NCKU
Aim for the Top University Project
2013 Annual Report

Research Project Title（Chinese）：發展標靶治療腫瘤微環境的新策略
Research Project Title（English）：Novel targeting therapy to normalize tumor microenvironment

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Co-Principal Investigator（CO-PI）：洪建中
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Collaborative investigator：謝達斌
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Department/Institute/Center：Institute of Oral Medicine and Department of Stomatology

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Signature of Principal Investigator：

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I. **Abstract** (1 page maximum, including 4~5 keywords)

Aberrant activation of signal transducer and activator of transcription 3 (Stat3) occurs in many cancers and tumor associated microenvironment. Therefore, Stat3 appears to be a novel cancer drug target. In this study, we developed a novel targeting therapy against Stat3 activation to normalize tumor microenvironment via Neuropilin-1 (NRP1) binding peptide-conjugated PLGA (poly (D, L-lactide-co-glycolide)) nanoparticles. We showed that NRP1 is regulated by IL-6/Stat3 signaling in various lung cancer cells and animal models. Inhibition of Stat3 activation by JAKs inhibitor (AG490) or S1PR1 agonist (FTY720) decreased NRP1 expression. PLGA-encapsulated FTY720 (PLGA-FTY720) or AG490 (PLGA-AG490) significantly suppressed more Stat3 activation compared to that of free inhibitors in lung cancer cells. We also showed NRP1 binding peptide (DG2) have better binding ability with NRP1 highly expressed lung cancer cells. Moreover, DG2 conjugation could enhance anti-cancer efficacy of PLGA-FTY720 and PLGA-AG490 nanoparticles. We found that FTY720 inhibits JAK1 phosphorylation and IL-6 secretion to suppress Stat3 activation in lung cancer cells. FTY720 treatment could also suppress oncogenic Kras4bG12D induced lung cancer in mice. Moreover, PLGA-FTY720 and PLGA-AG490 outperform free inhibitors in the suppression of de novo lung cancer generation. DG2 conjugation increased anti-cancer efficacy of PLGA-FTY720 and PLGA-AG490 were also found in vivo. Numbers of tumor associated macrophages (TAMs) infiltrated into tumors were decreased and the expression of PD-L1 in tumor cells was also declined by Stat3 inhibition. Token together, we developed a novel targeting therapy against Stat3 activation in tumor microenvironment via NRP1-guided PLGA nanoparticles.

Keywords: lung cancer, tumor microenvironment, Stat3 inhibitor, PLGA nanoparticles
II. Content of Research Proposal

Background and Significance of Research

Activation of Signal transducer and activator of transcription 3 (Stat3) in cancer cells induces cell cycle progression, cellular proliferation, survival, carcinogenesis, angiogenesis and immune evasion (Bue et al., 2002). Constitutively activated Stat3 has been found in many cancers, including lung cancer. We have demonstrated that autocrine secretion of IL-6 induces Stat3 activation in lung cancers and subsequently leads to the generation of malignant effusions via increase of VEGF production and vascular permeability (Yeh et al., 2006). Furthermore, we found autocrine IL-6 production from lung cancer cells is regulated by Stat3 in a positive feedback loop and the autocrine IL-6 contributes to tumor drug resistance (Huang et al., 2010). In addition to tumor cells, Stat3 is also constitutively activated in immune cells, stromal cells and endothelial cells of tumor microenvironment. Stat3 activation promotes the production of immunosuppressive programs to restrain anti-tumor immune responses (Yu et al., 2007). Yu’s group further showed that Stat3-induced S1PR1 expression and the S1P-S1PR1pathway reciprocally regulate Stat3 activity. This system may be a major positive feedback loop for persistent Stat3 activation in cancer cells and the tumor microenvironment (Lee et al., 2010). Therefore, Stat3 participates in the crosstalk between cancer cells and cells in tumor microenvironment leading to tumor progression. To control tumor progression we have to suppress IL-6/Jak/Stat3 pathway in cancer cells and cells of tumor microenvironment simultaneously. Our preliminary data showed that lung cancer cells with high levels of IL-6 or active Stat3 are more resistant to AG490 (JAK2 inhibitor) and S3I-201 (Stat3 inhibitor). In this study, we developed a novel targeting therapy against Stat3 activation both in tumors and tumor microenvironment via PLGA nanoparticles.

