再生医療実用化を促進するセルプロセシングセンター運用のための人材育成プロジェクト

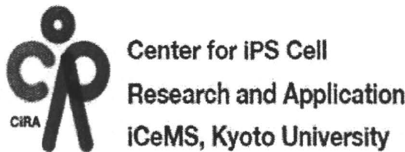
（公募番号21110301）

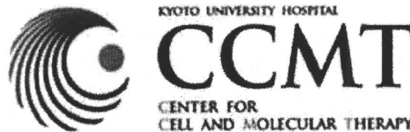
研究代表者　前川 平

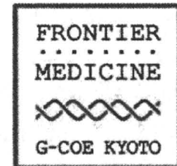
京都大学医学部附属病院

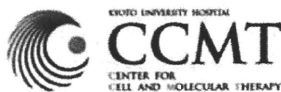
輸血細胞治療部

分子細胞治療センター









実施体制

研究代表者

前川　平	京大輸血細胞治療部・教授	総括、GMP教育、CPC運用実習

研究分担者

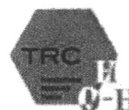
高桑徹也	京大人間健康・教授	カリキュラム作成、基礎細胞学教育、実習
青山朋樹	京大人間健康・教授	教育コース作成、基礎細胞学教育、実習
伊吹謙太郎	京大人間健康・教授	基礎細胞学、感染学教育、実習
笠井泰成	京大CCMT・主任技師	GMP教育、CPC運用教育、実習
川真田伸	先端医療財団・リーダー	GMP、GCP教育、CPC運用教育

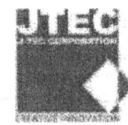
研究協力者

木村貴文	京大iPS研究所・教授	CPC運用教育、実習
青井貴之	京大iPS研究所・教授	CPC運用教育、実習
細田公則	京大人間健康・教授	基礎細胞学教育
門脇則光	京大血液腫瘍内科・准教授	細胞治療臨床指導
森本尚樹	京大形成・講師	細胞治療臨床指導
興津　輝	京大移植外科・助教	細胞移植治療臨床指導
伊藤達也	京大探索医療センター・助教	GMP教育

研究協力機関

京都大学iPS研究所
京都大学探索医療センター
先端医療財団
J-TEC





進捗状況

講義風景

人間科学専攻検査技術科学コース
（学部定員40→30, 修士13, 博士15）

- 学部学生時にGMP品質管理、安全教育、医療教育を学ぶ
- 学部卒業→臨床検査技師の国家資格取得
- 以上の基礎教育を受けた者に、修士大学院時代にさらにGMPに関する専門教育を行う

【平成21年度】

・基礎教育トレーニング
・GMP, GCP教育プログラム
・基礎細胞生物学講義
・品質検査学講義
・生命倫理・医療倫理講義

【平成22年度】

・セルプロセシング実地演習
（京大CPC, iPS-CPC, 神戸CPC, TRI）
・基礎細胞培養実習
・品質管理検査実習

京都大学iPS研究所
FiTスタートアップ
骨再生プロジェクトスタートアップ

【平成23年度】

・セルプロセシング実地演習
・学会資格等の検討

再生医療開発現場で即戦力となる細胞プロセシングの知識と経験をもった人材を供給する

2、3年後より毎年5～6名程度の人材供給が可能になると試算

『 ICRの推進による再生医療の実現 』

A.　再生医療事業化の推進（大学、研究所、企業）

B.　再生医療の実現化を阻む技術課題の解決（大学、研究所、企業）

CPCの即戦力として本スーパー特区に参画する大学、研究所、企業、あるいは行政（PMDAなど）へ人材供給することにより、特区内における再生医療開発の迅速化、品質管理・安全性の向上に資する。

細胞育成学総論：系統講義

平成22年度より「臨床検査展開学」大学院正式カリキュラムに昇格

細胞治療・再生治療開発への挑戦

細胞育成学連続講演会2010

このシリーズでは、京都大学内外で細胞治療・再生治療の研究、臨床をされている先生方に、最先端の話題を提供していただきます。また、細胞治療を支える細胞治療センターの重要な役割にスポットをあてます。学生、教員の皆様の聴講を歓迎致します。

場所：人間健康科学科 高井ホール（171号室）
日時：毎週水曜日 16:30～18:00（計14回）

◆ 9月29日(水)：前川　平（京都大学医学部附属病院　輸血細胞治療部　教授）
京都大学における細胞治療・再生治療開発への挑戦－概論－

◇ 10月6日(水)：伊藤　達也（京都大学医学部附属病院　探索医療センター　助教）
治験、臨床試験に関わる規制について

◇ 10月13日(水)：笠井　泰成（京大病院　分子細胞治療センター　主任技師）
細胞治療における臨床検査技師の役割

◇ 10月20日(水)：青井　貴之（京都大学　iPS細胞研究所　教授）
細胞治療に向けたiPS細胞の現状と課題

◇ 10月27日(水)：興津　輝（京都大学医学部附属病院　臓器移植医療部　助教）
臨床膵島移植

◆ 11月10日(水)：神田　輝（愛知県がんセンター研究所・腫瘍ウイルス学部　室長）
ウイルス抗原・がん抗原に特異的なT細胞を用いた細胞療法

◆ 11月17日(水)：川真田　伸（(財)先端医療振興財団　再生医療支援グループ　GL）
CPCの運営コストと事業化について　－神戸での取り組み－

◇ 12月1日(水)：門脇　則光（京都大学医学部附属病院　血液・腫瘍内科　講師）
癌免疫療法としての細胞療法

◇ 12月8日(水)：森本　尚樹（京都大学医学部附属病院　形成外科　講師）
自家培養真皮を用いた皮膚潰瘍治療

◇ 12月15日(水)：岩田　博夫（京都大学　再生医科学研究所　教授）
人工材料への細胞の接着

◇ 12月22日(水)：井家　益和（(株)J-TEC製品開発部　部長）
ヒト細胞を組み込んだ日本初の再生医療製品の開発

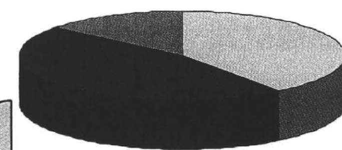
◆ 1月7日(金)：青山朋樹（人間健康科学系専攻　准教授）
間葉系幹細胞を用いた臨床応用

