Spheroid Culture of Primary Lung Cancer Cells with Neuregulin 1/HER3 Pathway Activation

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Introduction: Primary culture of cancer cells is expected to be useful for investigating the biology of cancer and predicting chemosensitivity for individual patients, yet has been hampered by technical difficulties. We recently developed the cancer tissue–originated spheroid (CTOS) method for the primary culture of colorectal cancer cells. In the present study, we applied this system to the primary culture of non–small-cell lung cancer.

Methods: We used 125 surgical specimens and 18 pleural effusions for CTOS preparation. Partially digested tumor fragments were cultured in a medium for embryonic stem cells. CTOSs were subjected to sensitivity assay and signal transduction assay for the epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitor (TKI) erlotinib. We also investigated the effects of growth factors in culturing lung cancer CTOS.

Results: The success rate of CTOS preparation from surgical specimens was 80.0%. The CTOS method was also suitable for culturing tumor spheroids from pleural effusions. CTOSs from lung cancer consisted mostly of pure cancer cells. CTOSs and CTOS-derived xenografts retained the characteristics of the original tumors. In vitro assay results showed that *EGFR* mutation status and expression levels corresponded with erlotinib sensitivity, confirming previous clinical findings. Furthermore, we found that neuregulin 1, a ligand of HER3, potently induced CTOS growth.

Conclusions: The CTOS method enables us to obtain primary lung tumor cells of high viability and purity. CTOS could be a new platform for studying lung cancer biology.

Key Words: Primary culture, Non–small-cell lung cancer, EGFR tyrosine kinase inhibitor, Neuregulin 1, HER3.

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Despite constant efforts to improve diagnostics and therapeutics, cancer remains a leading cause of death in developed countries, and pulmonary malignancies are the leading cause of cancer-related death worldwide. Studies have shown that some lung adenocarcinomas are addicted to epidermal growth factor receptor (*EGFR*) signaling, and EGFR tyrosine kinase inhibitors (TKIs) are effective treatment in such cases.[1,2] However, these therapies are challenged by primary and acquired resistance, including additional point mutations[3–5] and alternative pathways that bypass the targets of therapeutic reagents.[6–8] Other ErbB family tyrosine kinase receptors are also involved in non–small-cell lung carcinoma (NSCLC);[9] human epidermal growth factor receptor 3 (HER3) is reportedly involved in the acquired resistance to EGFR TKI, whereas HER2 is overexpressed in 10% to 20% of NSCLC and carrying mutations in approximately 2%.[10,11] Bypassing EGFR signaling can be achieved through HER3 phosphorylation by overexpressed hepatocyte growth factor receptor (MET),[6] or by the induction of HER3 ligand as an autocrine loop.[12] Targeting of HER2 is already in clinical use and that of HER3 is under clinical trial.

Much of the above described data have been obtained through preclinical cancer therapy research performed using cancer cell lines. Although cancer cell lines are originally established from patient tumors, they have adapted to the culture conditions, that is, serum-supplemented medium.[13] Long-term cultivation in serum-containing medium reportedly leads to the accumulation of genetic alterations;[14,15] therefore, it must be considered whether cancer cell lines continue to accurately represent the parental cancer cells.

Nevertheless, primary culture of cancer cells is expected to be useful for investigating cancer biology and predicting chemosensitivity for individual patients. However, it has been hampered by technical difficulties, including poor cell viability, weak growth, and contamination by host cells, especially fibroblasts. We recently reported a novel primary culture system for colorectal cancer.[16] The principle of this method is to retain cell–cell contact during preparation. Tumor biopsy specimens are partially digested into fragments and cultured in a serum-free medium for embryonic stem (ES) cells; within a few hours, the irregularly shaped tumor fragments spontaneously form spheroids, termed cancer tissue–originated spheroids (CTOSs). This CTOS method

enables preparation and culture of multiple spheroids consisting of highly pure and viable colorectal cancer cells.

In the present study, we applied the CTOS method to the primary culture of non–small cell lung cancer (NSCLC). We were able to prepare and culture CTOSs from surgical specimens and pleural effusion. CTOSs from NSCLC showed individual responses to EGFR tyrosine kinase inhibitor in vitro and in vivo. CTOSs were also applicable in a signal transduction assay. Moreover, we identified neuregulin 1 (NRG1, also known as heregulin β1), a ligand for HER3, as a prominent growth factor for CTOS growth.

MATERIALS AND METHODS

CTOS Preparation

Surgical specimens and pleural effusion samples from lung cancer patients were obtained from Osaka Medical Center for Cancer and Cardiovascular Diseases, with the patients' informed consent. Surgically resected tissues were minced with a scalpel into approximately 1-mm^3 pieces, and washed with Hank's balanced salt solution (HBSS, Invitrogen, Carlsbad, CA). Specimens were transferred to a 100-ml glass flask and digested in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.26 U/ml Liberase DH (5401089; Roche, Basel, Switzerland) and 1% PenStrep (Invitrogen), at 37°C for 1 to 2 hours with gentle stirring by a magnetic bar. Digested tissue suspensions were passed through 500-μm and 250-μm metal mesh filters to remove large masses of undigested fragments. Suspensions were further filtered through 100-μm and 40-μm cell strainers (BD FALCON, Franklin Lakes, NJ). Fragments on the cell strainer and cells in the flow-through fractions were collected separately, and were each washed with HBSS and cultured in StemPro hESC medium (GIBCO, Carlsbad, CA) in a non-treated dish (EIKEN, Tokyo, Japan). Pleural effusions were transferred to 50-ml tubes and centrifuged at 200 *g*. Pellets were resuspended in HBSS, filtered through 40-μm cell strainers, and collected and cultured in the same manner as surgical specimens.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tumors and CTOSs. CTOSs were embedded in Matrigel growth factor reduced (GFR) (BD Biosciences, Bedford, MA) before fixation in formalin. Sections were dewaxed, rehydrated, and subjected to antigen retrieval by autoclave incubation in citrate buffer (pH 6.0). The primary antibodies used are listed in the Supplementary text (Supplemental Digital Content, http://links.lww.com/JTO/A386). After secondary antibody incubation, sections were visualized with a fluorescence method or biotin-amplified method (Nova RED, VECTOR, Burlingame, CA).