An ideal drug delivery system should present the capability to protect the carried drugs or macromolecular payload from premature degradation or release before reaching the target sites. Nanocapsule formulation technologies have attracted significant global attention in pharmaceutical industry due to their capability to improve pharmacokinetic characteristics of the carried drugs, their solubility, bioavailability, and the possibility to combine targeting strategy and intelligent release control (Dobson, 2006; Hans and Lowman, 2002; Wissing et al., 2004). Integration of nano formulation with conventional drugs not only increases therapeutic efficacy and decreases side effects, but also broaden its therapeutic indications. PLGA is an FDA approved polymeric material emerging as an important excipient of drug formulation. Besides, preclinical biocompatibility and toxicology validation of the candidate API and formulation is equally important as their therapeutic efficacy. Therefore, targeting therapy to normalize tumor microenvironment via PLGA nanoparticles was developed.

So far, targeted delivery of therapeutic agents to tumors is still a challenge in cancer treatment, in which the efficacy is improved while side effects are reduced by increasing the amount of a drug reaching the tumor. Neupilin-1 (NRP1) was originally identified as a receptor for the semaphorin 3 subfamily mediating neuronal guidance and axonal growth (Soker et al., 1998) and subsequently found to specifically bind VEGF165 on endothelial cells and tumor cells.
NRP1 has been also frequently observed in high grade and metastatic tumors (Bagri et al., 2009; Yaqoob et al., 2012). Our previous study showed that NRP1 is a cancer invasion and angiogenesis enhancer. NRP1 plays a critical role in tumorigenesis, cancer invasion, and angiogenesis through VEGF, PI3K, and Akt pathways (Hong et al., 2007). Therefore, NRP1 may be a promising therapeutic target in solid tumors. In this project, we established NRP1-guided tumor homing nanodevices delivered with Stat3 inhibitors as novel ideal nanodevices which increased the anti-tumor efficacy and decreased toxicity for cancer treatments.

An important scenario encompassing the interaction between tumor cells and immune cells is the inflammatory process. Chronic inflammation is associated with malignant development of many cancers (Bromberg and Wang, 2009). Activation of Stat3 is an important molecule involved in tumor-induced immune suppression at many levels. For example, abnormal activation of Stat3 signaling leads to suppression of dendritic cell (DC) differentiation (Nefedova et al., 2005). Interference of DC differentiation or activation is connected with failure of successful development of tumor immunity. The IL-6, Janus kinase (Jak) and Stat3 activities are also involved in the expansion and function of myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAMs) which contribute to negative regulation of immune responses during cancer development (Gabrilovich and Nagaraj, 2009). Therefore, targeting Stat3 in the tumor microenvironment not only exert effects on tumor cells but also inflammatory and negative elements of the immune system. In addition to tumors, NRP1 can be expressed on several immune cells presented in the tumor microenvironment, including DC, regulatory T cells, MDSC and TAM (Glinka and Prud'homme, 2008; Ji et al., 2009; Potiron et al., 2009). We have found that macrophages treated with tumor-conditioned medium express a higher level of NRP1 and that both DG1 and DG2 (NRP1 binding peptides) can efficiently bind to these macrophages. Since NRP1 is regulated by Stat3, suppressive or tolerogenic immune cells, such as TAMs, MDSC, regulatory T cells and inactivated DC, that are characterized with an activated status of Stat3 also express NRP1. Therefore, NRP1 may serve as a good molecule for targeting Stat3-suppressing materials to TAMs, MDSC, Treg and inactivated DC in the tumor microenvironment for the conversion of a pro-cancer environment to an anti-cancer status. Thus, the application of NRP1-targeting nanodevices to revert immune suppression in tumor microenvironment for the prevention and treatment of cancer was evaluate.
III. Results and Discussions

FTY720 inhibits Jak1, Stat3 and AKT activation and suppresses IL-6 secretion in lung cancer cells.

