An ideal cancer therapeutic strategy involves the selective killing of cancer cells without affecting the surrounding normal cells. However, researchers have failed to develop such methods for achieving selective cancer cell death because of shared features between cancerous and normal cells. In this study, we have developed a therapeutic strategy called the cancer-specific insertions–deletions (InDels) attacker (CINDELA) to selectively induce cancer cell death using the CRISPR-Cas9 system. CINDELA utilizes a previously unexplored idea of introducing CRISPR-mediated DNA double-strand breaks (DSBs) in a cancer-specific fashion to facilitate specific cell death. In particular, CINDELA targets multiple InDels with CRISPR-Cas9 to produce many DNA DSBs that result in cancer-specific cell death. As a proof of concept, we demonstrate here that CINDELA selectively kills human cancer cell lines, xenografts human tumors in mice, patient-derived glioblastoma, and lung patient-driven xenograft tumors without affecting healthy human cells or altering mouse growth.

CRISPR / insertion/deletion | cancer / double-strand breaks

Genomic instability is a hallmark of most cancers (1–3). The Pan-Cancer Analysis of Whole Genomes study of the International Cancer Genome Consortium and The Cancer Genome Atlas have identified the genomic instabilities most frequently found in tumors (4, 5). In addition to single-nucleotide mutations, most cancer cells contain somatic mutations, which are primarily small insertions and deletions (InDels) that do not exist in neighboring normal cells. Whole-exome sequencing of solid tumors revealed that ~5% of the total somatic variation in cancer cells are InDels, thus suggesting that InDels can also be candidates for tumor-specific neo-antigen immunotherapy (6). A more recent study that analyzed 2,658 whole-cancer genomes reported that most tumors had 100 to 1,000 InDels (5). Incorrect DNA repairs by nonhomologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) might be a major cause of InDel formation in proliferating tumors (7). Recently, other DNA repair pathways including transcription-coupled repair and mismatch repair pathways were also suggested, which have contributed to InDel formation in different cancer cells (8).

Conventional cancer therapies target uncontrolled cancer cell growth (9). Owing to the continuous DNA replication required for proliferation, DNA damaging agents that inhibit DNA replication have been used as part of many conventional cancer therapies, including radiation and chemotherapeutic regimens. However, these treatments also damage the DNA in neighboring healthy cells, which then causes undesirable side effects including cell death and mutations (10). Therefore, an ideal cancer therapy would only target features that are unique to cancer cells. Recently, selective cancer cell killing was reported in cells that were addicted to a mutation or a cancer-specific protein produced by chromosomal translocation. The mutation or translocation breakpoint junctions were targeted with CRISPR-Cas9 (11, 12). Although these novel approaches were used to achieve selective cancer cell death, they are not extensively applicable because it is difficult to identify critical mutations or fusion proteins produced by translocation in most cancer cells.

CRISPR-Cas9 is a bacterial native immune system that inhibits bacteriophage infection by inducing sequence-specific DNA double-strand breaks (DSBs) in the bacteriophage...
genome (13). CRISPR RNA guides Cas9 endonuclease to specific DNA sequences in a bacteriophage to induce DNA DSBs. A DNA DSB is one of the most harmful types of DNA damage. A single unrepaired DSB can kill bacteria, and similar effects are predicted in other single-cell organisms including yeast (14–17). These drastic cell death effects in bacteria and yeast could be attributed to the preferential homologous recombination (HR) process to repair DNA DSBs. However, unlike bacteria and yeast, mammalian cells rely heavily on an alternative DSB repair pathway commonly referred to as NHEJ (18). NHEJ has two primary advantages for repairing sequence-specific DNA DSBs when compared with HR. First, NHEJ does not require a repair template. Thus, NHEJ can be used to repair DNA DSBs in all cell cycle phases. Secondly, owing to its mutagenic characteristics, NHEJ adds or removes nucleotides at the broken DNA site, thus destroying the enzymatic recognition sites used for sequence-specific DNA DSBs. Despite the improved repair pathway, mammalian cells still cannot tolerate high quantities of simultaneous DNA DSBs.

