

Efficient primary culture model of patient-derived tumor cells from colorectal cancer using a Rho-associated protein kinase inhibitor and feeder cells

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Received January 30, 2019; Accepted June 24, 2019

DOI: 10.3892/or.2019.7285

Abstract. *In vitro* culture of patient-derived tumor cells offers many advantages in the development of novel therapies for colorectal cancer. Although various culture systems have been developed, the long-term expansion of patient-derived tumor cells remains challenging. The present results suggested that tumor cells isolated from colorectal cancer patient-derived xenografts can be efficiently immortalized in conditioned medium from irradiated feeder cells containing Y-27632, a rho-associated coiled-coil containing protein kinase (ROCK) inhibitor. Patient-derived tumor cells proliferated rapidly, reaching 90-95% confluence in ~6 days. Short tandem repeat analysis suggested that these tumor tissues and cultured cells presented 13 identical short tandem repeat loci, including Amelogenin, Penta E, Penta D, D2S1338 and D19S433. Their epithelial phenotype was confirmed by staining for epithelial cell adhesion molecule and cytokeratin 20, whereas vimentin was used as a mesenchymal marker. When cells were transferred to 3D cultures, they continued to proliferate, forming well-defined tumor spheroids. Expression levels of human telomerase reverse transcriptase and C-Myc mRNA were increased in cultured cells. Finally, immortalized cells were used for the screening of 65 anticancer drugs approved by the

Food and Drug Administration, allowing the identification of gene-drug associations. In the present study, primary culture models of colorectal cancer were efficiently established using a ROCK inhibitor and feeder cells, and this approach could be used for personalized treatment strategies for patients with colorectal cancer.

Introduction

Colorectal cancer is one of the most common malignancies in the world. It is the third most common cancer in men and the second most common cancer in women worldwide according to the Global Cancer Statistics 2018 (1). In addition, the incidence rates of colorectal cancer are ~3-fold higher in developed countries compared with developing countries (1). To understand the molecular and cellular mechanism of colorectal cancer, previous studies have tried to establish several reliable *in vitro* cancer models (2-5). For decades, primary patient-derived tumor xenografts have been one of the most widely used cancer models (6). However, the use of animals can be challenging, laborious and time-consuming. *In vitro* culture systems for patient-derived intestinal epithelial cells have many advantages and may facilitate development of novel therapies for colorectal cancer (7,8). Long-term expansion of patient-derived tumor cells is challenging; however, conditional reprogramming methodologies for cell culture with a combination of irradiated feeder cells and a rho-associated coiled-coil containing protein kinase (ROCK) inhibitor, Y-27632, have been proposed to overcome the limitations of traditional methods, including the short lifespan of cancer cells and the low success rates of long-term expansion without the use of viral infection or gene transduction (9-12). Moreover, Clevers (13) have successfully obtained *in vitro* expansion of epithelial cells in a three-dimensional matrix by designing an organoid culture that can faithfully recapitulate the physiology and functionality of intestinal epithelia, although these cells are not readily applicable to high-throughput drug screening for evaluating drug responses. In the present study, it was observed that tumor cells isolated from colorectal cancer

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Key words: primary culture model, rho-associated coiled-coil containing protein kinase inhibitor, irradiated feeder cells, conditionally reprogramming, patient-derived tumor cells, patient-derived xenografts

patient-derived xenograft (PDX) could be efficiently immortalized in conditioned medium from irradiated feeder cells containing a ROCK inhibitor Y-27632.

The present results suggested that genes related to the ROCK signaling pathway playing a major role in the mechanism of conditional reprogramming were also associated with the immortalization of colorectal cancer cells from human-derived xenograft tumors. Conditional reprogramming models could be effectively used to evaluate drug response for personalized therapeutic approaches, and the present study performed a screening for anticancer drugs approved by the Food and Drug Administration. Finally, the potential applications of this established model for detecting gene-drug interactions and making clinical decisions were investigated.

Materials and methods

Establishment of PDXs. PDXs were established and analysis of mutation profiles was performed as previously described (11). Briefly, fresh tumor specimens were obtained from male and female patients (female to male ratio, 1:1) with colorectal cancer treated with surgical resection in Samsung Medical Center from December 2011 to February 2013. Clinical characteristics are presented in Table I. The specimens were maintained in RPMI medium (Gibco; Thermo Fisher Scientific, Inc.) containing 3% penicillin streptomycin, cut into 2-3 mm³ sized pieces, and embedded in high-concentrated Matrigel (BD Biosciences). Tumor fragments embedded in Matrigel were implanted into subcutaneous pockets of female BALB/c nude mice (age, 6-8 weeks old; weight, 16-18 g; Orient Bio, Inc.), which were made in each side of the lower back (n=5-6 for each tumor sample). Mice were maintained under standard conditions of temperature (20-26°C), relative humidity (30-70%) under a 12-h light/dark cycle with free access to food and water. When tumors reached 1,000 mm³ in volume, tumor tissues were collected for primary cell culture. All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Samsung Biomedical Research Institute. The present study performed in patients and animals was approved by the Institutional Review Board of Samsung Medical Center.

Cell isolation from xenograft tumors. Tumor tissue from xenografts was cut into small pieces (~1 mm³), washed with 70% ethanol and ice-cold PBS, mechanically dissociated using gentleMACS Dissociator (Miltenyi Biotec) for 45 sec at room temperature, and subsequently incubated at 37°C in S/F M199 media (Gibco; Thermo Fisher Scientific, Inc.) containing 200 U/ml collagenase (Gibco; Thermo Fisher Scientific, Inc.) and 0.1 mg/ml DNase (Roche Diagnostics) for 90 min at 37°C on a tube rotator. The dissociated cell suspension was passed through a cell strainer (Falcon; BD Biosciences; cat. no. 352340; pore size, 40 µm), washed with RPMI (Gibco; Thermo Fisher Scientific, Inc.) containing 20% FBS (Biowest LLC), and centrifuged at 1,000 rpm for 3 min. Viable cells were counted using a hemocytometer and a light microscope (magnification, x10) and subjected to cell culture.