It has been shown that Stat3-induced sphingosine-1-phosphate receptor 1 (S1PR1) expression is crucial for persistent Stat3 activation in tumors and tumor microenvironment. FTY720, an agonist of S1PR1, binds to S1PR1 and then S1PR1 is degraded by endocytosis. In this study, we found that FTY720 inhibits Stat3 activation as well as AKT activation in A549 cells (Figure 1A). The suppression of Stat3 may result from the JAK1 inhibitory activity of FTY720 (Figure 1B). Since Stat3/S1PR1 signaling positively regulates IL-6 expression, we then evaluated the effect of FTY720 on IL-6 secretion using ELISA. The results show that IL-6 secretion was reduced by FTY720 treatment in CL1-5 (Figure 1C) and A549 cells (Figure 1D). Taken together, the suppression of S1PR1 could disrupt IL-6/Stat3 positive feedback loop.

PLGA-encapsulated Stat3 inhibitors outperform free inhibitors in suppressing Stat3 activation and cell death.

PLGA (poly (D, L-lactide-co-glycolide) is approved to be used in human by the U.S. Food and Drug Administration (FDA). The PLGA nanoparticles have been shown to have higher efficiency than microparticles in transfection and enhancement of uptake by tissue and cells (Wang and Thanou, 2010). We used PLGA nanoparticles to encapsulate FTY720 or AG490 and found that free FTY720 did not suppress phosphorylation of Stat3 and AKT at the concentration of 6 \( \mu \text{M} \) in PC14PE6AS2 (AS2) lung cancer cells, whereas PLGA-FTY720 very effectively suppressed the phosphorylation of them (Figure 2A). We next examined the effects of PLGA-encapsulated AG490 (PLGA-AG490) on Stat3 activation and cell survival in AS2 cells. Consistently, cells treated with free AG490 showed a decreasing of phosphorylated Stat3 and PLGA-AG490 had a better inhibition on Stat3 activation, while PLGA control treatment did not alter Stat3 phosphorylation (Figure 2B). Stat3 downstream genes, which participate in cell survival, were further analyzed. Our data showed that expression of Bcl-xL, Survivin and Mcl-1 were suppressed by AG490 and these genes expression were further decreased by PLGA-AG490 treatment (Figure 2C). We also found AG490 treatment suppressed cell proliferation as demonstrated by MTT assay, and PLGA-AG490 showed more potent effect than free AG490 did (Figure 2D). Taken together, PLGA encapsulated FTY720 and AG490 outperform free inhibitors in suppressing Stat3 activation and cell proliferation.

Stat3 regulates NRP1 expression in various lung cancer cells.

To develop NRP1-guided anti-Stat3 nanodivices, we have confirmed that NRP1 expression is indeed regulated by Stat3. Stable cell lines overexpressing dominant negative Stat3 (S3D) in the Stat3 constitutively activated AS2 cells were established, a decreased level of NRP1 expression in S3D overexpressed cells than that in vector control (Vec) cells was shown (Figure 3A). We used shRNA of Stat3 retrovirus to further confirm the regulation of NRP1 by Stat3 in
CL1-5 cells (Figure 3B). Our data shows that knockdown of Stat3 expression in CL1-5 cells diminished NRP1 expression. Furthermore, the levels of NRP1 expression was rescued by overexpression of active-form Stat3 (Stat3C) in the Stat3 knockdowned CL1-5 cells (Figure 3C). Collectively, our data clearly demonstrate that NRP1 is a downstream target gene of Stat3 in lung cancer cells. The experiments using pharmaceutical inhibitors-INC424 and FTY720 confirm the regulation of NRP1 by Stat3 in various lung cancer cell lines. In CL1-5 cells, NRP1 expression was down-regulated by INC424 (Figure 3D) or FTY720 (Figure 3E) treatment dose-dependently. Same effects of FTY720 on NRP1 suppression was shown in AS2 cells (Figure 3F).

