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13. ABSTRACT (Maximum 200 Words)

During the three year funding period, we have well developed a novel and potent Stat3 inhibitor, which represents a promising class of anti-cancer drug in treatment of human cancers, including prostate, breast, head and neck, and long cancers. We appreciate to get a DOD funding for supporting this project. However, the funding period is too short to fulfill the preclinical drug development.

14. SUBJECT TERMS
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I. Introduction.

Our goal is to develop novel chemotherapeutic treatments for multiple human cancers by utilizing the newly developed anti-cancer agent, GQ-ODN. Innovative treatment approaches, including new agents with different therapeutic mechanisms of action and novel molecular targets, are urgently needed for many human cancers (e.g., prostate cancer, non-small cell lung cancer [NSCLC], and squamous cell carcinoma of head and neck [SCCHN]). In recent years, mounting evidence has indicated that signal transducer and activator of transcription 3 (Stat3) could be an important target for cancer therapy, since Stat3 participates in oncogenesis through the upregulation of genes encoding apoptosis inhibitors (Bcl-x_L, Mcl-1 and survivin), cell-cycle regulators (cyclin D1 and c-Myc), and inducers of angiogenesis (VEGF). Stat3 is constitutively activated in many human cancers—including more than 90% of SCCHN, 54% of NSCLC and 82% of prostate cancers. However, no anti-tumor drug whose primary mode of action is to target Stat3 has yet reached the clinic. To this end, we have laid the initial groundwork to develop G-quartet oligodeoxynucleotide (GQ-ODN), which forms G-quartet helical DNA structures, into a potent inhibitor of Stat3 activation in cancer cells and tumors. In our studies, we have: (1) demonstrated that GQ-ODN specifically inhibits Stat3 DNA binding activity and induces apoptosis in human cancer cells; (2) developed a novel delivery system for GQ-ODN, to increase drug activity in cells and *in vivo*; and (3) shown that GQ-ODN T40214 and T40231 significantly suppress tumor growth and greatly increase the survival time of nude mouse xenografts with tumors, in which Stat3 is activated (e.g., prostate, SCCHN and NSCLC tumors). Compared to other JAK/STAT inhibitors, the use of GQ-ODN as a Stat3 inhibitor is advantageous, in that it selectively targets Stat3 and demonstrates strong drug effectiveness *in vivo*. The hypothesis proposed in this study is that the critical oncogenic signaling pathway of Stat3 strongly influences the progression of SCCHN, prostate cancer, and NSCLC, and that targeting the Stat3 molecule will produce a novel chemotherapeutic treatment for these three human cancers. To determine the validity of this hypothesis, we have designed broadly therapeutic experiments.

II. Body of research accomplishments in PI's group from 4/1/05 to 3/31/06.

1. Publication: Targeting Stat3 with G-quartet oligonucleotides: a potential novel therapy for head and neck cancer (Jing et al. *Mol. Cancer therapeutics* 5:279-286 (2006), Appendix 1).

***ABSTREAT*:** Signal transducer and activator of transcription 3 (Stat3) is a critical mediator of oncogenic signaling activated frequently in many types of human cancer where it contributes to tumor cell growth and resistance to apoptosis. Stat3 has been proposed as a promising target for anti-cancer drug discovery. Recently, we developed a series of G-quartet oligodeoxynucleotides (GQ-ODN) as novel and potent Stat3 inhibitors, which significantly suppressed the growth of prostate and breast tumors in nude mice (Jing et al. *Cancer Res*. 2004; 64:6603-9). In the present study, we demonstrated that GQ-ODN specifically inhibited DNA-binding activity of Stat3 as opposed to Stat1. Computer-based docking analysis revealed that GQ-ODN predominantly interacts with the SH2 domains of Stat3 homodimers to destabilize dimer formation and disrupt DNA-binding activity. We employed five regimens in the treatment of nude mice with tumors of squamous cell carcinoma of the head and neck (SCCHN): placebo, paclitaxel, GQ-ODN T40214, GQ-ODN T40231, and T40214 plus paclitaxel. The mean size of SCCHN tumors over 21 days only increased by 1.7 fold in T40214-treated mice and actually decreased by 35% in T40214 plus paclitaxel-treated mice while the mean size of SCCHN tumors increased 9.4 fold in placebo-treated mice in the same period. These findings demonstrate that GQ-ODN has potent activity against SCCHN tumor xenografts alone and in combination with paclitaxel.

Conclusion 1: Here we demonstrated the selective inhibition of Stat3 rather than Stat1 for GQ-ODN. In addition to Stat3, constitutive activation of Stat1 are also active in head and neck cancer. Stat1, which acts in a pro-apoptotic and anti-proliferative manner, seems to be a tumor suppressor—but functions in a manner that is contrary to Stat3. Thus, specifically targeting Stat3, rather than Stat1, is a critical issue in the development of a

potent Stat3 inhibitor. Compared to other JAK/STAT inhibitors, specifically targeting Stat3 is advantageous for GQ-ODN as an anti-cancer agent. Based upon computational analysis we have demonstrated the mechanism and structures of specifically targeting Stat3 by GQ-ODN. Also, we employed five regimens in the treatment of nude mice with SCCHN tumors. As another advantageous, GQ-ODN has significant effect on tumor suppression in SCCHN. Therefore, GQ-ODN is not only a potent anti-cancer agent anole but also has great potential to combine with other drugs (e.g., paclitaxel) for novel and effective combined chemotherapy options.

2. Manuscript: **Stat3 is a critical therapeutic target for non-small cell lung cancer (NSCLC) and GQ-ODN, as a Stat3 inhibitor, represents a promising treatment in NSCLC** (Weerasinghe et al. *submitted* (2006), Appendix 2).

ABSTRACT: Worldwide, lung cancer is the leading cause of cancer mortality and the majority of lung cancers are non-small cell lung cancers (NSCLC). Despite some advancement in alternative therapies over the past decade, the overall prognosis for NSCLC patients remains very poor. Thus, innovative therapeutic approaches employing new agents are urgently needed. Signal transducer and activator of transcription 3 (Stat3)—which participates in oncogenesis through the upregulation of genes encoding apoptosis inhibitors, cell-cycle regulators, and inducers of angiogenesis—provides a rational molecular target for cancer therapy. Recently, we developed G-quartet oligodeoxynucleotide (GQ-ODN) as a potent anticancer agent. Our studies show that GQ-ODN T40214 and T40231 significantly suppress the growth of NSCLC tumors in nude mice by specifically inhibiting the activation of Stat3 and its regulated proteins (e.g., Bcl-2, Bcl-x_L, Mcl-1, VEGF, survivin, Cyclin D1, and c-*myc*); thereby, greatly promoting apoptosis and reducing angiogenesis and cell proliferation in NSCLC tumors. These findings validate Stat3 as an important molecular target for NSCLC therapy and provide evidence that targeting Stat3 signaling may constitute a novel and potent therapeutic treatment option for NSCLC. Our results, which demonstrate the efficacy of GQ-ODN in blocking p-Stat3 DNA-binding and inhibiting Stat3 phosphorylation, correspond well with the mechanism of Stat3 activation and inactivation proposed in 2005 by the Darnell group.

Conclusion 2: Worldwide, lung cancer is the leading cause of cancer mortality and the majority of lung cancers are non-small cell lung cancers (NSCLC). Despite some advancement in alternative therapies over the past decade, the overall prognosis for NSCLC patients remains very poor. Innovative treatment approaches that employ new agents with different mechanisms of action and novel molecular targets are urgently needed for lung cancer therapy. Stat3 has been identified as an important target for cancer therapy, since it participates in oncogenesis through the upregulation of genes encoding apoptosis inhibitors, cell-cycle regulators, and inducers of angiogenesis. In our studies, the results show that GQ-ODN T40214 and T40231 significantly suppress the growth of NSCLC tumors in nude mice by specifically inhibiting the activation of Stat3 and its regulated proteins (e.g., Bcl-2, Bcl-x_L, Mcl-1, VEGF, survivin, Cyclin D1, and c-*myc*); thereby, greatly promoting apoptosis and reducing angiogenesis and cell proliferation in NSCLC tumors. Our findings also validate Stat3 as an important molecular target for NSCLC therapy and provide evidence that targeting Stat3 signaling may constitute a novel and potent therapeutic treatment option for NSCLC. Moreover, our results, which demonstrate the efficacy of GQ-ODN in blocking p-Stat3 DNA-binding and inhibiting Stat3 phosphorylation, correspond well with the mechanism of Stat3 activation and inactivation proposed in 2005 by the Darnell group.

3. Ongoing project: **GQ-ODN T40214 is a potent anti-cancer agent for prostate cancer therapy**.

***RESULTS*:** After tumors were established (vol. 50~150 mm^3), treatment of the nude mice with prostate (PC3) tumors was performed by IP injection. The nude mice were randomly assigned to 3 groups (5 mice in each group): Group 1 was treated with paclitaxel (a clinical drug) at 10mg/kg; and Groups 2 were treated with GQ-ODN T40214 (10mg/kg), and Groups 3 were treated with combination of T40214 (5mg/kg) and paclitaxel (5mg/kg). Paclitaxel and T40214 were administered every two days. The results demonstrate that, over the 18-day treatment period, the mean size of prostate tumors in the paclitaxel-treated mice increased from 71 to 509

(mm^3), whereas, the mean size of prostate tumors in T40214 -treated mice decreased from 91 to 33 (mm^3) (Fig.1&2). Significant differences in tumor growth were observed between placebo-treated mice and T40214-treated (p=0.0005) and between placebo+T40214-treated mice and T40214-treated (p=0.0007).

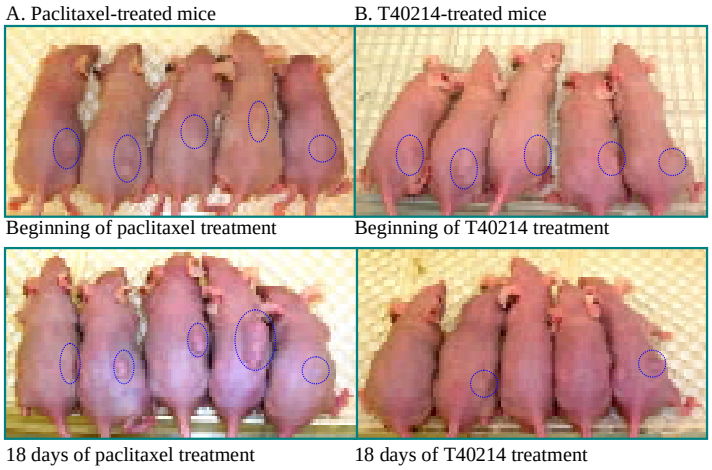

Figure 1. The nude mice with prostate tumors (PC3) were treated by paclitaxel (A) and GQ-ODN T40214 (B). Up-panels showed the tumors sizes of mice at beginning of treatment and down-panels showed tumor sizes of miceafter 18 day treatments.

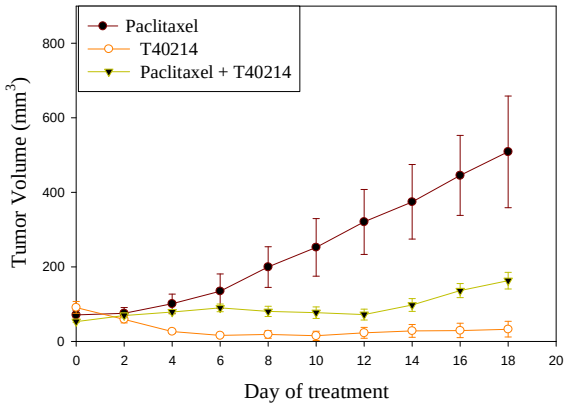

Figure 2. Plot of tumor volume vs day of treatment.

Conclusion 3: We demonstrated that GQ-ODN significantly suppresses the growth of prostate tumors in nude mice xenografts. In our previous studies (Appendices 1&2), we also know that GQ-ODN inhibits Stat3 DNA-binding activity and blocks its transcription. The activation of several critical proteins regulated by Stat3 is totally prohibited in tumors, including apoptosis inhibitors (Bcl-x_L, Mcl-1, and survivin), cell-cycle regulators (cyclin D1 and c-*myc*), and inducers of angiogenesis (VEGF). The inhibition of these proteins induces a tremendous increase in apoptosis and a concomitant decrease in angiogenesis and cell proliferation in tumors—all of which strongly deter tumor growth. We have also found that paclitaxel, a wildly used clinical drug, has a mild effect on tumor growth, due to the fact that this agent does not inhibit Stat3 activation, but only partially blocks expression of the proteins Bcl-x_L, survivin, and c-*myc*. These results provide solid evidence that Stat3 is an important molecular target for human cancer therapy and that targeting Stat3 signaling may constitute a novel and potent therapeutic treatment for human cancers.

III. Reportable outcomes.

Publications and manuscripts:

1. Shao, H. Xu, X., **Jing, N.**, Tweardy, D. J. "Structure requirements for signal transducer and activitor of transcription (STAT) 3 binding to granulocyte colony-stimulating factor receptor (G-CSFR) phosphotyrosine ligands 704 and 744" *Journal of Immunology* (2006) 176:2933-41.
2. **Jing N**, Zhu Q, Yuan P, Li Y, Mao L, Tweardy DJ. "Targeting Stat3 with G-quartet oligonucleotides: a potential novel therapy for head and neck cancer" *Mol. Cancer Therapeutics* (2006) 5:279-286.
3. Tweardy, DJ, **Jing N.** "Enhancing or eliminating signals for cell survival to treat disease" (2005) *Transactions of the American Clinical and climatological Associate* (in press).
4. Nussenzveig RH, Lingam HB, Gaikwar A, Zhu, Q, **Jing N**, Prchal JT. "A novel mutation of the cytochrome-b5 reductase gene in an Indian patient. Molecular basis of type I methemoglobinemia" (2006) (submitted).
5. Weerasinghe P, Garcia GE, Yuan P, Feng L, Mao L, **Jing N**. "Stat3 is a critical therapeutic target for non-small cell lung cancer (NSCLC) and GQ-ODN, as a Stat3 inhibitor, represents a promising treatment in NSCLC (2006) (submitted).

AACR conferences:

1. **Naijie Jing***, Yidong Li, Qiging Zhu, Ping Yuan, Yun Oh, Li Mao and David J. Tweardy "Suppression of growth of squamous cell carcinoma of head and neck xenografts in nude mice by G-quartet oligonucleotides that target Stat3" 96th AACR conference April 16-21, 2005, Anaheim CA.
2. Qiqing Zhu, Yidong Li, and **Naijie Jing*** "Structure-based drug design of G-quartet Oligonucleotides as a new class of Stat3 inhibitors for cancer therapy" 96th AACR conference April 16-21, 2005, Anaheim CA.
3. **Jing N**, Zhu Q, Li Y, Weerasinghe Y. "Targeting Stat3 with G-quartet Oligonucleotides: A Potential Novel Therapy for Human Cancers" EORTC-NCI-AACR meeting, 2005, Philadelphia, PN.

Invited presentation:

1. China Pharmaceutical University, Nanjing, China, Dec. 25, 2005.
2. Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Dec. 20, 2005.
3. Hormel Institute, University of Minnesota, Austin, MN, Aug. 26, 2005.
4. School of Medicine, University of Utah, Salt Lake City, July 27, 2005.

IV. Conclusion of the researches from 4/1/05 to 3/31/06.

Several significant progresses of human cancer therapeutics with a novel anti-cancer agent have made in PI laboratory. **(i)** We have demonstrated that Stat3 is critically therapeutic target for several human cancers, such as head and neck cancer, non-small lung cancer, and prostate cancer (see Appendices 1&2, part II of this report), and targeting Stat3 signaling can constitute a novel and potent therapeutic treatment for human cancers. **(ii)** Comparing with other JAK/STAT inhibitors, GQ-ODN has an advantage in specific inhibition of Stat3 activation in cells and *in vivo* (1). The selective targeting of Stat3 rather than Stat1 becomes a key factor in the development of a potent Stat3 inhibitor, however, the structure of Stat1 is very similar to that of Stat3, which makes the design of a potent Stat3 inhibitor even more challenging (2, 3). **(iii)** Importantly, comparing with the effect of clinical drug, paclitaxel, GQ-ODN T40214 and T40231 have a strong effect on suppressing tumor growth in nude mice, including head and neck tumors, non-small lung tumors and prostate tumors, showing that GQ-ODN represents a novel and promising class of anti-cancer drug in the treatment of metastatic tumors (see Appendices 1&2, part II of this report). **(iv)** The mechanism of the inhibition of tumor growth by GQ-ODN has been demonstrated in Figure 3. GQ-ODNs are delivered into the cytoplasm by PEI/DNA complexes. Induced by the elevated K^+ concentration within the cytoplasm, ODNs form G-quartet structures that diffuse into the nucleus and inhibit DNA binding, as previously described (4, 5); subsequently, they dissociate Stat3 dimer from DNA, inducing p-Stat3 dephosphorylation. The inhibition of Stat3 activation by GQ-ODN blocks the translation of Stat3-regulated genes; notably, those encoding anti-apoptotic proteins (Bcl-2, Bcl-x_L, Mcl-1, and survivin), cell-cycle regulators (cyclin D1 and c-*myc*), and inducers of angiogenesis (VEGF). Consequently, the inhibition of Stat3 activation—which significantly promotes apoptosis and reduces angiogenesis and cell proliferation—strongly suppresses tumor growth.