In this study, we developed a method called cancer-specific InDel attacker (CINDELA) that selectively kills cancer cells. CINDELA targets multiple InDel mutations produced during cancer development with CRISPR-Cas9 enzymes to only kill cancer cells. The CINDELA method was successfully applied to kill cancer cell lines, xenografted cancer cells in mice, patient-derived glioblastoma, and in a patient-driven xenograft (PDx) lung cancer model without affecting normal cells or mice.

**Results**

**Induced Cancer Cell Death Based on Multiple Simultaneous DNA DSBs.** We hypothesized that targeting InDels would produce high quantities of cancer cell-specific DNA DSBs to kill cancer cells (Dataset S1). First, we tested whether enzymatically induced DSBs can be sufficient to kill proliferating cancer cells using ER-AsiSI U2OS cells. ER-AsiSI U2OS cells express the AsiSI restriction enzyme that translocates into the nucleus in the presence of tamoxifen (4OHT) (19) (Fig. 1B). The current version of the human genome (GRCh38) contains 1,225 AsiSI recognition sites. Given that only 10 to 20% of AsiSI recognition sites can be cut in vivo, owing to their epigenetic status of DNA methylation (20, 21), we predicted that ~100 to 200 DSBs can occur depending on the cell type. When AsiSI was translocated into the nucleus after 4OHT treatment, almost all the ER-AsiSI U2OS cells were dead compared with U2OS cells treated with 4OHT (Fig. 1B). The DNA DSB marker γ-H2AX and apoptosis were induced in ER-AsiSI U2OS cells after 4OHT treatment (Fig. 1C and D). These data demonstrated that large quantities of enzymatically produced DSBs can kill proliferating cancer cells.

To test whether DNA DSBs induced by CRISPR-Cas9 can also kill cancer cells, we selected well-known CRISPR-Cas9–targeting sequences that appear repeatedly that range from a single to 20,000 targets in the human genome (Dataset S1). We observed that human embryonic kidney cells (HEK293T) started to die when the number of synthetic guide RNA (sgRNA) targets increased to more than 10, which created 20 DSBs in the genomes of diploid cells (Fig. 2A). HEK293T cell death was further enhanced when the transfected plasmid expressed the sgRNA and targeted a higher number of genomic locations (Fig. 2A). We delivered the same, multiple-target sgRNA using a lentiviral delivery into other types of tumor cells: HCT-116, MDA-MB-231, and K562. When the cells were cultured in competitive conditions (22), we observed gradual increases in cell death when the sgRNA that targeted a higher number of genomic locations was expressed in all these cells (Fig. 2B and C).

**Fig. 1.** CINDELA concept and cancer cell death induced by simultaneous multiple DSBs. (A) Schematic of the CINDELA approach. (B) Simultaneous, multiple DNA DSBs (generated with the restriction enzyme AsiSI) induced cell death in osteosarcoma cells (U2OS). (C) Time-dependent DSB occurrence caused by AsiSI localization confirmed by the signal of γ-H2AX. (D) The relative cell viability outcomes indicated that a significant number of cells died when AsiSI induced multiple DSBs. We quantified the cell viability and compared it with nontreated controls 6 d after the induction. *P* values were calculated using an unpaired Student’s two-sided t test. (E) The proportion of apoptotic cells confirmed that multiple simultaneous DSBs induced active cell death. *P* values were calculated using an unpaired two-sided Student’s t test.