◆ 1月12日(水)：一山　智（京都大学医学部附属病院　検査部　教授）
免疫不全患者における感染症の診断と治療

◆ 1月19日(水)：細田公則（人間健康科学系専攻　教授）
iPS細胞由来脂肪細胞を用いた脂肪萎縮症の成因解明、および細胞治療法の開発

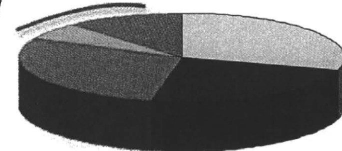
*本講演は修士選択科目「細胞育成学」を広く一般公開するものです。　臨床検査展開学, 細胞育成士養成プロジェクト　TEL/FAX: 075-751-3931

平成21年度参加者



受講者の多様化

平成22年度参加者



平成23年度は社会健康医学系の協力を得て、生命倫理の講義も組み込む予定

細胞育成学実践論：実習

「臨床検査展開学」カリキュラムとして平成23年2月14〜18日予定

細胞育成学実践論				区分		
				所属	職名	氏名
				医療検査展開学講座	教授	高桑徹也
				医療検査展開学講座	准教授	伊吹謙太郎
単位数	2単位	授業形態	講義	理学療法学講座	准教授	青山朋樹
時間数	30時間	対象回生	1,2回生	人間健康科学系専攻	教務補佐	上田路子
開講期	後期	選択	選択	輸血細胞治療部 iPS細胞研究所	主任技師 教授	笠井泰成 木村貴文

授業概要と学習目標

細胞治療とは、ヒトの細胞を輸注、移植することによって行う治療法の総称であり、従来から行われている輸血治療を原型とし、造血幹細胞移植、細胞移入免疫療法、遺伝子治療、再生医療などがこれに含まれます。細胞治療の今後の発展性を考えると、「細胞育成」という役割を持つ人材が必要不可欠で、これは臨床検査技術学を主体とした領域となると考えられます。求められる能力は、単に細胞が培養できるというだけでなく、1.細胞治療の基礎知識、理解、2.細胞治療センター(CPC)の運営管理の考え方の理解と実践、3.安全な細胞の育成、調製法の理解と実践、さらには4.細胞治療研究に必要な基礎技術の修得、5.細胞治療研究の実際と多岐にわたります。当コースでは、細胞治療における品質管理、培養法の実習、細胞治療を背後で支える細胞治療センターの紹介、見学、実際の運営法等について学びます。

授業計画と内容

1. 品質管理法実践論1　グラム染色による細菌感染否定試験
2. 品質管理法実践論2　マイコプラズマ否定試験
3. 品質管理法実践論3　エンドトキシン測定
4. 品質管理法実践論4　セルプロセシングセンターにおけるモニタリング
5. 細胞培養法実践論1　無菌培養手技
6. 細胞培養法実践論2　細胞数カウント、増殖曲線作成
7. 細胞培養法実践論3　細胞数カウント、増殖曲線作成
8. 細胞培養法実践論4　Cell sorting
9. 細胞調整室運営実践論　セルプロセシングセンター見学

(2コマ連続で行う回があります)

成績評価	レポート、出席
教科書	無し
参考書等	適宜テキストを配布します。
学生へのメッセージ	細胞治療の最先端とそれを支える細胞治療センター、細胞育成士の役割という主題で、配当いたします。 検査技術科学コースを含め、すべてのコースの学生の聴講を歓迎致します。 細胞育成学を受講していなくても受講可能です。 実習的要素もありますので、受講希望者は事前に担当教官と連絡を取ってください。

京都大学人間科学科

京都大学医学部附属病院 CCMT

京都大学iPS研究所　FiT

今後の活動と課題

標準化による安全性確保

（再生医療開発の制度的枠組みの基盤）

← CPC標準化
細胞培養技術標準化
細胞搬送技術標準化

再生医療実用化を促進するセルプロセシングセンター運用のための人材育成プロジェクト

他施設における人材育成教育カリキュラムとの融合（学-学連携、産-学連携）

PMDAとの人材交流およびPMDAへの人材供給（官-学連携）← 次の課題

神戸先端医療財団
— 細胞培養、品質管理教育カリキュラム

iPSアカデミアジャパン
— iPS細胞培養実習

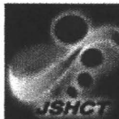
J-TEC
— 細胞培養、品質管理教育カリキュラム

→ **学会認定へ働きかけ**

テキスト作成
実習システム構築
DVD作成中

再生医療学会
造血細胞移植学会
輸血細胞治療学会

学会合同シンポを3月、4月に予定



第33回日本造血細胞移植学会総会

The 33rd Annual Meeting of the Japan Society for Hematopoietic Cell Transplantation

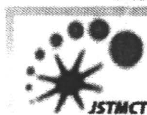
将来を見つめて移植の原点を考える

3．日本造血細胞移植学会/日本輸血細胞治療学会/日本再生医療学会 合同シンポジウム 細胞移植・細胞治療に関する国・学会の指針と基盤整備

3月10日（木）08:30-10:00　**第1会場 メインホール（1F）**

座長	加藤 俊一	（東海大学医学部基盤診療学系再生医療科学）
	前川 平	（京都大学医学部附属病院　輸血細胞治療部）
演者	豊嶋 崇徳	（九州大学病院　遺伝子・細胞療法部）
	田野崎 隆二	（国立がん研究センター中央病院　臨床検査科）
	森尾 友宏	（東京医科歯科大学大学院医歯学総合研究科　発生発達病態学分野）
	澤 芳樹	（大阪大学大学院医学系研究科外科学講座　心臓血管外科学）
	田邊 裕貴	（厚生労働省医政局研究開発振興課）

The 59th Annual Meeting of the Japan Society



第59回 日本輸血・細胞治療学会総会

3）シンポジウム

1. 将来の輸血医療を見据えて／職種間の連携によるチーム医療の構築（会長シンポジウム）
2. 細胞移植、細胞治療にかかわる国と学会の指針（造血細胞移植学会、再生医療学会との合同）
3. 世界の血漿分画製剤治療の現況
4. 我が国の輸血安全はどこまで確保されたか
5. 輸血のトリガー値を検証する
6. 輸血検査の変遷と今後の進歩
7. 新興輸血感染症の疫学と病態
8. 造血幹細胞移植のためのCell Processingの過去；基礎研究を振り返る
9. 基礎から臨床へ／学術振興委員会企画

今までの研究費の使途

(1) 備品

米国ベクトン・ディッキンソン　BDフローサイトメーター	15,618,750円
BD FACSCanto II フローサイトメーターオプション405nmレーザー	5,801,250円
米国ライフテクノロジーズ　Applied Bio リアルタイムPCRシステム	5,197,500円
バイオラッド　ChemiDoc XRS Plus	2,992,500円
微量サンプル分光光度計(GEヘルスケアバイオサイエンス社製)	1,606,500円
三洋電機バイオハザード対策用　キャビネット	1,411,200円
三洋電機　CO2インキュベーター	1,092,000円
微量高速冷却遠心機一式(トミー工業株式会社製)	1,041,600円 ほか

