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NF-κB signaling is activated and confers resistance to apoptosis in three-dimensionally cultured *EGFR*-mutant lung adenocarcinoma cells

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ABSTRACT

Epidermal growth factor receptor (*EGFR*)-mutant lung adenocarcinoma cells in suspension undergo apoptosis to a greater extent than adherent cells in a monolayer when EGFR autophosphorylation is inhibited by EGFR tyrosine kinase inhibitors (TKIs). This suggests that cell adhesion to a culture dish may activate an anti-apoptotic signaling pathway other than the EGFR pathway. Since the microenvironment of cells cultured in a monolayer are substantially different to that of cells existing in three-dimension (3D) *in vivo*, we assessed whether two *EGFR*-mutant lung adenocarcinoma cell lines, HCC827 and H1975, were more resistant to EGFR TKI-induced apoptosis when cultured in a 3D extracellular matrix (ECM) as compared with in suspension. The ECM-adherent *EGFR*-mutant cells in 3D were significantly less sensitive to treatment with WZ4002, an EGFR TKI, than the suspended cells. Further, a marked degradation of IκBα, the inhibitor of nuclear factor (NF)-κB, was observed only in the 3D-cultured cells, leading to an increase in the activation of NF-κB. Moreover, the inhibition of NF-κB with pharmacological inhibitors enhanced EGFR TKI-induced apoptosis in 3D-cultured *EGFR*-mutant cells. These results suggest that inhibition of NF-κB signaling would render ECM-adherent *EGFR*-mutant lung adenocarcinoma cells *in vivo* more susceptible to EGFR TKI-induced cell death.

1. Introduction

Gain-of-function mutations in the epidermal growth factor receptor (*EGFR*) gene are detected in ~30–50% of non-small cell lung cancers (NSCLCs) in East Asia and in ~10% of cases in North America and Western Europe. Most *EGFR*-mutant NSCLCs display adenocarcinoma histology. These *EGFR*-mutant lung adenocarcinomas depend on constitutively active EGFR signaling for survival [1,2]. When compared with cells in a monolayer on culture plastic, cancer cells in suspension show a markedly increased resistance to conventional chemotherapeutic drugs, which predominantly affect cells that are rapidly dividing, possibly due to cell cycle arrest in the suspended cells [3,4]. Our previous *in vitro* experiments, however, have shown that suspended *EGFR*-mutant lung adenocarcinoma cells undergo apoptosis to a greater extent than do adherent cells in a monolayer when treated with EGFR tyrosine kinase inhibitors (TKIs) [5]. This suggests that *EGFR*-mutant lung adenocarcinoma cells critically depend on constitutive EGFR activation to thrive in anchorage-independent conditions, and *EGFR*-mutant cells floating in circulation or pleural effusion *in vivo* may be vulnerable to EGFR TKIs. Alternatively, some anti-apoptotic signaling, which is probably derived from the adhesion of cells to culture dishes, may be activated in adherent tumor cells cultured in a monolayer in addition to signaling via the EGFR pathway.

Since the microenvironment of cells in a monolayer culture condition is considerably different to that of cells *in vivo* existing in a three-dimensional (3D) structure, it remains unclear whether extracellular matrix (ECM)-adherent *EGFR*-mutated carcinoma cells *in vivo* are less sensitive to EGFR TKIs than cells floating in circulation or pleural effusion. The aim of this study was 1) to clarify whether ECM-adherence in a 3D culture model renders *EGFR*-mutant lung adenocarcinoma cells more resistant to EGFR TKI-induced apoptosis than those cells treated in suspension; and, if this is the case, 2) to determine what molecule(s) contribute to this increased resistance to apoptosis in ECM-adherent cells.

2. Materials and methods

The experimental procedures were approved by the Institutional Review Board at the Kanagawa Cancer Center, Japan.

