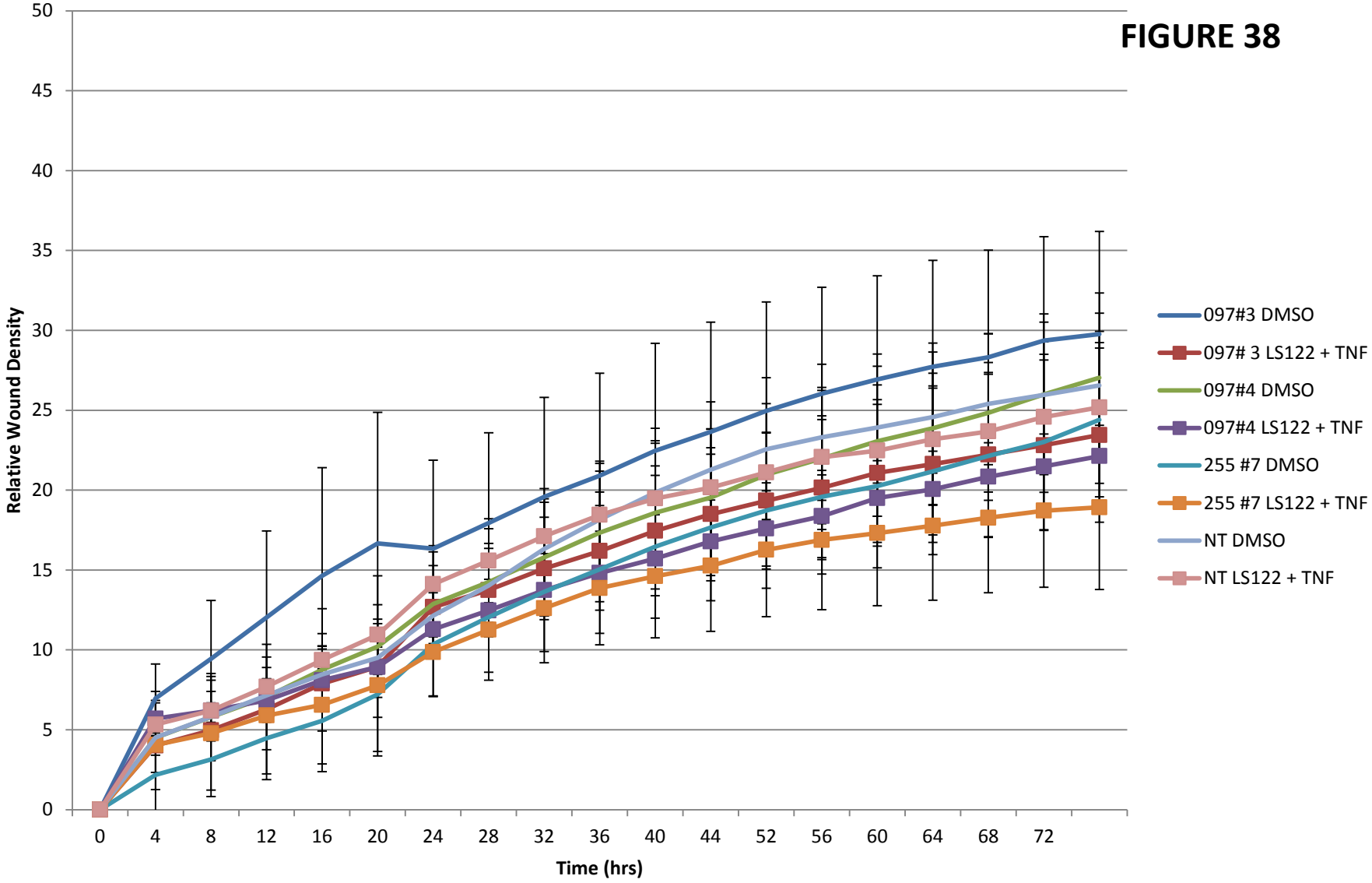


FIGURE 37

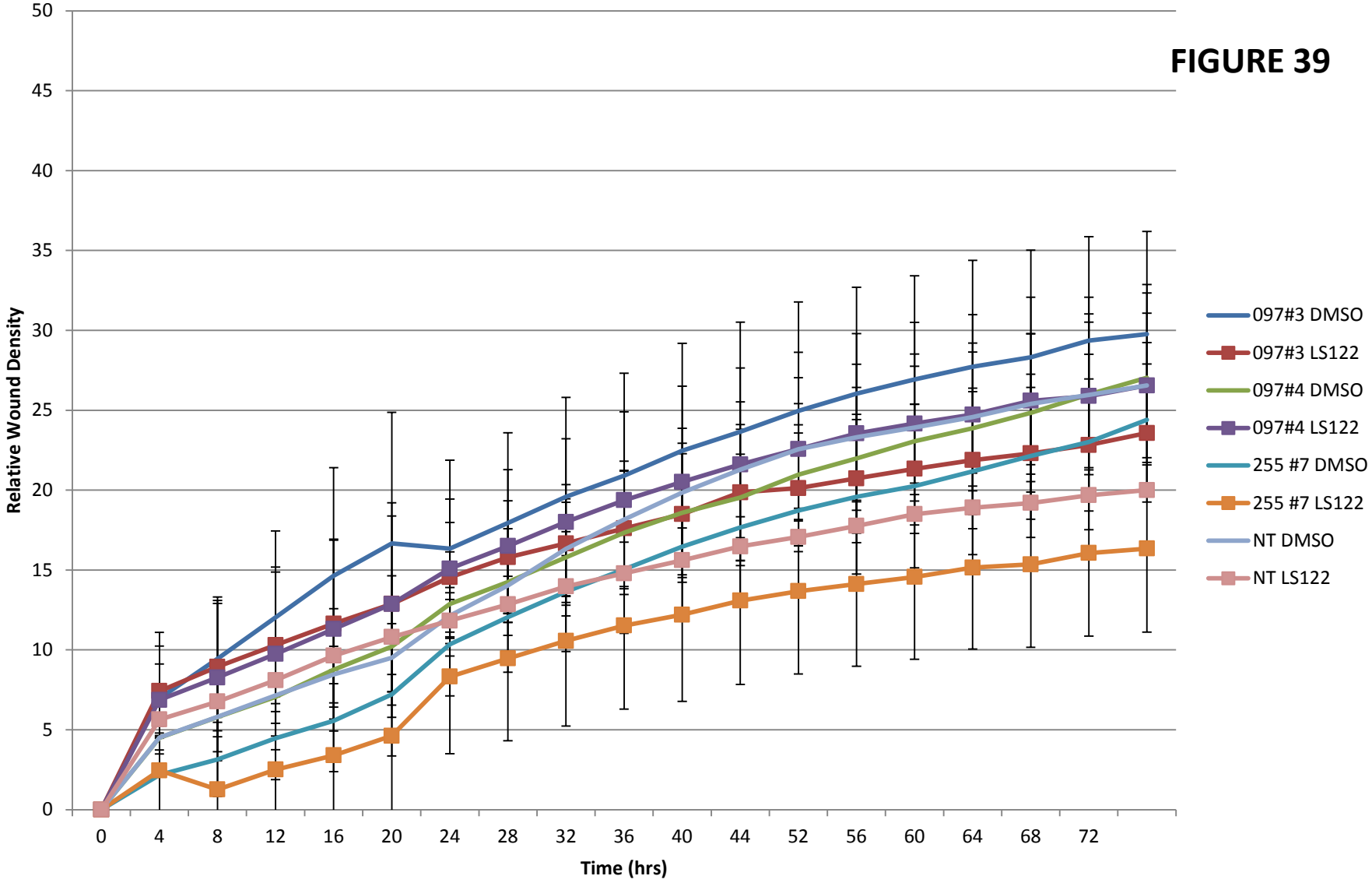