CTOS Culture

For assessing the effects of growth factors, CTOSs were embedded in Matrigel GFR as described above, and cultured in 100 μl of basal medium containing 10 ng/ml or 100 ng/ml of the following growth factors: NRG1 (Peprotech, Rocky Hill, NJ), Long-IGF1 (Gropep, Thebarton SA, Australia), bFGF (Invitrogen), Activin A (R&D Systems, Minneapolis, MN), or EGF (Invitrogen). Basal medium consisted of DMEM/F12, 2% BSA fraction V, 1× nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Invitrogen), 50 μg/ml ascorbic acid (SIGMA, St. Louis, MO), 10 μg/ml human transferrin (SIGMA), 0.1 mM β-mercaptoethanol (Wako, Osaka, Japan), and 1× trace elements A, B, C (Mediatech, Manassas, VA).

For assessment of the inhibitory effect of anti-HER3 antibodies on CTOS growth, CTOSs were embedded in Matrigel GFR as described above, cultured in medium containing 10 ng/ml NRG1 or in StemPro hESC, and treated with anti-HER3 antibody (cloneH3.105.5; Calbiochem, La Jolla, CA) at the indicated doses.

CTOS Sensitivity Assay In Vitro

For the erlotinib sensitivity assay, each CTOS was embedded in a gel droplet of Matrigel GFR, and cultured in StemPro hESC containing erlotinib (Toronto Research Chemicals, North York, ON, Canada) at the indicated doses. CTOSs were exposed to erlotinib for 7 days. CTOS viability was evaluated based on CTOS size at day 7, corrected for the CTOS size at day 0. CTOS size was measured using image analysis software (Image J; National Institutes of Health, Bethesda, MD). Half maximal (50%) inhibitory concentration values were calculated with GraphPad Prism 4 software, using the sigmoidal dose-response function.

Animal Studies

Animal studies were performed in compliance with the guidelines of the institutional animal study committee of Osaka Medical Center for Cancer and Cardiovascular Diseases. Primary xenograft tumors were generated by inoculating small pieces of patient tumors into non-obese diabetic/severe combined immunodeficient (NOD/Scid) mice. One hundred CTOSs were suspended in 50 μl of Matrigel GFR, and transplanted subcutaneously into the flanks of NOD/scid mice. CTOSs prepared from mouse xenografts are designated with the postfix "m" in the figures. Treatment was started when the tumor volume reached approximately 65 mm^3. Erlotinib (Tarceva, CHUGAI Pharmaceutical, Tokyo, Japan) was suspended in 0.5% methyl cellulose, and administered at a dose of 100 mg/kg orally once a day, ×5, for 2 weeks.

Western Blot

CTOSs were cultured overnight in growth factor-free DMEM-F12, and pulsed with StemPro hESC supplement or 10 ng/ml of the indicated growth factors for 15 minutes. CTOSs were lysed with cell lysis buffer (10 mM Tris (pH 7.4), 0.15 M NaCl, 1% NP40, 0.25% sodium deoxycholate, 0.05 M NaF, 2 mM EDTA, 0.1% SDS, 2 mM $NaVO_4$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF). Western blot was performed as previously described.[17] For signal transduction assays under erlotinib treatment, CTOSs were cultured overnight under growth-factor–starved conditions, incubated for 1 hour with erlotinib, and pulsed with 10 ng/

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ml epidermal growth factor (EGF) for 15 minutes. Primary antibodies used are listed in the Supplemental text.

Statistical Analysis

Data are expressed as mean, SD. Statistical analysis was performed using Student's *t* test. Differences were considered significant at *p* values less than 0.05.

RESULTS

Preparation and Characterization of CTOSs from Lung Cancer Patients

To apply the CTOS technology[16] to NSCLC, surgically resected tumor tissues were mechanically minced and enzymatically digested. Samples were then filtered through cell strainers and divided into two fractions: the organoid fraction (tissue fragments on the cell strainer) and the flow-through fraction. The irregular-shaped fragments in the organoid fraction formed spheroids within a few hours in a serum-free medium for ES cell culture (Fig. 1A, Supplementary Fig. 1A, http://links.lww.com/JTO/A355). The flow-through fraction mostly consisted of single cells, small-cell aggregates, and debris; we occasionally found small CTOS-like structures in the flow-through fraction, probably derived from cell aggregates (Supplementary Fig. 1B, http://links.lww.com/JTO/A355). CTOSs were also prepared from pleural effusions, without any dissociation process (Fig. 1B). When CTOSs were dissociated into single cells by trypsin treatment, the dead cells increased over time (Supplementary Fig. 1C, http://links.lww.com/JTO/A355). Thus, retaining cell–cell contact was crucial for preparation and maintenance of viable lung cancer cells, as previously observed in CTOSs of colorectal cancer.[16]

Flow cytometric analysis revealed that CTOSs from adenocarcinoma mostly consisted of epithelial cell adhesion molecule (EpCAM)–positive epithelial cells. CD45-positive hematopoietic cells were barely detectable in CTOSs (Fig. 1C). In contrast, cells in the flow-through fraction consisted of various types of cells, including EpCAM-negative nonepithelial cells and CD45-positive cells (Fig. 1C). Next, we assessed the purity of CTOSs by immunohistochemistry. The original patient tumors consisted of various type of cells, including epithelial cells (E-cadherin positive), myofibroblasts (α-smooth muscle actin [α-SMA] positive), and macrophages (CD68 positive). In contrast, the cells in CTOSs were almost exclusively E-cadherin positive (Fig. 1D). Thus, highly pure epithelial cells could be prepared from bulk tumor tissues by the CTOS method.