**PLGA-FTY720 outperforms free FTY720 in suppressing lung cancer formation in vivo.**

We have successfully established the transgenic mice bearing lung tumor induced by oncogenic Kras4bG12D under the control of doxycycline. We found that NRP1 and Stat3 phosphorylation are increased in Kras-induced lung cancer (Figure 4A). Because Stat3 could support Ras-dependent oncogenic transformation (Gough et al., 2009), we then further investigated the therapeutic effects of Stat3 suppression by FTY720 on Kras-induced lung tumorigenesis. We found that the lung nodules were significantly reduced by free FTY720 treatment at 0.25 and 0.5 mg/kg twice a week for 2 months. We have showed that PLGA encapsulated FTY720 is more potent than free inhibitors in vitro. We then test the efficacy of PLGA-FTY720 in suppressing lung cancer generation in vivo. Consistently, PLGA-FTY720 significantly reduced more de novo lung cancer generation than free FTY720 did was observed (Figure 4B). Expression levels of pStat3 inhibited by free FTY720 and PLGA-FTY720 was confirmed by Western blot (Figure 4C). IHC staining also showed pStat3 levels were decreased by free FTY720 and PLGA-FTY720 treatment (Figure 4D). PD-L1 is a Stat3 target gene and anti-PD-L1 therapies produce impressive clinical response in patients with advanced lung cancers recently (Brahmer et al., 2012; Topalian et al., 2012). We also found that PD-L1 expression in lung cancer cells was inhibited by free FTY720 and PLGA-FTY720 (Figure 4C and 4D). Taken together, PLGA-FTY720 outperforms free FTY720 in suppressing Stat3 activation and de novo lung cancer generation induced by mutation Kras.

**NRP1-binding peptide (DG2) conjugation enhanced anti-cancer efficacy of PLGA-FTY720 on lung cancer cells.**

We have showed that NRP1 expression is regulated by IL-6/Stat3 signaling in lung cancer cell lines. To develop NRP1-guided PLGA nanoparticles, NRP1-binding peptide (DG2) was conjugated to PLGA and then loaded with Nile red dye. To evaluate the binding ability of DG2 with lung cancer cells, low invasive CL1-0 and high invasive CL1-5 lung adenocarcinoma cell lines were used. We found that the expression of NRP1 is higher in CL1-5 cells and the NRP1 expression correlates well with phosphor Stat3 level (Figure 5A). Immunofluorescent staining also showed higher NRP1 expression in CL1-5 cells, whereas CL1-0 cells showed undetectable level of NRP1 (Figure 5B). We then evaluated the binding activity of DG2 conjugated PLGA with lung cancer cells. DG2 was conjugated to PLGA and then Nile red was loaded into the
nanoparticles. The nanodevice was applied to CL1-0 cells and CL1-5 cells for an hour. Cells were then washed and signals were detected by fluorescent microscopy. The data showed that CL1-5 cells contained more Nile red signals than CL1-0 cells, indicating the DG2 targeting peptide effectively bring the nanoparticles to NRP1 overexpressed cells (Figure 5C). DG2 conjugated PLGA-FTY720 (DG2-PLGA-FTY720) was then applied to the same cells, Figure 4D showed that DG2 conjugated PLGA-FTY720 enhanced anti-cancer efficacy of PLGA-FTY720 as demonstrated by cell survival (Figure 5D).

**DG2 conjugated PLGA-Stat3 inhibitors enhanced anti-tumor activity on mutant Kras-induced lung cancer.**

Because NRP1 expression was elevated in mutant Kras-induced lung cancers and we have demonstrated DG2-PLGA nanoparticle could specifically bind to NRP1 highly expressed lung cancer cells. We then examined the effects of DG2-PLGA-FTY720 on mutant Kras-induced lung cancer in mice model. We found that DG2-PLGA-FTY720 (0.1 mg/kg) treatment reduced more de novo lung cancer generation than that of PLGA-FTY720 (0.5 mg/kg) did, indicating DG2 conjugation enhanced the potency of the PLGA-FTY720 (Figure 6A). Expression levels of pStat3 inhibited by DG2-PLGA-FTY720 was confirmed by Western blot (Figure 6B). Consistently, DG2-PLGA-AG490 significantly reduced more de novo lung cancer generation and pStat3 levels of tumor cells than PLGA-AG490 did at the dose of 0.1 mg/kg was also observed (Figure 6C and 6D). These results indicate that DG2 conjugation enhanced anti-tumor efficacy of PLGA-FTY720 and PLGA-AG490 on mutant Kras-induced lung cancers.