(2) 消耗品

(3) 人件費

(4) その他

京都大学 大学院医学研究科 人間健康科学系専攻 臨床検査展開学分野
病理学研究室 (Laboratory of Pathology）

2010年度の修士論文ができました »

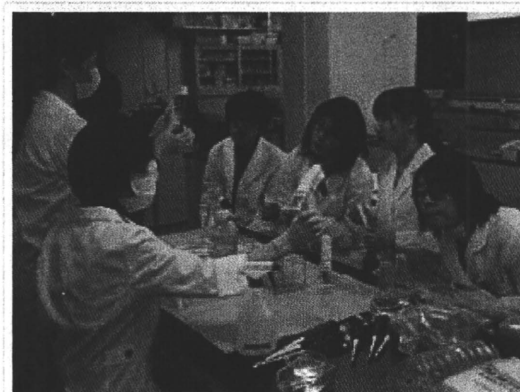
細胞育成学実践論が行われました…

平成23年2月14，16，18日（月、水、金）、細胞育成学実践論が行われました。

再生医療を展開する人材育成を目的に行われた実習ですが、今回はその基礎として下記の３項目を行いました。人間健康科学科の他、附属病院分子細胞治療センター、iPS研究所のスタッフの協力を得て充実したカリキュラムとなりました。

1.無菌性細胞製造実習ー細胞培養の基礎を学ぶ

- バイオハザード対策キャビネットの使用法
- 培養液調製
- 付着細胞剥離
- 細胞数カウント
- 細胞播種
- 細胞観察
- 付着細胞剥離
- 細胞数カウント
- 倍加時間算定



2.品質管理ー

- エンドトキシン測定オリエンテーション
- エンドトキシン測定実践

3.CPC管理

検査技術専攻の他、リハビリテーション科学、看護科学専攻の修士、学部生計８名が参加しました。

実施場所：人間健康科学科 培養室）実施場所：分子細胞治療センター）実施場所：iPS研究所 FiT）

.pdf 細胞育成学実践論実施要項

Site

- 病理学研究室へようこそ….
  - ご挨拶
  - 教育の概要
    - 1.学部教育
    - 2.大学院教育
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研究成果の刊行に関する一覧表（英文）

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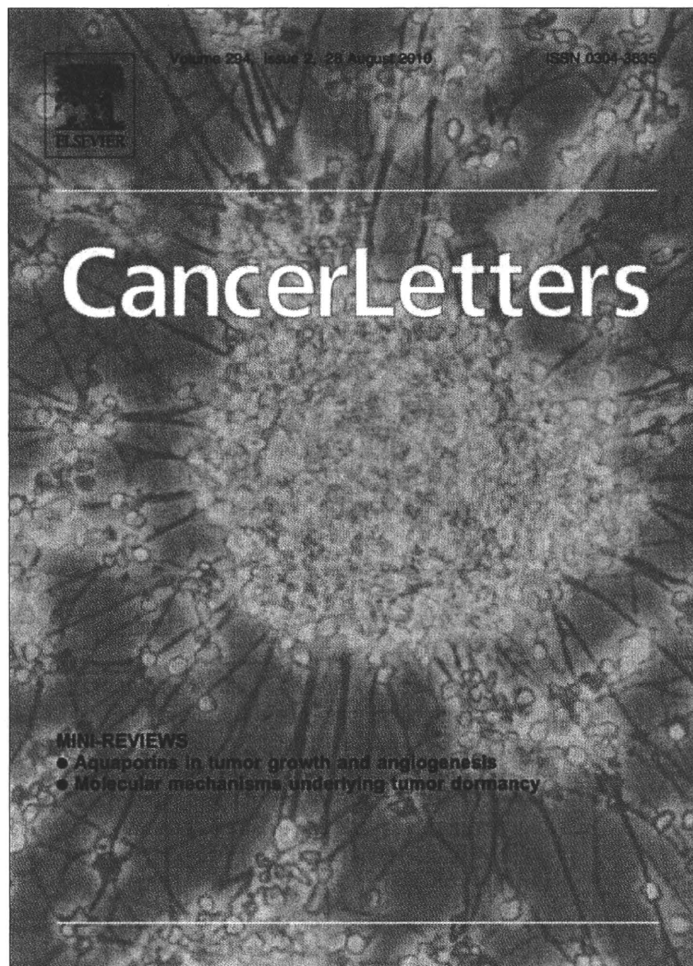

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A combination of a DNA-chimera siRNA against PLK-1 and zoledronic acid suppresses the growth of malignant mesothelioma cells *in vitro*

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ABSTRACT

Although novel agents effective against malignant mesothelioma (MM) have been developed, the prognosis of patients with MM is still poor. We generated a DNA-chimeric siRNA against polo-like kinase-1 (PLK-1), which was more stable in human serum than the non-chimeric siRNA. The chimeric PLK-1 siRNA inhibited MM cell proliferation through the induction of apoptosis. Next, we investigated the effects of zoledronic acid (ZOL) on MM cells, and found that ZOL also induced apoptosis in MM cells. Furthermore, ZOL augmented the inhibitory effects of the PLK-1 siRNA. In conclusion, combining a PLK-1 siRNA with ZOL treatment is an attractive strategy against MM.

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1. Introduction

Malignant mesothelioma (MM) is an aggressive tumor, which develops from the mesothelial surface of the pleural and peritoneal cavities. Asbestos is well-known as a carcinogen in MM and the incidence of MM is increasing worldwide [1]. Although several surgical approaches have been proven to be effective [2,3], a combination of therapies including chemotherapeutic agents, radiation, and immunotherapy are required to fight the disease. However, in spite of the emergence of novel effective anticancer agents such as pemetrexed [4,5] and raltitrexed [6,7], the prognosis of patients with MM is still poor [8,9]. Therefore, the development of novel effective therapeutic strategies is essential to improve the prognosis of this disease.

RNA interference (RNAi) is a process involving sequence specific post-transcriptional gene silencing induced by double-stranded (ds) RNA. It is widely applied as a powerful tool in postgenomic research, and has been experimentally introduced into the field of cancer therapy. Synthetic, short interfering RNAs (siRNAs) for inducing RNAi are 19- to 21-nucleotide dsRNAs with two-nucleotide 3′ overhangs at either end [10,11]. Unfotunately, siRNAs are degraded by endogenous nucleases when administered *in vivo*. Many techniques, including the use of DNA-chimeric siRNAs, have been developed to protect siRNAs from such degradation [10,12,13]. Previous investigations have revealed that their silencing activity is as powerful as that of non-chimeric siRNAs [12–14].