Cell cultures. Dissociated cells were cultured in a mixture of conditioned medium from irradiated 3T3-J2 fibroblasts and

fresh F medium (3:1 ratio), which contained three parts of DMEM (Thermo Fisher Scientific, Inc.), one part of Ham's F-12 Nutrient Mixture (Gibco; Thermo Fisher Scientific, Inc.) and the following supplements: 5% FBS (Biowest LLC), 0.4 µg/ml hydrocortisone (Sigma-Aldrich; Merck KGaA), 5 µg/ml insulin (Sigma-Aldrich; Merck KGaA), 8.4 ng/ml cholera toxin (Sigma-Aldrich; Merck KGaA), 10 ng/ml epidermal growth factor (EGF; Thermo Fisher Scientific, Inc.), 10 µmol/l Y-27632 (Enzo Life Sciences), 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.), 20 µg/ml gentamicin and 500 ng/ml Fungizone. For 3D culture, cells were embedded in Matrigel (BD Bioscience) on ice and plated into 24-well plates (1x10⁴ cells with 50 µl of Matrigel per well). Matrigel was polymerized at 37°C for 15 min. In each well, 1 ml of a mixture of conditioned medium and fresh F media (3:1 ratio) supplemented with 5 µmol/l Y-27632 was added. The medium was replaced every other day. All cells were maintained at 37°C in a humidified incubator with 5% CO₂ and passaged at a ratio of 1:4 when they reached 80-90% confluency. The number of cells was counted every 3 days using the C-Chip hemocytometer (NanoEnTek, Inc.).

Differential trypsinization. Cells at 95% confluence were washed with PBS and treated with 1 ml trypsin/EDTA (0.25%; Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 1 min. Fibroblasts were efficiently detached from the bottom of the flask, whereas epithelial cells remained attached the flask. Trypsin digestion was terminated by the addition of complete culture media, and the fibroblasts were removed using PBS.

Epithelial cell enrichment. Dissociated cells were incubated with anti-epithelial cell adhesion molecule (EpCAM) microbeads (cat. no. 130-061-101; Miltenyi Biotec, Inc.) for 30 min at 4°C. Subsequently, labeled cells were collected on a magnetic separation column. After removal of the column from the magnetic field, EpCAM⁺ cells were eluted with PBS for cell culture. Dissociated cells or EpCAM⁺ cells were cultured in conditioned medium.

Conditioned medium from irradiated 3T3-J2 fibroblast. The fibroblast cell line 3T3-J2 was obtained from the American Type Culture Collection. Cells were maintained at 37°C with 5% CO₂ in DMEM containing 10% bovine calf serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.). To prepare conditioned medium from irradiated 3T3-J2 fibroblast, suspension cells were irradiated at 30 Gy and plated at a density of ~70% in T-175 flasks with 30 ml F medium. After 3 days, the medium was collected, and fresh F medium was replaced for an additional 3 days. The collected medium was passed through a Stericup filter unit (pore size, 0.22 µm; EMD Millipore) and stored at -80°C. Conditioned medium was mixed with fresh F medium at a ratio of 3:1 and supplemented with 5 µmol/l Y-27632.

Immunohistochemistry and hematoxylin and eosin (HE) staining. Cells were fixed with 4% paraformaldehyde for 1 h at room temperature and embedded in paraffin. Paraffin-embedded sections (thickness, 4 µm) were dewaxed

Table I. Clinical information of PDXs used for cell culture.

Sample	Age	Sex	Tumor	Cell type	Stage ^a	Preoperative chemotherapy
PDX1	51	F	Adenocarcinoma	MD	IV	No
PDX2	57	M	Adenocarcinoma	MD	IV	No
PDX3	74	F	Adenocarcinoma	WD	IV	No
PDX4	46	F	Adenocarcinoma	MD	IV	Yes

^aThe American Joint Committee on Cancer 7th staging system was used (46). PDX; patient-derived xenograft, MD; moderately differentiated, WD; well differentiated; F, female; M, male.

in xylene for 30 min at room temperature and rehydrated in a graded alcohol series. Endogenous peroxidase was blocked with 3% H₂O₂ for 30 min at room temperature. Before incubating the samples with the primary antibodies, sections were immersed in 10 mM citrate buffer (pH 6.0), rinsed in TBS. The following primary antibodies were incubated with the samples for 1 h at room temperature: Anti-cytokeratin 20 (CK20; 1:100; Dako; cat. no. M7019, Agilent Technologies, Inc.) and Ki67 (1:100; Dako; cat. no. M7240, Agilent Technologies, Inc.). Sections were subsequently treated with a biotin-labeled secondary antibody (cat. no. B2763; Thermo Fisher Scientific, Inc.) for 30 min at room temperature and incubated with an avidin-biotin-peroxidase complex for 30 min at room temperature. 3,3'-Diaminobenzidine was used as the chromogen followed by HE counterstaining. The sections were stained with HE for 5 min at room temperature to examine cell morphology. Stained sections were visualized and captured using a slide scanner (Aperio ScanScope slide scanner; Leica Microsystems, Inc.) with a 20X objective (final magnification, x200).