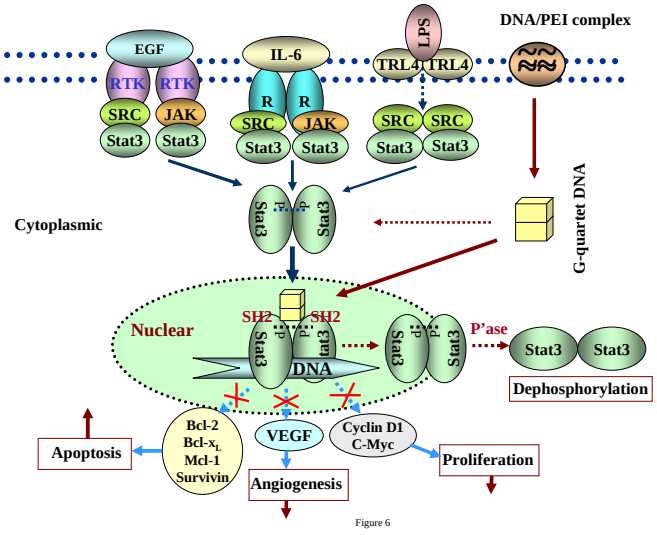

Figure 3. The pathway of inhibiting Stat3 with GQ-ODN in cancer therapy.

V. References.

1. Jing N, Tweardy DJ. Targeting Stat3 in cancer therapy. *Anti-Cancer Drugs* (in press) (2005)
2. Becker S, Groner B, Muller C. Three-dimensional structure of the Stat3β homodimer bound to DNA. *Nature* 394:145-150, 1998.
3. Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE, Kuriyan J. Crystal structure of a tyrosine phosphorylated Stat-1 dimer bound to DNA. *Cell* 93:827-839, 1998.
4. Jing N, Sha W, Li Y, Xiong W, Twaerdy DJ. Rational drug design of G-quartet DNA as anti-cancer agents" *Curr. Pharma. Design* 11:2841-2854, 2005.
5. Jing N, Xiong W, Guan Y, Pallasch L, Wang S. Potassium dependent folding: a key to intracellular delivery of G-quartet oligonucleotides as HIV inhibitors. *Biochemistry* 41:5397-5403, 2002.

VI. Appendices (attached).

1. Jing N, Zhu Q, Yuan P, Li Y, Mao L, Tweardy DJ. "Targeting Stat3 with G-quartet oligonucleotides: a potential novel therapy for head and neck cancer" *Mol. Cancer Therapeutics* (2006) 5:279-286.
2. Weerasinghe P, Garcia GE, Yuan P, Feng L, Mao L, Jing N. "Stat3 is a critical therapeutic target for non-small cell lung cancer (NSCLC) and GQ-ODN, as a Stat3 inhibitor, represents a promising treatment in NSCLC (submitted 2006).

Targeting signal transducer and activator of transcription 3 with G-quartet oligonucleotides: a potential novel therapy for head and neck cancer

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Abstract

Signal transducer and activator of transcription 3 (Stat3) is a critical mediator of oncogenic signaling activated frequently in many types of human cancer where it contributes to tumor cell growth and resistance to apoptosis. Stat3 has been proposed as a promising target for anticancer drug discovery. Recently, we developed a series of G-quartet oligodeoxynucleotides (GQ-ODN) as novel and potent Stat3 inhibitors, which significantly suppressed the growth of prostate and breast tumors in nude mice. In the present study, we showed that GQ-ODN specifically inhibited DNA-binding activity of Stat3 as opposed to Stat1. Computer-based docking analysis revealed that GQ-ODN predominantly interacts with the SH2 domains of Stat3 homodimers to destabilize dimer formation and disrupt DNA-binding activity. We employed five regimens in the treatment of nude mice with tumors of head and neck squamous cell carcinoma (HNSCC): placebo, paclitaxel, GQ-ODN T40214, GQ-ODN T40231, and T40214 plus paclitaxel. The mean size of HNSCC tumors over 21 days only increased by 1.7-fold in T40214-treated mice and actually decreased by 35% in T40214 plus paclitaxel–treated mice whereas the mean size of HNSCC tumors increased 9.4-fold in placebo-treated mice in the same period. These findings show that GQ-ODN has potent activity against HNSCC tumor xenografts alone and in combination with paclitaxel. [Mol Cancer Ther 2006;5(2):279–86]

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Introduction

Signal transducer and activator of transcription 3 (Stat3), a critical mediator of oncogenic signaling, is constitutively activated in many human cancers (1–4) including 82% of prostate cancers (5), 70% of breast cancers (6), >90% of head and neck cancers (7), and over half of lung cancers (8). Stat3 plays a critical role in oncogenic signaling through the up-regulation of genes encoding apoptosis inhibitors (*Bcl-x_L*, *Mcl-1*, and *survivin*), cell cycle regulators (*cyclin D1* and c-*myc*), and inducers of angiogenesis (*VEGF*; ref. 3). Therefore, Stat3 is considered to be an important molecular target for human cancer therapy (4).

Head and neck squamous cell carcinoma (HNSCC) is believed to arise via multistep carcinogenesis (9–11). Common molecular events that contribute to the emergence of the tumor cell clone and its survival may be considered as potential therapeutic targets. Increasing evidence has emerged from studies examining tumor cells in culture and clinical samples that show Stat3 is a critical mediator of oncogenic signaling. In a recent report, 74 tumor specimens from 90 HNSCC patients were found to have constitutive levels of Stat3 activation (12). Within these patient samples, 74% and 47% showed high levels of Stat3 activity in the early and late classification of carcinogenesis, respectively. Previous studies using antisense treatment and transfection of dominant-negative Stat3 constructs have shown that Stat3 plays a crucial role in HNSCC cell growth *in vitro* (13, 14). Cumulatively, these results indicate that activation of Stat3 signaling contributes to the development of HNSCC and provide a strong rationale for targeting Stat3 in the treatment of head and neck cancer (15).

G-rich DNA sequences have been identified, cloned, and characterized in the telomeres of many organisms, such as fungi, ciliates, vertebrates, and insects (16). G-quartets arise from the association of four G-bases into a cyclic Hoogsteen H-bonding arrangement. G-quartets stack on top of each other to give rise to tetrad-containing helical structures. G-rich DNA can form different G-quartet structures, including intramolecular G-quartets, hairpin dimers, and parallel-stranded tetramers (17–19). Based on the G-quartet structure and its physical properties, GQ-ODNs act as non-antisense agents that directly interact with a target protein to interfere with its function. G-quartet forming oligodeoxynucleotides (GQ-ODN) have been developed to modulate several biological processes such as telomerase activity (20), human thrombin activity (21), HIV infection (22, 23), and HIV-1 integrase activity (24–27). GQ-ODNs were also developed as anticancer agents to inhibit DNA replication and induce S-phase cell cycle arrest (28, 29) and inhibit topoisomerase I (30). Topoisomerase I plays a crucial role in DNA replication, RNA transcription, and other cellular functions (31).

Recently, we developed GQ-ODNs, T40214, and T40231, which serve as potent inhibitors of Stat3 DNA-binding activity thereby suppressing expression of Stat3-regulated, antiapoptotic genes such as *Bcl-x_L* and *Mcl-1* in cancer cells (32). Computational methods predicted that GQ-ODNs insert between the two SH2 domains of the Stat3 homodimer, resulting in dimer destabilization. When administered i.v. through a novel drug delivery system, T40214 and T40231 dramatically inhibited the growth of prostate and breast tumor xenografts in nude mice. A biochemical examination of tumors from GQ-ODN–treated mice showed a significant decrease in the expression of antiapoptotic proteins, Bcl-2 and Bcl-x_L, and a marked increase in apoptosis of tumor cells (33).

In the present study, we showed that GQ-ODN selectively inhibits the DNA-binding activity of Stat3, but not Stat1, derived from HNSCC cells and that it significantly increases apoptosis in these cells. Our *in vivo* results showed that the growth of HNSCC tumors in nude mice xenografts was strongly suppressed by treatment with GQ-ODN alone or combined with paclitaxel.

Materials and Methods

Materials

Oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX) and used without further chemical modification. The HNSCC cell lines MDA-1986, Tu167, and B4B8 were provided by Dr. Li Mao (Thoracic/Head and Neck Medical Oncology, M.D. Anderson Cancer Center, Houstin, TX). The cells were grown in DMEM medium containing 10% fetal bovine serum with penicillin and streptomycin. Polyethylenimine (~25K) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Interleukin 6 (IL-6) and IFN-γ were purchased from R&D Systems, Inc. (Minneapolis, MN). Antibodies to Stat1 and Stat3 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay was done as previously described (32). Briefly, IL-6 (25 ng/mL) or IFN-γ (25 ng/mL) was added into wells containing 5×10^5 to 7×10^5 HNSCC cells. Cells were washed and extracted using high-salt buffer. The protein concentrations of the extracts were determined using a Bradford assay (Bio-Rad Laboratories, Hercules, CA). The ^{32}P-labeled duplex DNA probe (hSIE, 5′-AGCTTCATTTCCCGTAAATCCTA) was purified using G-25 columns (GE Healthcare/Amersham Biosciences, Piscataway, NJ). Labeled hSIE probe was mixed with 5 μg of cell protein in 1× binding buffer and 2 μg of poly(deoxyinosinic-deoxycytidylic acid) and incubated at room temperature for 15 minutes with or without GQ-ODN. Samples were loaded onto 5% polyacrylamide gel containing 0.25× Tris-borate EDTA and 2.5% glycerol. The gel was run at 160 to 200 V for 2 to 3 hours at room temperature, dried, and autoradiographed.

Computational Analysis

GRAMM and HEX docking programs were employed to predict the interaction between GQ-ODN T40214 and Stat3 dimer. GRAMM uses a geometry-based algorithm to generate quantitative data that will predict the structure of a protein-protein complex and forecast the quality of the contact between molecules of known structures (34). The GRAMM docking program constructed the complexes of GQ-ODN/Stat3 dimer, without any prior restriction for the binding site, and generated a low-energy conformation as an initial orientation for HEX. An advanced docking technology tool, HEX can rapidly search a high-resolution structure in six dimensions for rigid ligand docking based on its spherical polar Fourier correlations (35). We systematically rotated (180 degrees) each molecule about its centroid and searched the structure of 980 candidates for those with the lowest binding energy for shape complementarities and electrostatic contribution. The final docking structure was determined through GRAMM and HEX.

In vivo Delivery of Fluorescent-Labeled GQ-ODN

We administered 5′-fluorescent-labeled T40214 (10 mg/kg) plus polyethylenimine (2.5 mg/kg) via i.p. injection into nude mice with HNSCC tumors. After injecting GQ-ODN/polyethylenimine complex at 24, 48, and 72 hours, the mice were sacrificed and the tumor tissues were harvested and frozen. Frozen tissues were sectioned for histopathologic analysis with a cryostat microtome; subsequently, the sections were lightly fixed and the tumors were examined via fluorescent microscopy.

In vivo Drug Tests with Xenograft Models

Athymic nude mice (Balb-*nu/nu*, 4 weeks old, and weighing ~20 g; Charles River Laboratories, Inc., Wilmington, MA) were injected s.c. into the right (or left) flank with 1 million HNSCC cells (MDA1986) in 200 μL PBS. After tumors were established, nude mice with HNSCC tumors were randomly assigned to five groups with five mice in each group: group 1 (placebo) was treated by polyethylenimine alone; group 2 was treated with paclitaxel (a toxicity drug); groups 3 and 4 were treated with T40231/polyethylenimine and T40214/polyethylenimine, respectively; and group 5 was treated with T40214/polyethylenimine plus paclitaxel. Polyethylenimine and GQ-ODN were administered every 2 days and paclitaxel was injected i.p. every 4 days. Tumors were measured every 2 days; tumor sizes were calculated by using the function $[a \times (0.5b)^2]$, where a and b are the length and width of tumors, respectively.

H&E Staining and Terminal Deoxyribonucleotidyl Transferase–Mediated dUTP Nick End Labeling Analysis

To determine potential morphologic changes in tumors derived from cells treated with GQ-ODNs, we collected tumor tissues from each treatment group, fixed them with 10% formaldehyde in paraffin, and sectioned them. For morphologic examination, 5-μm tissue sections were stained with H&E. For terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis, 5-μm tissue sections were mounted on siliconized glass slides, air-dried, and heated at 45°C overnight. After deparaffinization and rehydration, the sections were digested with proteinase K (120 μg/mL) for 20 minutes at room temperature. Following quenching of the endogenous peroxidase activity, the sections were washed in PBS and

subsequently incubated with equilibration buffer for 10 minutes at room temperature. After blotting, we applied 50 μL of a mix containing terminal deoxynucleotidyl transferase and reaction buffer containing dATP and digoxigenin-11-dUTP. The sections were covered with a plastic coverslip and placed in a humidified chamber at 37°C for 1 hour. After removal of the coverslip, the sections were washed in stop/wash buffer for 10 minutes at room temperature and subsequently in PBS. The sections were then incubated with antidigoxigenin-peroxidase for 30 minutes at room temperature and washed in PBS. Finally, color development was accomplished by immersion of the slides in 3.3′-diaminobenzidine/0.1% H_2O_2 for 3 to 7 minutes. Sections were counterstained with ethyl green, washed in butanol, cleared in xylol, and mounted with Permount.

Results

Inhibition of Stat3 DNA-Binding Activity by GQ-ODNs in Head and Neck Cancer Cells

Recently, we developed G-rich oligodeoxynucleotides, T40214 and T40231, as lead compounds that would serve as potent inhibitors of Stat3 activity (Fig. 1A); T40214 and T40231 both form G-quartet DNA structures (GQ-ODN) inside cells (32). The difference between the two structures is that T40214 is composed of two G-quartets in the center and two G-C-G-C loop domains on the top and bottom, whereas T40231 is composed of two G-quartets with one T-G-T-G loop on the bottom and one G-T loop on the top (Fig. 1B). The GQ-ODN T40214 forms very stable molecular structures, ~15 Å in length and 15 Å in width. The results obtained from electrophoretic mobility shift assay showed that IL-6 activated Stat3 DNA-binding activity within three HNSCC cells, TU167, B4B8, and MDA1986 (Fig. 1C), and that GQ-ODNs T40214 and T40231 strongly inhibited the Stat3 DNA-binding activity with IC_{50}s of 5 and 7 μmol/L, respectively (Fig. 1D). Nonspecific ODN, which does not form G-quartet structures, was used as a control; nonspecific ODN showed no inhibition of Stat3 DNA-binding activity even when the concentration of ODN was increased up to 285 μmol/L, showing that G-quartet structure is essential to the inhibition of Stat3 DNA-binding activity.

GQ-ODN Specifically Targets Stat3 among STAT Proteins

To confirm that the GQ-ODN was specific to Stat3, as opposed to Stat1, we examined the ability of GQ-ODN to inhibit the DNA-binding activity of IL-6-activated Stat3 and IFN-γ-activated Stat1, respectively, via electrophoretic mobility shift assay (Fig. 2A). The Stat1-DNA and Stat3-DNA complexes were identified using antibodies against Stat3 (Ab3) and Stat1 (Ab1). We found that T40214 strongly inhibited the DNA-binding of Stat3 (IC_{50}, 5 μmol/L) whereas 50% inhibition of Stat1 DNA-binding was not achieved under the same conditions using concentrations of T40214 up to 142 μmol/L. These results provide solid evidence that GQ-ODN preferentially targets Stat3 rather than Stat1.

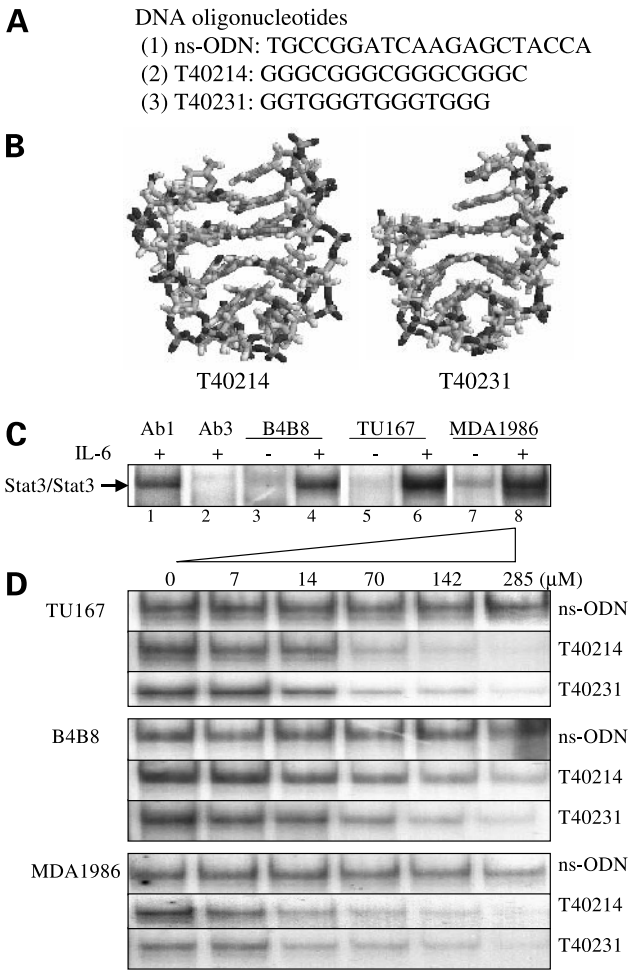

Figure 1. Inhibition of Stat3 DNA-binding activity in HNSCC cells by GQ-ODN. The sequences (**A**) and structures (**B**) of GQ-ODN, T40214, and T40231 constructed from nuclear magnetic resonance data (39). Stat3 is constitutively activate in all three HNSCC cells and shows increased activity following IL-6 exposure (25 ng/mL, 20 min): B4B8, TU167, and MDA1986 (**C**). IC_{50}s for inhibiting Stat3 DNA-binding activity of T40214 and T40231 in HNSCC were determined via electrophoretic mobility shift assay; nonspecific ODN (*ns-ODN*) is the control (**D**).