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2.1. 3D on-top culture

We cultured *EGFR*-mutant lung adenocarcinoma cells above a thin layer of 100% ECM in medium containing 2% ECM, termed "3D on-top culture", as previously described [6,7]. Briefly, culture dishes of 94-mm in diameter (Greiner Bio-One, Tokyo, Japan) were coated with 2350 μL/dish of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), containing a laminin-rich mixture of ECM proteins produced by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, and incubated at 37 °C for 30 min to allow the Matrigel to solidify. Subsequently, spheroids, which were formed from $\sim 60 \times 10^5$ tumor cells in a suspension culture system using 90-mm low cell binding dishes (Nalge Nunc International KK, Tokyo, Japan), were resuspended in 10 mL of 3D culture medium (RPMI 1640 medium containing 2% Matrigel, and supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin) and added on top of the Matrigel (Fig. 1A). For 3D on-top culture with 24-well culture plates, Matrigel matrix 24-well plates (BD Biosciences) were used, and spheroids were seeded onto the gel at an initial density of $\sim 2 \times 10^5$ cells per well in 500 μL of 3D culture medium.

2.2. Cell culture and drugs

Two *EGFR*-mutant lung adenocarcinoma cell lines, HCC827 (del E746-A750) and NCI-H1975 (L858R/T790 M), were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37 °C in a humidified incubator with 5% CO_2 in air. HCC827 cells or H1975 cells were seeded on low cell binding dishes and then cultured in 10 mL medium without 2% Matrigel for 24 h. In these conditions, the suspended cells aggregated to form tight, densely packed spheroids (Fig. 1A) [5,8]. The spheroids were then cultured in 3D on-top or suspension culture conditions for another 48 h. Subsequently, the spheroids were untreated or treated with the small-molecule inhibitor(s) for another 24 h. The pharmacological agents used in this study were the third-generation EGFR TKI, WZ4002 (Selleck Chemicals, Houston, TX, USA) [9] and a potent, specific IKKβ inhibitor, TPCA-1 (Tocris Bioscience, Ellisville, MO, USA) [10]. As a control, cells were treated with the same concentration of dimethyl sulfoxide (Sigma–Aldrich, St. Louis, MO, USA). Stock solutions of the drugs were prepared at 10 mM in dimethyl sulfoxide.