**Inhibition of Stat3 activation blocked tumor associated macrophage (TAMs) infiltration to lung cancer.**

Stat3 activation is involved in the expansion and function of tumor-associated macrophages (TAMs) which contribute to negative regulation of immune responses during cancer development (Gabrilovich and Nagaraj, 2009). NRP1 is known to be expressed on several immune cells presented in the tumor microenvironment, including TAMs (Glinka and Prud'homme, 2008; Ji et al., 2009; Potiron et al., 2009). Therefore, we examined the effect of Stat3 inhibition on TAMs expression in the tumor microenvironment. Abundant expression of TAMs, which were positively stained with CD163 and CD68 by IHC were found in the mutant Kras-induced lung tumors. We found that inhibition of Stat3 activation by DG2-PLGA-FTY720 and DG2-PLGA-AG490 blocked TAMs infiltration to the lung nodules. These results indicate that targeting Stat3 in the tumor microenvironment not only exert effects on tumor cells but also TAMs.
Discussion

In this study, we demonstrated that NRP1 expression is regulated by IL-6/Stat3 signaling in various lung cancer cells and a mutant Kras-induced lung cancer mice model. When cells were treated with IL-6, Stat3 was activated and NRP1 expression was also elevated. Blockage of Stat3 activation by dominant negative Stat3 or blockage of Stat3 expression by Stat3 specific shRNA showed a decreasing level of NRP1 expression. The regulation of NRP1 by Stat3 was further confirmed by using JAKs inhibitor (INC424) and S1PR1 agonist (FTY720) to inhibit Stat3 activation. In mutant Kras transgenic mice, elevated NRP1 expression correlates with Stat3 activation in Kras-induced lung cancer. Therefore, NRP1-guided nanodevices could be developed to suppress IL-6/JAKs/Stat3 pathway in cancer cells as well as cells of tumor microenvironment.

FTY720, an agonist of S1PR1, binds to S1PR1 and then S1PR1 is degraded by endocytosis. S1PR1 is found as a direct downstream target of Stat3. It has been shown that Stat3-induced sphingosine-1-phosphate receptor 1 (S1PR1) expression is crucial for persistent Stat3 activation in tumors and tumor microenvironment. We found that FTY720 treatment inhibits Stat3 activation in various lung cancer cells. FTY720 treatment also inhibits JAK1/2 phosphorylation and IL-6 secretion in lung cancer cells. Therefore, FTY720 suppressed Stat3 activation could be due to inhibition of JAK1 activation and IL-6 secretion. Moreover, AG490 suppressed Stat3 phosphorylation through inhibition of JAKs activation. Synergistic effect of cytotoxicity on lung cancer cells was showed by combination of FTY720 and AG490.

PLGA (poly (D, L-lactide-co-glycolide) is approved to be used in human by the U.S. Food and Drug Administration (FDA) and PLGA nanoparticles have been shown to have higher efficiency than microparticles in transfection and enhancement of uptake by tissue and cells (Wang and Thanou, 2010). We used PLGA to encapsulate FTY720 and AG490 and found that PLGA encapsulated inhibitors suppressed more Stat3 activation compared to that of free inhibitors in lung cancer cells. Similar effects were also demonstrated in animal models. We conjugated NRP1 binding peptides (DG2) on PLGA to develop NRP1-guided targeting nanoparticles. We found that DG2 conjugated PLGA nanoparticle have a better binding ability to NRP1 highly expressed CL1-5 cells than lower NRP1 expressed CL1-0 cells did, which correlates with the NRP1 expression levels in these two cells. Consistently, DG2 conjugated PLGA-FTY720 were more cytotoxic to CL1-5 cells than that of PLGA-FTY720. In mutant Kras-induced lung cancer mice model, tumor number reduced more by DG2-PLGA-FTY720 treatment than that of PLGA-FTY. These data supports above idea of treating lung cancers via NRP1-guided delivery in Stat3 activated cells.

Mutations in kinase domain of EGFR and Kras were the most common population with lung cancer in western and eastern countries, respectively. We found elevated Stat3 phosphorylation in mutant Kras-induced lung cancer. Administration of AG490 (1 mg/kg) or S3I-201 (5 mg/kg) intraperitonealy to mice reduced lung nodules formation, suggesting blockage of Stat3 activation suppressed mutant Kras-induced lung cancer. We also showed PLGA-AG490 treatment more significantly reduced de novo lung cancer formation than free form AG490. MMP7 is an important downstream target of Stat3 to mediate invasion and metastasis of cancer cells.
levels of MMP7 were decreased by inhibition of Stat3 in mutant Kras-induced lung nodules.