Immunofluorescence staining. Cells were grown on poly-L-lysine coated coverslips. After reaching 50–60% confluence, cells were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature and incubated in blocking buffer (2% BSA and 0.2% Triton X-100 in PBS) for 1 h at room temperature. These cells were labeled with primary antibodies against EpCAM (Biolegend; cat. no. 324210; 1:20 dilution), Vimentin (Santa Cruz Biotechnology, Inc.; cat. no. SC6260; 1:100 dilution) and CK20 (Dako; Agilent Technologies, Inc.; cat. no. M7019; 1:200 dilution) overnight at 4°C. Samples were incubated for 1 h at room temperature with secondary antibody (Alexa Fluor 546 goat anti-mouse IgG; Gibco; Thermo Fisher Scientific, Inc.; cat. no. MOP-A-11003; 1:40 dilution). Coverslips were mounted onto glass slides using VECTASHIELD mounting media (Vector Laboratories), sealed with nail polish to prevent drying, and stored at 4°C. Slides were analyzed with a laser scanning confocal microscope (magnification, x20). DAPI and protein signals were detected at excitation wavelengths of 633 and 488 nm, respectively.

Short tandem repeat (STR) analysis. STR analysis was performed using PowerPlex 18D system (Promega Corporation) according to the manufacturer's instructions.

DNA extracted from tumor tissues and cell pellets was amplified by multiplex PCR for 18 loci, including 17 STR loci (D3S1358, tyrosine hydroxylase 1, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, von Willebrand factor type A, D8S1179, TPOX, fibrinogen α chain, D19S433 and D2S1338) and amelogenin. Primers and DNA polymerase were included in the kit (cat. no. DC1802; Promega Corporation). The internal lane standard was labeled with the dye WEN (included in the kit). PCR products were electrophoresed on an ABI 3130xL Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) and analyzed with GeneMapper 4.0 software (Thermo Fisher Scientific, Inc.) using allelic ladders supplied by Applied Biosystems (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from patient-derived cancer cells (PDCs) and tumor tissues from xenografts using a RNeasy Mini kit (Qiagen). In total, 500 ng RNA was reverse transcribed using murine leukemia virus reverse transcriptase (cat. no. M0253S; New England Biolabs, Inc.) primers and reaction buffer were included in the kit (New England Biolabs, Inc.) for 1 h at 42°C. The RT-qPCR amplification was performed using a SYBR Green Master Mix (Roche Diagnostics) in a real-time system under the following conditions: Initial denaturation at 95°C for 60 sec, followed by 45 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, with a final elongation step at 72°C for 2 min. Human-specific PCR primers (Roche Diagnostics) were used to analyze expression levels of the following genes: Human telomerase reverse transcriptase (hTERT), C-Myc, p16, CDK4 and GAPDH. The primers used for RT-qPCR analysis were the following: hTERT forward, 5'-CTACTCCTCAGG CGACAAGG-3' and reverse 5'-TGGAACCCAGAAAGA TGGTC-3'; C-Myc forward, 5'-TCAAGAGGCGAACACACA AC-3' and reverse, 5'-GGCCTTTTCATTGTTTTCCTA-3'; p16 forward, 5'-GCACCAGAGGCAGTAACCAT-3' and reverse, 5'-GGAATCCCGTAGCTTCCCTA-3'; CDK4 forward, 5'-GAAACTCTGAAGCCGACCAG-3' and reverse, 5'-CCT GGGTTCAGCAGAAAGAG-3'; GAPDH forward, 5'-CTC AGACACCATGGGGAAGGTGA-3' and reverse, 5'-ATGATC TTGAGGCTGTTGTCATA-3'. mRNA levels of specific genes were calculated using the 2^{- $\Delta\Delta C_q$} method (14) and normalized to GAPDH.

Mutation profiling. Mutational analysis of PDXs was performed as previously described (15).

Anticancer drug screening. PDCs grown in conditioned medium were collected and seeded into 384-well plates at a density of 500 cells/well. After plating, cells were treated with 65 different drugs under clinical and preclinical investigation in seven serial 4-fold dilutions ($n=2$ for each condition) using a Janus Automated Workstation (PerkinElmer, Inc.). The same drug library was used in a previous study (16). After 6 days of incubation at 37°C in a humidified incubator, cell viability was analyzed using an ATP monitoring system based on Firefly luciferase enzymatic activity (ATPLite 1step; PerkinElmer, Inc.). Viable cells were measured using an EnVision Multilabel Reader (PerkinElmer, Inc.). Drug sensitivity was analyzed by assessing the IC_{50} , slope of the dose-response curve and area under the dose-response curve (AUC). Drugs were stored and diluted according to the manufacturer's instructions (Selleck Chemicals).

Statistical analysis. All experiments were repeated three times for each sample. Data are presented as the mean \pm SEM. To compare gene expression levels between the two samples in the PCR analysis, expression levels derived from the PDX samples were set to zero, and relative quantification was calculated. Data were analyzed using Student's t-test. In the drug screening analysis, the mean and SD of the AUC for every drug were calculated using a reference samples containing 462 patient-derived tumor cells across 14 cancer types (16), and Z-scores or standard scores of AUC were calculated for each drug. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using Graphpad Prism (version 6; GraphPad Software, Inc.).

Results

Primary culture of epithelial cells from colon cancer PDXs.

In our previous studies, a total of 97 PDXs were successfully established from 143 colorectal cancer specimens (15,17). Among 18 xenografts, 10 (55.5%) led to the establishment of PDCs. In the present study, four PDCs were selected and characterized since their genomic profiles was previously identified (Table I). In addition, PDCs could grow over 10 passages. Tumor tissues derived from xenografts were dissociated mechanically and enzymatically. Dissociated cells were cultured for >5 passages (>30 days) at 37°C in a conditioned medium collected from cultures of irradiated 3T3-J2 cells with 5 $\mu\text{mol/l}$ of Y-27632. PDCs were grown in epithelial cell colonies (Fig. 1) and rapidly proliferated to reach 90% confluence after ~6 days (Fig. 2). Primary cultures of human tumor fragments are frequently contaminated by rapidly-proliferating tumor-associated stromal fibroblasts (18-21). After 3 days, small clusters of epithelial cells began to be attached to the Petri dish, and large colonies of epithelial cells surrounded by stromal fibroblasts were detected after 14 days. For a pure culture of PDCs, differential trypsinization methods were used to remove stromal fibroblasts (Fig. 3A). PDC3 floated as small spheroid cultures for 3 days during initial plating. However, they adhered when floating cells were seeded into new flasks (Fig. 3B).