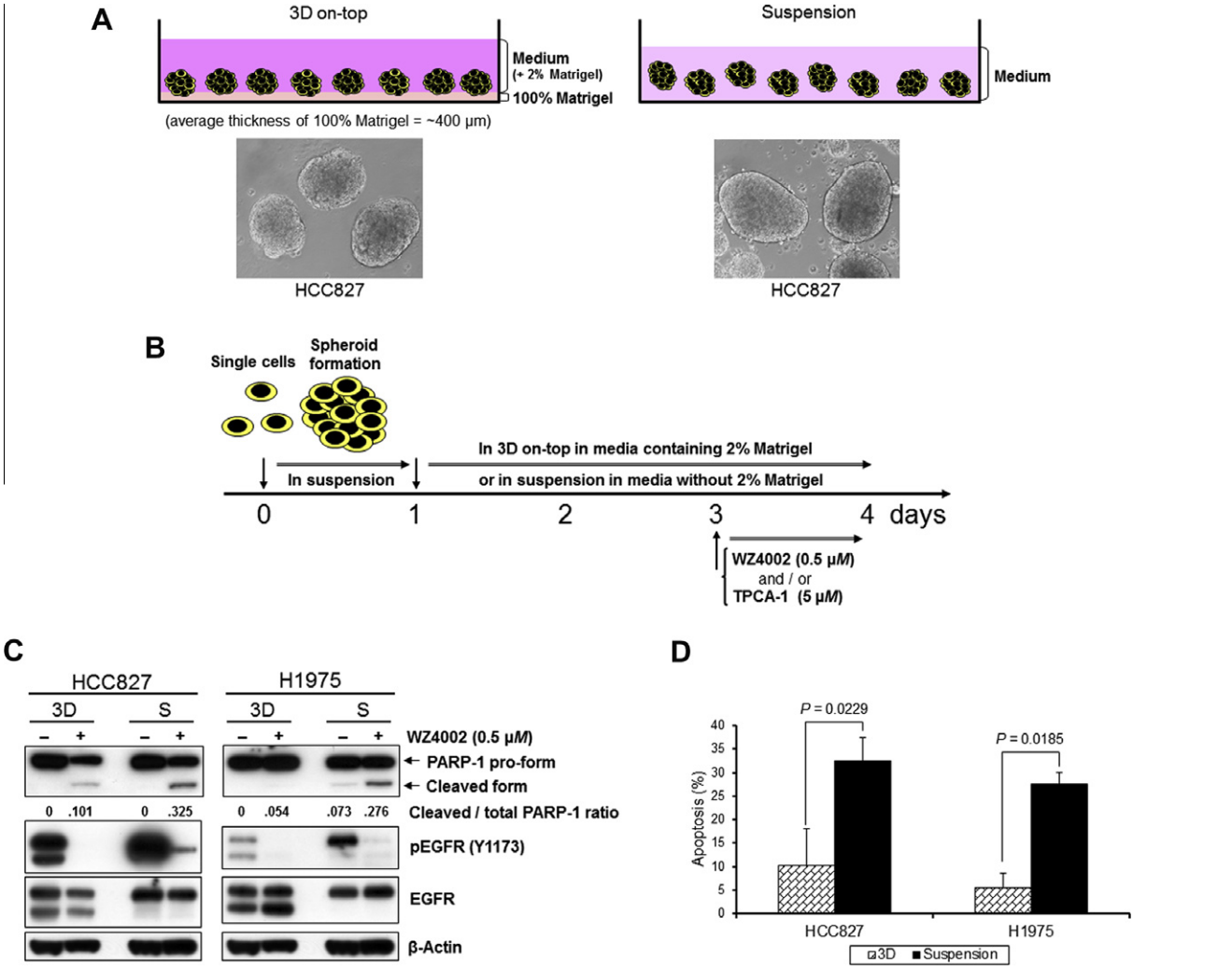

Fig. 1. Sensitivity of *EGFR*-mutant cells to EGFR TKI when cultured in 3D or suspension. (A) Schematic description of culture methods used, and representative images of spheroids of HCC827 cells in the 3D 'on-top' Matrigel or suspension culture conditions. (B) Experimental schema. (C) Western blots examining the effects of WZ4002 treatment on apoptosis in 3D-cultured or suspended cells. Cells were grown and treated with WZ4002 for 24 h as shown in panel B. The cleaved/total PARP-1 ratio determined for each cell line using Image J software is also represented. The values are the means of three independent experiments. S, suspension culture. (D) Quantification of apoptotic cells assessed by PARP-1 cleavage from each cell line treated in 3D or in suspension culture conditions. Columns, means ($n = 3$); bars, SD.

2.3. Western blotting

3D-cultured cells were harvested by treatment with Dispase (BD Biosciences) to resolve Matrigel, according to the manufacturer's instructions. The cells, untreated or treated with the indicated agent(s) and cultured in 3D or suspension conditions, were then lysed in NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA). The whole cell lysates were subjected to SDS–PAGE (NuPAGE 4–12% Bis-Tris Gel; Invitrogen) followed by blotting with specific antibodies, and detection using the Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). The types and dilutions of primary antibodies used were: anti-PARP-1 (p116/p85) (E78; 1:1000 dilution; Epitomics, Burlingame, CA, USA), anti-EGFR (1:1000 dilution), anti-phospho-EGFR (Y1173) (53A5; 1:2000 dilution), anti-IKKβ (2C8; 1:1000 dilution), anti-phospho-IKKα (S176)/IKKβ (S177) (C84E11; 1:500 dilution), anti-IκBα (44D4; 1:1000 dilution), anti-NF-κB p65 (1:2000 dilution), or anti-phospho-NF-κB p65 (S536) (93H1; 1:2000 dilution), and anti-β-actin (AC–15; 1:10,000 dilution; Sigma–Aldrich). All antibodies used, except anti-PARP-1 and anti-β-actin, were purchased from Cell Signaling Technology Japan (Tokyo, Japan).