To test whether DSBs at specific InDels produced by CRISPR-Cas9 can selectively kill cancer cells (CINDELA method), we determined whole-genome nucleotide sequences of U2OS and HCT-116 cells derived from osteosarcoma and colon cancer, respectively. For comparison, the whole genome of RPE1, a non-tumor cell line that originated from retinal pigment epithelium and immortalized by telomerase expression, was also sequenced. For the InDels identified in each cell line, we designed sgRNAs targeting cell line–specific InDels (see Materials and Methods and Datasets S2–S4 for details) and validated by in vitro cleavage (SI Appendix, Fig. S1). To investigate whether these sgRNAs can induce cell-specific DNA DSBs, we transfected 30 CRISPR-SpCas9 (Streptococcus pyogenes Cas9) ribonuclease protein (RNP) complexes that targeted U2OS-specific InDels into U2OS cells. The death of U2OS cells with U2OS-specific, InDel-targeting CRISPR-RNP complexes began at 72 h post-transfection. No cell death was observed when HCT-116–specific, InDel-targeting CRISPR-RNP complexes were transfected into U2OS cells (Fig. 3A). Furthermore, dose-dependent cell death was observed when the number of guide RNAs (gRNAs) was reduced from 30 to 18, 12, or 6 for RNP delivery (SI Appendix, Fig. S2). We also delivered cancer–cell–specific or multiple target sgRNAs in HCT-116 or U2OS cells using lentivirus. Unlike sgRNA targeting wherein either no-target or two-target loci were identified, sgRNA targeting affected 50 loci in the genome that killed both HCT-116
CINDELA Treatments Selectively Kill Patient-Derived Primary Tumors.

To prove that CINDELA treatment can be applied to actual tumor samples, we used the primary tumor cell line GBL-67 derived from a Korean glioblastoma patient. Based on whole-genome sequencing data, we designed 26 SaCas9 CINDELA gRNAs that targeted specific GBL-67 InDels. Because we did not have access to normal cells from the same individual, NSC-10 neural stem cells, which do not have the targeted InDels, were used as the control. AAVs expressing sgRNA and SaCas9 were generated and simultaneously transduced into primary glioblastoma GBL-67 and NSC-10 cells. We found that ~50 cell-specific DSBs induced by CINDELA treatment were sufficient to enhance apoptosis and kill glioblastoma cells (Fig. 4A). In contrast, the NSC-10 cells did not exhibit any growth attenuation after the same transduction.

CINDELA Treatment Suppresses Xenograft Tumor Growth and PDX In Vivo.

We subsequently tested whether CINDELA can inhibit in vivo tumor growth. We created a xenograft model with HCT-116 cells and injected lentivirus (2 × 10^7 viral particles/injection) with SpCas9 and sgRNA targeting multiple loci (MT2 for two loci, MT50 for 50 loci) or sgRNAs targeting HCT-116–specific 23 InDels (HMX23) for 2 wk on a daily basis. As expected, lentiviral treatment of MT50 and HMX23 significantly inhibited tumor growth compared with that of MT2 (Fig. 4B). When we treated higher titer lentivirus (2 × 10^8 viral particles/injection) expressing SpCas9 and MT50 sgRNA every 3 d for four times, the tumor sizes were not increased compared with the case in which the lentivirus was expressing SpCas9 and sgRNA with no predicted target locus (NT) (Fig. 4C). Mice did not show any prominent symptoms during lentiviral treatments, thus suggesting that lentiviral-delivered CINDELA did not affect normal mice tissues and cells.