Characterization of immortalized PDCs. PDCs grew in monolayers and displayed epithelial morphology.

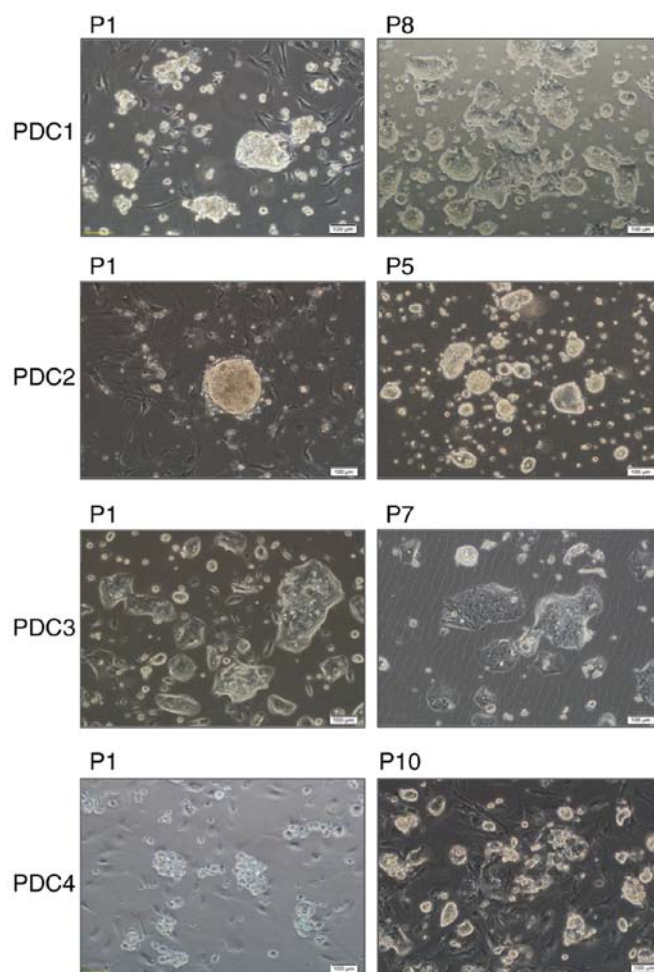


Figure 1. Propagation of colorectal cancer PDCs. Cells isolated from patient-derived xenografts tissues were cultured in conditioned medium from irradiated cells containing 10 μM ROCK inhibitor (Y27632). Small colonies of epithelial cells could be observed after 1 day. Scale bar, 100 μm ; magnification, $\times 10$. PDC, patient-derived cells; P, passage number.

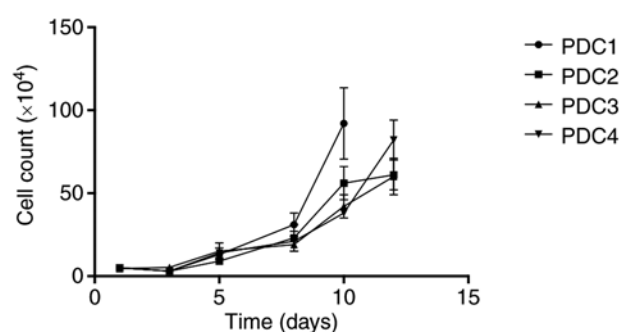


Figure 2. Growth curve of epithelial cells isolated from different PDXs. PDX, patient-derived xenograft; PDC, patient-derived cells.

Immunocytochemical analysis revealed that epithelial colonies expressed high levels of membrane-localized EpCAM (Fig. 4). Colon epithelial cell-specific marker CK20 was observed in the cytoplasm and cell membranes of epithelial colonies (Fig. 4). In contrast, the mesenchymal marker vimentin was shown to specifically stain fibroblasts (22), and showed negative staining for all four PDCs (Fig. 4). Epithelial cells were grown in 2D cultures on stromal cells (Fig. 5A). When they