CTOSs were successfully prepared from 100 of 125 NSCLC samples (80.0%) from surgically resected tumors, and in eight of 18 pleural effusion samples (44.4%), including all histologic subtypes (Table 1, Supplementary Table 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A356). In most of the failed cases, tumor fragments were not observed in the effusion. CTOSs were also prepared from small-cell lung cancer (Supplementary Table 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A356). CTOSs were successfully prepared from all (5 of 5) primary xenograft

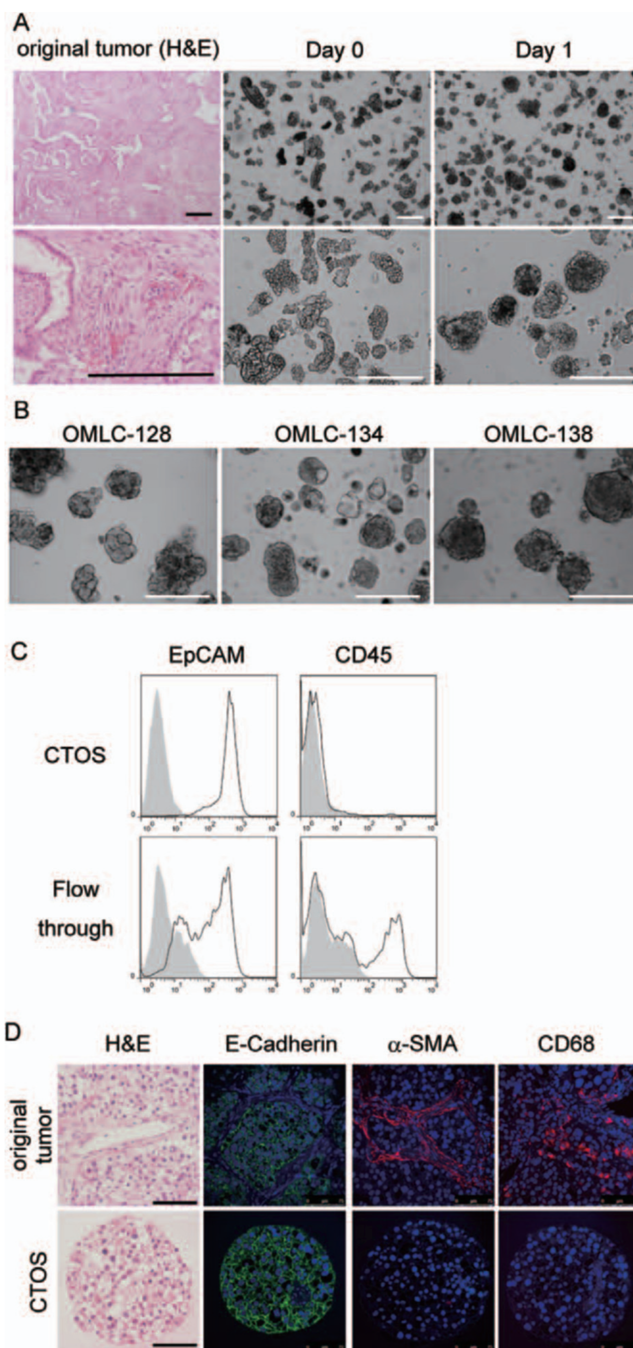

FIGURE 1. Preparation and characterization of CTOSs from patients' lung tumors. *A*, H&E staining of original tumor and phase contrast images of organoid fraction at the indicated times after OMLC-58 preparation. Low- (upper panels) and high-magnification (lower panels) images are shown. Scale bar: 200 μm. *B*, Phase contrast images of CTOSs from three independent pleural effusion samples. Scale bar: 200 μm. *C*, Flow cytometric analysis of the indicated cell-surface antigens by specific antibodies (solid line) or isotype controls (gray filled). *D*, OMLC-40 subjected to H&E staining and immunohistochemistry of E-cadherin (green), α-SMA (red), CD68 (red), and Hoechst33342 (blue). Scale bar: 75 μm. H&E, hematoxylin and eosin.

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tumors, which were generated by inoculating small pieces of patient tumors into NOD/scid mice.

CTOS Preserved the Original Tumor Characteristics

To examine the tumorigenic capacity of CTOS, 100 CTOSs (approximately 1×10^4 cells) were transplanted into the flanks of NOD/scid mice. When primary CTOSs prepared from patient tumors were subcutaneously injected into immunodeficient mice, the CTOSs from six of 18 cases (33.3%) formed xenograft tumors (Supplementary Table 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A356). However, CTOSs from primary xenograft tumors formed xenograft tumors in four of five cases (80%). Histologic analysis revealed that CTOSs and mouse xenograft tumors exhibited characteristics of the original patient tumors, at least of some parts in mixed subtype tumors (Fig. 2A). Xenografts or CTOSs also preserved the immunohistochemical characteristics of the original tumors, including staining patterns of EGFR, CK7, TP53, and neural cell adhesion marker (Fig. 2B, Supplementary Fig. 2, Supplemental Digital Content 3, http://links.lww.com/JTO/A357). The ratio of CD133-positive cells, a reported marker of cancer stem cells (CSCs) in lung cancer,[18] was not enriched in CTOSs (Supplementary Fig. 3, Supplemental Digital Content 4, http://links.lww.com/JTO/A358).