Polo-like kinase-1 (PLK-1) belongs to the PLK family of serine/threonine kinases and is highly conserved among eukaryotes. PLK-1 regulates cell division at several points during the mitotic phase of the cell cycle, including: mitotic entry through CDK1 activation, bipolar spindle

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formation, chromosome alignment, segregation of chromosomes, and cytokinesis [15,16]. Previous studies have reported that PLK-1 is overexpressed in cancerous tissues and that PLK-1 expression levels are tightly correlated with histological grades of tumors, clinical stages, and the patients' prognosis [17–20]. Thus, PLK-1 is considered to be a suitable target for cancer therapy, and several small molecular targeting agents have been used in clinical trials [21,22], while siRNAs against PLK-1 have been investigated in preclinical studies [19,20,23].

Bisphosphonates (BPs) are inhibitors of bone-resorption, and second- and third-generation BPs have been developed primarily to treat benign and malignant bone disease [24]. This class of drugs inhibits the proliferation of cancer cells by preventing the post-translational prenylation of small GTPases including the Ras family proteins [25]. We have demonstrated previously that third-generation BPs such as zoledronic acid (ZOL) and minodronic acid (YM529) have direct anti-tumor effects against different cancer cells [26–30].

In the present study, we have investigated the effects of a DNA-chimeric PLK-1 siRNA and ZOL on MM cells *in vitro*. Our results show that these agents induce apoptosis and inhibit the proliferation of MM cells. In addition, we found that ZOL enhances the inhibitory effects of the PLK-1 siRNA.

2. Materials and methods

2.1. Cell lines, reagents, and animals

The human MM cell lines H2452, H2052, H28, and 211H were cultured in RPMI1640 medium (Gibco, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Tokyo, Japan), *l*-glutamine (Gibco), and 1% penicillin–streptomycin (Gibco). The normal human dermal fibroblast (NHDF) cells were cultured in Dulbecco's Modified Eagle medium (DMEM; Gibco) containing 10% FCS, *l*-glutamine, and 1% penicillin–streptomycin. All cell lines were maintained at 37 °C in a fully humidified atmosphere of 5% CO_2 in air. All four MM cell lines were obtained from the American Type Culture Collection (Rockville, MD). Normal fibroblast NHDF cells were purchased from Kurabo (Osaka, Japan). LIC™ Transfection Reagent (Hayashi Kasei, Tokyo, Japan) was used for transfection into MM cells. ZOL (1-hydroxy-2-[1*H*-imidazole-1-yl]ethylidene-bisphosphonic acid) was obtained from Novalits Pharma AG (Basel, Switzerland).

We generated two types of siRNA against PLK-1 (GenBank accession number NM_005030) using siDIRECT™ (alphaGEN Co, Ltd, Tokyo, Japan). One of the siRNAs contained of ribonucleotides and the other was a DNA-chimeric siRNA consisting partially of deoxyribonucleotides. The oligonucleotide sequences of the non-chimeric PLK-1 siRNA against PLK-1 were: sense strands, 5′- GCACCGAAACCG AGUUAUUCA-3′ and that antisense strand, 5′-AAUAACUC GGUUUCGGUGCAG-3′. The sequences of the DNA-chimeric siRNA against PLK-1 were: sense strand, 5′-GCACCGAAAC CGAgttattca-3′, and antisense strand, 5′-aataacUCGGUUU CGGUGCAG-3′. This DNA-modified siRNA was constructed by substituting six ribonucleotides at the 5′ end of the guide strand and the 3′ end of the passenger strand with the cognate deoxyribonucleotides (designated in lower case). The oligonucleotide sequences for the chimeric siRNA controls were: sense strand, 5′-GUACCGCACGUCAttcgtatt-3′, and antisense strand, 5′- tacgaaUGACGUGCG GUACGU-3′. The sequences for the non-chimeric control siRNA were: sense strand, 5′-GUACCGCACGUCAUUC GUAUU-3′, and antisense strand, 5′-UACGAAUGACGUGCG-GUACGU-3′. All siRNAs used were chemically synthesized (Hokkaido System Science Co. Ltd., Hokkaido, Japan).

2.2. Stability of the DNA-chimera siRNA in human serum

We investigated the stability of the DNA-chimeric and non-chimeric siRNAs in human serum. Each siRNA was incubated in human serum (95%) at 37 °C. Serum RNase was inactivated by adding SDS and proteinase K, and then digested samples were loaded onto 15% polyacrylamide gel, which was then stained using SYBR Gold (Invitrogen).

2.3. Growth inhibitory effects of PLK-1 siRNA

Cell proliferation was determined by the modified MTT assay using the Cell-Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) as previously described [19,20]. Cells were seeded in a flat-bottomed 96-well plate (Becton Dickinson, Tokyo, Japan) at 3×10^3 cells in 100 µl of medium per well and incubated with serial dilutions of the DNA-chimeric siRNA for 72 h. The mean of four samples was calculated. Half-maximal inhibition constants (IC_{50}s) were determined with the nonlinear regression program CalcuSyn (Biosoft, Cambridge, UK).

2.4. Growth inhibitory effects of zoledronic acid

Cell proliferation was determined by the modified MTT assay using the Cell-Counting Kit-8 as mentioned above. Cells were seeded in a flat-bottomed 96-well plate (Becton Dickinson, Tokyo, Japan) at 3×10^3 cells in 100 µl of medium per well and incubated with serial dilutions of ZOL for 72 h. The mean of four samples was calculated. Half-maximal inhibition constants (IC_{50}s) were determined with the nonlinear regression program CalcuSyn. We also evaluated the combined effects of concurrent PLK-1 siRNA and ZOL treatment on H2452 and H28 mesothelioma cell lines and the analyzed data is shown by the combination index (CI). CI is a method for quantifying drug cytotoxic synergism based on the mass-action law principle derived from enzyme kinetic models. This method was developed by Chou and Talalay [31,32] which has been widely used to evaluate interactions of antineoplastic agents [33–36]. Cells were incubated for 72 h with six concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 times the IC_{50}) of each agent or both in combination using the constant ratio design followed by the modified MTT assay. We calculated the combination indexes (CIs) as reported previously [33–36], and calculated the fraction affected (Fa) at each dilution (for example, Fa of 0.25 equals 75% viable cells). This method provides a quantification of the synergism (CI < 1), additive effect

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(CI = 1), and antagonism (CI > 1) at different dose and effect levels [31]. Calculations of the CI were made under the assumption that the mechanisms of action of the evaluated drugs were not mutually exclusive.