LS122 + TNF and DMSO vs Time, invasion

FIGURE 38



LS122 and DMSO vs Time, invasion

FIGURE 39



LS122 and DMSO vs Time, invasion

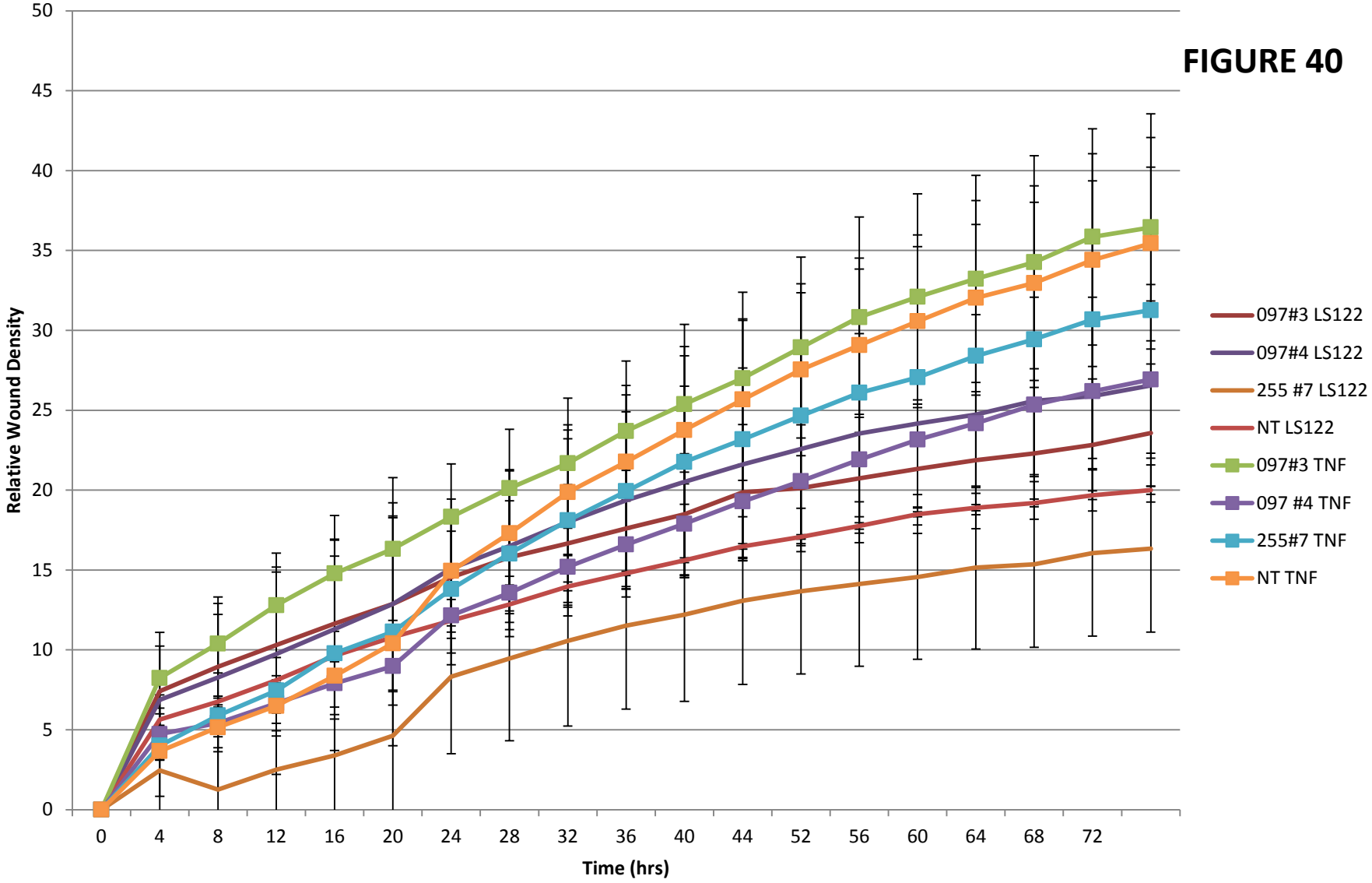
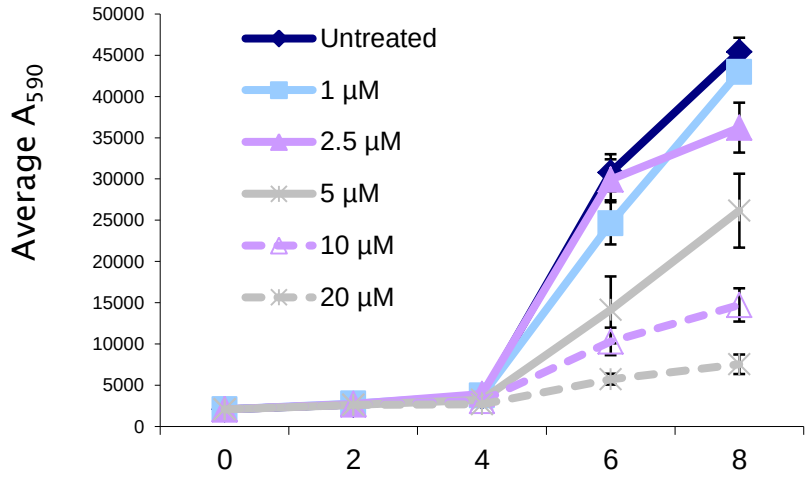