TABLE 1. Success Rate of CTOS Formation from Lung Cancer Specimen

		Sample Number	CTOS Formation	Success Rate (%)
Surgical sample	Ad	82	64	78.0
	AdSq	6	6	100
	Sq	31	25	80.6
	Large	4	4	100
	Pleomorphic	2	1	50.0
	Total	125	100	80.0
Pleural effusion	Ad	15	7	47
	AdSq	1	1	100
	Sq	2	0	0
	Total	18 (8)[a]	8 (8)[a]	44.4 (100)[a]

CTOS, cancer tissue–originated spheroid; Ad, adenocarcinoma; Sq, squamous cell carcinoma; AdSq, adenosquamouse cell carcinoma,; Large, large-cell carcinoma, Pleomorphic, pleomorphic carcinoma.

[a]Excluding samples without any tumor fragments after filtration of the pleural effusion

Response of Lung CTOSs to EGFR TKI

We successfully grew lung CTOSs in vitro in StemPro medium, embedded in Matrigel GFR (Supplementary Fig. 4A, Supplemental Digital Content 5, http://links.lww.com/JTO/A358). Under these culture conditions, 67.2% of CTOSs grew to more than 1.2 times their original size after 7 days (Supplementary Fig. 4B, Supplemental Digital Content 5, http://links.lww.com/JTO/A359). CTOSs from adenocarcinomas tended to grow better compared with those from squamous cell carcinomas.

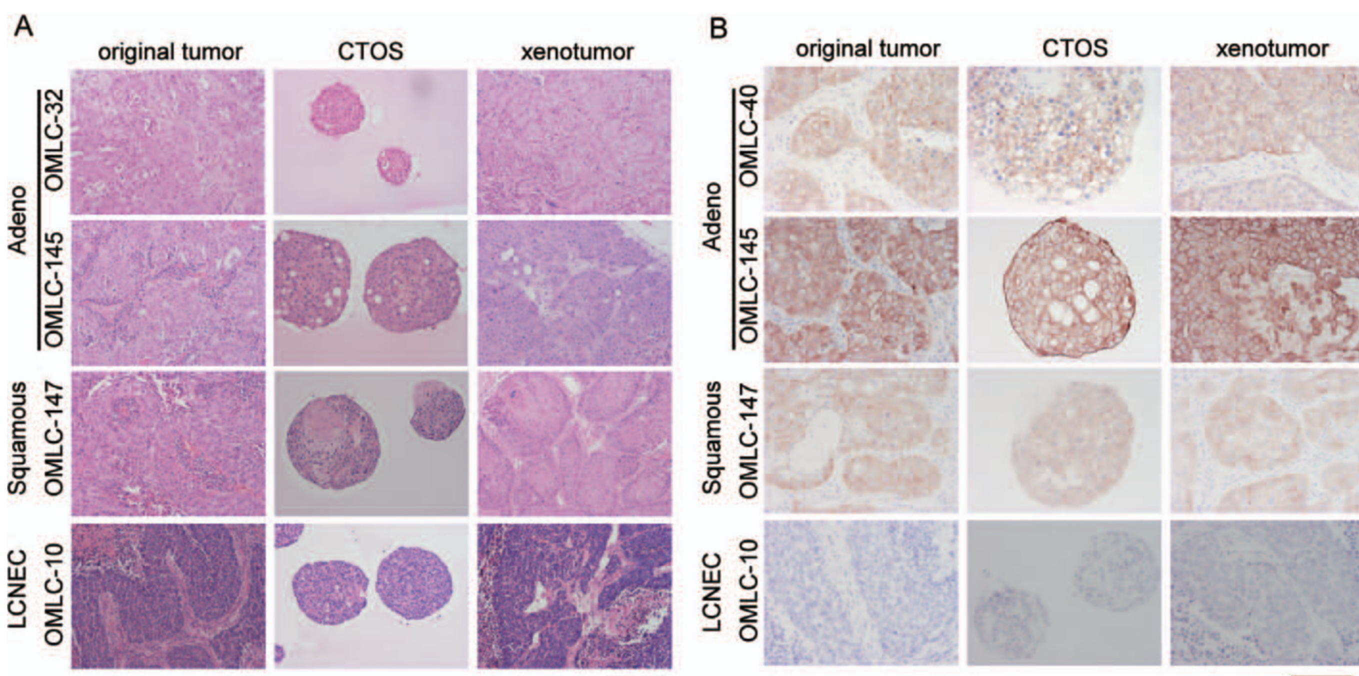

FIGURE 2. CTOS-derived xenotumors preserved the original characteristics of parental tumors. *A*, H&E staining of the indicated cases. *B*, Immunohistochemistry of EGFR from the indicated cases. Original tumor (left column), CTOS (middle column), and xenograft tumor (right column). Adeno, adenocarcinoma; Squamous, squamous cell carcinoma; LCNEC, large-cell neuroendocrine carcinoma. Scale bar: 100 μm.