2.5. Cell cycle and apoptosis analysis

Cell cycle analysis using propidium iodide (PI) was performed as previously described [20]. Apoptosis induced by each siRNA treatment or ZOL treatment was determined using the Annexin-V-FITC Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA) as recommended by the manufacturer. Cells were analyzed with FACS CANTO II using Diva software (BD Bioscience).

2.6. Western blotting analysis

Following the transfection of cells with PLK-1 siRNA, or treatment with ZOL, as described above, the medium was aspirated and the cells were washed with ice-cold PBS (−). The cells were lysed with ice-cold RIPA buffer (50 mM Tris–HCl [pH 7.4], 0.25 M NaCl, 5 mM EDTA, 20 mM NaF, 1% NP-40) with PMSF (1 mM) and protease inhibitor (10 μg/ml). The cells were then scraped off the plate, and the suspension of cells in lysis buffer was transferred to a centrifuge tube, which was placed on ice for 15 min with an occasional vortex to ensure complete lysis. The cell suspension was then cleared by centrifugation at 14,000g for 30 min at 4 °C, and the supernatant (total cell lysis) was either used immediately or stored at −80 °C. The protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories, Osaka, Japan).

Immunoblotting was performed as previously described [20]. The following primary antibodies (Abs) were used: rabbit polyclonal anti-PLK-1 Ab (Upstate Biotechnology Inc., Charlottesville, VA); rabbit polyclonal anti-caspase-3 Ab; rabbit polyclonal anti-cleaved caspase-3 Ab (Cell Signaling Technology, Danvers, MA); polyclonal anti-Rap1A Ab (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-RhoA Ab (Santa Cruz Biotechnology); mouse monoclonal anti-Ras Ab (BD Bioscience), and rabbit polyclonal anti-actin Ab (Sigma–Aldrich, Tokyo, Japan).

3. Results

3.1. Stability of the DNA-chimera siRNA in human serum

An siRNA can be protected from RNase or nuclease cleavage by the partial substitution of ribonucleotides with deoxyribonucleotides at the 5′ end of the guide strand and the 3′ end of the passenger strand. Therefore, we first designed the PLK-1 siRNA using siDIRECT™ and then converted this siRNA into a DNA-chimeric siRNA. We incubated the DNA-chimeric, or non-chimeric siRNAs against PLK-1 in 95% human serum and investigated their degeneration. The non-chimeric siRNA degenerated in a time-dependent manner, while the DNA-chimeric siRNA did not degenerate for at least 120 min (Fig. 1A). This result shows that the chimeric siRNA is more stable in human serum than the non-chimeric siRNA.

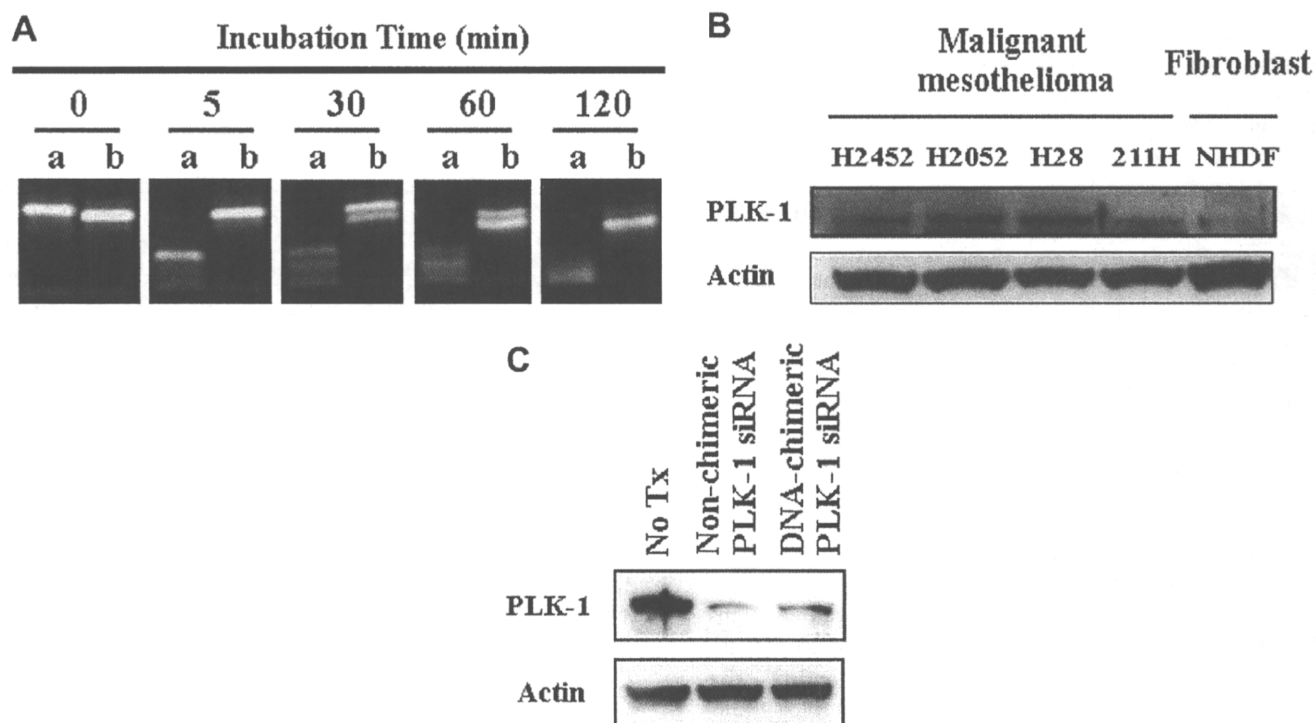

Fig. 1. DNA-chimeric siRNA against PLK-1 is more stable in human serum than a non-chimeric siRNA. (A) Each siRNA was incubated in human serum (95%) at 37 °C. Serum RNase was inactivated by adding SDS and proteinase K, and then digested samples were loaded onto a 15% polyacrylamide gel. The gel was stained by SYBR Gold. (a): non-chimeric PLK-1 siRNA, (b): DNA-chimeric PLK-1 siRNA. (B) PLK-1 expression in MM cells. Immunoblotting of whole cell lysates obtained from MM cell lines and normal NHDF human fibroblast cells. (C) Depletion of PLK-1 expression in H2452 MM cells in response to treatment with non-chimeric or DNA-chimeric PLK-1 siRNA. We obtained whole cell lysetas from H2452 MM cells 72 h after the transfection of non-chimeric or DNA-chimeric PLK-1 siRNA (50 nM), and immunoblotting was performed as described in Section 2.