2.4. Apoptosis assays

Apoptosis was examined by western blot analysis of the cleaved poly(ADP-ribose) polymerase-1 (PARP-1) and Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA). PARP-1 is an intracellular "death substrate" and its cleaved-form is used as a representative marker of apoptosis [11]. For the PARP-1 cleavage assay, the band intensities of the pro- and cleaved-forms of PARP-1 were measured on X-ray films using Image *J* software. For Caspase 3/7 activation, tumor spheroids of the two cell lines were seeded on Matrigel matrix 24-well plates for 3D culture at an initial density of $\sim 2.0 \times 10^5$ cells per well in 500 μL of 3D culture medium, and allowed to adhere to the gel for 24 h. The cells were then untreated or treated with the indicated drug(s) for another 24 h. After 48 h, cell apoptosis was assessed using the Caspase-Glo 3/7 Assay, according to the manufacturer's instructions.

2.5. Statistical analysis

Differences in rates of apoptosis between treated cells in 3D or suspension culture conditions, or between 3D-cultured cells treated with WZ4002 alone or in combination with TPCA-1, were evaluated by paired t-tests. *P*-values of less than 0.05 were considered significant. All statistical calculations were performed with the JMP software system (JMP for Windows version 7; SAS Institute Japan; Tokyo, Japan).

3. Results

3.1. Three dimensionally cultured EGFR-mutant lung adenocarcinoma cells are more resistant to WZ4002-induced apoptosis than suspended cells

Our 3D culture model seems to recapitulate well carcinoma tissue architecture *in vivo* with evidence of tumor cell spheroids, where cells are interconnected with one another and adhering to adjacent ECM. During the period of observation, spheroids of HCC827 or H1975 cells in 3D culture settings maintained almost the same round shape as suspended spheroids (Fig. 1A, 1B). The microenvironment of 3D-cultured spheroids was thus similar to that of spheroids in suspension, with the exception that 3D-cultured spheroids interacted with the ECM. However, the PARP-1 cleavage assay demonstrated that 3D-cultured *EGFR*-mutant cells were significantly less sensitive to WZ4002-induced apoptosis than suspended cells (Fig. 1C, 1D). Phosphorylation of EGFR expressed in *EGFR*-mutant cells in the 3D culture was completely suppressed by WZ4002 treatment (Fig. 1C), indicating that the adhesion of spheroids to the ECM did not elevate drug efflux activity, and that the 2% Matrigel within the media did not affect drug diffusion. These observations suggest that some anti-apoptotic signaling, probably derived from the interaction between the spheroids and the ECM, was activated only in 3D-cultured spheroids.

3.2. NF-κB is activated in 3D-cultured EGFR-mutant lung adenocarcinoma cells

We found that the expression of IκBα, the inhibitor of nuclear factor-κB (NF-κB), was markedly reduced in 3D-cultured cells, as compared with suspended cells (Fig. 2A). As a result, the activation of NF-κB was enhanced in 3D-cultured cells, as confirmed by the increased expression of phosphorylated NF-κB component p65 (also termed RelA). The marked reduction in the levels of IκBα in 3D-cultured cells seemed to be due to activation of IκB kinase (IKK) β (Fig. 2A), which phosphorylates IκB. This phosphorylation, in turn, causes IκB to become ubiquitylated and rapidly degraded in proteasomes [12]. In the absence of IκB, NF-κB is free to move into the nucleus, where it activates the expression of anti-apoptotic genes. We surmised that these anti-apoptotic genes activated by the NF-κB transcription factor play an important role in the increased resistance of 3D-cultured *EGFR*-mutant cells to WZ4002-induced apoptosis. The obvious decrease in IκBα expression levels, leading to increased NF-κB activation, was observed as early as 6 h after tumor cell spheroids were cultured in 3D conditions (Fig. 2B).