U2OS cell xenografts were created in nonobese diabetic severe combined immunodeficient (NOD-SCID) mice and monitored until the tumor size reached ~1 cm. Either AAVs expressing U2OS-specific CINDELA sgRNAs and SaCas9 or AAVs expressing only SaCas9 were then injected into the mice five times within a 3-d interval for 2 wk. As shown in Fig. 4C, we observed that tumor growth was selectively inhibited by the injection of the AAVs that expressed U2OS-specific CINDELA sgRNAs and SaCas9. The HA-tag of SaCas9 was detected in tumor sections from mice injected with a) SaCas9 only and b) with SaCas9 with sgRNAs (SI Appendix, Fig. S3A). Mice did not show any prominent symptoms during AAV treatment, which suggested that AAV-delivered CINDELA did not affect normal tissues and cells (SI Appendix, Fig. S3B). To check the specific cell death pathway induced by the CINDELA treatment, we harvested the remaining tumors and stained them for γ-H2AX or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). As anticipated, CINDELA-targeted tumors led to a significant induction of γ-H2AX (Fig. 4D) and apoptotic cell death (SI Appendix, Fig. S3C) in addition to TUNEL signaling (Fig. 4E) when compared with control tumors.
the control (with no gRNA), a significant number of cells died. When we pooled AAV with 30 different gRNAs targeting U2OS-sgRNAs. (Fig. 3. A) CINDELA-induced cancer cell death introduced by the SpCas9 RNP complex with 30 gRNAs targeting U2OS-specific InDels. (Scale bar, 300 μm.) (B) CINDELA-induced cell death introduced by the lentivirus-delivered SpCas9. We tested by using multiple target gRNAs in conjunction with cell type-specific gRNAs (23 sgRNAs for HCT-116 and 21 sgRNAs for U2OS). The relative cell viability and the proportion of apoptotic cells confirmed the specific cell death by CINDELA both on multiple target gRNAs and cell type-specific InDel targeting gRNAs. (C) CINDELA-induced cell death introduced by the AAV-delivered SaCas9. When we pooled AAV with 30 different gRNAs targeting U2OS-specific InDels and SaCas9 and delivered it to U2OS cells. Compared with the control (with no gRNA), a significant number of cells died.

Lastly, we tested CINDELA’s efficacy in PDXs, which are predictive preclinical models for cancer treatment (24). After sequencing tissue from an established lung cancer PDX, we identified ~30 CINDELA targets (29 gRNAs for SPX4-073 and 22 gRNAs for SPX4-318). Xenograft tumor size was monitored for 2 wk after the injection of AAVs (five times within a 3-d interval) that expressed SaCas9 with the targeted sgRNAs. PDX tumor growth was inhibited by the AAVs that expressed sgRNAs and SaCas9. In contrast, AAVs that only expressed SaCas9 did not inhibit tumor growth (Fig. 4D and SI Appendix, Fig. S4A and B). In addition, the mice did not exhibit any significant symptoms during the AAV treatment (SI Appendix, Fig. S4C).

Discussion
Selective cancer cell killing is considered the best therapeutic strategy against cancer; however, the implementation has remained elusive. Inhibitors of DNA replication, DNA repair, and cell metabolism have been investigated as unique cancer cell targets (25). However, shared mechanisms between cancer and normal cells, including progenitor and stem cells, remain a considerable hurdle. In this study, we developed a method to induce targeted cancer cell death by utilizing CRISPR-Cas to introduce simultaneously multiple DNA DSBs. To overcome the risk of targeting the DSBs in normal cells, we focused on short InDel that CRISPR-Cas can distinctively recognize. In the future, an engineered CRISPR-Cas system with high fidelity (26, 27) can be used to achieve CINDELA targeting to single base-level changes with increased precision.

Cell death caused by multiple DSBs using CRISPR-Cas was previously reported as an unintended side effect of genome-wide CRISPR screens. Hart and colleagues reported that promiscuous gRNAs that targeted at least 20 genomic loci can introduce significant growth defects, similar to sgRNAs that targeted the essential genes (28). Similarly, genes with higher copy numbers exhibited strong lethal effects in the CRISPR screen, which can be considered as false positives (29, 30). In our experiments, CINDELA required ~10 to 30 sgRNAs to kill cancer cells, which is similar to previous reports. The requirement for such a high number of DSBs is primarily attributed to the highly effective NHEJ and potentially MMEJ DNA repairs in mammalian cells (31).