Tumor cells expressing the ligand for the receptor programmed death-1 (PD-1), PD-L1, have been shown to increase apoptosis of antigen-specific human T-cell to evade the immune system. In this study, we determined the influence of disrupting Stat3-S1PR1 vicious cycle by FTY720 and AG490 on tumor formation and PD-L1 expression in mutant Kras-induced lung cancer. Inhibition of Stat3 activation suppressed lung cancer formation and decreased PD-L1 expression in tumor tissues, indicating that PD-L1 expression could be regulated by Stat3 and inhibiting persistent Stat3 activation could suppress tumor formation via down-regulating tumor PD-L1 expression.

Taken together, we have developed an ideal NRP1-guided PLGA nanoparticle with DG2 conjugation for Stat3 targeting in tumors and efficiently delivering to the tumor microenvironment.
References


Figure 1. FTY720 inhibits Stat3 activation and decreases IL-6 secretion in lung cancer cells. (A) A549 cells were treated with FTY720 of left untreated for various time points. The p-Stat3 and p-AKT were analysis by Western blotting. (B) AS2 cells were treated with various dosages of FTY720 for 48 h. The cell lysate were prepared to detect JAK1 and phosphor-JAK1 expression levels. CL1-5 cells (C) and A549 cells (D) were treated with various dosages of FTY720 for 48 hours, conditional medium were collected and IL-6 levels were detected using ELISA.
Figure 2. PLGA-encapsulated FTY720 or AG490 outperform free drugs in suppressing Stat3 activation and cell death. (A) PC14PE6/AS2 cells were treated with various dosages of PLGA-encapsulated FTY720 (PLGA-FTY720) or free FTY720 for 24 h. Cell lysates were prepared and proteins levels were analyzed by Western blot against antibodies as indicated. (B) PC14PE6/AS2 cells were treated with 100 μM of PLGA-encapsulated AG490 (PLGA-AG490) or free AG490 for 24 h. Cell lysates were prepared and pStat3 and Stat3 levels were analyzed by Western blot. PLGA treated alone was used as the control of PLGA-AG490. (C) The same cell lysate as described as (B) were analyzed of Bcl-xL, Survivin and Mcl-1 expression levels by Western blot with specific antibodies. (D) PC14PE6/AS2 cells, after seeding in 96-well tray for 24 h, were treated with AG490, PLGA, PLGA-AG490 or left untreated for 48 h. Cell survival were detected using MTT assay.
Figure 3. **NRP1 expression is regulated by Stat3 activation in lung cancer cells.** (A) AS2 cells stably expressing dominant negative Stat3 (S3D) or vector control (Vec) were established. The expression levels of S3D and NRP1 were detected by anti-HA and NRP1 antibodies. Both of dominant negative Stat3 (S3D) expressing clones (S3D(C) and S3D(F)) show decreased NRP1 expression level compared to parental (P) and vector control (Vec1 and Vec2) cells. (B) The expression of Stat3 in CL1-5 cells was knockdown by lentivirus infection of Stat3 shRNA. Cells were selected by antibiotics for two weeks and cell lysates were prepared to detect NRP1 and Stat3 expression using specific antibodies by Western blot. (C) Stat3 expression in shStat3 knockdowned CL1-5 cells was rescued by overexpression active-form Stat3C plasmid. NRP1 and Stat3 expression levels were determined using Western blotting. (D) CL1-5 cells were treated with various dosages of JAKs inhibitor (INC424) for 48 h. The NRP1 expression and Stat3 activation were analysis by Western blot. The CL1-5 cells (E) and AS2 cells (F) were treated with various dosages of FTY720 for 48 h, the cell lysates were prepared to examine expression levels of NRP1 and p-Stat3.
Figure 4. PLGA-FTY720 outperforms free FTY720 in the suppression of de novo lung cancer generation induced by Kras-mutation. (A) Lung cancer in the bitransgenic mice were induced by administration of doxycycline (0.5 g/liter) to the drinking water starting at the age of 2 months. Lungs were excised for paraffin embedding, slice preparation, and immunohistochemical staining with various antibodies. (B) Transgenic mice were treated with various dosages of FTY720 or PLGA-FTY720 followed by intraperitoneal injection for 2 months in the presence of doxycycline (0.5g/L) by oral administration. Surface pulmonary nodules of mice were calculated. (C) Excised lungs were homogenized for protein preparation, and proteins were analyzed by Western blotting using antibodies against PD-L1, pStat3 (Y705) and Stat3. (D) PD-L1 and pStat3 (Y705) were also analyzed by IHC staining.
Figure 5. DG2 conjugation enhances anti-cancer efficacy of PLGA-FTY720 on cell survival.
(A) After CL1-0 and CL1-5 cells were seeded in 10 cm dishes for 24 hours, medium was replaced and cell lysates were collected as indicated time. The cell lysates were subjected to detect NRP1, Stat3 and pStat3-Y705 expression using specific antibodies. β-actin was used as internal control. (B) Expression of NRP1 in CL1-0 and CL1-5 cells was detected by immunofluorescent staining. (C) NRP1-binding peptide (DG2) was conjugated with PLGA and then Nile red dye were embed into the nanoparticle. The nanoparticles were applied to CL1-0 or CL1-5 cells and incubation for 1 h. After PBS wash, cells were fixed and examined by fluorescent microscope. (D) CL1-5 cells were treated with various dosage of PLGA, PLGA-FTY720 and DG2-PLGA-FTY720 for 24 h, cell survival was assayed by MTT assay.
Figure 6. DG2 conjugation enhances anti-tumor efficacy of PLGA-FTY720 and PLGA-AG490 in Kras-induced lung tumors. (A) Transgenic mice were treated with various dosages of FTY720, PLGA-FTY720 or DG2-PLGA-FTY720 followed by intraperitoneal injection for 2 months in the presence of doxycycline (0.5g/L) by oral administration. Surface pulmonary nodules of mice were calculated. (B) Excised lungs were homogenized for protein preparation, and proteins were analyzed by Western blotting using antibodies against pStat3 (Y705), Stat3 and CD274. (C) Transgenic mice were treated with various dosages of AG490, PLGA-AG490 or DG2-PLGA-AG490 followed by intraperitoneal injection for 2 months in the presence of doxycycline (0.5g/L) by oral administration. Surface pulmonary nodules of mice were calculated. (D) Excised lungs were homogenized for protein preparation, and proteins were analyzed by Western blotting using antibodies against pStat3 (Y705), Stat3 and CD274.
Figure 7. Inhibition of Stat3 activation by DG2-PLGA-AG490 and DG2-PLGA-FTY720 prevented infiltration of tumor associated macrophages (TAMs). Transgenic mice were treated with DG2-PLGA-AG490 or DG2-PLGA-FTY720 by intraperitoneal injection for 2 months in the presence of doxycycline (0.5g/L) by oral administration. Subsequently, lungs were excised for paraffin embedding and slices were prepared for HE staining and IHC staining of CD163 and CD68 expression.
IV. Contributions (Please complete the table as below and provide a description of the major contributions.)