3.3. TPCA-1(a specific inhibitor of IKKβ) treatment enhances the levels of apoptosis induced by EGFR TKI in 3D-cultured EGFR-mutant lung adenocarcinoma cells

The findings mentioned above suggested that NF-κB activation played a role, at least in part, in the increased resistance of 3D-cul-

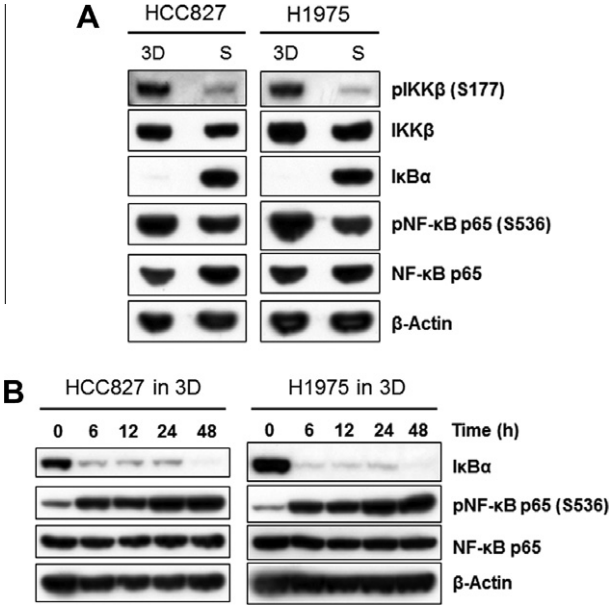

Fig. 2. NF-κB activation induced in 3D culture conditions. (A) Western blots examining the effects of culture conditions (3D or suspension) on HCC827 cells and H1975 cells. Cells were harvested and lysed in LDS sample buffer at day 3 (schema is shown in Fig. 1B). S, suspension culture. (B) Western blots examining the effects of 3D culture on NF-κB activation in each cell line. Cells were seeded and left in suspension to form spheroids for 24 h, and then maintained in 3D settings for another 0, 6, 12, 24 or 48 h.

tured cells to WZ4002-induced apoptosis. NF-κB pathway inhibition can be achieved through the inhibition of IKKβ, the primary kinase that promotes IκB instability and leads to NF-κB activation. We thus tested whether a potent, selective IKKβ inhibitor, TPCA-1 [10], could enhance the susceptibility of *EGFR*-mutant lung adenocarcinoma cells to WZ4002 in our 3D-culture. The action of TPCA-1 treatment was confirmed by the increased levels of IκBα. Fig. 3A shows that caspase 3/7 activation induced by the combined treatment of WZ4002 plus TPCA-1 was significantly greater than that induced by WZ4002 alone in *EGFR*-mutant cells in 3D culture. Further, the combination treatment induced apoptosis to a greater degree than WZ4002 alone in terms of PARP-1 cleavage (Fig. 3B). The efficacy of the combined treatment of WZ4002 plus TPCA-1 was also supported by a significant increase in the number of fragmented cells observed in the treated spheroids (Fig. 3C). Another IKKβ inhibitor, BMS-345541 [10], also increased the level of apoptosis in 3D-cultured cells treated with WZ4002 (data not shown). These findings indicate that enhanced NF-κB activation in 3D settings plays a certain role in the decreased vulnerability of 3D-cultured *EGFR*-mutant spheroids to WZ4002, as compared with suspended spheroids.