Mechanism of Stat3-Specific Targeting by GQ-ODN

To determine the mechanism of selective Stat3 targeting by GQ-ODN, we randomly docked GQ-ODN T40214 (1,000 times) onto the dimer structures of both Stat3 and Stat1, without setting any constraints, and analyzed a distribution of H-bonds formed between GQ-ODN and the dimer of Stat3 or Stat1. The number of H-bonds formed between GQ-ODN and Stat3 dimer was 2-fold greater than that formed between GQ-ODN and Stat1 dimer, showing strong binding interaction between GQ-ODN and Stat3 dimer. The histograms of H-bond distribution show that the interaction between GQ-ODN and Stat3 dimer was highly concentrated on the binding site composed of amino acids 638 to 652, especially in the residues of Q643, N646, and N647. However, strong binding interaction did not occur within the region of the Stat1 dimer (Fig. 2B and C). The

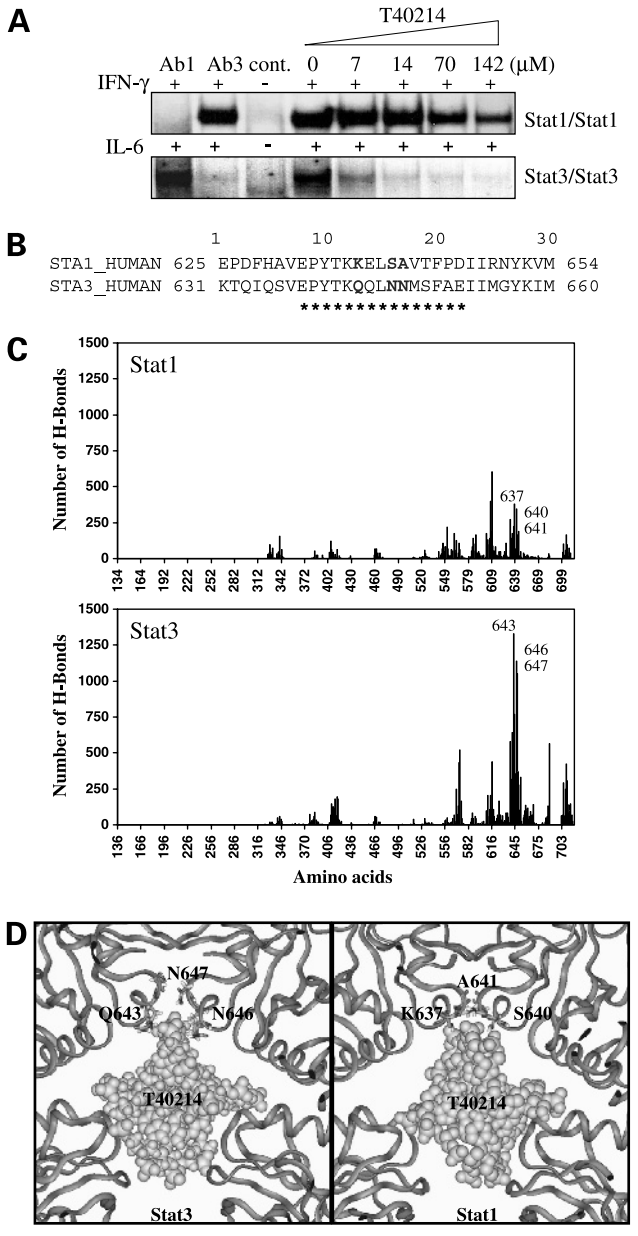

Figure 2. GQ-ODN specifically targets Stat3 dimer. **A,** electrophoretic mobility shift assays show the inhibition of IL-6-activated Stat3 and IFN-γ-activated Stat1 DNA-binding activity by GQ-ODN T40214 in the same conditions. **B,** alignment of the partial sequences in SH2 domains of Stat1 and Stat3. The GQ-ODN binding site within Stat3 composed of residues E638 to E652 (*). **C,** the histograms showed the distributions of H-bonds formed between GQ-ODN and Stat1 or Stat3 [i.e., the number of H-bonds versus amino acids of Stat1 (*top*) and Stat3 (*bottom*)], based on docking 1,000 complexes. A total of 15,935 H-bonds formed between T40214 and Stat3 dimer and 39% of the H-bonds distributed into the binding site (residues E638-E652). No specific binding site of GQ-ODN was observed in Stat1. **D,** docking complexes of T40214/Stat3 (*left*) and T40214/Stat1 (*right*) showed that (*a*) the paired residues of Q643 and N646 repel one another to form an open channel and the distance of Q643 of one monomer to N646 of another is about 7.81 Å; (*b*) GQ-ODN extends into the channel to form seven H-bonds with residues Q643 to N647; and (*c*) GQ-ODN cannot extend into SH2 domains of Stat1 dimer because the side chain of K637 of one monomer is in close proximity to a hydroxyl group of S640 of another.

lowest energy complex obtained from high-resolution GRAMM docking was selected as the initial ligand for HEX. HEX docking scanned 2,520,089,600 orientations; the complex of GQ-ODN/Stat3 dimer is depicted in Fig. 2D. The crystal structures of Stat3 and Stat1 dimers (36, 37) show that in Stat3 dimer, the residue Q643 of one monomer repels N646 of another due to negative-charged polar side chains, thus opening a channel in the SH2 domains. In contrast, in Stat1, K637 of one monomer interacts with S640 of another, which locks the dimer together. These docking results show that residues in the loop domains of GQ-ODN form seven H-bonds with the residues of Q643 to N647 and tightly bind into the site of Stat3, thereby destabilizing the dimer formation and disrupting Stat3 DNA-binding; in contrast, GQ-ODN cannot interact with Stat1.

Delivery of GQ-ODNs into Tumors of Xenograft Models

An effective drug delivery system is essential to the development of nucleic acid based drugs targeting intracellular signaling proteins as cancer therapeutic agents. The principal difficulty of delivering GQ-ODN into cells arises from the physical and structural properties of GQ-ODN because it cannot directly penetrate cell membranes (38). To determine if GQ-ODN is actually delivered to HNSCC tumors of nude mice and how long it persists within tumors, we administered, via i.p. injection, 5′-fluorescent-labeled T40214 (10 mg/kg) plus polyethylenimine (2.5 mg/kg). After these injections, we harvested the tumors at 24, 48, and 72 hours and examined the tumors under fluorescent microscopy. The level of GQ-ODN in tumors at 48 and 72 hours was ~60% and 20%, respectively, of GQ-ODN in tumors at 24 hours (Fig. 3A). The results clearly showed that GQ-ODN was effectively delivered within the tumors and that GQ-ODN had prolonged half-life within the tumors.

Suppression of HNSCC Tumor Growth by GQ-ODN

The assessment of the effectiveness of a drug in an animal model is an important step toward establishing the potential clinical utility of the drug. To this end, we used the nude mice tumor xenograft model to evaluate the potential of GQ-ODNs as anticancer agents. First, nude mice were injected s.c. with HNSCC cells (MDA1986). When tumors were established (volume of 50–150 mm^3), treatment of the nude mice with HNSCC tumors was administered by i.p. injection. The nude mice were randomly assigned to five groups: group 1 (placebo) was treated by polyethylenimine (2.5 mg/kg) alone; group 2 was treated with paclitaxel at 10 mg/kg (39); groups 3 and 4 were treated with T40231/polyethylenimine and T40214/polyethylenimine (10 + 2.5 mg/kg), respectively; and group 5 was treated with T40214/polyethylenimine (10 + 2.5 mg/kg) plus paclitaxel (10 mg/kg). Polyethylenimine and GQ-ODN were administered every 2 days and paclitaxel was injected every 4 days. Over the course of the 21-day treatment period, we found that the mean size of HNSCC tumors in the placebo-treated mice increased from 90 to 850 mm^3 whereas the mean size of HNSCC tumors in the T40214-treated mice

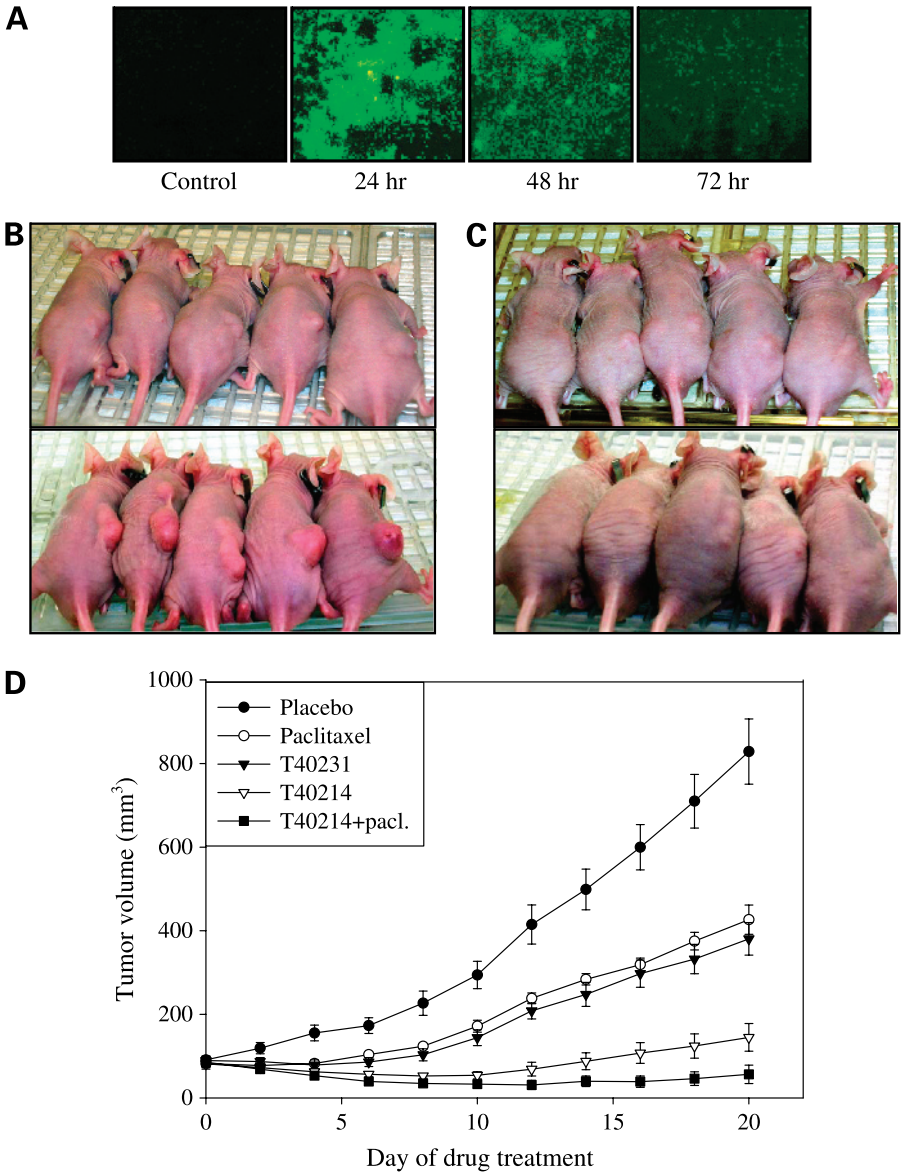

Figure 3. GQ-ODN suppressed the growth of HNSCC in xenograft models. **A,** fluorescent micrographs show the distribution of labeled T40214 in head and neck tumors after 24 (*left middle*), 48 (*right middle*), and 72 (*right*) h. **B** and **C,** mice with HNSCC tumors were treated by placebo and T40214 + paclitaxel, respectively. *Top,* mice at the beginning of treatment; *bottom,* mice at the end of treatment. **D,** tumor volumes versus day of drug treatment for the five groups of nude mice.

only increased from 84 to 145 mm^3; in the T40214 plus paclitaxel–treated mice, tumors decreased from 86 to 56 mm^3 (Fig. 3B-D). Our statistical analyses indicated significant differences in tumor growth between (*a*) placebo-treated and combination-treated mice ($P < 0.001$), (*b*) placebo-treated and T40214-treated mice ($P = 0.001$), (*c*) placebo-treated and T40231-treated mice ($P = 0.037$), (*d*) paclitaxel-treated and combination-treated mice ($P < 0.001$), and (*e*) paclitaxel-treated and T40214-treated mice ($P = 0.005$).

GQ-ODN Suppression of Tumor Growth through Apoptosis

To determine the mechanism of tumor growth suppression by GQ-ODNs, we analyzed HNSCC tumors microscopically following H&E staining (Fig. 4, *top*) and TUNEL staining (Fig. 4, *bottom*). HNSCC tumors were harvested from placebo-treated and drug-treated (GQ-ODN or paclitaxel) nude mice under the same experimental conditions. Tumor cell histologic features of apoptosis on H&E-stained slides were observed only in drug-treated tumors and not in tumors from placebo-treated mice (Fig. 4A). TUNEL staining showed no TUNEL-positive cells in tumors from placebo-treated mice (Fig. 4B, *left*). A few TUNEL-positive cells were detected in the tumors from paclitaxel-treated mice (Fig. 4B, *middle*). However, the greatest number of TUNEL-positive cells was observed in tumors from GQ-ODN–treated mice (Fig. 4B, *right*). A count of the TUNEL-positive cells among total cells in the tumors revealed that the mean percentage of TUNEL-positive cells in the tumors from placebo-treated mice was 1.4% whereas that from T40214-treated mice was 12.3% ($P = 0.023$, Wilcoxin rank sum test).

Discussion

Stat3 has been proposed as a target for development of novel treatments for cancers including HNSCC (32, 33). Stat3 is constitutively activated in HNSCC as results of increased epidermal growth factor receptor or IL-6 receptor (gp130) activity, mediated by increased epidermal growth factor receptor expression and autocrine production of transforming growth factor-α (40, 41) or IL-6 (42), respectively. Inhibition of Stat3 activity—by targeting epidermal growth factor receptor, transforming growth factor-α (43, 44), and gp130 (42) or by directly targeting Stat3 via antisense oligodeoxynucleotides, dominant-negative Stat3 constructs, or antisense constructs—has been shown to inhibit tumor cell growth *in vitro* (45) and increase tumor cell apoptosis *in vivo* (13, 46). These findings indicate the importance of increased Stat3 activity in HNSCC and confirm that Stat3 is a critical oncogenic signaling protein involved in the proliferation and apoptosis prevention of HNSCC. Our previous studies have shown that GQ-ODN strongly suppresses the growth of prostate and breast tumors in nude mice xenografts by inhibiting Stat3 activity, which blocks transcription of the antiapoptotic proteins Bcl-x_L and Bcl-2 and triggers apoptosis of cancer cells (33). Cumulatively, these findings show that Stat3 strongly influences tumor cell proliferation and apoptosis in multiple types of human cancer, making it an important therapeutic target. The development of a potent inhibitor of Stat3 may therefore be an important step toward formulating a novel chemotherapeutic treatment for HNSCC.

To determine whether targeting Stat3 with GQ-ODN will produce a novel chemotherapeutic treatment for head and neck cancers, we did *in vitro* and *in vivo* experiments with GQ-ODN using several HNSCC cell lines. The results clearly show that GQ-ODN significantly inhibits the DNA-binding activity of Stat3 but not that of Stat1. Specifically targeting Stat3 among other STAT proteins is highly desirable. In addition to Stat3, constitutive activation of Stat1 protein has been shown in certain human cancer cells and tumor tissues, including breast cancer and HNSCC. Stat1 acts in a proapoptotic and antiproliferative manner, which denotes a function that is in opposition to Stat3 (3). Consequently, specifically targeting Stat3, rather than Stat1, is advantageous in the development of a potent Stat3 inhibitor.