Cancer cells in tumors are heterogeneous, owing to the accumulation of mutations during their rapid proliferation. InDel mutations targeted by CINDELA can only affect a subset of the cancer cells in a tumor. Thus, the choice of the InDel mutations that need to be targeted is critical. Most abundant InDels, possibly generated at an early tumorigenesis stage, would likely be the most effective at irradiating the most abundant tumor population. Any cancer cells that survived through an initial CINDELA treatment can be retargeted by choosing additional InDel mutations in successive treatments.

Multiple sgRNA deliveries would be a critical part of the implementation of the CINDELA treatment. Although we could not directly measure the number of sgRNAs in each cell, a gradual increase in cell death with increased CINDELA sgRNAs was observed. In general, the cell death effect is directly attributed to increased DNA DSB events. However, different DNA repair capacities in different cancer cells can cause differential cell death effects. Acute cell death caused by the introduction of multiple DSBs follows an intact apoptotic cell death pathway in most cases. However, it is possible that several cancer cells, especially those carrying a p53 mutation, may require several days for cell death to occur, owing to the lack of proper apoptotic pathways. We speculate that multiple DNA DSBs may trigger a senescent type of cell death characterized by growth inhibition within these cancer cells. The detailed mechanisms of CINDELA-induced cell death should be investigated further.

Combinatorial treatment with other drugs, such as DNA DSB response inhibitors or other chemotherapeutic reagents, can be considered to enhance the efficiency of CINDELA methods. We tested the ATM inhibitor (KU60019) and cisplatin with CINDELA treatment and observed the synergistic improvement of specific cell death (SI Appendix, Fig. S5). Many inhibitors of NHEJ might also be beneficial to increase the DSB effect by CRISPR-Cas (32–34). PARP1 recognizes DNA DSBs in G1 cells to direct both NHEJ and strand-break repairs. PARP1 inhibitors are approved for clinical treatments of ovarian tumors. They undergo clinical trials for breast tumors that have lost the homologous recombination repair pathway (25). Thus, PARP1 inhibition might increase CINDELA’s efficacy for cancer treatment.

In this study, we presented a therapeutic approach to selectively kill cancer cells with minimal side effects to healthy
cells using the CRISPR-Cas system. Most cancers have high mutational burdens. Therefore, our method is a practical cancer treatment that warrants consideration for future cancer therapy.

Materials and Methods

Cell lines. We purchased the following cell lines from American Type Culture Collection (ATCC): HCT116 (CCL-247), U2OS (HTB-96), HEK293T (CRL-3216), and RPE1 (CRL-4000). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), which contained 10% fetal bovine serum (FBS) (General Electric ≥ Healthcare), 100 μM penicillin G (Life Technologies), and 100 μg/mL streptomycin (Life Technologies). The patient-driven glioblastoma cell line GB-L-67 and neural stem cell NSC-10 were established and maintained (35) and used in this study following the approval of the Institutional Review Board (IRB) at the Seoul National University Hospital (IRB No. H-2014-117-1028). These cells were cultured in DMEM, which contained 20% FBS (GE Healthcare), 100 μM penicillin G (Life Technologies), and 100 μg/mL streptomycin (Life Technologies) in a 5% hypoxia chamber.

Cell Death Induced by Simultaneous Multiple DSBs Using AsI. U2OS cells with ER-AuSi (19) were kindly provided as a gift by Tanya Paull (University of Texas at Austin, Austin, TX). ER-AuSi U2OS cells were grown in DMEM, which contained 10% FBS (GE Healthcare). Subsequently, 1 × 10^6 cells were seeded into a 6-well plate, and 4-hydroxytamoxifen (4-OHT) (catalog [cat] no. H7904, Merck) was added to obtain a final treatment concentration of 300 nM. After 4 h of 4-OHT treatment, the media were refreshed, and the cells were incubated for 6 additional days. Cell survival was determined with either a colony-forming assay with methylene blue staining (cat no. M9140, Merck) or the CellTiter-Glo assay, performed according to the manufacturer’s protocol (GS241, Promega).