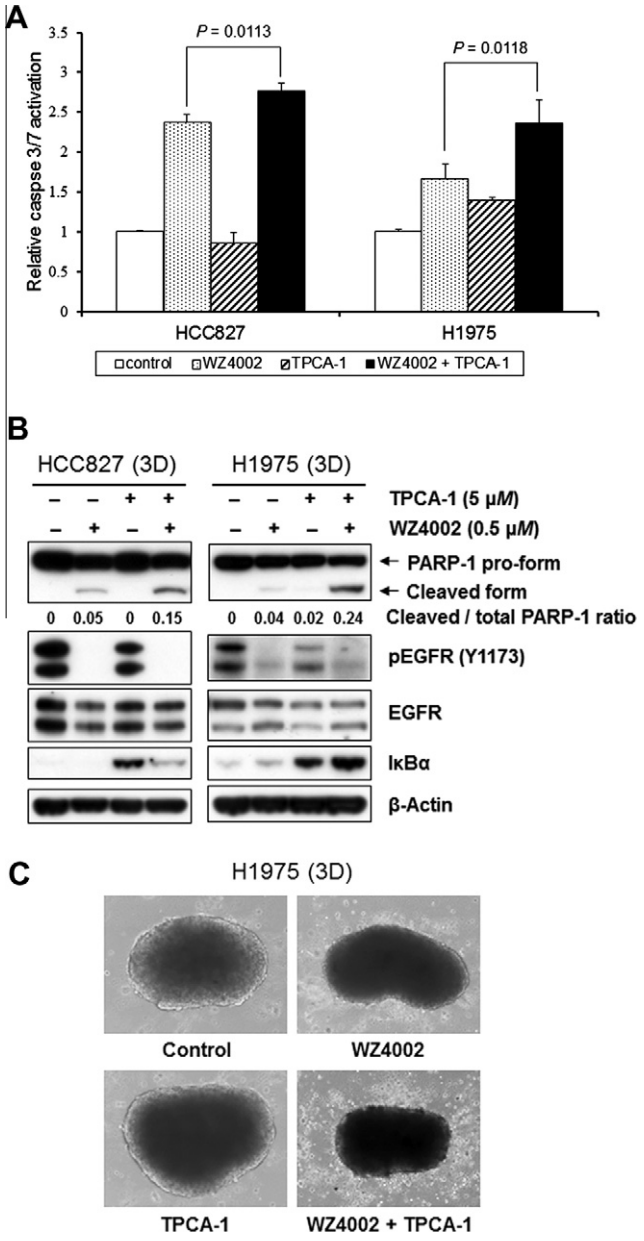

Fig. 3. EGFR TKI-induced apoptosis enhanced by the inhibition of NF-κB. (A) Caspase 3/7 activation in HCC827 and H1975 cells in 3D culture conditions. Cells were grown in suspension to form spheroids for 24 h, and then the spheroids were maintained in 3D culture for 24 h, before being untreated or treated with the indicated drug(s) for another 24 h. Caspase activity was evaluated using the Caspase-Glo 3/7 assay and normalized to an arbitrary unit of 1.0 for the mean of three untreated wells. The means ($n = 3$) and SDs are plotted. (B) Western blots examining the effects of the EGFR TKI, WZ4002, the IKKβ inhibitor TPCA-1, or a combination of WZ4002 and TPCA-1, or neither in 3D-cultured HCC827 or H1975 cells. Cells were grown and treated with the indicated pharmacological agent(s) for 24 h (see Fig. 1B). The cleaved/total PARP-1 ratio determined for each cell line was measured using Image J software. (C) Representative morphology of spheroids of untreated or treated H1975 cells in 3D culture conditions. Cells were grown and treated with the indicated drug(s) for 24 h, and representative microscopic images were then photographed at day 4 (see Fig. 1B). Original magnification × 100.