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SCI papers:
1. Hou CC, Tsai TL, Su WP, Hsieh HP, Yeh CS, Shieh DB, Su WC*. Pronounced induction of endoplasmic reticulum stress and tumor suppression by surfactant-free poly(lactic-co-glycolic

## II. Expenses

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<td><strong>Capital Expenses</strong></td>
<td>900,000</td>
<td>900,000</td>
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</tr>
</tbody>
</table>

博士後研究員薪資第七年薪資(一名含年終1.5*12/12=1、勞健保、離職儲金)
薪資(含年終)70985*6.5=461403
勞健保(2805+3646)*5=32255
公提離職儲金4368*5=21840
補充保險費(雇主負擔)2,129元
共計:517,627

細胞與細胞培養試劑、細胞培養與保存用相關耗材、一般性化學試劑、實驗室一般消耗性耗材、實驗用消耗型器皿、組織包埋切片及染色費、Western Bolt 實驗耗材、抗體、實驗動物及飼養費等
共計:1,936,372
<table>
<thead>
<tr>
<th>Equipment Expenses</th>
<th>900,000</th>
<th>900,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>小鼠新陈代谢笼组 5 组 99,000 元</td>
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</tr>
<tr>
<td>超高速共焦扫描器影像分析软件 434,000 元</td>
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<tr>
<td>固态光源超高速共焦扫描器光路系统及自动化模组升级 350,000 元</td>
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<tr>
<td>小鼠新陈代谢笼组 1 组 17,000 元</td>
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<td>共计:900,000</td>
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<tr>
<td>Subtotal (NTD)</td>
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