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3.2. DNA-chimeric PLK-1 siRNA inhibited the growth of mesothelioma cells

We examined the PLK-1 expression in four MM cell lines: H2452, H2042, H28, and 211H cell lines. All cell lines examined expressed a higher level of PLK-1 than normal NHDF fibroblast cells (Fig. 1B). Next we confirmed the knockdown effects of both DNA-chimeric and non-chimeric PLK-1 siRNAs in MM cells. We transfected both types of siRNAs into H2452 MM cells, and both siRNAs effectively knocked down PLK-1 expression (Fig. 1C). Then we investigated the inhibitory effects of the DNA-chimeric PLK-1 siRNA on MM cells *in vitro*. Western blot analysis showed that the transfection of the DNA-chimeric PLK-1 siRNA suppressed PLK-1 expression in H2452 mesothelioma cells in a dose-dependent manner, whereas the nonsense chimeric siRNA (100 nM) did not (Fig. 2A). The IC_{50} values for H2452 and H28 cells at 72 h exposure were 1.6 nM and 38.7 nM, respectively. Out next step was to examine the growth inhibitory effects of the DNA-chimeric siRNA against PLK-1 on H2452 and H28 mesothelioma cells using a modified MTT assay. As shown in Fig. 2B, the chimeric PLK-1 siRNA inhibited cell growth in a dose-dependent manner, whereas no significant inhibitory effects were detected in normal NHDF cells (Fig. 2C).

3.3. The mechanisms of cell death induced by PLK-1 depletion

Next we investigated the mechanisms of cell death caused by PLK-1 siRNA transfection. Cell cycle analysis confirmed that PLK-1 siRNA treatment induced G2/M arrest as previously reported [19,20], and revealed an increase in the subG1 fraction 72 h after transfection with the DNA-chimeric PLK-1 siRNA (Fig. 3A, upper panel). Early apoptotic cells (Annexin – V+/PI – fraction), and late apoptotic cells and necrotic cells

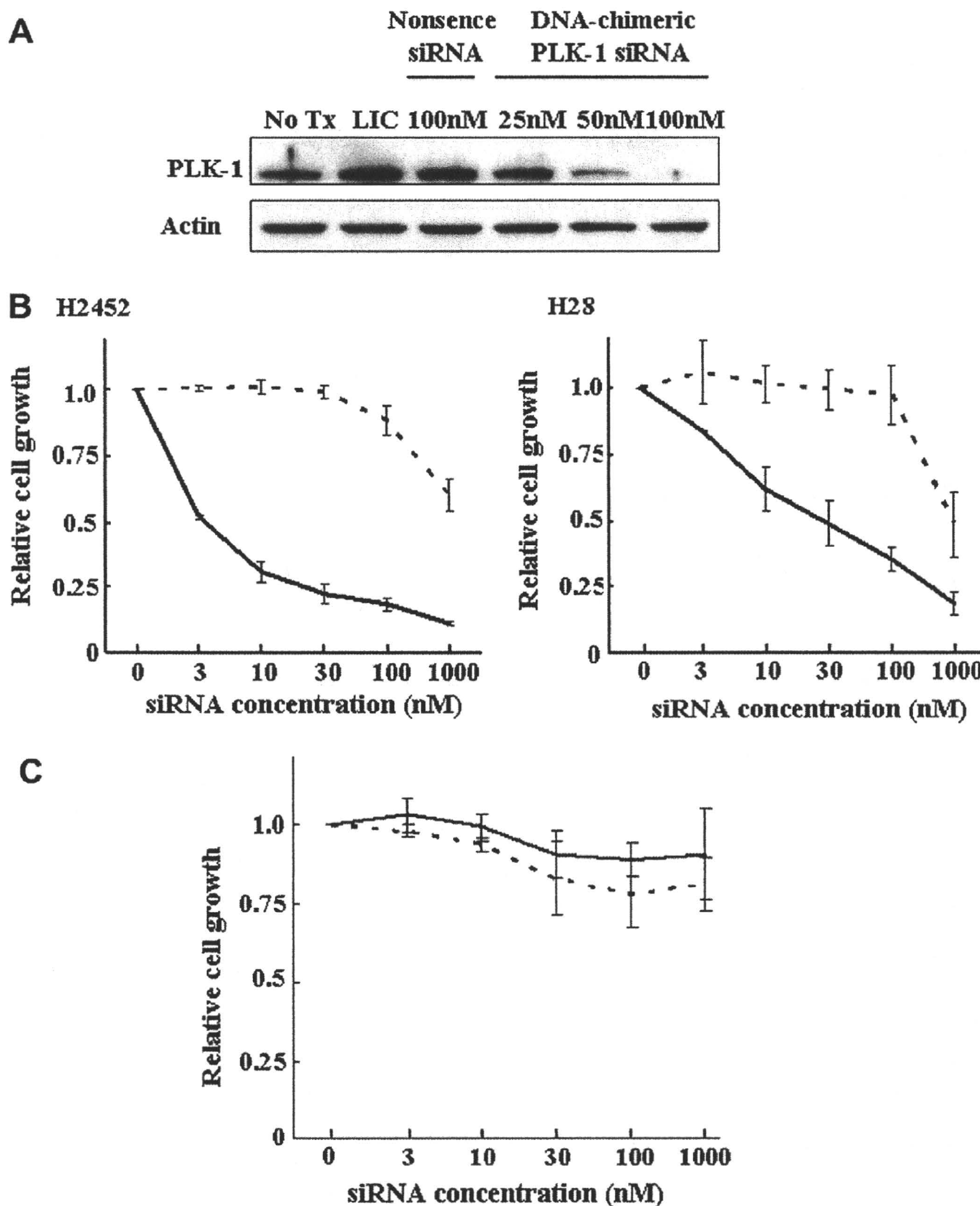

Fig. 2. DNA-chimeric PLK-1 siRNA inhibits the proliferation of MM cells, but not NHDF normal fibroblast cells. (A) Expression of PLK-1 in H2452 MM cell lines. H2452 cells were incubated with serial dilutions of DNA-chimeric PLK-1 siRNA and LIC transfection reagent for 72 h. Whole cell lysates were obtained and immunoblotting was performed as described in Section 2. (B) Cell proliferation was determined by the modified MTT assay as described in Section 2. DNA-chimeric PLK-1 siRNA shows inhibitory growth effects on H2452 and H28 MM cells in a dose-dependent manner. (C) DNA-chimeric PLK-1 siRNA does not inhibit the proliferation of normal NHDF fibroblast cells. Data represents the means ± standard deviations (SD) of three independent experiments. Solid and dotted lines indicate chimeric PLK-1 siRNA and chimeric control siRNA, respectively.