4. Discussion

Although the 3D on-top culture model exploited in this study does not fully recapitulate all aspects of the *in vivo* microenvironment, it appears to offer a more natural culture setting than a monolayer culture [6,7]. In particular, our 3D culture model seems to be similar, at least partly, to carcinoma tissue architecture *in vivo* where developed tumor cell nests, in which cells interconnect with each other, adhere to the ECM through hemidesmosomes. This is because tumor cell spheroids, where cells are interconnected with one another, adhere to adjacent ECM in our 3D model. Here, we have demonstrated that 3D-cultured spheroids of two *EGFR*-mutant lung adenocarcinoma cell lines (HCC827 and H1975) were significantly less sensitive to EGFR TKI-induced apoptosis than suspended spheroids. Interestingly, IκBα was markedly degraded only in the 3D culture, probably mediated by IKKβ, leading to aberrant NF-κB activation. IKKβ phosphorylates IκB, and the phosphorylated IκB then becomes ubiquitylated and rapidly degraded in proteasomes [12]. It seems that such activation of the NF-κB pathway was mainly caused by interactions between tumor spheroids and the ECM (100% Matrigel), because only spheroids in the 3D culture, and not those in suspension, adhered to and interacted with the ECM. More importantly, we confirmed that the pharmacological inhibition of the enzymatic activity of IKKβ enhanced the efficacy of EGFR TKI in 3D-cultured *EGFR*-mutant cells. The findings on the NF-κB activation induced in 3D culture obtained in the present study are essentially in accord with those established by the seminal study of Weaver and colleagues in breast epithelial cells [13]: Weaver et al. previously clarified that 3D cell organization and polarity were responsible for the activation of NF-κB and regulation of cellular sensitivity to drug-induced apoptosis. However, our study is the first report to demonstrate that NF-κB is activated and confers resistance to EGFR TKI-induced apoptosis in 3D-cultured, polarized spheroids of *EGFR*-mutant lung adenocarcinoma cells.

Although only a minority of cells constituting spheroids attached themselves to the ECM in our 3D on-top culture system,

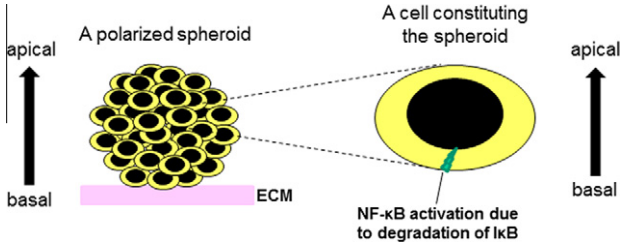

Fig. 4. Schema of a polarized spheroid in 3D on-top culture conditions and a cell constituting the spheroid.

we observed a marked degradation of IκBα in these 3D-cultured spheroids. This suggests that IκBα was degraded in most, if not all, cells constituting spheroids. Adhesion of a spheroid to the ECM probably induces spheroid polarization, and we surmise that the ECM may also induce polarization of the cells constituting the spheroid, with IκB being degraded in the polarized cells (Fig. 4). It remains to be elucidated precisely whether and to what degree IκB is degraded; in other words, the degree to which NF-κB is activated in individual cells constituting a spheroid.

NF-κB activation has been shown to be essential for the development of K-*ras* driven lung adenocarcinomas [14]. More recently, the transcription factor has been reported to play a certain role in non-uniform response to EGFR TKI treatment in *EGFR*-mutant lung cancers [15]. The results presented here clearly reveal that NF-κB is activated and significantly correlated with increased resistance to EGFR TKI-induced apoptosis in *EGFR*-mutant lung adenocarcinoma cells cultured in 3D conditions that better reflect the *in vivo* microenvironment. Moreover, clinical data exist showing a positive association between low IκB expression (=high NF-κB activation state) and worse progression-free survival and decreased overall survival in *EGFR*-mutant lung cancer patients treated with EGFR TKI [15]. These data collectively suggest that pharmacological inhibition of NF-κB using an IKKβ inhibitor may improve the clinical outcome for *EGFR*-mutant lung adenocarcinoma patients treated with EGFR TKIs.

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