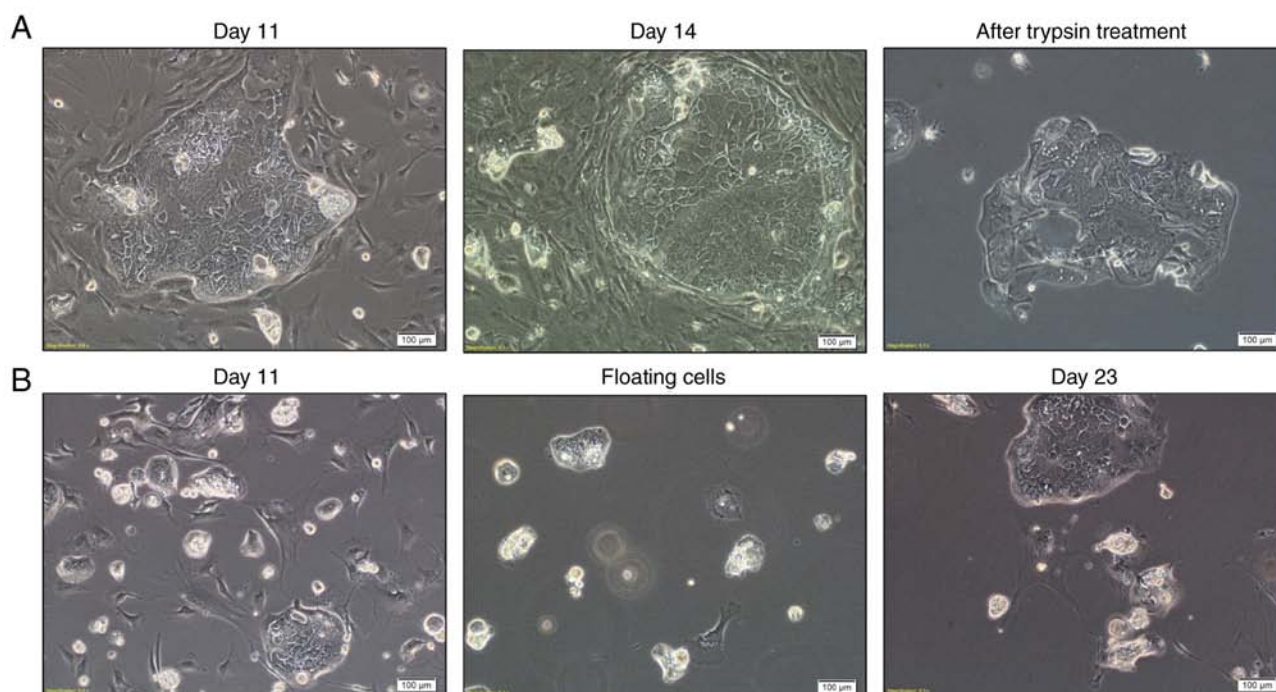


Figure 3. Growth of epithelial cell colonies. Epithelial cells isolated (A) without EpCAM microbeads from PDX3 tissue. Epithelial cell colonies firmly attached to the flask surface and differential trypsinization was used to separate stromal cell and epithelial cells. (B) Epithelial cells isolated with EpCAM microbeads from PDX3 tissue. EpCAM⁺ epithelial cells proliferated as floating cells without stromal cell contamination. Scale bar, 100 µm; magnification, x10. PDX, patient-derived xenograft; EpCAM, epithelial cell adhesion molecule.

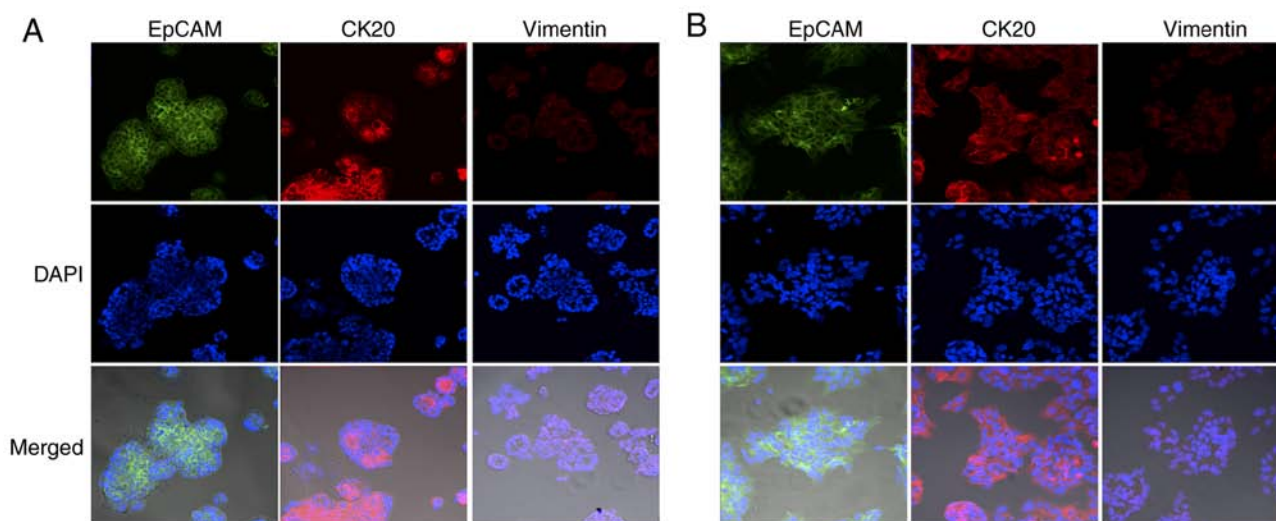


Figure 4. Immunofluorescence staining of epithelial cells isolated from (A) PDX2 and (B) PDX3. Cells were stained by anti-EpCAM, an epithelial cell marker, in green, by anti-CK20, a colon cancer marker, in red, and by anti-vimentin a fibroblastic/mesenchymal marker, in red. Nuclei, in blue, were stained by DAPI. Scale bar, 100 µm; magnification, x10. EpCAM, epithelial cell adhesion molecule; PDX, patient-derived xenograft; CK20, cytokeratin 20.

were transferred to Matrigel, they formed well-defined spheres without stromal fibroblasts (Fig. 5B). Lumen formation was observed in PDC spheres in cells stained with proliferation marker protein Ki67 and CK20 (Fig. 5C). STR analysis was performed for 18 loci on different chromosomes to verify that these PDCs were derived from PDXs without contaminations from other cells during passaging (Table II).

Cooperative effect of feeder cells and ROCK inhibitor on primary culture. Previous studies have shown that

a combination of feeder cells and ROCK inhibitor is important for primary cell immortalization (11). Irradiated feeder cells can induce pronounced telomerase activity and hTERT expression in keratinocytes (12). To determine whether PDCs exhibited a similar phenomenon, RT-qPCR was used to measure the expression levels of hTERT. The present RT-qPCR analysis suggested that hTERT and CDK4 mRNA expression was increased in PDCs grown in conditioned medium on irradiated feeder cells with Y-27632 (Fig. 6A). It has been shown that ROCK inhibitor