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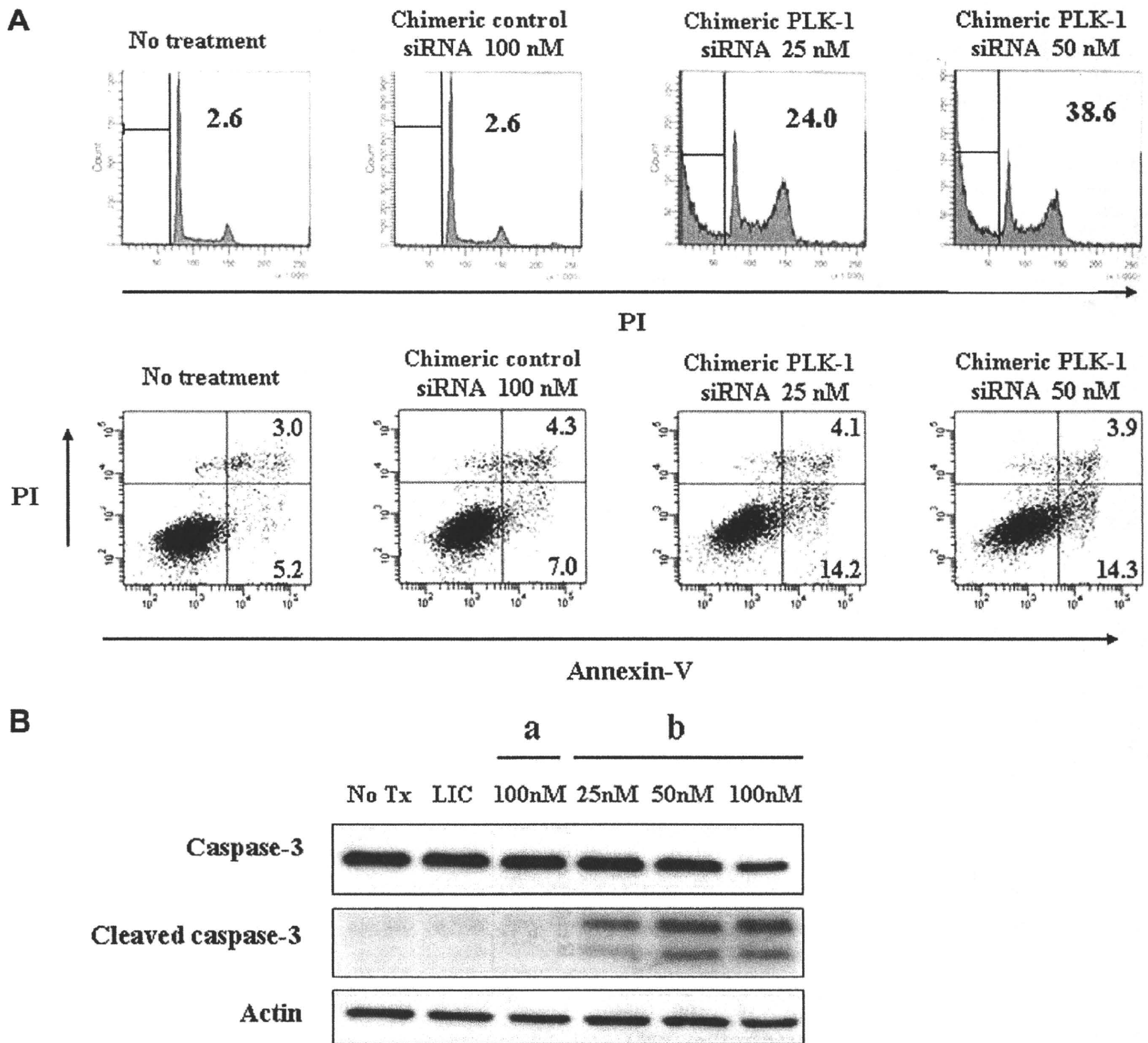

Fig. 3. DNA-chimeric PLK-1 siRNA treatment induces apoptosis in H2452 MM cells by activating caspase-3. (A: upper panels) Cell cycle analysis in H2454 MM cells using propidium iodide (PI) was performed after 72 h of treatment with the chimeric control, or chimeric PLK-1 siRNAs, at the concentration indicated. Results are representative of three independent experiments. The numbers inside each histogram indicate the percentage of the subG1 fraction. (A: lower panels) Determination of apoptosis induced by each siRNA treatment. Results are representative of three independent experiments. The numbers inside each quadrant indicate the percentage of the cell population with the quadrant characteristic. (B) Cleavage of caspase-3 by DNA-chimeric PLK-1 siRNA treatment. H2452 cells were incubated with serial dilutions of DNA-chimeric PLK-1 siRNA and LIC transfection reagent for 72 h. Whole cell lysates were obtained and immunoblotting was performed as described in Section 2. (a): chimeric control siRNA, (b): chimeric PLK-1 siRNA.

(Annexin – V+/PI + fraction) also increased after DNA-chimeric PLK-1 siRNA transfection (Fig. 3A, lower panel). In addition, Western blotting analysis demonstrated an increase in cleaved caspase-3 activity following PLK-1 siRNA transfection (Fig. 3B). Thus, transfection with a PLK-1 siRNA transfection resulted in the induction of apoptosis in mesothelioma cells through the activation of caspase-3.

3.4. ZOL inhibits the growth of mesothelioma cells and synergistically augments with the effects of the PLK-1 siRNA

We examined the inhibitory effects of ZOL on H2452 and H28 mesothelioma cells using a modified MTT assay. ZOL inhibited cell growth in a dose-dependent manner, and the IC_{50} values for H2452 and H28 cells at 72 h exposures were 11.4 μM and 58.1 μM, respectively (Fig. 4A). ZOL treatment increased the subG1 fractions (Fig. 4B) and the number of apoptotic cells (Fig. 4C) in a dose-dependent manner. Furthermore, we found that caspase-3 was cleaved by ZOL treatment (Fig. 5A).

Next we investigated the unprenylation of Rap1A, RhoA, and Ras proteins. MM cell lysates were analyzed by Western blotting using Abs against Ras and the unprenylated form of Rap1A and RhoA. ZOL treatment resulted in an increase in unprenylated Rap1A and RhoA in MM cells (Fig. 5B). The anti-Ras Ab recognizes both a slower migrating band, representing the unprenylated Ras, and a faster migrating band representing the prenylated Ras [37]. After ZOL treatment, there was an increase in the unprenylated form of Ras in MM cells which was accompanied by a reduction in the prenylated form (Fig. 5B). Taken together, the results indicate that ZOL treatment induced apoptosis through the cleavage of caspase by blocking the prenylation of small GTP-binding proteins, which resulted in the inhibition of cell growth of MM cells.