FIGURE 40

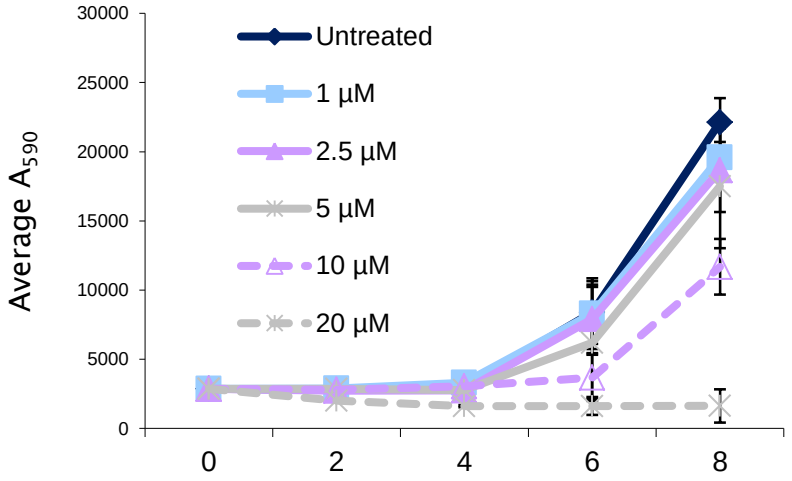
LS122 blocks key steps in both osteoblast and osteoclast activation

FIGURE 41

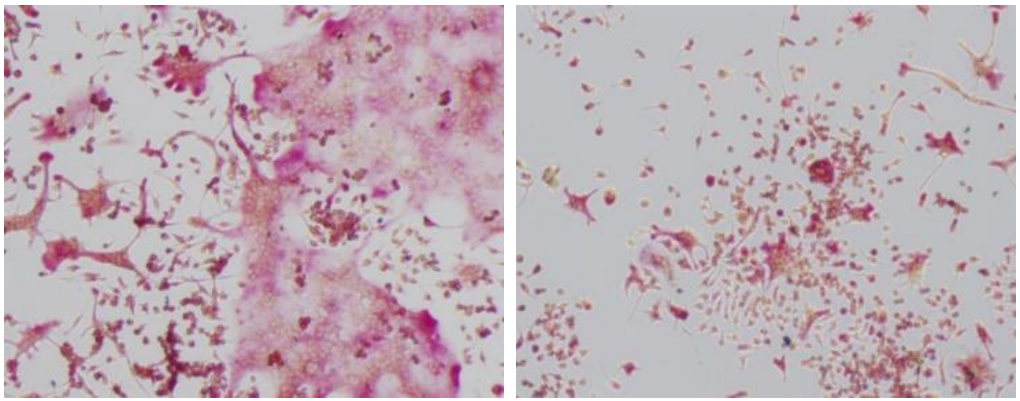
A



B



C



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