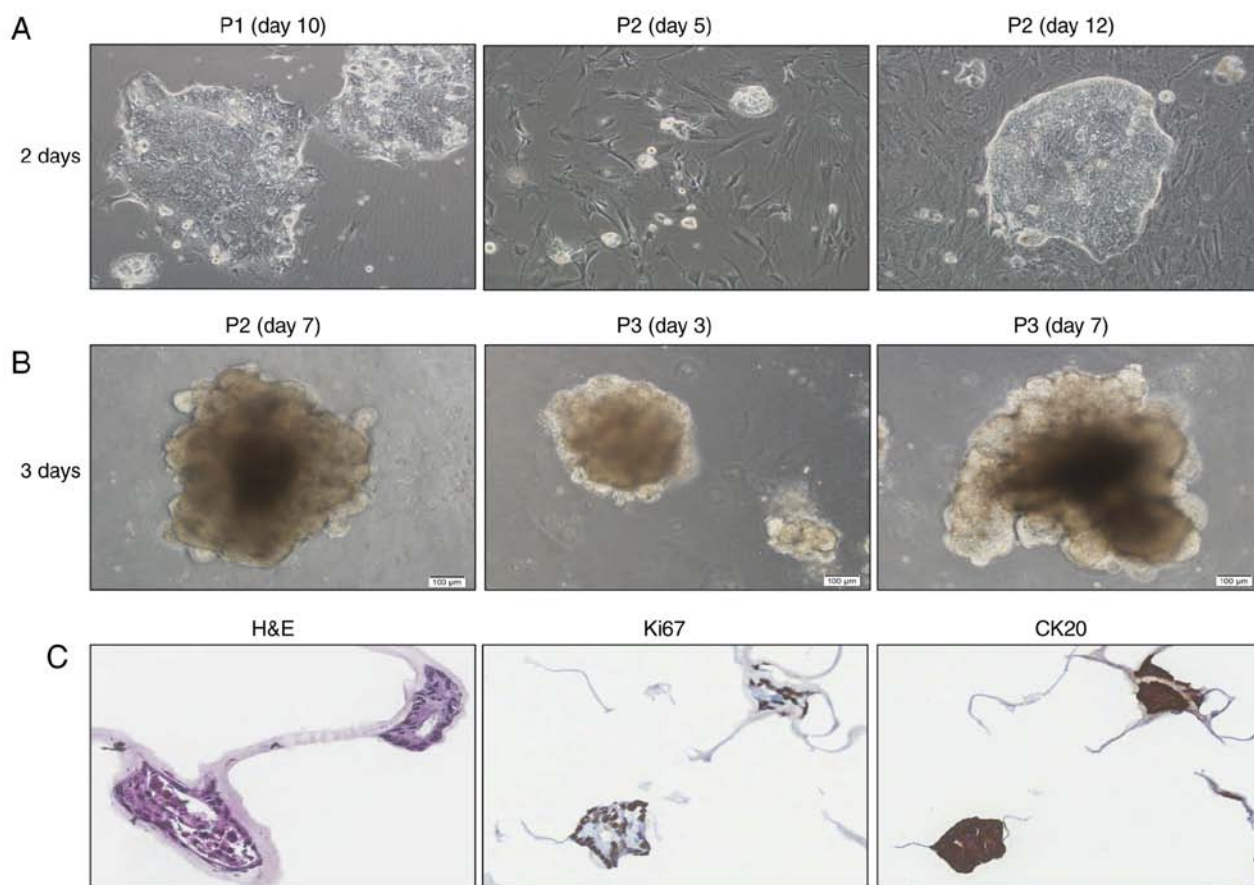


Figure 5. Characterization of 3D cultures of epithelial cells in conditioned medium. (A) Epithelial cells proliferated on stromal cells in 2D cultures. (B) Epithelial cells formed spheres without stromal cells in 3D cultures. (C) Immunohistochemistry staining of 3D cultures of epithelial cells with anti-Ki67, a proliferation marker, and anti-CK20, a colon cancer marker. Scale bar, 100 μ m; magnification, x10. CK20, cytokeratin 20; Ki67, proliferation marker protein Ki67; P, passage; H&E, hematoxylin and eosin.

induces C-Myc mRNA expression in human keratinocytes and that long-term increase in C-Myc expression is associated with increased TERT expression (23). C-Myc mRNA level was also increased in PDCs. In immortalized human keratinocyte, the RB transcriptional corepressor (RB)/p16 signaling pathway is inactivated (12). The present results suggested that, in PDCs, p16 mRNA level was also decreased (Fig. 6B).

Screening of 65 FDA-approved anticancer drugs. To validate the applicability of PDCs for preclinical drug screening of personalized anticancer therapeutics, 65 FDA-approved anticancer drugs were screened using seven different concentrations, and cell viability was measured after 6 days of drug exposure. The drug library consisted of agents targeting 20 molecular targets, including compounds that are undergoing clinical trial and preclinical investigations (16). The library also included three first line chemotherapeutics for the treatment of colorectal cancer (24), such as fluorouracil (5FU), oxaliplatin and irinotecan. In the present study, drug screening was performed for all PDCs established, and the results showed a diverse range of drug sensitivities (Figs. S1-S4). Following quantitative analysis, various effective drugs showing statistically significant response scores were selected for each PDC (Table SI). A small Z-score indicates the effectiveness of a drug, as previously described (16).

PDC3, being derived from PDX3, was the most sensitive cell line to various EGF receptor (EGFR) inhibitors such as afatinib, lapatinib, CI-1033, dacomitinib and gefitinib, in line with the mutation profiling results of PDXs in Table III indicating that PDX3 was the only cell line carrying wild-type KRAS and EGFR. The present results suggested that PDCs could be used to identify effective drugs against colorectal cancer cells. In addition, the PDC *in vitro* model established in the present study may be suitable for personalized medicine approaches.

Discussion

In the past, the propagation of adult neoplastic epithelial cells required the use of specific media that led to early onset senescence (25,26). To bypass senescence, the overexpression of viral oncogenes such as simian virus 40 large T antigen or E6/E7 proteins of oncogenic human papillomaviruses were necessary, resulting in genomic alterations and antigenicity (27-32). In the present study, colorectal cancer cells derived from xenograft tissue could be conditionally immortalized by combining the ROCK inhibitor Y-27632 and irradiated fibroblast feeder cells. Based on STR analysis, cultured cells were identified to be genetically identical to tumor tissues, suggesting that continuous proliferation was not due to genomic instability, but caused by the immortalization of cells. Previous studies

Table II. Short tandem repeat profiling of PDX tissues and cultured cells.

