A liquid culture cancer spheroid model reveals low PI3K/Akt pathway activity and low adhesiveness to the extracellular matrix

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Introduction

Adherent cells, both cancerous and noncancerous, grow in the form of clusters when cultured in three-dimensional (3D) condition in vitro [1]. If sufficient extracellular matrix (ECM) is provided and cells maintain a strong apical–basal polarity, the clusters present a cyst-like structure, also known as acini or spheres, comprising a closed cell monolayer surrounding a lumen [2]. The cyst cells are orientated such that Three-dimensional (3D) cultures of cancer cells in liquid without extracellular matrix (ECM) offer in vitro models for metastasising conditions such as those in vessels and effusion. However, liquid culturing is often hindered by cell adhesiveness, which causes large cell clumps. We previously described a liquid culture material, LA717, which prevents nonclonal cell adhesion and subsequent clumping, thus allowing clonal growth of spheroids in an anchorage-independent manner. Here, we examined such liquid culture cancer spheroids for the acquisition of apical–basal polarity, sensitivity to an Akt inhibitor (anticancer drug MK-2206) and interaction with ECM. The spheroids present apical plasma membrane on the surface, which originated from the failure of polarisation at the single-cell stage and subsequent defects in phosphorylated ezrin accumulation at the cell boundary during the first cleavage, failing internal lumen formation. At the multicellular stage, liquid culture spheroids presented bleb-like protrusion on the surface, which was enhanced by the activation of the PI3K/Akt pathway and reduced by PI3K/Akt inhibitors. Liquid culture spheroids exhibited slow proliferation speed and low endogenous pAkt levels compared with gel-cultured spheroids and 2D-cultured cells, explaining the susceptibility to the Akt-inhibiting anticancer drug. Subcutaneous xenografting and in vitro analysis demonstrated low viability and adhesive property of liquid culture spheroids to ECM, while migratory and invasive capacities were comparable with gel-cultured spheroids. These features agree with the low efficacy of circulating tumour spheroids in the settling step of metastasis. This study demonstrates the feature of anchorage-independent spheroids and validates liquid cultures as a useful method in cancer spheroid research.
the basal plasma membrane out-faces and interacts with ECM, whereas the apical membrane forms the lining of the internal lumen for secretion and absorption, thus mimicking glandular or ductal structure [3]. In the absence of ECM or anchorage, noncancerous epithelial cells either undergo apoptosis by anoikis [4] or, often in vitro, use other cells as an anchorage and thus form apical plasma membrane out-facing (apical-out) spheroids [5]. Cancer cells also prefer to grow on the scaffold if it is available, tending to congregate together in the fluid, forming spheroids without a clear cyst-like structure. As most cancerous cells maintain apical-basal polarity only weakly if any, the surface of tumour spheroids may be apical, basal or possibly neither, depending on the cell type, the extracellular environment and degree of malignancy [1,6–10]. Presentation of functionally distinct apical or basal features on the surface is crucial, especially in vivo where the spheroids interact with cells, matrices and fluid. For example, circulating tumour spheroids undergo dynamic cytoarchitectural changes during metastasis [6]. In another example, apical-out metastatic cancer spheroids are found in the peritoneal cavity of colon cancer patients in vivo and are associated with a high potency of invasion [10].

While the process of apical–basal polarisation has been extensively studied in MDCK cells using gel matrices based on the physiological relevance, studies of cancer cells require a contrasting analysis both in the liquid and in the matrix because of their metastatic potential and subsequent interaction with fluidal or matrix environment in vivo. Because of this, it has been much desired to have an in vitro system where an apical-out or basal-out polarity is fully controllable. Liquid cultures facilitate apical-out polarity and reflect cells traversing in vessels or cavities, whereas gel matrices facilitate basal-out polarity mimicking growth in ECM. Suspension cultures on low attachment plates or agarose-coated plates have been employed for apical-out, anchorage-independent growth of many types of cells; however, the spheroids often result in large clumps with a highly necrotic centre. We have recently developed a culture supplement of low molecular weight agar, named LA717, which facilitates the effective dispersion of cell suspension in the medium [11]. Application of LA717 also permits cell growth only as clones by inhibiting interspheroidal adhesion, thus preventing clumping [11]. Another advantage of LA717 is that ECM components may be added at variable concentrations as required to induce anchorage-dependent growth. As such, LA717 offers many potentials for the study of spheroid interaction with the microenvironment reflecting various in vivo context. It was also noted that LA717-grown spheroids showed marked sensitivity to specific anticancer drugs such as Akt inhibitor than 2D-grown cells, suggesting that specific pathways are differentially modulated between the anchorage-independent and anchorage-dependent growths.