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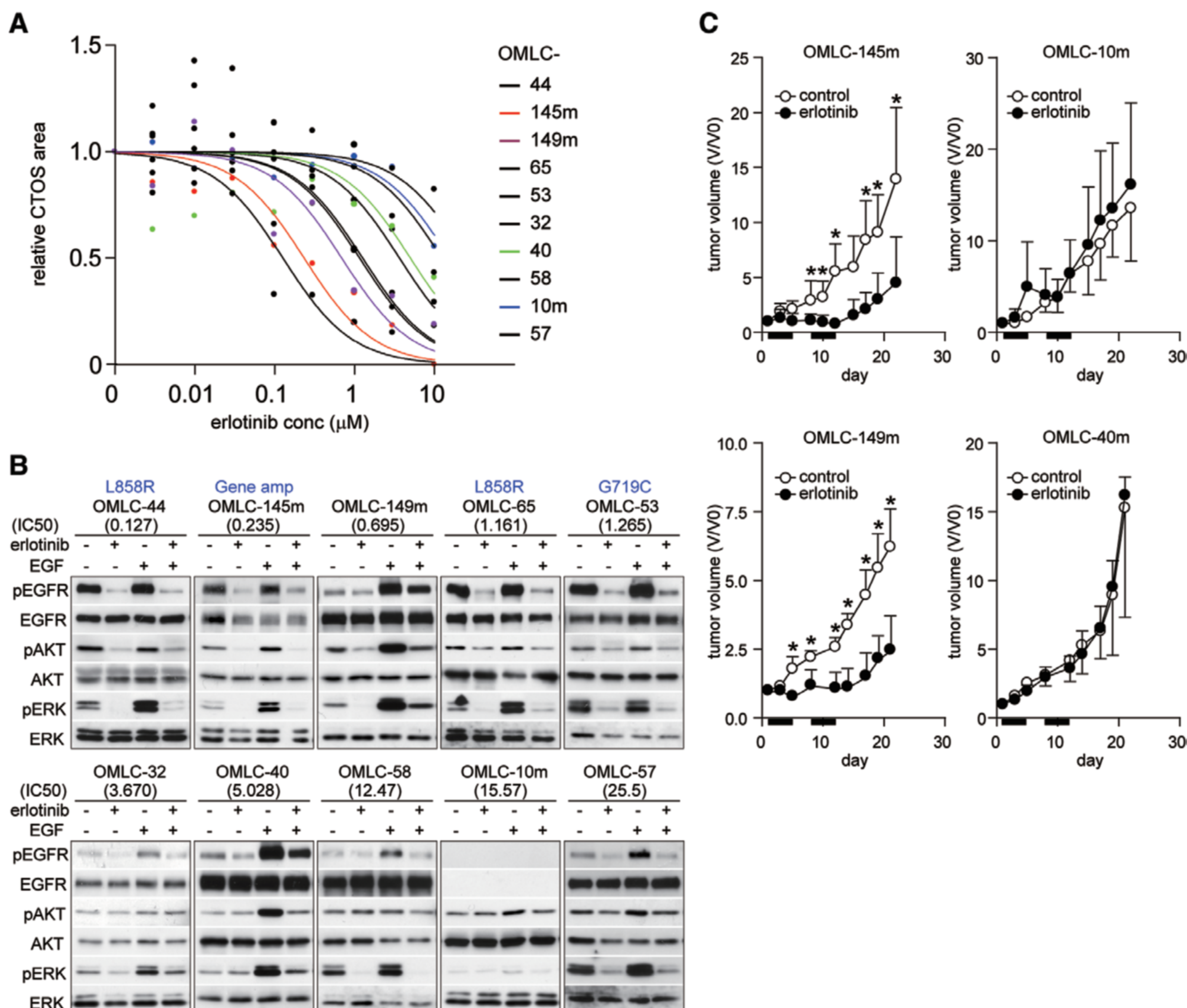

FIGURE 3. Effect of erlotinib on CTOS growth and signal transduction. *A*, In vitro dose–response curve of erlotinib sensitivity with 10 clinical cases. Origins of the cases are indicated in ascending order of IC50. *B*, Western blot of EGFR signaling pathway from 10 clinical cases, in ascending order of IC50. CTOSs were cultured under growth-factor–starved conditions and pulsed for 15 minutes with 10 ng/ml EGF in the presence or absence of 1 μM erlotinib. *C*, Effects of erlotinib on the growth of xenograft tumors derived from OMLC-145 (EGFR overexpresser), OMLC-10 (EGFR-nonexpresser), OMLC-149 (*EGFR* wt), and OMLC-40 (*EGFR* wt). The CTOSs were transplanted in NOD/SCID mice ($n = 5$). Erlotinib was administered orally once a day ×5 for 2 weeks, indicated as black bars. Tumor volume at each time point (V) was corrected by that on day 1 (V0). Open circles, vehicle-treated controls; closed circles, erlotinib-treated groups. IC50, inhibitory concentration. *$p < 0.05$

The EGFR TKIs gefitinib and erlotinib are known to be effective against lung cancer harboring active *EGFR* mutations[1,19] or EGFR gene amplification.[20,21] We examined whether the response of CTOSs to EGFR TKI was consistent with these clinical findings, and found that erlotinib sensitivity varied among individual patients (Fig. 3A). Three EGFR active mutant cases and one gene amplification case were among the top five most sensitive cases (Supplementary Fig. 5A and B, Supplemental Digital Content 6, http://links.lww.com/JTO/A360). One advantage of analyzing CTOSs is the ability to assess the status of intracellular signaling and the change of status after drug treatment in individual patient samples. We subjected the CTOSs to immunoblot analysis to investigate the effect of erlotinib on intracellular signaling. *EGFR* mutants (OMLC-44, OMLC-53, and OMLC-65) and the *EGFR* overexpresser (OMLC-145) showed high levels of phosphorylation of EGFR and AKT, even under growth-factor–starved conditions, indicating constitutive activation of EGFR signaling (Fig. 3B). In these cases, the phosphorylation of EGFR, AKT, and extracellular signal-regulated kinase, was