The mechanism for the selective targeting of Stat3 by GQ-ODN was determined using computational analyses, which are based on statistical methods at the molecular level (47). We randomly docked GQ-ODN onto Stat3 or Stat1 dimers 1,000 times without setting any restrictions. The number of H-bonds formed between GQ-ODN and Stat3 dimer was 2-fold greater than those formed between GQ-ODN and Stat1 dimer, showing a stronger binding interaction

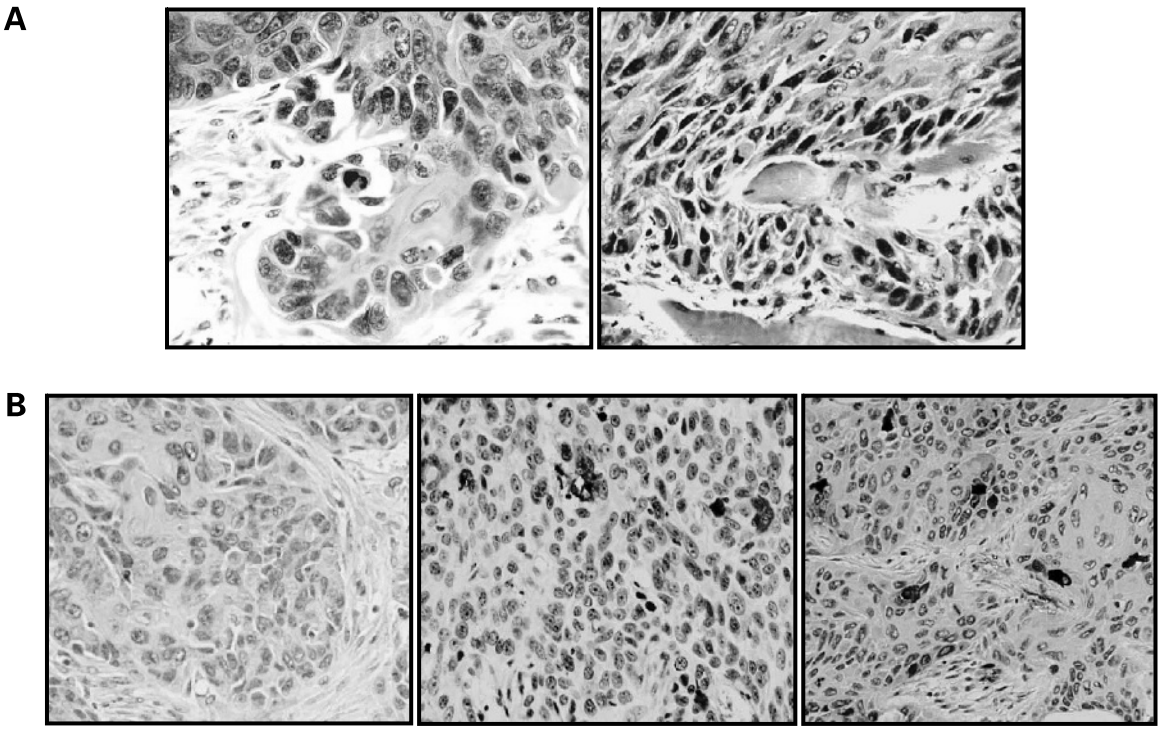

Figure 4. Apoptosis induced in HNSCC tumors. **A,** H&E images show that in placebo-treated tumors, all HNSCC cells are alive (*left*) whereas in GQ-ODN – treated tumors, most HNSCC cells are reduced in size and condensed, indicating apoptosis (*right*). **B,** TUNEL-stained slides show that in placebo-treated tumors, TUNEL-positive cells are not present (i.e., no cells are stained dark brown; *left*); a few TUNEL-positive cells are present in paclitaxel-treated tumors (*middle*); and in the GQ-ODN – treated tumors, the greatest number of TUNEL-positive tumor cells are presented (*right*).

between GQ-ODN and Stat3 dimer. The histograms of H-bond distribution clearly showed that GQ-ODN interacts predominantly with Stat3 dimer in the range of amino acid residues 638 to 652, which lie within the SH2 domains; these residues play a critical role in Stat3 dimerization. Although the sequences and structure of Stat3 dimer are very similar to the Stat1 dimer (36, 37), selective targeting of Stat3, and not of Stat1, by GQ-ODN was based on a few critical amino acid residues. The paired residues, Q643 and N646, repel one another due to their negatively charged polar side chains. The docking results showed that GQ-ODN extended into the SH2 domains and formed seven H-bonds with residues Q643 to N647, and predicted that GQ-ODN would destabilize Stat3 dimer formation and disrupt DNA-binding in Stat3.[5] However, GQ-ODN did not interact with Stat1 due to the fact that the K637 of one monomer interacts with the S640 of the other, thereby locking the binding site and blocking the interaction of GQ-ODN with the Stat1 dimer.

The growth of HNSCC tumors in nude mice xenografts was significantly suppressed by GQ-ODN T40214, as well as by the combined treatment of GQ-ODN T40214 and paclitaxel. The tumors grew very fast when the mice were treated with polyethylenimine alone. However, for the five mice treated with T40214 and paclitaxel, virtually all tumors disappeared over the 21-day treatment period. Paclitaxel binds to microtubules, stimulates microtubule polymerization, and blocks the ability of cells to dismantle the mitotic spindle during mitosis (48, 49). GQ-ODN has been shown to inhibit Stat3 activation and suppress the expression of antiapoptotic proteins, such as Bcl-x_L and Bcl-2, inducing apoptosis in tumors (33). In our *in vivo* drug tests, we found that the combined treatment of T40214 and paclitaxel significantly suppressed HNSCC tumor growth during the entire study period, showing the synergy of this combination. GQ-ODN suppressed tumor growth, at least in part, because of increased apoptosis whereas paclitaxel presumably blocked tumor cell division. Possibly, the two activities, enhancing cell death and reducing cell proliferation, combined to effectively block tumor growth.

GQ-ODN, as a novel class of anticancer agent, has several specific features (47). GQ-ODNs such as T40213 and T40231 form the G-quartet structures within cells, which prevent single-strand endonucleases from accessing their cleavage sites, leading to a long oligonucleotide half-life inside cells (50). In addition, G-quartet ODNs show low toxicity. An analogue of T40214 (51) did not exhibit genetic toxicity in three different mutagenesis assays: the Ames *Salmonella* mutagenesis assay, the Chinese hamster ovary/hypoxanthine-guanine phosphoribosyltransferase mammalian cell mutagenesis assay, and the mouse micronucleus assay. In addition, this GQ-ODN had an $LD_{50} \geq 1.5$ g/kg body weight, which is 150-fold greater than the therapeutic dose used in our studies (10 mg/kg). Therefore, GQ-ODNs represent promising novel agents for cancer therapy either alone or in combination with other drugs such as paclitaxel.

[5] Q. Zhu and N. Jing, unpublished data.

References

1. Broberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. Cell 1999;98:295 – 303.
2. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19:2474 – 88.
3. Yu H, Jove R. The STATS of cancer—new molecular targets come of age. Nat Rev Cancer 2004;4:97 – 105.
4. Darnell JE. Validating Stat3 in cancer therapy. Nat Med 2005;11: 595 – 6.
5. Mora LB, Buettner R, Seigne J, et al. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res 2002; 62:6659 – 66.
6. Dolled-Filhart M, Camp RL, Kowalski DP, Smith BL, Rimm DL. Tissue microarray analysis of signal transducers and activatior of transcription 3 and phospho-state 3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. Clin Cancer Res 2003;9:594 – 600.
7. Nagpal JK, Mishra R, Das BR. Activation of Stat3 as one of early events in tobacco chewing-mediated oral carcinogenesis. American Cancer Sociaty 2002;94:2393 – 4000.
8. Song L, Turkson J, Karras JG, Jove R, Haura EB. Activation of Stat3 by receptor tyrosine kinases and cytokines regulates survival in human non-small cell carcinoma cells. Oncogene 2003;22:4150 – 65.
9. Field JK. The role of oncogenes and tumor-suppressor genes in the aetiology of oral, head and neck squamous cell carcinoma. J R Soc Med 1995;88:35 – 9P.
10. Wong DT, Todd R, Tsuji T, et al. Molecular biology of human oral cancer. Crit Rev Oral Biol Med 1996;7:319 – 28.
11. Santini J, Formento JL, Francoul M, et al. Characterization, quantification, and potential clinical value of the epidermal growth factor receptor in head and neck squamous cell carcinomas. Head Neck 1991; 13:132 – 9.
12. Nagpal JK, Mishra R, Das BR. Activation of Stat3 as one of early events in tobacco chewing-mediated oral carcinogenesis. Cancer 2002; 94:2393 – 400.
13. Grandis JR, Drenning SD, Zeng Q, et al. Constitutive activation of stat3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. Proc Natl Acad Sci U S A 2000;97:4227 – 32.
14. Leong PL, Andrews GA, Johnson DE, et al. Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. Proc Natl Acad Sci U S A 2003;97:4138 – 43.
15. Turkson J, Jove R. STAT proteins: novel molecular target for cancer drug discovery. Oncogene 2000;19:6613 – 26.
16. Henderson E. Telomere DNA structure. In: Blackburn, EH, Greider, CW, editors. Telomeres. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1995. p. 11 – 34.
17. Williamson JR. G-quartet structures in telomeric DNA. Annu Rev Biophys Biomol Struct 1994;23:703 – 30.
18. Gilber DE, Feigon J. Multistranded DNA structures. Curr Opin Struct Biol 1999;9:305 – 14.
19. Jing N. Developing G-quartet oligonucleotides as novel anti-HIV agents: focus on anti-HIV drug design. Expert Opin Investig Drugs 2000;9: 1777 – 85.
20. Shafer RH, Smirnov I. Biological aspects of DNA/RNA quadruplexes. Biopolymers 2001;56:209 – 27.
21. Sen D, Gilbert W. Formation of parallel four-stranded complexes by guanine-rich motif for meiosis. Nature 1998;334:364 – 6.
22. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature 1992;355:564 – 6.
23. Marshall WS, Beaton G, Stein CA, Matsukura M, Caruthers MH. Inhibition of HIV activity by phosphodithioate oligonucleoxytidine. Proc Natl Acad Sci U S A 1992;89:6256 – 9.

24. Wyatt JR, Vickers TA, Roberson JL, et al. Combinatorially selected guanosine-quartet structure is a potent inhibitor of HIV envelope-mediated cell fusion. Proc Natl Acad Sci U S A 1994;91:1356 – 60.

25. Rando RF, Ojwang J, Elbaggari A, et al. Suppression of human immunodeficiency virus type 1 activity *in vitro* by oligonucleotides which form intramolecular tetrads. J Biol Chem 1995;270:1754 – 60.

26. Jing N, Rando FR, Pommier Y, Hogan ME. Ion selective folding of loop domains in a potent anti-HIV oligonucleotide. Biochemistry 1997;36: 12498 – 505.

27. Jing N, Hogan ME. Structur-activity of tetrad-forming oligonucleotides as a potent anti-HIV therapeutic drug. J Biol Chem 1998;273: 34992 – 9.

28. Bates PJ, Kahlon JB, Thomas SD, Trent JO, Miller DM. Antiproliferative activity of G-rich oligonucleotides correlates with protein binding. J Biol Chem 1999;274:26369 – 77.

29. Xu X, Hamhouyia F, Thomas SD, et al. Inhibition of DNA replication and induction of S phase cell cycle arrest by G-rich oligonucleotides. J Biol Chem 2001;276:43221 – 30.

30. Marchand C, Pourquier P, Laco GS, Jing N, Pommier Y. Interaction of human topoisomerase I with guanosine quartet-forming and guanosine-rich single-stranded DNA and RNA oligonucleotides. J Biol Chem 2002; 277:8906 – 11.

31. Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol 2002;3:430 – 40.

32. Jing N, Li Y, Xeu X, Li P, Feng L, Tweardy D. Targeting Stat3 with G-quartet oligonucleotides in human cancer cells. DNA Cell Biol 2003;22: 685 – 96.

33. Jing N, Li Y, Xiong W, Sha W, Jing L, Tweardy DJ. G-quartet oligonucleotides: a new class of Stat3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis. Cancer Res 2004;64:6603 – 9.

34. Vakser IA. Long-distance potentials: an approach to the multiple-minima problem in ligand-receptor interaction. Protein Eng 1996;9:37 – 41.

35. Ritchie DW, Kemp GJL. Fast computation, rotation, and comparison of low resolution spherical harmonic molecular surfaces. J Comput Chem 1999;20:383 – 95.

36. Becker S, Groner B, Muller C. Three-dimensional structure of the Stat3β homodimer bound to DNA. Nature 1998;394:145 – 50.

37. Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE, Kuriyan J. Crystal structure of a tyrosine phosphorylated Stat-1 dimer bound to DNA. Cell 1998;93:827 – 39.

38. Jing N, Xiong W, Guan Y, Pallansch L, Wang S. Potassium dependent folding: a key to intracellular delivery of G-quartet oligonucleotides as HIV inhibitors. Biochemistry 2002;41:5397 – 403.

39. Wang H, Yu D, Agrawal S, Zhang R. Experimental therapy of human prostate cancer by inhibiting MDM2 expression with novel mixed-backbone antisense oligonucleotides: *in vitro* and *in vivo* activities and mechanisms. Prostate 2003;15:194 – 205.

40. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor α and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 1993;53: 3579 – 84.

41. Grandis JR, Melhem MF, Barnes EL, Tweardy DJ. Quantitiative immunohistochemical analysis of transforming growth factor-α and epidermal growth factor receptor in patients with squamous cell carcinoma of the head and neck. Cancer 1996;78:1284 – 92.

42. Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS. Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. Cancer Res 2003;63:2948 – 56.

43. Grandis JR, Chakraborty A, Melhem MF, Zeng Q, Tweardy DJ. Inhibition of epidermal growth factor receptor gene expression and function decreases proliferation of head and neck squamous carcinoma but not normal mucosal epithelial cells. Oncogene 1997;15:409 – 16.

44. Grandis JR, Chakraborty A, Zeng Q, Melhem MF, Tweardy DJ. Down-modulation of TGF-α protein expression with antisense oligonucleotides inhibits proliferation of head and neck squamous carcinoma but not normal mucosal epithelial cells. J Cell Biochem 1998;69:55 – 62.

45. Grandis JR, Drenning SD, Chakraborty A, et al. Requirement of Stat3 but not Stat1 activation for EGFR-mediated cell growth *in vitro*. J Clin Invest 1998;102:1385 – 92.

46. Kijima T, Niwa H, Steinman RA, et al. STAT3 activation abrogates growth factor dependence and contributes to head and neck squamous cell carcinoma tumor growth *in vivo*. Cell Growth Differ 2002;13: 355 – 62.

47. Jing N, Sha W, Li Y, Xiong W, Twaerdy DJ. Rational drug design of G-quartet DNA as anti-cancer agents. Curr Pharm Des 2005;11:2841 – 54.

48. Taghian AG, Abi-Raad R, Assaad SI, et al. Paclitaxel decreases the interstitial fluid pressure and improves oxygenation in breast cancers in patients treated with neoadjuvant chemotherapy: clinical implications. J Clin Oncol 2005;23:1951 – 61.

49. Fanucchi M, Khuri FR. Chemotherapy for recurrent or metastatic squamous cell carcinoma of the head and neck. Semin Oncol 2004;31: 809 – 15.

50. Bishop JS, Guy-Caffey JK, Ojwang JO, et al. G-quartet motifs confer nuclease resistance to a potent anti-HIV oligonucleotide. J Biol Chem 1996;271:5698 – 703.

51. Wallace TL, Gamba-Vitalo C, Loveday KS, Cossum PA. Acute, multipe-dose, and genetic toxicology of AR177, an anti-HIV oligonucleotide. Toxicol Sci 2000;53:63 – 70.

Stat3 is a critical therapeutic target for non-small cell lung cancer (NSCLC) and GQ-ODN, as a Stat3 inhibitor, represents a promising treatment in NSCLC

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ABSTRACT

Worldwide, lung cancer is the leading cause of cancer mortality and the majority of lung cancers are non-small cell lung cancers (NSCLC). Despite some advancement in alternative therapies over the past decade, the overall prognosis for NSCLC patients remains very poor. Thus, innovative therapeutic approaches employing new agents are urgently needed. Signal transducer and activator of transcription 3 (Stat3)—which participates in oncogenesis through the upregulation of genes encoding apoptosis inhibitors, cell-cycle regulators, and inducers of angiogenesis—provides a rational molecular target for cancer therapy. Recently, we developed G-quartet oligodeoxynucleotide (GQ-ODN) as a potent anticancer agent. Our studies show that GQ-ODN T40214 and T40231 significantly suppress the growth of NSCLC tumors in nude mice by specifically inhibiting the activation of Stat3 and its regulated proteins (e.g., Bcl-2, Bcl-x_L, Mcl-1, VEGF, survivin, Cyclin D1, and c-*myc*); thereby, greatly promoting apoptosis and reducing angiogenesis and cell proliferation in NSCLC tumors. These findings validate Stat3 as an important molecular target for NSCLC therapy and provide evidence that targeting Stat3 signaling may constitute a novel and potent therapeutic treatment option for NSCLC. Our results, which demonstrate the efficacy of GQ-ODN in blocking p-Stat3 DNA-binding and inhibiting Stat3 phosphorylation, correspond well with the mechanism of Stat3 activation and inactivation proposed in 2005 by the Darnell group (17).