Locus	PDX1		PDX2		PDX3		PDX4	
	Tissue	Cell	Tissue	Cell	Tissue	Cell	Tissue	Cell
AMEL	X	X	X,Y	X,Y	X	X	X	X
CSF1PO	11,12	11,12	11	11	10,12	10,12	11,13	11,13
D13S317	8,9	8,9	8,9	8,9	8,10	8,10	11,12	11,12
D16S539	11	11	9,10	9,10	11,13	11,13	9	9
D18S51	14	14	14	14	16	16	18	18
D19S433	14	14	13,15	13,15	13,2,16	13,2,16	13	13
D21S11	30	30	29,30	29,30	30	30	22,28.2	22,28.2
D2S1338	18	18	23	23	19	19	17,18	17,18
D3S1358	15	15	16	16	15,17	15,17	15	15
D5S818	10	10	12	12	10,12	10,12	10,13	10,13
D7S820	11,12	11,12	10,12	10,12	8,12	8,12	12	12
D8S1179	10,13	10,13	10,12	10,12	11,13	11	13,14	13,14
FGA	23	23	22	22	26	26	22,23	22,23
Penta D	10,12	10,12	9,13	9,13	8,11	8,11	9,10	9,10
Penta E	11	11	17	17	11,8	11,8	24	24
TH01	9,9.3	9,9.3	9	9	9	9	8	8
TPOX	8	8	8	8	8	8	8,11	8,11
vWA	16	16	16,18	16,18	17,18,19	17,18	17	17,18

PDX, patient-derived xenograft; AMEL, Amelogenin; vWA, von Willebrand factor type A.

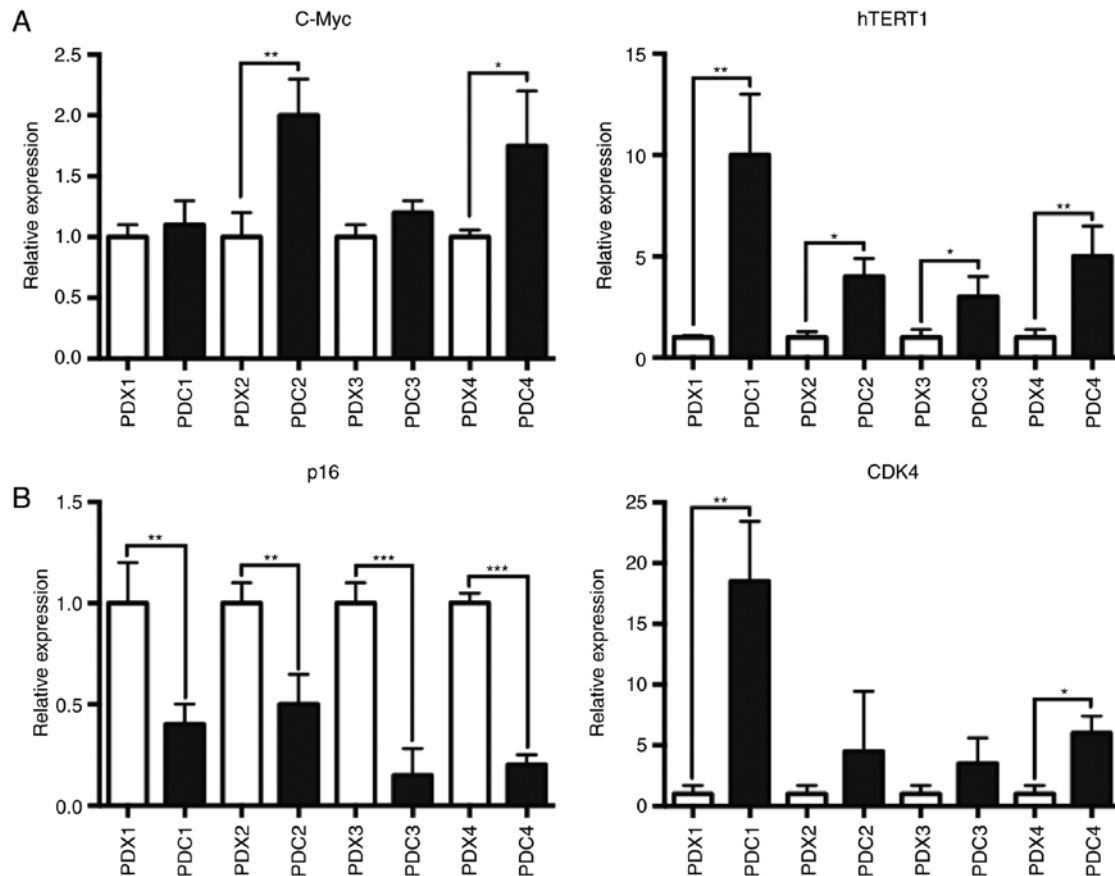


Figure 6. Reverse transcription-quantitative PCR analysis. (A) Genes involved in telomerase activity, such as C-Myc and hTERT1. (B) Genes involved in the p16/RB transcriptional corepressor signaling pathway, such as p16 and CDK4. Gene expression was analyzed in both PDX tissues and PDC cells. *P<0.05, **P<0.01, ***P<0.001. PDX, patient-derived xenograft; PDC, patient-derived cells; hTERT, human telomerase reverse transcriptase.

Table III. Mutation profiling of PDXs.