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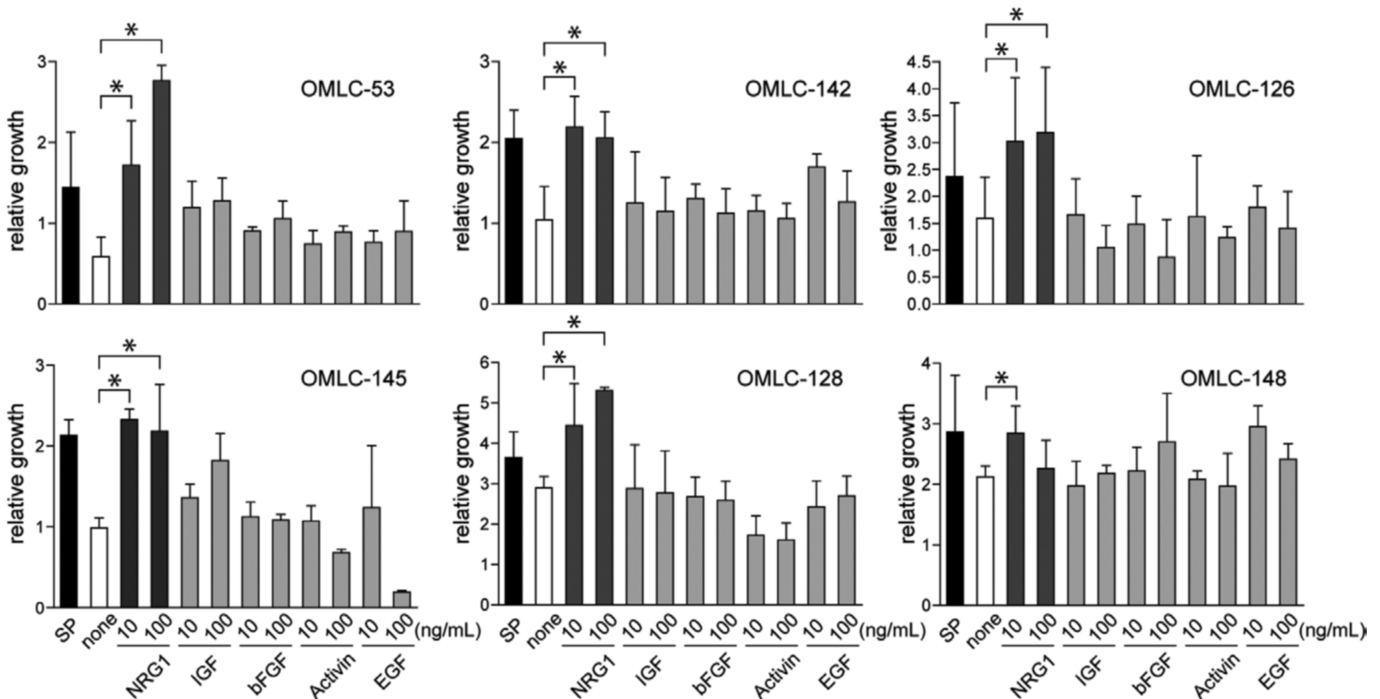

FIGURE 4. NRG1stimulated CTOS growth in vitro. Growth ratio of CTOSs from six cases at day 7 relative to day 0. The CTOSs were embedded in Matrigel growth factor reduced and cultured in StemPro hESC or defined conditions containing NRG1, Long-IGF (IGF), bFGF, Activin A (Activin), or EGF at 10 ng/mlL or 100 ng/ml. *$p < 0.05$.

diminished after erlotinib treatment, even in the presence of the EGFR ligand EGF.

One case of CTOS (OMLC-149), in which we could detect neither known *EGFR* mutations nor EGFR gene amplification, showed high sensitivity to erlotinib and gefitinib, whereas other CTOSs with wild-type *EGFR* were resistant to erlotinib (Fig. 3A, Supplementary Fig. 5C and D). The phosphorylation of EGFR under starved conditions was not high; however, in contrast with other *EGFR* wild-type cases, the phosphorylation of both AKT and ERK was substantially high, which was suppressed by erlotinib treatment.

Next, we assessed whether the results of the CTOS sensitivity assay or the signal analysis in vitro reflected the sensitivity to erlotinib in vivo using the following four CTOSs: OMLC-145 (erlotinib sensitive, EGFR gene amplification), OMLC-10 (erlotinib resistant, EGFR not expressed), OMLC-149 (erlotinib sensitive, *EGFR* wild-type), and OMLC-40 (erlotinib resistant, *EGFR* wild-type). We transplanted these CTOSs into the flanks of NOD/SCID mice. Consistent with the results of the in vitro experiments, growth of the tumors derived from erlotinib-sensitive CTOSs was suppressed by erlotinib treatment, whereas no effect was observed in the tumors derived from erlotinib-resistant CTOSs (Fig. 3C). Thus, the results of the in vitro CTOS sensitivity assay and signal transduction assay with an EGFR inhibitor were in parallel with the therapeutic efficacy in vivo in the four examined cases.

Neuregulin 1 Stimulated CTOS Growth In Vitro

To determine which growth factors contributed to the CTOS growth in culture, we performed an assay in which the growth factors NRG1/heregulinβ1, insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), activin A, and epidermal growth factor (EGF), in StemPro hESC[22] were added one at a time to the basal medium. In 10 of 13 cases (71.4%) tested, the HER3 ligand NRG1 showed the most potent effect on CTOS growth at the dose compatible to that of StemPro hESC (Figure 4, Supplementary Table 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A356). In contrast, EGF showed only a minimal effect on CTOS growth, and occasionally showed a toxic effect (Fig. 4; OMLC-145). Among the tested cases, two CTOSs from squamous cell carcinoma did not respond to NRG1 (Supplementary Table 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A356).

Analysis of intracellular signaling revealed that NRG1 was the most potent inducer of AKT phosphorylation (Fig. 5A). Next, we analyzed the localization of HER3 phosphorylation by NRG1 in CTOSs by immunohistochemistry (Fig. 5B). Phosphorylation of HER3 and AKT was detected after NRG1 stimulation. As demonstrated by ZO-1/E-cadherin and EGFR staining, the CTOSs from OMLC-53 had polarity, in which the apical side was outside of CTOSs (Fig. 5B). HER3 phosphorylation was detected at the basolateral membrane of the cells in the CTOS. However, CTOSs from OMLC-128 did not show clear polarity.