INTRODUCTION

Lung cancer is one of the most prevalent cancers, and a leading cause of cancer mortality worldwide. In the United States, approximately 170,000 people are diagnosed with lung cancer each year (1,2); of those who are diagnosed, approximately 85% die of the disease. Moreover, the number of lung cancer deaths exceeds the combined deaths attributable to breast, prostate, and colon cancers (3). Lung cancer has been the leading cause of cancer death in men for years and, since 1988, it has also become the number one cause of cancer death in women. The majority of lung cancers are non-small cell lung cancers (NSCLC). More than 60% of all NSCLC patients have advanced or metastatic disease; at this stage, surgery is not a suitable option (3). Despite some improvement in alternative treatments over the past decade, the overall prognosis for NSCLC patients remains very poor (4). Innovative treatment approaches that employ new agents with different mechanisms of action and novel molecular targets are urgently needed for lung cancer therapy. In this regard, Stat3—a critical mediator of oncogenic signaling that is highly activated in a wide variety of human tumors (5)—may hold promise.

Signal transducer and activator of transcription (STAT) proteins were discovered as latent cytoplasmic transcription factors (6). Seven known mammalian STAT proteins (i.e., Stat1, 2, 3, 4, 5a, 5b, and 6) are involved in immune response, inflammation, proliferation, differentiation, development, cell survival, and apoptosis (5). These proteins contain several domains: a tetramerization domain, a coil-coil domain, a DNA-binding domain, a linker domain, an Src-homology 2 (SH2) domain, a critical tyrosine residing near the C-terminal end, and a C-terminal transactivation domain (7, 8). STAT proteins are activated in response to the binding of a number of ligands—including cytokines (e.g., IL-6) and growth factors (e.g., EGF)—to their

cognate cell surface receptors, and are recruited to specific phosphotyrosine residues within receptor complexes through their SH2 domains; they subsequently become phosphorylated on the tyrosine residue within their C-terminus and dimerize through reciprocal interactions between the SH2 domain of one monomer and the phosphorylated tyrosine of the other. The activated dimers translocate to the nucleus, where they bind to DNA-response elements in the promoters of target genes and activate specific gene expression programs (9).

Stat3 has been identified as an important target for cancer therapy, since it participates in oncogenesis through the upregulation of genes encoding apoptosis inhibitors (Bcl-x_L, Mcl-1, and survivin), cell-cycle regulators (cyclin D1 and c-*myc*), and inducers of angiogenesis (VEGF) (9). Mounting evidence has shown that Stat3 is also constitutively activated in many human cancers, including: 82% of prostate cancers, 70% of breast cancers, over 90% of head and neck cancers, and more than 50% of lung cancers (10-13). These findings indicate that the activation of Stat3 signaling is essential to the development of human cancer cells; thus, providing a strong rationale for targeting Stat3 to treat human cancers in which constitutive Stat3 activation plays a critical role.

Recently, we laid the groundwork to develop G-quartet oligodeoxynucleotide (GQ-ODN)—which forms G-quartet helical DNA structures—as a potent inhibitor of Stat3 activation. In our preliminary studies, we have: (1) demonstrated that GQ-ODN specifically inhibits Stat3 activation in cancer cells; (2) developed a novel delivery system for GQ-ODN, to increase drug activity in cells and *in vivo*; and (3) shown that GQ-ODN T40214 and T40231 significantly suppress tumor growth and greatly increase the survival of nude mice with tumors in which Stat3 is activated (e.g., prostate, breast, and SCCHN tumors) (14-16). Based on the results of our

previous studies, we have determined that GQ-ODN is a novel and promising class of anti-cancer drug in the treatment of metastatic tumors.

In the present report, we have demonstrated that: 1) as a critical oncogenic signaling pathway, Stat3 strongly influences the progression of NSCLC and 2) targeting the Stat3 molecule with GQ-ODN constitutes a novel and potent therapeutic treatment for NSCLC. We also provide experimental evidence for the proposed mechanism, that a tyrosine-phosphorylated STAT dimer is quickly dephosphorylated when the STAT dimer is dissociated from DNA in cells (17, 18).

MATERIALS AND METHODS

***Materials*.** The following polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-Stat3; anti-Stat1; anti-cyclin D1 against amino acids 1–295, which represents full-length cyclin D1 of human origin; anti-VEGF; anti-Bcl-x_L; and anti-Bcl-2. Phospho-specific antibodies, p-Stat1 and p-Stat3, were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit horseradish peroxidase (HRP) conjugate was purchased from Bio-Rad Laboratories (Hercules, CA), goat anti-mouse HRP conjugate was purchased from BD Transduction Laboratories (Lexington, KY). Penicillin, streptomycin, RPMI-1640 medium, fetal bovine serum (FBS), and 0.4% trypan blue vital stain were obtained from Invitrogen Corporation/Life Technologies, Inc. (Grand Island, NY). Oligonucleotides were synthesized by The Midland Certified Reagent Company, Inc. (Midland, TX), dissolved in PEI as a 1 μg/μl stock solution, and stored at room temperature (RT).

Cell Lines and Cell Culture. The cell lines used in our studies included: A 549 (human non-small cell lung carcinoma); H292 (human lung epithelial cell carcinoma); and H359, H596, H1792, and H1299 cells, which were purchased from ATCC (Manassas, VA). These cell lines were cultured in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Electrophoretic Mobility Shift Assay (EMSA). An EMSA assay was performed, as previously described (14). Briefly, 7x 10^5 NSCLC cells were stimulated with EGF or IL-6 (25ng/ml) for 20 minutes. Cells were washed twice, and proteins were extracted using high-salt buffer. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories). P^{32}-labeled duplex DNA probe (hSIE 5'-AGCTTCATTTCCCGTAAATCCTA) was purified using G-25 columns (GE Healthcare/Amersham Biosciences; Piscataway, NJ). Labeled hSIE probe was mixed with 5 μg of cell protein in 1x binding buffer and 2 μg of polydeoxyinosinic-deoxycytidylic acid, then incubated at room temperature for 15 minutes, with or without GQ-ODN oligos. Samples were then loaded into wells in 5% polyacrylamide gel (0.25x Tris borate, EDTA, and 2.5% glycerol). The gel was run at 160 V for 2-3 hours at room temperature and dried, followed by autoradiography.

Western Blot Analysis. To determine the effect of GQ-ODN on Stat3 phosphorylation, cytoplasmic extracts were prepared, as previously described (14), from murine tumor tissue or A549 lung cancer cells that had been pretreated with GQ-ODN. To determine the effects of GQ-ODN on p-Stat3, lung tumor cells (1 million cells per well) were first pre-treated with IL-6 (25 ng/ml) or EGF (25 ng/ml) for 30 minutes. Cells were then washed in serum-free medium and

incubated with various concentrations (0.5, 1, 5, 10 and 20 μg/μl) of GQ-ODN/PEI complexes for 24 hours. Cells were lysed and 30 micrograms of whole cell protein was resolved on 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with specific antibody against Stat3 and phosphorylated Stat3 (p-Stat3). To determine the expression of Stat3, p-Stat3, cyclin D1, Bcl-x_L, Bcl-2, VEGF, and survivin in tumor tissue, 50 μg of protein was resolved on SDS-PAGE and probed by Western blot with specific antibodies, in accordance with the manufacturer's recommended protocol. The blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h, and finally detected by ECL reagent (GE Healthcare/Amersham Biosciences). The bands were quantitated using a Personal Densitometer Scanner (version 1.30) and ImageQuant software (version 3.3) (GE Healthcare/Amersham Biosciences).

Animal/Xenograft Model. Athymic nude mice (Balb-*nu/nu*, 4 weeks old, weighing approximately 20g) were ordered from Charles River Laboratories, Inc. (Wilmington, MA); 2.5 million A549 NSCLC cells in 200 μl of PBS were then injected subcutaneously into the right flank of each mouse. After the NSCLC tumors were established (50-150mm^3), the nude mice were randomly assigned to 4 groups of 5 (or 4): Group 1, was treated with polyethylenamine (PEI) (vehicle) alone; Group 2 was treated with paclitaxel; Group 3 was treated with GQ-ODN T40214/PEI; and Group 4 was treated with GQ-ODN T40231/PEI. PEI and GQ-ODN were administered every other day and paclitaxel was injected intraperitoneally (IP) every 4 days. Weight and tumor size were measured every other day. Tumor size was calculated by using the function (a x $(0.5b)^2$), where *a* equals the length and *b* equals the width of tumors.

RNase Protection Assay (RPA). RPA was performed, as previously described (19, 20). Briefly, for each sample prepared from NSCLC tumor tissues, five micrograms of total RNA were used in the RNase protection assay. Riboprobes specific to survivin, c-*myc*, and Mcl-1 mouse genes were prepared. Mouse Angio-1, Apo-2, and CYC-1 multi-probes were obtained from BD Biosciences/Pharmingen (San Diego, CA). An RNase protection assay was performed using a kit (Torrey Pines Biolabs, Inc.; Houston, TX), in accordance with the manufacturer's instructions. The ^{32}P[UTP] (3000 Ci/mmol, ICN)-labeled antisense RNA probes were synthesized using mCK5 multi-probes (BD Biosciences/Pharmingen) as templates, through an *in vitro* transcription system (Promega Corporation; Madison, WI). Antisense RNA probes were hybridized with the RNA samples at 90°C for 25 minutes. Unhybridized single-stranded RNA was digested by ribonuclease A/T1 (Sigma-Aldrich; St. Louis, MO) for 30 minutes. Double-stranded RNA was precipitated by stop solution at -80°C for 15-30 minutes, and centrifuged at maximum speed for 30 minutes. The samples were resolved by 6% sequencing gel. Subsequently, the gels were dried and exposed to X-ray film.

H and E Staining and Terminal Deoxyribonucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) Analysis. In order to determine the alterations in tumor tissue derived from athymic mice treated with vehicle alone (PEI), GQ-ODN T40214, GQ-ODN T40231, and paclitaxel, we collected, fixed (with 10% formaldehyde in paraffin), and sectioned the tumor tissue. For morphology, 5 μm tissue sections were stained with H and E. For TUNEL analysis, 5 μm tissue sections were mounted on siliconized glass slides, air dried, and heated at 45^{o}C overnight. After deparaffinization and rehydration, the sections were digested with proteinase K (120μg/ml) for 20 minutes at room temperature. Following quenching of the endogenous

peroxidase activity, the sections were washed in PBS, and subsequently incubated with equilibration buffer for 10 minutes at room temperature. Sections were boiled and 50 μl of a mix containing terminal deoxynucleotidyl transferase, reaction buffer containing dATP, and digoxigenin-11-dUTP was then added. The sections were covered with a plastic coverslip, washed in stop/wash buffer for 10 minutes at room temperature, and subsequently washed in PBS. The sections were then incubated with anti-digoxigenin peroxidase for 30 minutes at room temperature and washed in PBS. Finally, color development was accomplished through immersion of the slides in 3'3 diaminobenzidine/0.1% H_2O_2 for 3-7 minutes. Sections were counterstained with ethyl green, washed in butanol, cleared in xylol and mounted with permount.

RESULTS

Activation of Stat3 in NSCLC cells. To determine whether Stat3 is highly activated in NSCLC, we stimulated Stat3 phosphorylation with IL-6, EGF, and LPS in 6 different NSCLC cell lines: H292, H358, A549, H596, H1792, and H1299 (Fig.1A). Western blotting analysis of the A549, H596, and H1279 cell lines showed that Stat3 was highly activated when stimulated with IL-6; whereas, the H292, H358, and H1299 cell lines showed marginal or no Stat3 activation. When stimulated with EGF, the H358, A549, H596, H1792, and H1299 cell lines showed high activation of Stat3; whereas, the H292 cell line showed marginal activation of Stat3. Compared with total Stat3 levels, LPS stimulation resulted in high activation of Stat3 in all 6 cell lines. The kinetics of Stat3 activation (Fig.1B) were studied in A549 NSCLC cells. When exposed to EGF (or IL-6) in A549 cells for 5, 10, 30, and 60 mins, Stat3 was highly activated at 10 to 30 min (IL-6 data not shown). The stimulation of A549 cells with LPS (lipopolysaccharide, 5μg/ml) for 5, 10, 30, and 60 mins showed Stat3 activation at 60 minutes. These results indicate that: 1) as a

critical mediator of oncogenic signaling, Stat3 is highly activated in all NSCLC cells and 2) in NSCLC, Stat3 activation can be stimulated through several pathways.

Inhibition of Stat3 activation by GQ-ODN in NSCLC cells. EMSA and Western blot were employed to demonstrate the inhibition of Stat3 activation by GQ-ODN in NSCLC cells. The sequences and structures of GQ-ODN T40214 and T40231 have been previously delineated (14), and the inhibition of Stat 3 DNA binding activity by GQ-ODN T40214 and T40231 is shown in Fig.2A. DNA-binding in phosphorylated Stat3 (p-Stat3) dimers was identified through blot antibodies against Stat1 (Ab1) and Stat3 (Ab3). Using PEI (polyethylenimine) as a vehicle for intracellular delivery, the results showed inhibition of Stat3 activity, via T40214 and T40231, in NSCLC cell lines A549 and H292, which were stimulated by IL-6 and EGF, respectively. IC_{50}s of the inhibition of Stat3 DNA-binding activity for T40214 and T40231 are about 10 μM. Western blot was performed under the same conditions as EMSA in A549. The results demonstrate that GQ-ODN T40214 totally blocks Stat3 phosphorylation in NSCLC at 70 μM (Fig.2B); as a control, equal amounts of total Stat3 (T-Stat3) were loaded for each lane. As a vehicle, PEI does not inhibit p-Stat3. Also, in NSCLC cells, T40214 does not inhibit p-Stat1 expression, compared to T-Stat1, which shows that T40214 selectively targets p-Stat3, but not p-Stat1, in NSCLC cells.

GQ-ODN induced apoptosis in cancer cells, but not normal epithelial cells. Here, we employed TUNEL assays to determine the effect of GQ-ODN on apoptosis in normal versus malignant epithelial cells. As shown in Fig.3, normal epithelial cells (NRK-52E; upper panels) and cancer cells (HepG2; lower panels) were pre-incubated with PEI alone (middle panels) or GQ-ODN/PEI

complexes (right panels), under the same conditions, for 24 hours. Micrographs show that apoptotic cells were stained dark brown via TUNEL-positive staining and normal cells were unstained. Compared to cells incubated in control media (left panels), PEI did not induce apoptosis, either in cancer or normal epithelial cells (middle panels). However, GQ-ODN (right panels) significantly increased apoptosis in cancer cells, where Stat3 is overactive, but not in normal cells; thus, demonstrating that GQ-ODN has no toxicity in normal cells.

GQ-ODN significantly suppressed the growth of NSCLC tumors in vivo. Assessing the effectiveness of a drug in animal models is an important step toward establishing its potential clinical utility. To this end, we utilized nude mice xenografts as animal models of *in vivo* drug testing in order to evaluate the potential of GQ-ODN as an anti-cancer agent. First, nude mice were injected subcutaneously with NSCLC cells (e.g., A549) in which Stat3 is constitutively active. After tumors were established (vol. 50~150 mm^3), treatment of the nude mice with NSCLC (A549) tumors was performed by IP injection. The nude mice were randomly assigned to 4 groups (4 or 5 mice in each group): Group 1 (placebo) was treated with PEI (2.5 mg/kg) alone; Group 2 was treated with paclitaxel (a clinical drug) at 10mg/kg; and Groups 3 and 4 were treated with T40231/PEI and T40214/PEI (10mg/kg/+2.5mg/kg), respectively. PEI and GQ-ODN were administered every two days; paclitaxel was injected every four days, to ensure safety of the mice from toxicity. The results demonstrate that, over the 21-day treatment period, the mean size of NSCLC tumors in the placebo- and paclitaxel-treated mice increased from 93 to 1144 (mm^3) and from 88 to 519 (mm^3), respectively; whereas, the mean size of NSCLC tumors only increased from 89 to 204 (mm^3) and from 83 to 123 (mm^3) in mice treated with T40231 and

T40214, respectively (Fig.4). Significant differences in tumor growth were observed between placebo-treated mice and T40214-treated (p=0.002) or T40231-treated mice (p=0.004).

The mechanism of GQ-ODN suppressing NSCLC tumors in vivo. It is important to understand the mechanism of NSCLC therapy when using GQ-ODN as an anti-cancer agent. Here, we performed Western blots with tissue from NSCLC tumors of nude mice to demonstrate the expression of p-Stat3 versus p-Stat1 (Fig.5A). Xenografted tumors were harvested at the end of treatment, cut into small pieces, and sonicated or homogenized with POLYTRON aggregate in ice for 2 minutes. After centrifugation, the supernatants were harvested and concentrations of proteins were determined by Bradford assay. Western blots were obtained from murine tumor tissues pretreated with PEI alone, GQ-ODN (T40214 and T40231), and paclitaxel. To determine expression of the Stat3-regulated proteins—including cyclin D1, Bcl-x_L, Bcl-2, VEGF, survivin, and others—in whole cell extracts of treated tumor tissues, 30-50 μg of protein was resolved on SDS-PAGE and probed by Western blot with specific antibodies, in accordance with the manufacturer's recommended protocol.