This study aimed to characterise cancer spheroids grown in the liquid medium with LA717 by comparing them with those grown in gel matrices. We demonstrate that epithelium-derived breast cancer cell MCF7 presents apical-out phenotypes in the liquid culture with LA717, whereas apical-in phenotypes in ECM gel, with a graded shift depending on the gel concentration. The initial process of apical-basal polarity acquisition at 1- to 2-cell stages revealed that phosphorylated ezrin forms foci on the outer cell membrane in gel-grown cells, whereas the foci are not clearly formed in the liquid culture, suggesting that the membrane sorting mechanism is impaired. We found that liquid culture MCF7 spheroids had low endogenous Akt activities and that PI3K and Akt activators promoted presentation of apical markers on the surface of the spheroid, whereas the inhibitors increase them in the liquid culture. Furthermore, liquid culture spheroids exhibited a low adhesive ability to ECM and affected engrafting capacity in subcutaneous xenografting. LA717 has allowed us to produce apical-out or, in combination with gels, basal-out spheres in a controlled manner for analyses, which are useful for in vitro model systems representing interstitial and influid states, respectively.

Results

3D liquid cultures with LA717 form apical-out spheroids

First, MCF7 spheroids were grown in ECM gel (Matrigel® or Geltrex®, 100%, 60% or 25% v/v) or with LA717 and compared for the expression of apical–basal markers. The apical plasma membrane was detected by antibodies against phosphorylated ezrin/radixin/moesin (pERM), which is highly enriched on the apical side of polarised epithelial cells [12], whereas the basal plasma membrane was detected by antibodies against β1-integrin, which interacts with ECM [13]. In 3D cultures with 100% Geltrex, β1-integrin localised on cell–cell boundaries and on cell–matrix boundaries, whereas pERM was seen mainly at the lining of internal cavities, mimicking cyst structures, as anticipated (Fig. 1A; Movie S1). In 25–60% Geltrex, pERM was seen on the surface of the spheroids in addition to the lining of internal cavities (Fig. 1B,C; Movies S2 and...
S3). Spheroids grown with LA717 without gel (hereinafter called LA717-grown spheroids) showed pERM most prominently on the surface (Fig. 1D; Movie S4). pERM-positive small cavities were also observed internally, showing that luminal cavities can form in the multilayered liquid-grown spheroids (Fig. 1D, z = 21, 30). Quantification analysis showed a gradual increase in pERM expression on the spheroid surface, by the decreasing gel concentration in both Matrigel and Gel-trex (Figs S1 and S2). The increase in pERM on the