We then investigated the combined effects of ZOL treatment with the PLK-1 siRNA on H2452 and H28 MM cells. PLK-1 regulates RhoA in the mitotic phase [38,39]. The modified MTT assay with six concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 times the IC_{50}) of each agent or both in combination with the constant ratio was carried out. The values of the

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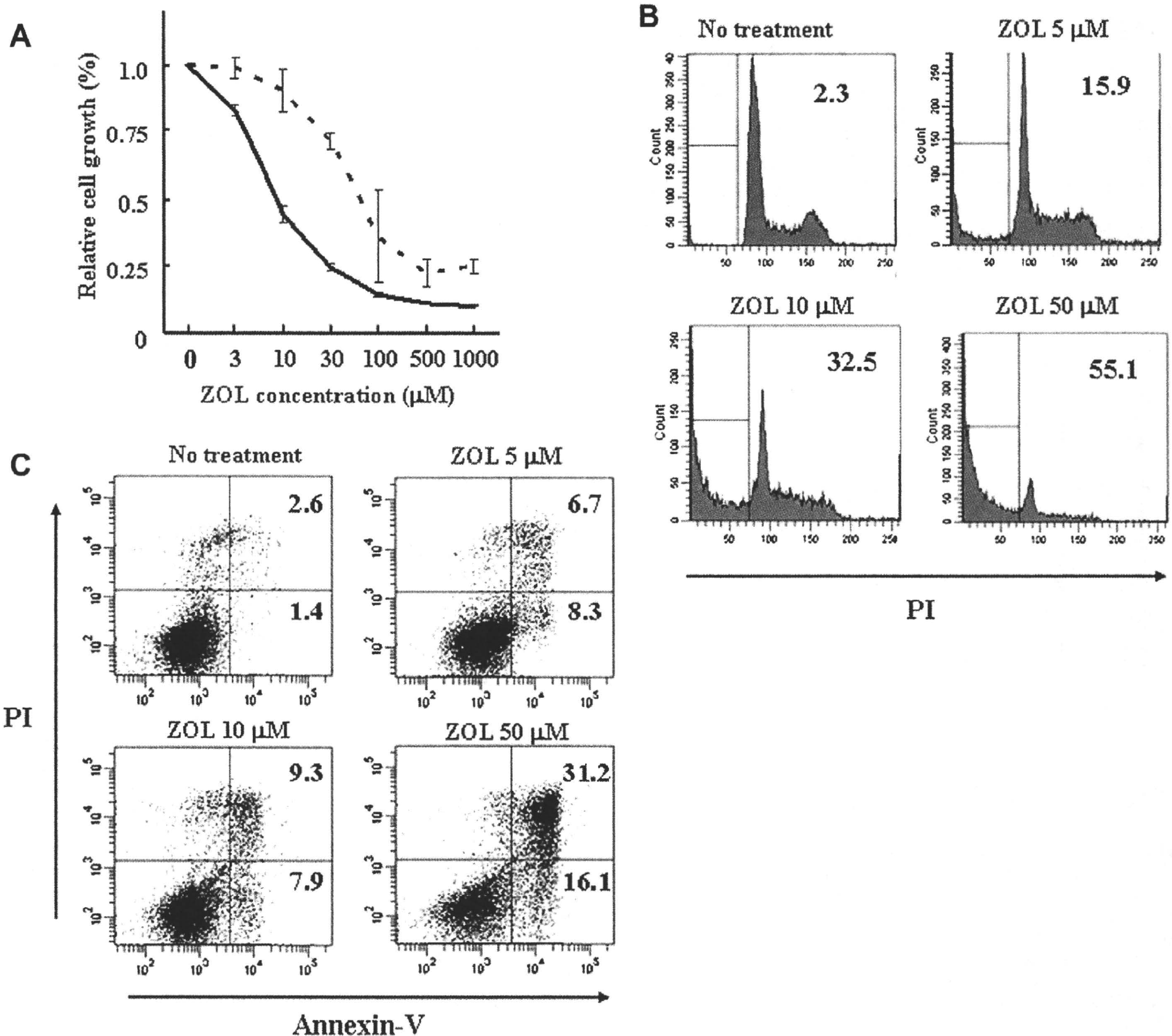

Fig. 4. ZOL treatment inhibits the proliferation of MM cells. (A) Cell proliferation was determined by the modified MTT assay as described in Section 2. ZOL treatment produced growth inhibitory effects in H2452 (solid line) and H28 (dotted line) MM cells in a dose-dependent manner. (B) Cell cycle analysis in H2452 MM cells using propidium iodide (PI) was performed after 72 h treatment with ZOL at the concentration indicated. Results are representative of three independent experiments. The numbers inside each histogram indicate the percentage of the subG1 fraction. (C) Determination of apoptosis induced by ZOL treatment at each concentration. Results are representative of three independent experiments. The numbers inside each quadrant indicate the percentage of the cell population with the quadrant characteristic.

IC_{50} which were obtained from the experiments above were used. We calculated the CIs and the Fa values at each dilution using the CalcuSyn soft as reported previously [33–36]. Dose-effect and CI-Fa plots illustrating the effects of PLK-1 siRNA and ZOL combinations are shown in Fig. 5C. As shown in the left panel of Fig. 5C, the treatment of PLK-1 siRNA combined with ZOL produced more growth inhibition than the treatments of each agent alone that are shown in Figs. 2B and 4A. The mathematically analyzed data of CI-Fa plots are shown in the right panel of Fig. 5C. In H2452 cells, the CI values at Fa 0.5, and 0.8 were 0.809 and 0.974, respectively; and in H28 cells, the CI values at Fa 0.5, and 0.8 were 0.082 and 0.836, respectively. These observations indicate that exposure to ZOL and the DNA-chimeric PLK-1 siRNA produced a synergistic effect on H2452 and H28 MM cell lines. We also investigated the alternation of the unprenylated RhoA expression in H2452 and H28 cells by the treatment of PLK-1 siRNA and ZOL. The combined treatment of concurrent PLK-1 siRNA and ZOL did not alter the unprenylated RhoA expression compared to the treatment of ZOL alone (Supplementary Fig. S1), suggesting that PLK-1 siRNA does not act on the prenylation of RhoA GTPase although PLK-1 siRNA diminishes PLK-1 expression.

4. Discussion

Synthetic siRNAs form complexes with liposomes, after which, the siRNA/liposome complex binds to the cell membrane and enters the cytoplasm via endocytosis. The complex then escapes from the endosome and releases its siRNA to the RNAi machinery [11,40]. Although single-stranded (ss) nucleic acids are rapidly degraded in serum or inside cells, ds nucleic acids are more stable than their