By loading equal amounts of total Stat3 (T-Stat3) in each sample as a control, we found that: 1) compared to the tumors treated by placebo (PEI alone, Lane 1), GQ-ODN T40214 and T40231 (Lanes 2 and 3) totally blocked expression of phosphorylated Stat3 (p-Stat3) and its down-regulated proteins (i.e., Bcl-2, Bcl-x_L, Mcl-1, survivin, VEGF, Cyclin D1, and c-*myc*) in NSCLC tumors and 2) compared to tumors treated by placebo (Lane 1), paclitaxel (Lane 4) did not inhibit Stat3 activation (p-Stat3), and only partially suppressed expression of Bcl-x_L, survivin, and c-*myc* in NSCLC tumors, thus showing a mild effect in NSCLC tumors. Finally, in

comparing the bends of total Stat1 (T-Stat1) and phosphorylated Stat1 (p-Stat1), we observed that phosphorylated Stat1 (p-Stat1) was not inhibited by GQ-ODN in tumors.

To determine whether the expression of Stat3-regulated proteins (e.g., Bcl-2, Bcl-x_L, Mcl-1, VEGF, and others) is inhibited through blocking Stat3 DNA transcription, or directly by GQ-ODN, an RNase protection assay (RPA) was employed to test the mRNA of the Stat3-regulated genes in NSCLC tumors. The results (Fig. 5B) obtained from the tumors of two placebo-treated mice (Lanes 1 and 2) and two T40214-treated mice (Lanes 3 and 4) clearly show that the mRNA levels of the Stat3-regulated genes (i.e., Mcl-1, VEGF, bcl-x, and bcl-2) in NSCLC tumors of T40214-treated mice are much lower than the mRNA levels of the placebo-treated mice. Fig. 5A and B demonstrate that GQ-ODN blocks the expression of Stat3-regulated proteins and suppresses the mRNA of these proteins. In sum, the RPA data demonstrate that the activation of Stat3-regulated proteins—such as Bcl-2, Bcl-x_L, Mcl-1, VEGF, and others—is inhibited, due to the fact that Stat3 DNA transcription is blocked by GQ-ODN.

The TUNEL kit used to quantify apoptosis was based on labeling the apoptotic cells with cleaved DNA fragments, at the single cell level; light microscopy was used for data analysis. The apoptotic tumor cells were stained dark brown via TUNEL-positive staining, and the normal tumor cells remained unstained. The results show that, compared with the NSCLC tumors treated by PEI alone (Figure 5C, upper left panel), significant apoptosis of tumor cells is observed in NSCLC tumors treated by GQ-ODN T40214 (Fig.5C, upper right panel). The pathological microscopy of H&E clearly shows that, in comparison with the placebo-treated H&E image (Figure 5C, lower left panel), the GQ-ODN-treated H&E image (Figure 5C, lower right panel)

display that tumor cells were shrunk with chromatin condensation and tumor tissue was partially resulted in the necrosis.

DISCUSSION

Despite some improvement in alternative therapies over the past decade, the overall prognosis for NSCLC patients remains very poor (21). Although chemotherapy provides a clinically significant benefit, the resultant effect is only modest (22). Thus, new agents—with different mechanisms of action and novel molecular targets—are urgently required to treat NSCLC. The recent report by Haura *et al*., that p-Stat3 was highly expressed in 54% of NSCLC primary tumors in 176 patients, suggests Stat3 is a promising molecular target for lung cancer therapy (23).

Our findings demonstrate that the phosphorylation of Stat3 in NSCLC cells can be stimulated by the cytokine, IL-6; epithelial growth factor (EGF); or lipopolysaccharide (LPS) (Fig.1). The kinetic results indicate EGF or IL-6 activate Stat 3 within a short time, and that LPS stimulates Stat 3 activation after a longer period, which suggests that IL-6 and EGF directly interact with the JAK/STAT system, as previously described (9); however, LPS seems to stimulate Stat3 activation indirectly and the mediators are, as yet, unknown. We also observed that, in NSCLC cells, when T40214 is increased to 70μM and the incubation is continued for 24 hours, Stat3 DNA-binding activity is significantly inhibited (Fig. 2A) and phosphorylation of Stat3 is totally blocked as well (Fig. 2B). It should be noted that our EMSA results are consistent with the data derived from Western blot analysis under the same conditions. These studies demonstrate that GQ-ODN not only blocks Stat3 DNA-binding activity, it also inhibits Stat3 phosphorylation. The

Darnell group recently proposed that a tyrosine-phosphorylated STAT dimer will be quickly dephosphorylated when the STAT dimer is dissociated from DNA in cells (17, 18). The simple process proposed is that, after dissociating with DNA duplex, the parallel p-STAT dimer—held together only by pY-SH2 interaction—spontaneously dissociates as monomers that are then targeted by phosphatase and return to the cytoplasm. Our results provide evidence that both functions (i.e., blocking Stat3 DNA-binding and inhibiting Stat3 phosphorylation) can be induced by one binding mechanism. GQ-ODN T40214 inhibits DNA-binding activity within p-Stat3 dimer and dissociates p-Stat3 dimer from DNA duplex, as demonstrated in EMSA, and subsequently induces p-Stat3 dephosphorylation (or attenuates p-Stat3), as shown in Western blot. Thus, our results correspond well with the mechanism of Stat3 activation and inactivation proposed by Darnell group; however, details at the molecular level remain unknown.

In addition, our results in cells and tumors clearly show that GQ-ODN selectively inhibits the activation of Stat3, but not Stat1, both *in vitro* and *in vivo* (Fig.2B & 5A). The selective targeting of Stat3 becomes a key factor in the development of a potent Stat3 inhibitor. Independent of Stat3, Stat1 and Stat5 are also active in many human cancers (5). Stat5 has a potent oncogenic role similar to Stat3. Stat1, which acts in a pro-apoptotic and anti-proliferative manner, seems to be a tumor suppressor whose functions differ from those of Stat3 (24). However, the crystal structures show that the dimer organization of Stat1 is very similar to that of Stat3 and also 51% of amino acids of Stat1 are identity with that of Stat3 (60% identity in the SH2 domain), which makes the design of a potent Stat3 inhibitor even more challenging (7, 8). In our previous studies (14, 16), we demonstrated that GQ-ODN predominantly interacts with the p-Stat3 dimer in the range of amino acid residues, 638 to 652, within the SH2 domains. The selective inhibition of p-

Stat3 activity by GQ-ODN is based upon a few critical residues that form a local structure different from that of the p-Stat1 dimer. In the p-Stat3 dimer, the paired residues of Q643 and N646 repel one other to form a channel conformation, in which GQ-ODN is held by seven H-bonds that disrupt DNA-binding interaction. However, the corresponding paired-residues of Stat1 dimer, K637 and S640, lock the dimer together; thereby, blocking the interaction of GQ-ODN with Stat1.

With a novel intracellular delivery system (25), we demonstrated that GQ-ODN significantly suppresses the growth of NSCLC tumors in nude mice xenografts. Importantly, we also know that GQ-ODN inhibits Stat3 DNA-binding activity and blocks its transcription. The activation of several critical proteins regulated by Stat3 is totally prohibited in NSCLC tumors, including apoptosis inhibitors (Bcl-x_L, Mcl-1, and survivin), cell-cycle regulators (cyclin D1 and c-*myc*), and inducers of angiogenesis (VEGF). The inhibition of these proteins induces a tremendous increase in apoptosis and a concomitant decrease in angiogenesis and cell proliferation in tumors—all of which strongly deter tumor growth. We have also found that paclitaxel has a mild effect on NSCLC tumor growth, due to the fact that this agent does not inhibit Stat3 activation, but only partially blocks expression of the proteins Bcl-x_L, survivin, and c-*myc*. These results provide solid evidence that Stat3 is an important molecular target for NSCLC therapy and that targeting Stat3 signaling may constitute a novel and potent therapeutic treatment for NSCLC.

The mechanism of Stat3 inhibition by GQ-ODN is depicted in Fig.6. Monomers of Stat3 in the cytoplasm of cancer cells become activated through the stimulation of IL-6, EGF, or LPS. Stat3

is also recruited by intrinsic or receptor-associated tyrosine kinases, such as JAK or Src. Tyrosine phosphorylation of Stat3 induces the formation of active Stat3 dimers through their SH2 domains; the activated Stat3 dimers then translocate to the nucleus, where they bind to DNA-response elements in the promoters of target genes and activate specific gene expression programs (5). GQ-ODNs are delivered into the cytoplasm by PEI/DNA complexes. Induced by the elevated K^+ concentration within the cytoplasm, ODNs form G-quartet structures that diffuse into the nucleus and inhibit DNA binding, as previously described (15, 25); subsequently, they dissociate Stat3 dimer from DNA, inducing p-Stat3 dephosphorylation. The inhibition of Stat3 activation by GQ-ODN blocks the translation of Stat3-regulated genes; notably, those encoding anti-apoptotic proteins (Bcl-2, Bcl-x_L, Mcl-1, and survivin), cell-cycle regulators (cyclin D1 and c-*myc*), and inducers of angiogenesis (VEGF). Consequently, the inhibition of Stat3 activation—which significantly promotes apoptosis and reduces angiogenesis and cell proliferation—strongly suppresses tumor growth. Therefore, GQ-ODN is believed to represent a novel and promising class of anti-cancer drug in the treatment of metastatic NSCLC tumors.

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REFERENCES

1. Lung cancer: In BW Stewart and P Kleihues (eds). *World Cancer Report*. Lyon, France: IARC Press, pp 182-187, 2003.

2. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. *CA Cancer J Clin*. 50:7-33, 2000.
3. Choen V, Khuri FR. Chemoprevention of lung cancer: Current status and future prospects. *Cancer and Metastasis Review* 21:349-362, 2002.
4. Ginsberg RJ, Goldberg M, Waters PF. Surgery in non-small cell lung cancer, in Roth JA, Ruckdeschel JC, Weisenburger TH (editors): *Thoracic Oncology* (2^{nd} edition). Philadelphia, PA: W.B. Saunders Company, pp.124-126, 1995.
5. Yu H, Jove R. The STATS of cancer-new molecular targets come of age. *Nature Rev/Cancer* 4:97-105, 2004.
6. Darnell Jr JE. STATs and gene regulation. *Science* 277:1630-1635, 1997.
7. Becker S, Groner B, Muller C. Three-dimensional structure of the Stat3β homodimer bound to DNA. *Nature* 394:145-150, 1998.
8. Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE, Kuriyan J. Crystal structure of a tyrosine phosphorylated Stat-1 dimer bound to DNA. *Cell* 93:827-839, 1998.
9. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumor provides novel molecular targets for therapeutic intervention. *Clin. Cancer Res.* 8:945-954, 2002.
10. Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, Bowman T, Falcone R, Fairclough R, Cantor A, Muro-Cacho C, Livingston S, Karras J, Pow-Sang J, Jove R. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. *Cancer Research* 62: 6659-66, 2002.
11. Dolled-Filhart M, Camp RL, Kowalski DP, Smith BL, Rimm DL. Tissue microarray analysis of signal transducers and activator of transcription 3 and phospho-stat3 (Tyr705)

in node-negative breast cancer shows nuclear localization is associated with a better prognosis. *Clinical Cancer Res*. 9: 594-600, 2003.

12. Nagpal JK, Mishra R, Das BR. Activation of Stat3 as one of early events in tobacco chewing-mediated oral carcinogenesis. *American Cancer Society* 94:2393-4000, 2002.
13. Song L, Turkson J, Karras JG, Jove r, Haura EB. Activation of Stat3 by receptor tyrosine kinases and cytokines regulates survival in human non-small cell carcinoma cells. *Oncogene*. 22:4150-65, 2003.
14. Jing N, Li Y, Xiong W, Sha W, Jing L, Tweardy, DJ. G-quartet oligonucleotides: a new class of Stat3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis" *Cancer Research* 64:6603-6609, 2004.
15. Jing N, Sha W, Li Y, Xiong W, Twaerdy DJ. Rational drug design of G-quartet DNA as anti-cancer agents" *Curr. Pharma. Design* 11:2841-2854, 2005.
16. Jing N, Zhu Q, Yuan P, Li Y, Mao L, Tweardy DJ. Targeting Stat3 with G-quartet oligonucleotides: a potential novel therapy for head and neck cancer *Mol. Cancer Therapeutics* 5:279-286, 2006.
17. Zhong M, Henriksen MA, Takeuchi K, Schaefer O, Liu B, ten Hoeve J, Ren Z, Mao X, Chen X, Shuai K, Darnell JE Jr. Implications of an antiparallel dimeric structure of nonphosphorylated STAT1 for the activation-inactivation cycle. *Proc Natl Acad Sci USA.* 102(11):3966-71, 2005.
18. Darnell JE. *Nat Med*. 11:595-596, 2005.
19. Feng L, Garcia GE, Yang Y, Xia Y, Gabbai FB, Peterson OW, Abraham JA, Blantz RC, Wilson CB. Heparin-binding EGF-like growth factor contributes to reduced glomerular filtration rate during glomerulonephritis in rats. *J Clin Invest* 105:341-350, 2000.

20. Chen S, Bacon KB, Li L, Garcia GE, Xia Y, Lo D, Thompson DA, Siani MA, Yamamoto T, Harrison JK, Feng L. *In vivo* inhibition of CC and CX3C chemokine-induced leukocyte infiltration and attenuation of glomerulonephritis in Wistar-Kyoto (WKY) rats by vMIP-II. *J Exp Med* 188:193-198, 1998.
21. Cohen V, Khuri FR. Chemopprovention of lung cancer. *Curr Opin Pulm Med* 10:279-283, 2004.
22. Reck M, Gatzemeier U. Chemotherapy in stage –IV NSCLC. *Lung Cancer* 45:S217-S222, 2004.
23. Haura EB, Zheng Z, Song L, Cantor A, Bepler G. Activated epidermal growth factor receptor-Stat-3 signaling promotes tumor survival *in vivo* in non-small cell lung cancer. *Clin Cancer Res.* 11:8288-94, 2005.
24. O'Shea J. J., Gadina M., Schreiber R. D. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* 109, Suppl:S121-31, 2002.
25. Jing N, Xiong W, Guan Y, Pallasch L, Wang S. Potassium dependent folding: a key to intracellular delivery of G-quartet oligonucleotides as HIV inhibitors. *Biochemistry* 41:5397-5403, 2002.

FIGURE LEGENDS

Figure 1. (A) Western blots show that, when stimulated by IL-6, EGF, and LPS, Stat3 is highly activated in NSCLC cells (e.g., H292, H358, A548, H596, H1792, and H1299). (B) Kinetic data show stimulation of Stat3 phosphorylation in A549 cells with EGF and LPS for 5, 10, 30, and 60 mins; p-Stat3 stimulated by EGF or LPS is activated in 10 or 60 mins, respectively.

Figure 2. (A) Results obtained from EMSA show the inhibition of Stat3 DNA-binding activity by GQ-ODNs T40214 and T40231 in NSCLC cells (e.g., A549 and H292); ns-ODN (non-specific ODN) is the control. (B) The Western blot shows that GQ-ODN significantly blocks phosphorylation of Stat3, but not Stat1, in NSCLC cells (e.g., A549); p-Stat3 (or p-Stat1) and T-Stat3 (or T-Stat1) are phosphorylated Stat3 (or Stat1) and total Stat3 (or Stat1), respectively. PEI does not inhibit p-Stat3.

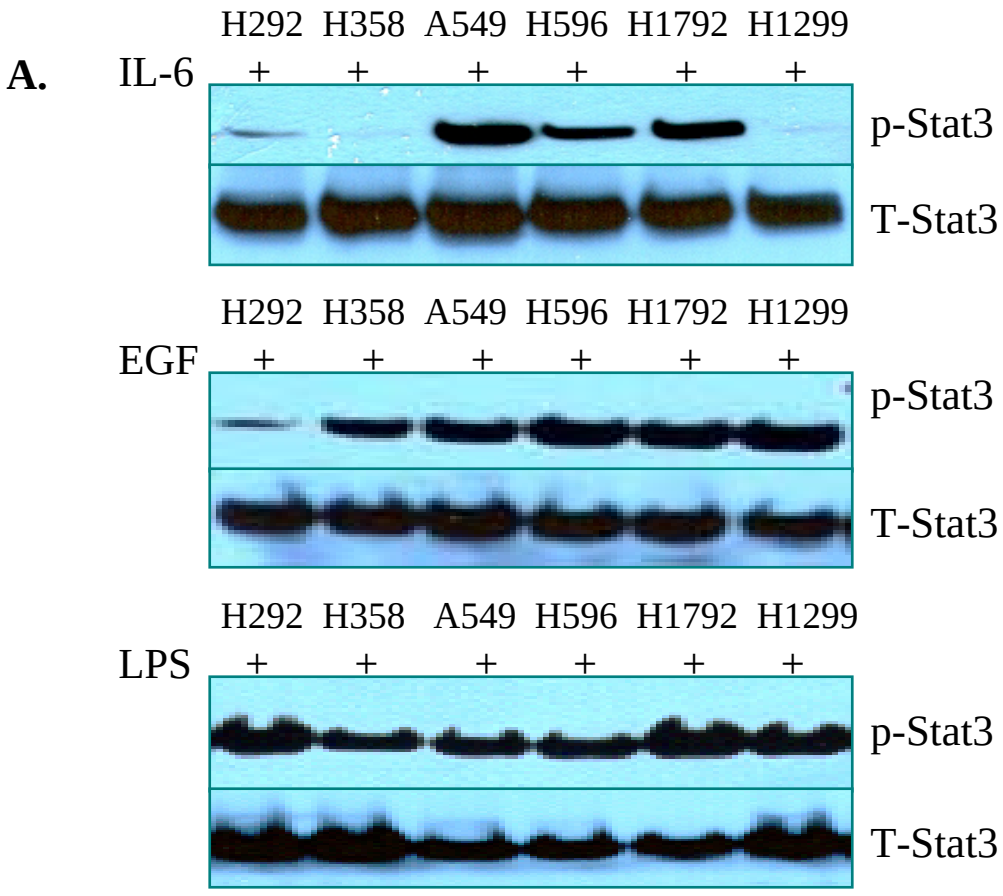
Figure 3. The photomicrographs show that apoptotic cells (stained in dark brown) are induced by GQ-ODN. The normal epithelial cells (NRK-52E) (upper panels) and the cancer cells (HepG2) (lower panels) were pre-incubated with PEI alone (middle panels) or GQ-ODN/PEI complexes (right panels) for 24 hours, under the same conditions.