Cell Death Caused by Simultaneous Multiple DSBs Using CRISPR. sgRNAs were designed to target multiple loci in the human genome with computational valuation for their targets with the use of the Cas-Offinder (36) against the human GRCh38 reference genome. Each sgRNA sequence was cloned into a sgRNA expression vector with a modified mouse U6 promoter from the pM179 plasmid (cat no. 85996, Addgene). A total of 1 d before transfection, 1.5 × 10^4 HEK293T cells were seeded in each well of a 96-well plate. Subsequently, 200 ng SpCas9 (cat no. 43945, Addgene) and sgRNA expression plasmids were transfected into the cells by using Lipofectamine 3000 (cat no. L3000015, Thermo Fisher Scientific). A total of 3 d post-transfection, cell viability was measured using the CellTiter-Glo Luminescence Cell Viability Assay (cat no. G7570, Promega) according to the manufacturer’s instructions. Luminescence was measured using a microplate reader and Gen5 software (BioTek).

CINDELA gRNA Design. We performed whole-genome sequencing of cell lines and xenograft tissues using an Illumina NovaSeq 6000, which targeted 30X coverage with 150-base pair paired-end reads according to the manufacturer’s instructions (Illumina). After trimming the adapter sequences with trimmomatic (version 0.39) (37), we mapped the reads against the human genome (GRCh38) with BMA-MEM (version 0.7.17-r1188) (38). The reads were mapped to the database with both human (GRCh38) and mouse (mm10) genomes. Filtered reads were then preferentially mapped to the human genome for further analysis with the xenograft samples. After removing the duplicated hits with Samtools (version 1.10) (39), we used Strelka2 (version 2.9.10) (40) with the default setting for calling germline variations. To design the sgRNAs for cancer cell line-specific InDels, we first filtered the InDel sequences that had lengths equal to 3 to 8 bps and designed sgRNAs with those InDels for either SpCas9 or SaCas9. The heterogeneity, allele depth, and InDel availability in either the gnomAD database (version 2.1.1) (41) or other cancer cells we had previously tested were also considered when sgRNAs were chosen. Finally, we checked the possibility of off targets using Exonerate (version 2.4.0) (42) with a two-mismatch allowance, and all final candidate targets were visually inspected. All code used in this analysis is available on GitHub (https://github.com/taejonlab/CINDELA-toolbox).

CINDELA Treatment with RNPs. We ordered the gRNAs that targeted cancer-specific InDels and the tracer RNAs (Integrated DNA Technologies). RNP complexes were formed by combining 20 μg equimolar mixture of tracer and gRNAs with 15 μg SpCas9 (43). We delivered RNPs with Lipofectamine CRISPRMAX Cas9 Transfection Reagent (cat no. CMAX00001, Thermo Fisher Scientific) according to the manufacturer’s protocol.

CINDELA Treatment with Lentivirus. The lentiviral plasmid expressing CRISPR/SpCas9 under the EF-1α promoter (Addgene, no. S2962) was used to produce lentiviral production as described previously (22). Lentiviral particles were harvested on the third day after transfection into HEK293T cells and were

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transduced to the K562, MDA-MB-231, or HCT-116 cell lines. For seven days, lentivirus-integrated cells were selected using blasticidin (concentration: 4 g/mL). The cell fitness was estimated by measuring the ratio of each fluorescence using flow cytometry cell sorting at days 3, 6, and 9 after the onset of the coculture.

CINDELA Treatment with AAV. The AAV plasmid expressing CRISPR/SaCas9 under the cytomegalovirus (CMV) promoter was purchased (Addgene, no. 61591). AAV virus particles were harvested on the third day after transfection into HEK293T cells and concentrated as described previously (44). The AAV serotype two was used for viral packaging, and 2.81 × 10^10 viral particles were used to transduce target cells for CINDELA.

Elevenchoice in somatic mammalian cells.

DNA double-strand break repair-pathway.

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