We then challenged the CTOSs cultured in NRG1-containing medium or StemPro hESC with a neutralizing antibody against HER3.[23] The growth (Fig. 5C) and the phosphorylation of HER3 and AKT (Fig. 5D) were partially suppressed by the antibody. Taken together, our findings show

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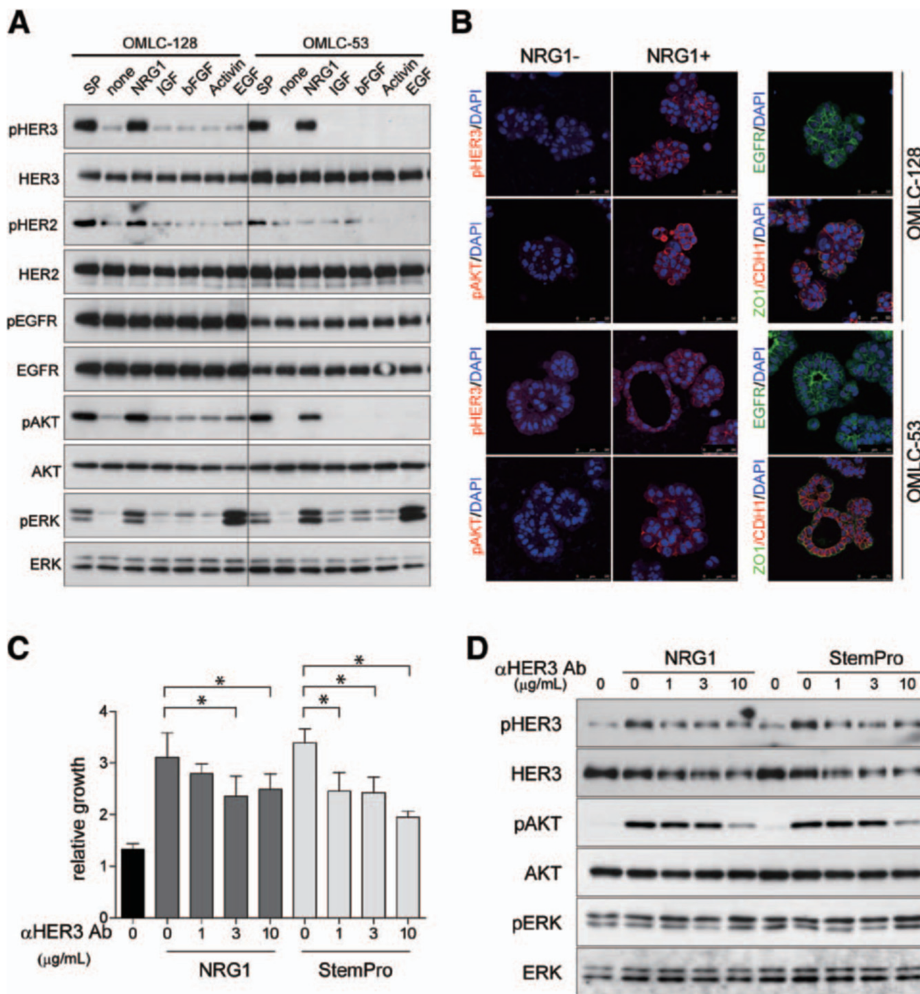

FIGURE 5. Effect of NRG1 on pathway activation in CTOSs. *A*, Western blot for ErbB family signaling pathway of OMLC-128 and OMLC-53. CTOSs were cultured under growth-factor–starved conditions and stimulated by 10 ng/ml of each indicated growth factor for 15 minutes. *B*, Immunohistochemistry of phospho-HER3 and phospho-AKT after NRG1 stimulation. OMLC-128 or OMLC-53 CTOSs were cultured under starvation conditions and stimulated by 10 ng/ml NRG1 for 6 hours (pHER3) or 1 hour (pAKT). EGFR, ZO1, and E-cadherin staining indicate the polarity of the CTOSs. *C*, Inhibitory effect of anti-HER3 antibody on growth of OMLC-128 CTOSs. CTOSs were embedded in Matrigel growth factor reduced, cultured in basal medium containing 10 ng/ml NRG1 or StemPro hESC, and treated with the indicated doses of anti-HER3 antibody. *$p < 0.05$. *D*, Effect of anti-HER3 antibody on signal transduction of OMLC-128. CTOSs were cultured in StemPro containing the indicated dose of anti-HER3 antibody for 24 hours. StemPro hESC; IGF; long-IGF1, Activin; Activin A.

that NGR1/HER3 signaling played a role in the culture of lung CTOSs.

DISCUSSION

Primary culture of lung cancer cells has been attempted for decades, using two basic approaches: histoculture and dissociation-based culture. In histoculture, tumors are cultured directly[24,25] or on gel matrices after being minced into small pieces.[26–28] In dissociation-based culture, tumors are enzymatically dissociated into single cells or cell aggregates of several cells; then the cells or cell aggregates are cultured on the dish[29] or in gel matrices.[30,31] In these methods, the cultured cells are mixtures of tumor cells and host cells in varying degrees. In contrast, CTOS technology enables us to culture mostly purified lung cancer cells as spheroids in a medium with defined growth factors, and allows us to obtain reproducible results; these features are essential for bioassays, such as treatment sensitivity assays and pathway-activation assays.

The success rate of preparing CTOSs from lung cancer was 80.0%, which is relatively low compared with the high success rate (98.7%) achieved with colorectal cancer.[16] This difference may be partly because of the relatively poor content of cancer cells in the lung cancer samples. In addition, as lung cancer tissues contain more fibrous component than colorectal cancer tissues do, optimizing the protocol of tissue dissociation might improve the CTOS preparation from tiny biopsy samples of lung cancer. The success rate of tumor formation with lung cancer CTOSs in NOD-scid mice was also relatively low, 33.3%, compared with the results with colorectal cancer.[16] Compared with colorectal cancer, lung cancer cell growth might be more dependent on the cancer-associated microenvironment, which might not be replicated in mouse subcutaneous tissue.