Figure 4. The photographs show nude mice with NSCLC tumors treated by placebo (PEI alone; left panels) and by GQ-ODN T40214 (right panels) over a period of 21 days. Mice with NSCLC tumors were treated by placebo and T40214, respectively, at the beginning of treatment (A) and at the end of treatment (B). (C) Tumor volumes versus day of drug treatment for the four treatment groups.

Figure 5. (A) Western blots obtained from NSCLC tumors demonstrate the expression of total Stat3 (T-Stat3), phosphorylated Stat3 (p-Stat3), T- Stat1, and p-Stat3 and its downregulated proteins, including Bcl-2, Bcl-x_L, Mcl-1, survivin, VEFG, Cyclin D1 and c-*myc*. Lane 1: tumor treated by placebo (PEI alone); Lane 2: tumor treated by T40214; Lane 3: tumor treated by T40231; and Lane 4: tumor treated by paclitaxel. (B) The results of RPA were obtained from the tumors of two placebo-treated mice (Lanes 1 and 2) and two T40214-treated mice (Lanes 3 and 4). The mRNA of the Stat3-regulated genes: Mcl-1 (left), VEGF (middle), Bcl-x_L, and Bcl-2 (right) in NSCLC tumors of T40214-treated mice are much weaker than those of placebo-treated

mice. L32 and GAP were the controls. (C) Apoptosis induced in NSCLC tumors. TUNEL-stained slides (upper panels) demonstrate that in placebo-treated tumors (left), TUNEL-positive cells are not present (i.e., no cells are stained dark brown), and in the GQ-ODN-treated tumors (right), the greatest number of TUNEL-positive tumor cells are present. H&E images (lower panels) show that in placebo-treated tumors (left), all NSCLC cells are alive, while in GQ-ODN-treated tumors (right), most NSCLC cells have shrunk, due to chromatin condensation, partially resulting in necrosis (the circled area).

Figure 6. The mechanism of GQ-ODN in inhibiting Stat3 activation for the purpose of cancer therapy (details are provided in the text).


A.
H292 H358 A549 H596 H1792 H1299
IL-6
+ + + + + +
p-Stat3
T-Stat3
H292 H358 A549 H596 H1792 H1299
EGF
+ + + + + +
p-Stat3
T-Stat3
H292 H358 A549 H596 H1792 H1299
LPS
+ + + + + +
p-Stat3
T-Stat3

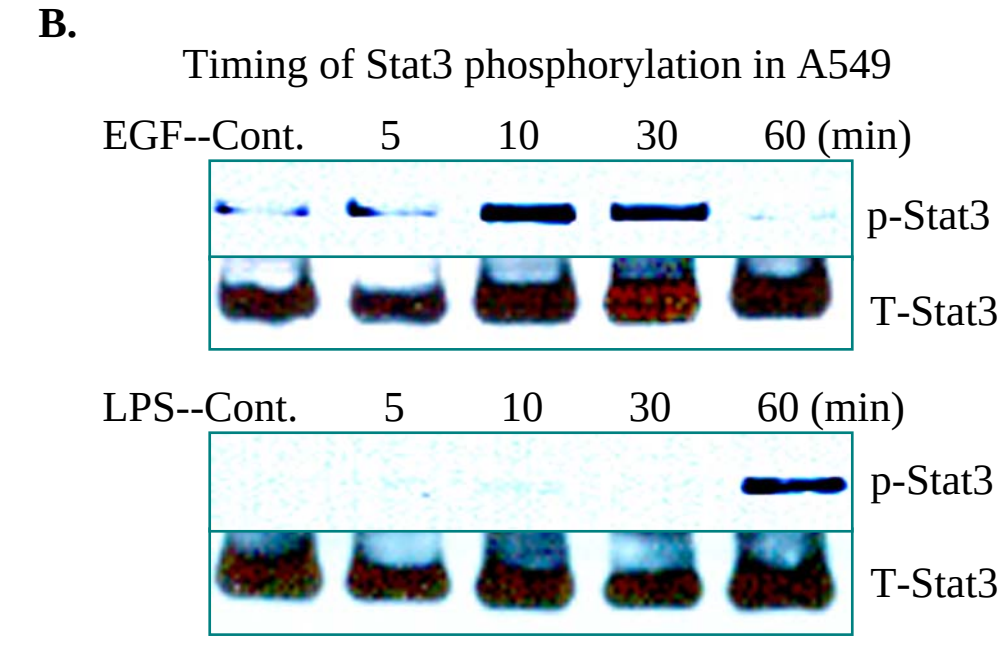

B.
Timing of Stat3 phosphorylation in A549
EGF--Cont. 5 10 30 60 (min)
p-Stat3
T-Stat3
LPS--Cont. 5 10 30 60 (min)
p-Stat3
T-Stat3

Figure 1

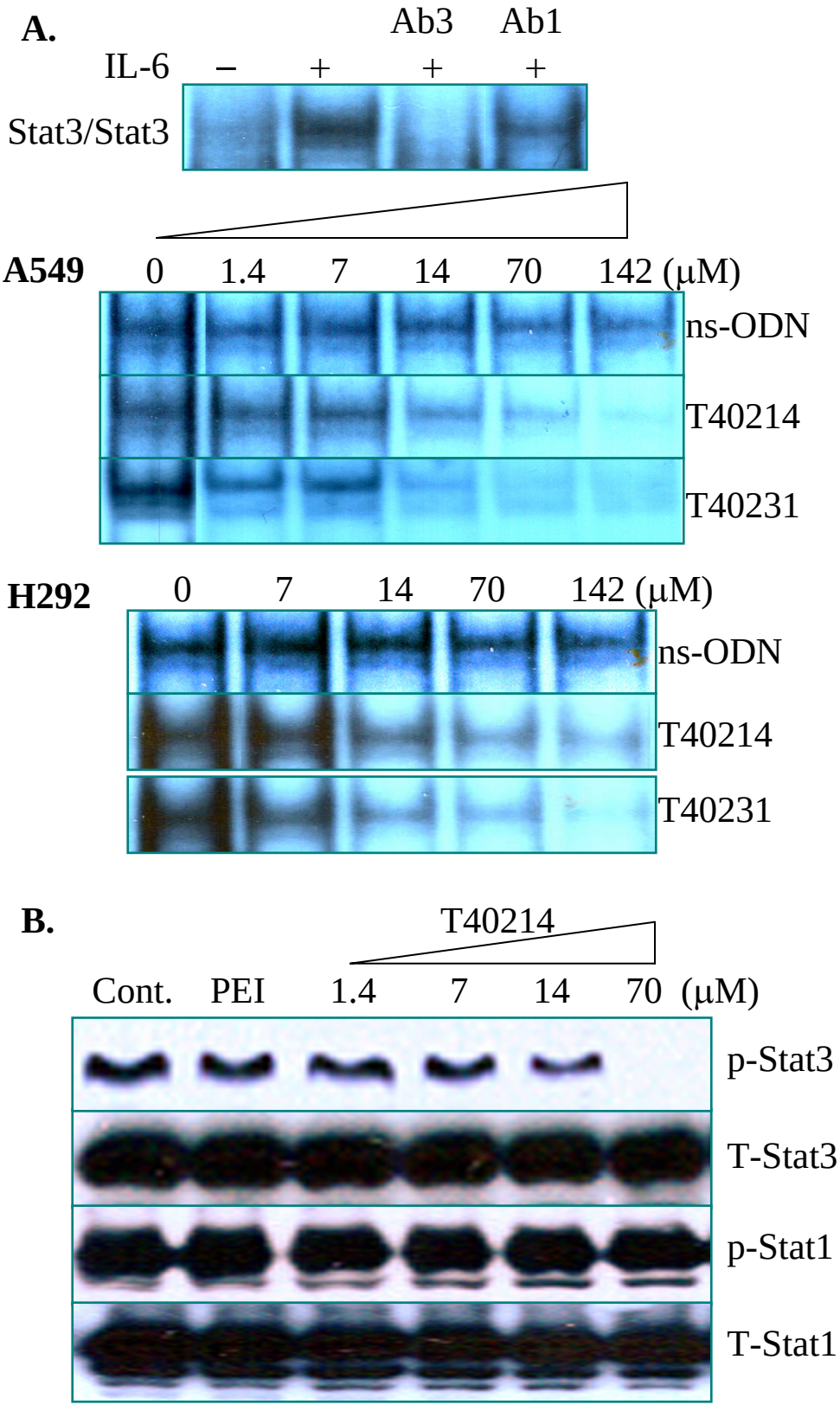

A.
Ab3
Ab1
IL-6
–
+
+
+
Stat3/Stat3
A549
0
1.4
7
14
70
142 (μM)
ns-ODN
T40214
T40231
H292
0
7
14
70
142 (μM)
ns-ODN
T40214
T40231
B.
T40214
Cont.
PEI
1.4
7
14
70
(μM)
p-Stat3
T-Stat3
p-Stat1
T-Stat1

Figure 2

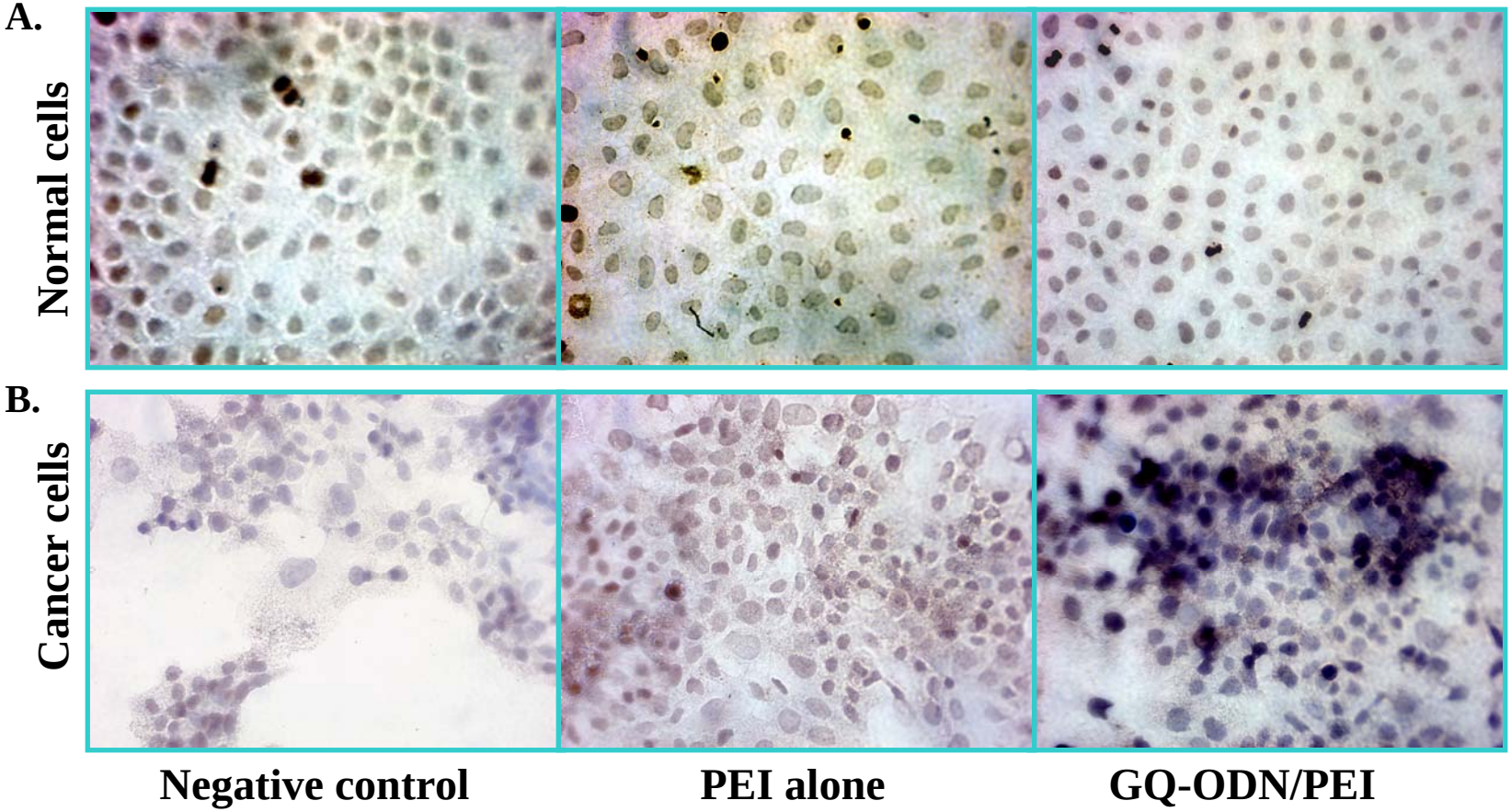
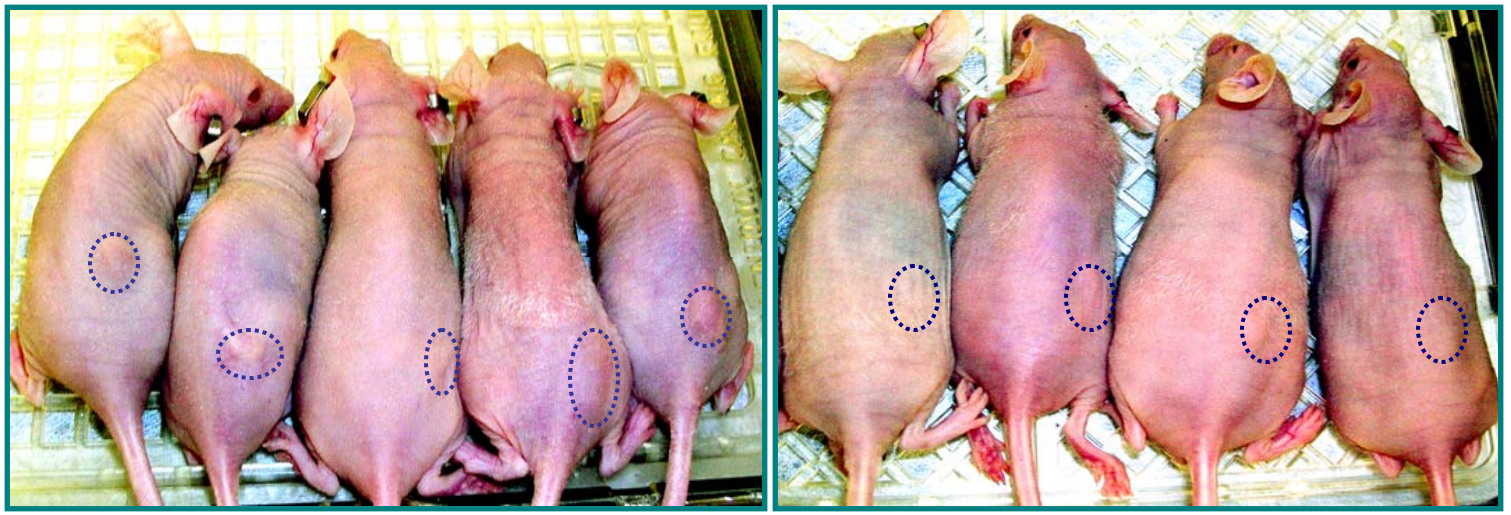

A.
Normal cells
B.
Cancer cells
Negative control
PEI alone
GQ-ODN/PEI

Figure 3

A. Beginning of placebo-treatment

Beginning of T40214-treatment



B. After placebo-treatment

After T40214-treatment

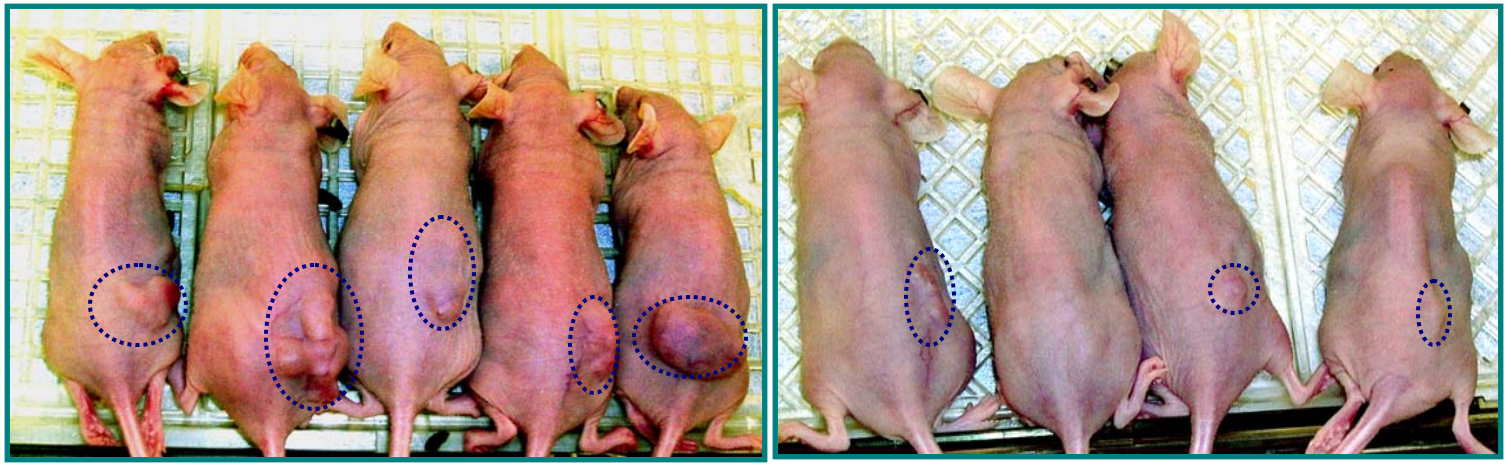


Figure 4

C.