Sample	Site	Mutation				
		APC	PI3KCA	p53	KRAS	EGFR
PDX1	Metastasis	R858X	WT	FS_DEL	G12C	R521K
PDX2	Primary	WT	WT	P72R	G12D	R521K
PDX3	Primary	WT	WT	Y31C	WT	WT
PDX4	Metastasis	R223X	WT	E285K	G13D	R199C

PDX, patient-derived xenograft; WT, wild-type; EGFR, epidermal growth factor receptor; APC, APC regulator of WNT signaling pathway; PI3KCA, PI3K catalytic subunit α .

have shown that cells from various human tissues including prostate, breast and colon could be conditionally immortalized with a ROCK inhibitor and irradiated fibroblast feeder cells (11). Similarly to prostasphere or mammosphere, PDCs formed well-defined spheres without contamination of fibroblasts following administration of Matrigel. In a previous study, contamination of fibroblasts has been suggested to limit conditional reprogramming (9). In the present study, EpCAM staining was used to selectively identify epithelial cells and it was confirmed that EpCAM-positive cells could be isolated and cultured without stromal cells. Differential trypsinization methods were also used to separate epithelial cells and stromal cells to avoid contaminations.

Although the mechanism of conditional reprogramming has not been fully elucidated, previous studies have suggested that increased activity of telomerase and dysregulation of the cytoskeleton and p16/RB signaling pathway are associated with conditional immortalization of epithelial cells (33-39). Liu *et al* (11) demonstrated that hTERT expression is increased in conditionally-reprogrammed prostate and breast cancer cells. In the present study, the expression levels of hTERT and C-Myc, genes involved in telomerase activity, were higher in cultured cells compared with tumor tissues. The present RT-qPCR results suggested that p16 expression in cultured cells was decreased, whereas CDK4 expression was increased compared with tumor tissues, suggesting that inactivation of the p16/RB signaling pathway and dysregulation of the cytoskeleton may be important mechanisms underlying conditional reprogramming.

Notably, this efficient primary culture model can be used for personalized treatment approaches. To assess the applicability of this model for preclinical screening of personalized anticancer therapeutics, 65 FDA-approved chemotherapeutic agents were screened in the present study. The drugs tested in the established *in vitro* model included compounds generally used in the first-line treatment of colorectal cancer, such as 5FU, oxaliplatin and irinotecan, and monoclonal antibodies prescribed for targeted therapies, such as lapatinib or imatinib.

In clinical settings, mutation profiles of KRAS and EGFR are reliable and validated predictors for the effect of EGFR inhibitors such as cetuximab (40,41). In the present study, all PDXs, with the exception of PDX3, were identified to carry mutations in KRAS and EGFR. In total, five EGFR inhibitors

were identified as effective drugs for PDC3, which were originated from PDX3 cells expressing wild-type KRAS and EGFR. Notably, EGFR inhibitors did not affect the growth of PDC1 and PDC4. However, two EGFR inhibitors were identified as effective drugs for PDC2, although PDX2, derived from PDC2, was identified to carry mutations in KRAS and EGFR genes, suggesting potential resistance to EGFR inhibitors. The present results may be explained by intra-tumor heterogeneity (ITH), which lead to different genotypes and phenotype of individual cancer cells within the same tumor (42). ITH is one of the major causes causing discordance between the results of drug screening assays using patient-derived cancer cells and chemotherapy response (43). Using multiple PDCs from a single tumor at the same time may help overcoming this limitation. Performing drug screening assays using the PDC platform established in the present paper, clinicians could not only predict the response to individual chemotherapeutic agents, but also consider a combination of targeted drugs effective in cancer cells isolated from patients in the first-line treatment (40,41).

The conditional reprogramming method described in the present study may have several advantages, including lack of genotypic drift, rapid proliferation, efficient immortalization and high success rate (~50%) compared with conventional traditional methods (1-10%) (44), and may allow experiments such as screening assays of anticancer drug. Recently, PDX and 3D organoid cultures have emerged as novel *in vitro* models to study cancer (45). Although PDXs present molecular and cellular features that reflect tumor heterogeneity, they are difficult and expensive to develop (6,15). By contrast, 3D organoid cultures are easy to transfect and can be useful for assessing drug responses; however, since 3D structures can be difficult to analyze by light microscopy and organoid culture medium contains many growth factors or small molecular inhibitors that can affect the response to the tested drugs, using organoid models in automated high-throughput drug screening is challenging. Considering that the timing for the analysis of cancer tissues and therapeutic decisions is key in clinical settings, conditional reprogramming could be the most appropriate model for the application of personalized medicine strategies. The high efficiency and robustness of conditionally reprogrammed cells may increase the importance of biobanking and facilitate the investigation of cancer cells for genetic and

molecular analysis allowing clinicians to make precise and prompt decisions for treating patients using personalized treatment approaches.

Acknowledgements

The authors would like to thank Ms. Yoo La Lee (Samsung Advanced Institute for Health Sciences and Technology) and Ms. Mi Na Hwang (Samsung Advanced Institute for Health Sciences and Technology) for PDC culture and PDX generation.

Funding

The present study was supported by grants of The Korea Health Technology R&D Project of The Korea Health Industry Development Institute, funded by The Ministry of Health and Welfare, Republic of Korea (grant. nos. HI14C3418 and HI15C1593), and by The Basic Science Research Program of the National Research Foundation of Korea, funded by The Ministry of Education (grant. no. NRF-2017R1D1A1B03034309). In addition, the present study was supported by The Samsung Medical Center (grant. no. OTC1190571).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HKH and YBC designed the experiments. DHP and HKH wrote the manuscript. HKH, DHP, TWK, NHY, YSL and SJS performed experiments. WYL collected the clinical data and reviewed the literature. YBC conceived the study and supervised the experiments and writing of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

Ethics approval and consent to participate

All procedures performed in the present study were in accordance with the ethical principles of the care and use of laboratory animals guidelines approved by the Institutional Animal Care and Use Committee of Samsung Biomedical Research Institute. The experiments conducted on animal and patient samples were approved by the Institutional Review Board of Samsung Medical Center. Written informed consents were obtained from all participating patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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