CSC theory has been recently proposed and widely investigated; this theory states that only a minor population of cancer cells has the capacity of self-renewal and multipotency of differentiation; therefore, cancers form with a hierarchy dominated by the CSCs.[18,32] CTOSs share some characteristics of CSCs, including sphere-forming capacity in suspension culture with serum-free medium, and tumorigenic capacity in immunodeficient mice. However, CTOSs and CSC-derived spheroids are different in several aspects. First, lung CSCs are reportedly CD133-positive cells, which are rare in tumors and enriched in CSC-derived spheroids,[18] whereas the level of CD133-positive cells was not enriched in CTOSs (Supplementary Fig. 3, Supplemental Digital Content 4, http://links.lww.com/JTO/A358). Second, the expression levels of

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differentiation markers, including cytokeratin and neural cell adhesion marker, are reportedly low in CD133-positive cells,[18] whereas the expression levels of these markers in CTOSs were similar to those in the original tumors (Supplementary Fig. 2, Supplemental Digital Content 3, http://links.lww.com/JTO/A357). Thus, the cells comprising the CTOS represented the major population of cancer cells within the patient's tumor, and are not likely derived from a minority cell population in the tumor, although our experiments do not exclude the existence of CSCs in CTOSs.

We showed that the CTOS method can be used to evaluate EGFR signaling and the response to an EGFR TKI in primary cultured lung cancer cells from individual patients. Tumors harboring EGFR-activating mutations and gene amplification are known to be clinically sensitive to EGFR TKIs,[1–3,20,21] and correspondingly, these cases were sensitive to erlotinib in the CTOS assay in vitro (Fig. 3A). In terms of intracellular signaling, the levels of AKT phosphorylation are reportedly high in EGFR TKI–sensitive patients or cell lines.[21,33] Indeed, in the erlotinib-sensitive CTOSs, the rates of phosphorylation of EGFR and AKT were high even under growth-factor–starved conditions, and were completely suppressed by erlotinib treatment. We also found one CTOS with wild-type *EGFR* and no gene amplification to be sensitive to erlotinib in vitro and in vivo. Thus, CTOS might provide an alternative method for selecting patients for molecular-targeted drugs, in addition to the *snapshot* of information given by assessing biopsy samples. Further studies are required to test the relationship between the sensitivity assay with CTOSs and the patients' clinical response to specific treatments and determine whether the CTOS method is useful for personalized medicine.

We found here that NRG1-HER3 signaling is important for CTOS culture from NSCLC. NRG1 showed the most potent effect on CTOS growth in 71.4% cases at the tested doses, yet other growth factors also promoted CTOS growth to some extent (Fig. 4). In some cases, the growth-promoting effect of NRG1 was saturated at 10 ng/ml. This might be because of the internalization and degradation of HER3 after ligand binding. HER3 is a member of ErbB family of transmembrane tyrosine kinase receptors. HER3 lacks tyrosine kinase activity, and transduces downstream signals by forming heterodimers with other tyrosine kinase receptors.[9] As HER3 has six docking sites for phosphatatidylinositol-3 kinase (PIK3) p85, it can potently activate the PI3K-AKT pathway.[34] Among the growth factors examined in this study, NRG1 most strongly induced AKT phosphorylation in CTOSs (Fig. 5A). Despite the growth-promoting effect of NRG1 on the CTOSs, we only observed a marginal inhibitory effect of the anti-HER3 antibody (Fig 5D). This might be because of the antibody's ability to block signaling; it is also possible that the tight junctions in CTOSs (illustrated by ZO-1 staining in Fig. 5B) stopped the antibody from penetrating sufficiently.

The NRG1-HER3 pathway plays important roles in the maintenance of normal airway epithelium.[35] HER3 and its ligands are both constitutively expressed in normal lung alveolar cells. NRG1 is secreted to the apical side, whereas HER3 is expressed in the basolateral side; thus, HER3 has no access to the ligands. Upon injury to the alveolar epithelium, NRG1 binds to HER3 and the signal contributes to wound healing. Lung cancer might use the wound healing system of normal lung epithelium. In our experiments with CTOSs, NRG1 was the most potent inducer of proliferation and AKT phosphorylation (Fig. 5A). The phosphorylation of HER3 after NRG1 treatment was observed on the basolateral membrane (Fig. 5B). In StemPro hESC, CTOSs from squamous cell carcinomas showed relatively poor growth compared with CTOSs from adenocarcinomas (Supplementary Fig. 4B, Supplemental Digital Content 5, http://links.lww.com/JTO/A359). In addition, two CTOSs from squamous cell carcinomas did not depend on any of the growth factors tested (Supplementary Table 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A356). These results suggest that CTOSs from squamous cell carcinomas require growth factors other than those tested in the present study. It was recently reported that NRG1-HER3 signaling promotes mammosphere formation from breast cancer–derived single cells.[36] NRG1 might exert an effect on the three-dimensional culture of cancer cells; although in CTOSs, NRG1 was not necessary for their formation.

It is reported that activation of PI3K-AKT by EGFR requires HER3, using gefitinib-sensitive cell lines.[37] NRG1 is also reported to promote tumor growth in two of eight lung adenocarcinoma cell lines (25%), and both of the responders were *EGFR* mutants.[38] However, in the present study, some of the *EGFR* wild-type CTOSs also responded to NRG1 (Supplementary Table 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A356), probably via phosphorylation of HER3 by HER2. These results suggested that the role of HER3 signaling might not be restricted to the *EGFR* mutants. Further studies are required to assess whether NRG1 alone can be substituted for StemPro hESC and allow not only growth, but also maintenance of original characters, and tumorigenicity of CTOSs.

In conclusion, the present findings show that the novel CTOS method can be successfully used to obtain primary lung tumor cells of high viability and purity. Thus, it is possible that CTOSs could be a new platform for studying lung cancer biology, and for examining an individual patient's response to clinical treatment.

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