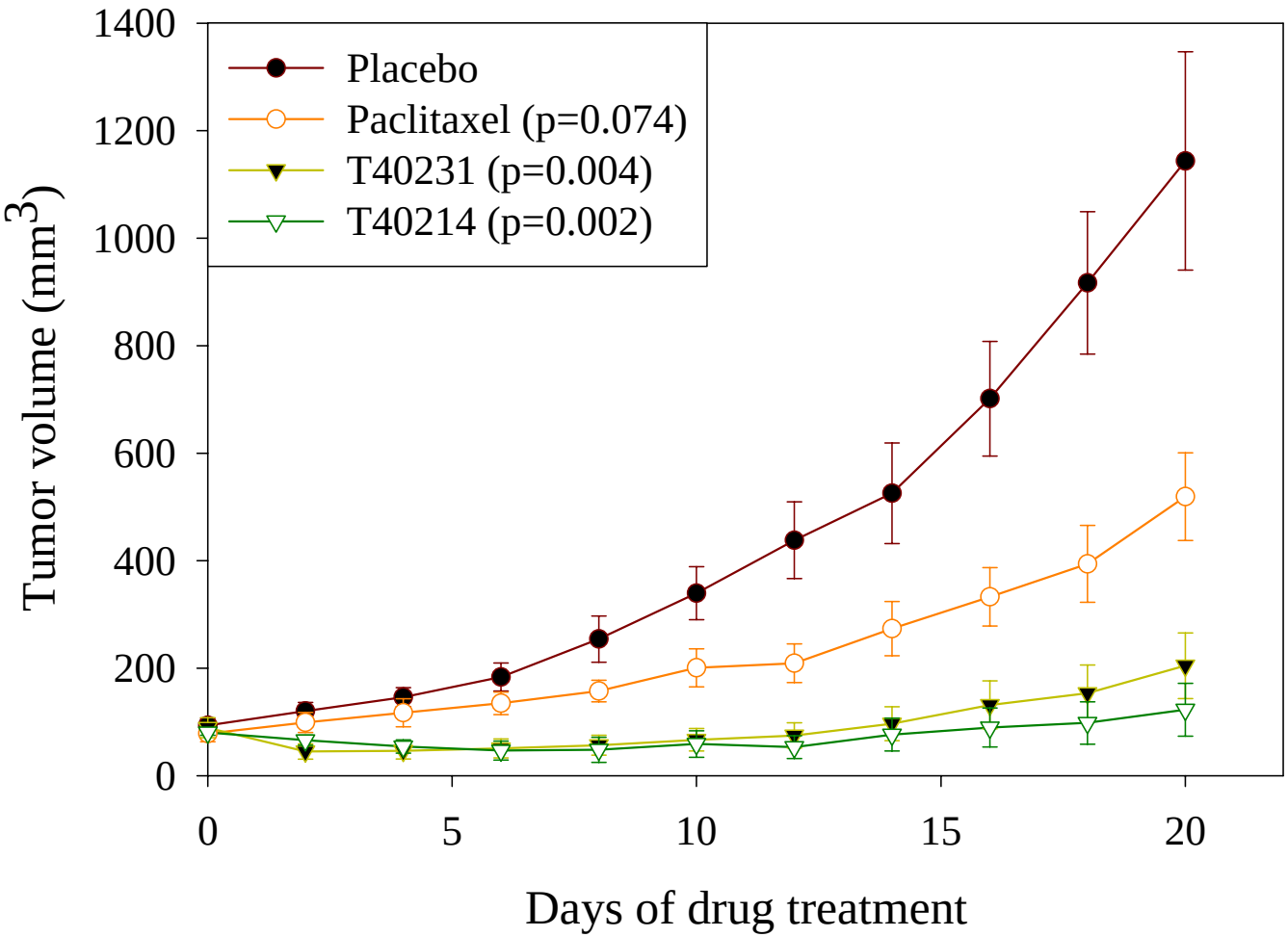


Figure 4(Cont)

A.

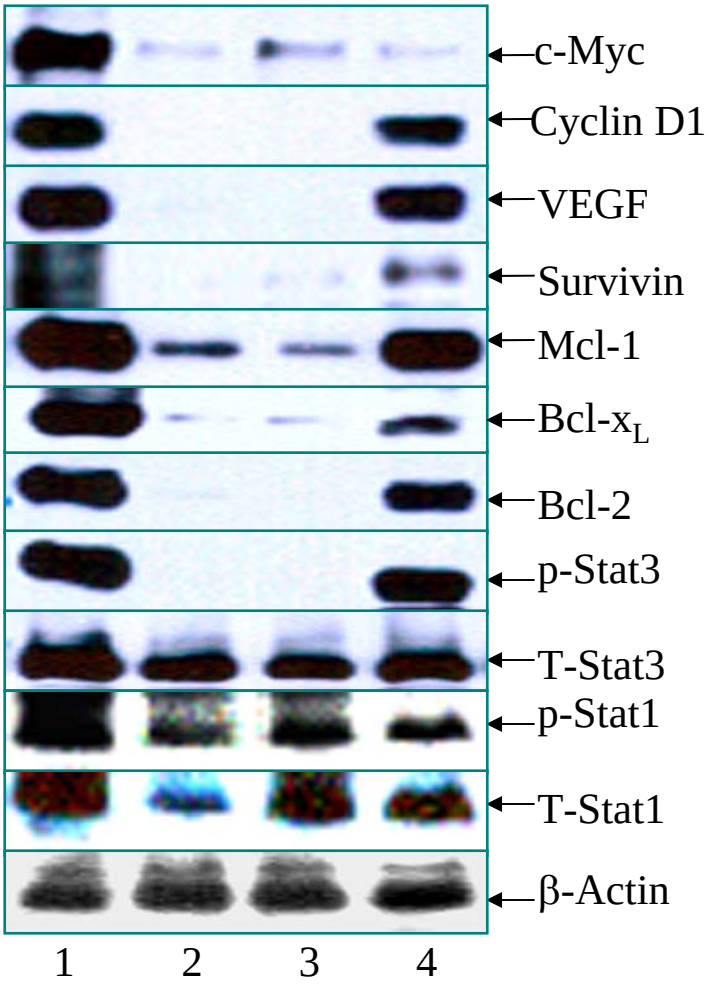
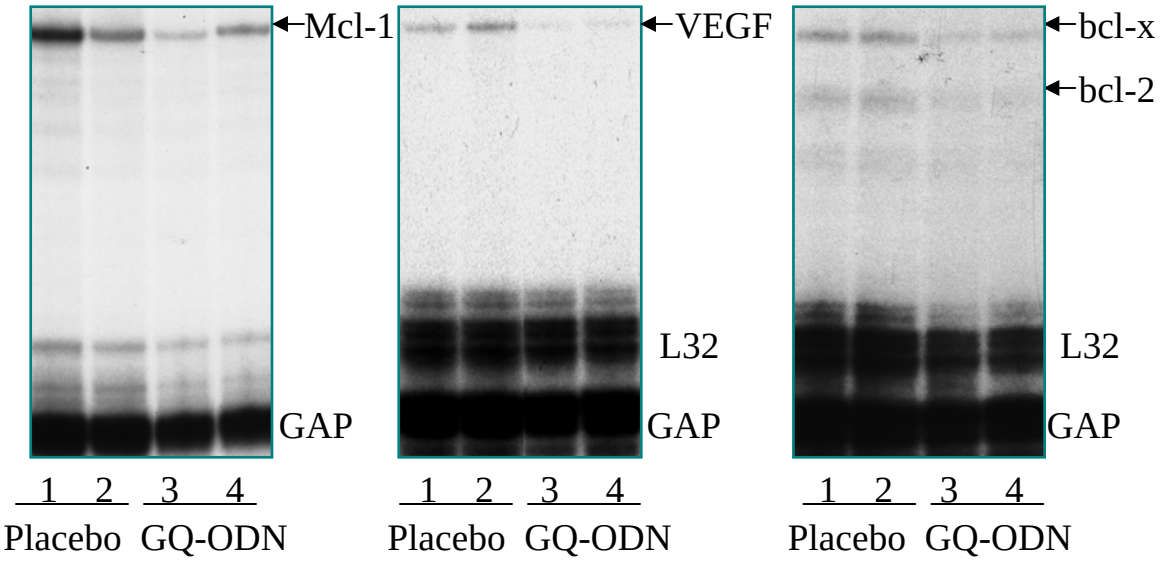

c-Myc
Cyclin D1
VEGF
Survivin
Mcl-1
Bcl-x$_L$
Bcl-2
p-Stat3
T-Stat3
p-Stat1
T-Stat1
β-Actin
1
2
3
4

Figure 5

B.



C.

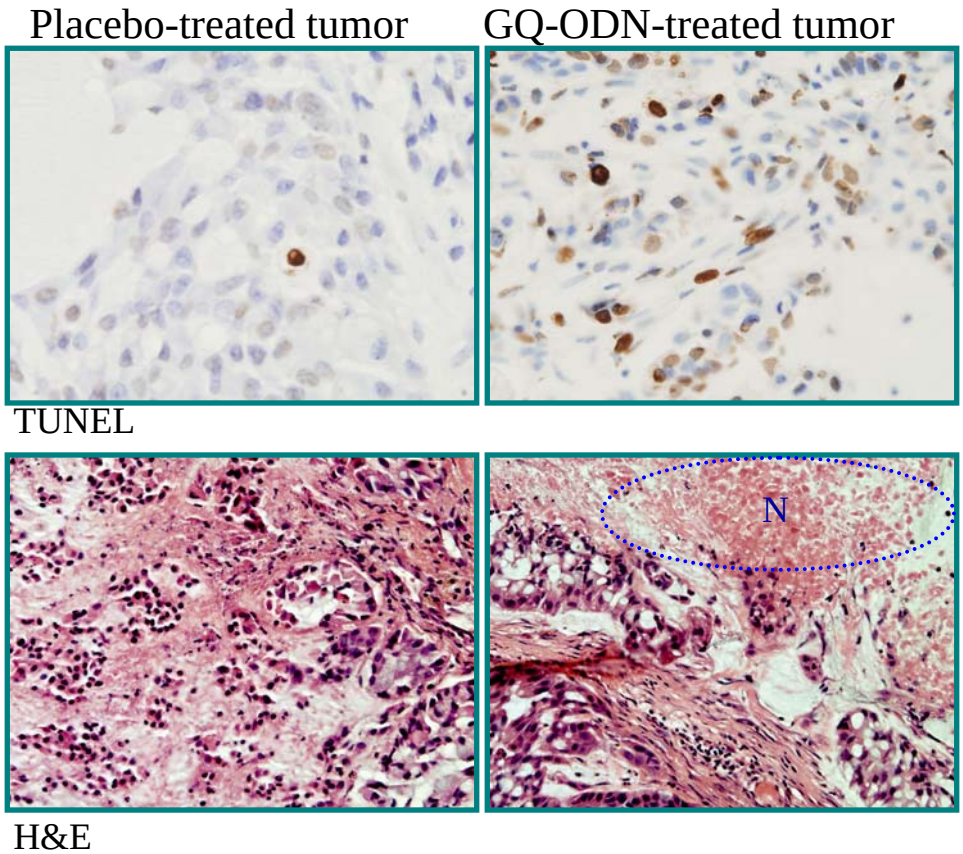


Figure 5(Cont)

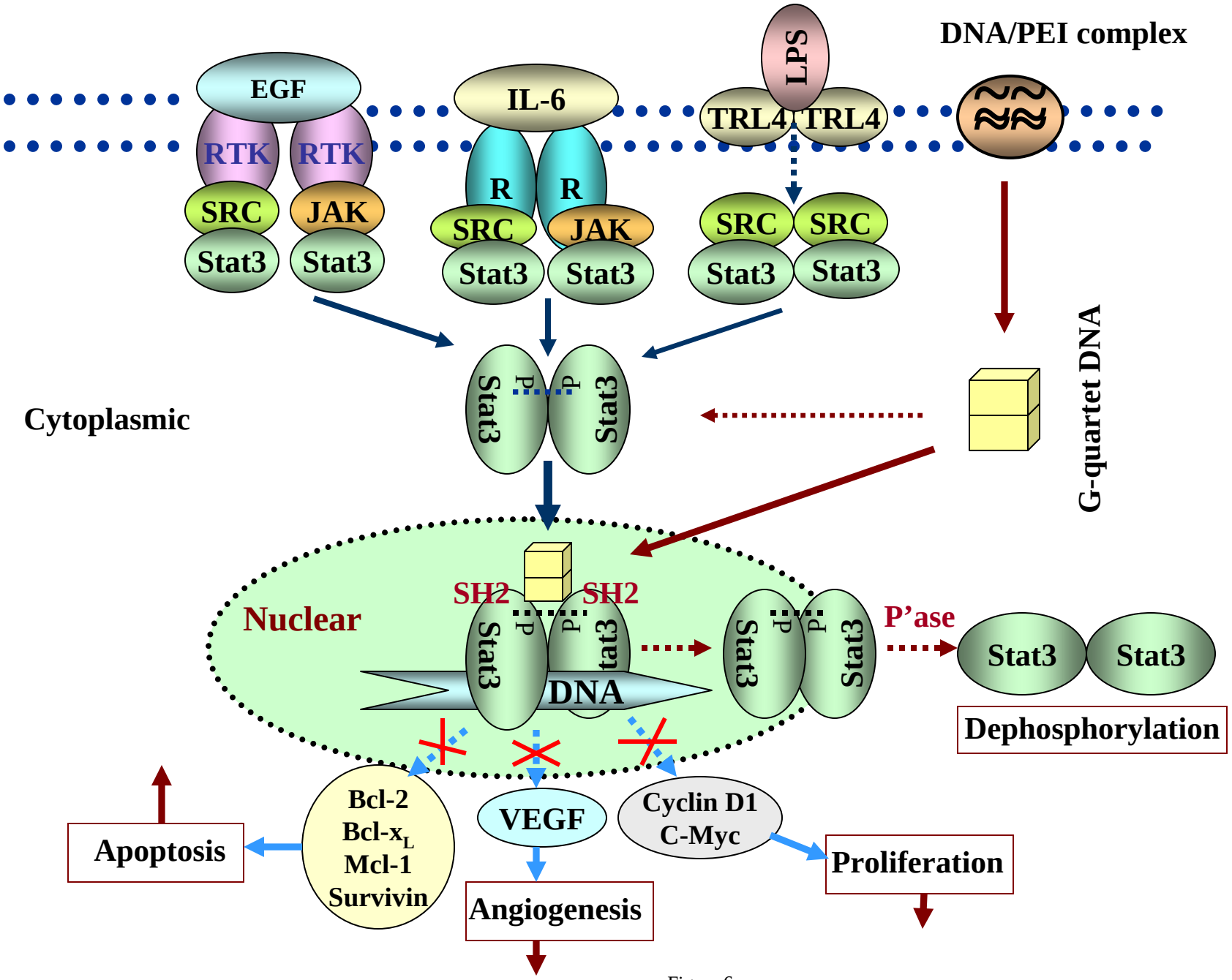

DNA/PEI complex
LPS
EGF
IL-6
TRL4
TRL4
RTK
RTK
R
R
SRC
JAK
SRC
SRC
SRC
JAK
Stat3
Stat3
Stat3
Stat3
Stat3
Stat3
P
P
Stat3
Cytoplasmic
G-quartet DNA
Nuclear
SH2
SH2
Stat3
Stat3
DNA
P'ase
Stat3
Stat3
Dephosphorylation
Apoptosis
Bcl-2
Bcl-x$_L$
Mcl-1
Survivin
VEGF
Cyclin D1
C-Myc
Proliferation
Angiogenesis

Figure 6