**Fig. 1.** Characterisation of MCF7 spheroids grown in the liquid culture or the gel matrix. (A–D) Confocal images of MCF7 spheroids grown in various conditions, with Geltrex at 100%, 60% and 25% or LA717 as indicated on the panel, for 5 days and stained for pERM (green), β1-integrin (red) and nuclei (blue). Five different z-planes are shown in each column. The whole Z scan is available in Movies S1–S4. Note pERM-positive cavity-like structures in all cases. (D) shows high pERM staining on the surface of the spheroid (z = 11, 59; arrow in Z = 45). β1-integrin (red) is on cell–cell and cell–ECM boundaries in 60–100% Geltrex, whereas in spheroids in 25% Geltrex or LA717, β1-integrin is mostly on internal cell–cell boundaries and not on the surface of the spheroids. Quantification of pERM staining on the spheroid surface and comparison between Geltrex and Matrigel are shown in Fig. S2. (E–G) MCF7 spheroids stained for E-cadherin (green), β1-integrin (red) and nuclei (blue). Note that spheroids that were grown with LA717 or 25% Geltrex show E-cadherin in spots underneath the cell boundary and on the cell membrane, whereas those grown in 100% Geltrex show E-cadherin mostly localised to the cell boundary. It is also noticeable in spheroids grown with LA717 that β1-integrin is partly missing on the surface (arrows). (H–J) MDCK spheroids in various conditions for comparison to (A–D). In 100% Geltrex, they present clear apical-in/basal-out acinar structure. The acinar structure is partly compromised in 25% Geltrex. In the LA717-containing medium, the surface of the spheroid is exclusively pERM-positive, and β1-integrin is seen only on cell–cell boundaries. Z scan of (J) is available in Movie S5. (K–P) Nonconfocal images of MDCK spheroids grown for 4 days with LA717 and additional Geltrex as indicated. The surface of spheroids grown without gel is exclusively pERM-positive (P). In 20% Geltrex, pERM is on the surface and on the lining of the internal cavities (O). In 30% Geltrex, the surface shows a mix of pERM-positive and β1-integrin-positive membranes (arrows and arrowhead, respectively, in N). As the percentage of the Geltrex concentration increases, the incidence of pERM-expressing plasma membrane on the surface of the spheroids decreases (K–N). Quantification of the incidence in both Geltrex and Matrigel is shown in Fig. S5. Scale bars in (A–P), 20 μm.
Matrigel at the 25% spheroid surface was more prominent in Geltrex than Matrigel at the 25–60% gel concentration, while there was no difference at 100% (Fig. S2). In LA717-grown spheroids, β1-integrin was detected only partly on the surface, in a manner complementary to pERM staining (Fig. 1D, z = 11, 59). These results demonstrate that MCF7 cells form apical-out spheroids in LA717, whereas in 100% gel matrices they form apical-in, basal-out spheroids, and that graded concentration of gels causes a graded amount of pERM expression on the surface.

Further analysis using other polarity markers confirmed the apical-out polarity in LA717-grown MCF7 spheroids. Podocalyxin, which colocalises with pERM on terminally polarised, well-segregated apical plasma membrane [14], was expressed on the spheroid surface similarly to pERM, although not clearly expressed in the luminal surface of the internal cavity (Fig. S3). A tight junction protein ZO-1 was expressed on the surface of spheroids grown with LA717 at the cell border, and not in spheroids grown in Geltrex or Matrigel (Fig. S3), showing a clear distinction in the formation of the tight junction on the liquid-grown and gel-grown spheroid surfaces. Cytokeratin 18 (CK18), expressed in luminal breast cancer cells [15], showed a similar expression pattern as β1-integrin, outlining the spheroids grown in Geltrex or Matrigel and partly on LA717-grown spheroids (Fig. S3). These results confirmed the apical-out polarity of LA717-grown MCF7 spheroids.

We additionally examined whether LA717-grown spheroids acquire apical-in, basal-out polarity when transferred to the gel matrix. MCF7 cells were first incubated with LA717 to form apical-out spheroids, which were then transferred to 100% of Matrigel or Geltrex. The result showed acquisition of apical-in, basal-out polarity with internal luminal cavity formation, demonstrating dynamic polarity changes in response to the provided gel matrix (Fig. S4).

The cell–cell boundary of LA717-grown MCF7 spheroids was examined by co-staining with E-cadherin and β1-integrin, lateral and basal markers, respectively. In spheroids cultured with 25–60% Geltrex, the two markers mostly overlapped on cell-cell boundaries, suggesting basolateral features, whereas the outer surface showed mainly β1-integrin-only, confirming cell–ECM boundary formation (Fig. 1E,F). The outer surface of LA717-grown spheroids was mostly negative for β1-integrin (Fig. 1G), agreeing with the aforementioned pERM expression on the surface. Endocytosed E-cadherin, shown as intracellular vesicular staining, was prominent in spheroids grown with LA717 and 25% Geltrex (Fig. 1F,G), suggesting that the spheroids were at mechanically dynamic state with weak cell–cell adhesion [16,17]. Collectively, these results demonstrate that MCF7 spheroids grown in liquid culture with LA717 mainly present apical plasma membrane on the surface with dynamic cell–cell assembly, different from gel-grown spheroids that present β1-integrin-positive basal plasma membrane on the surface with a static structure. When Matrigel or Geltrex is added to the LA717-containing medium, spheroids restore the basal-out structure.

As a control, well-studied noncancerous epithelial cells, MDCK, were grown in parallel. MDCK cells formed cyst structures with cavities, with clear apical-in/basal-out structure, when cultured in 100% Geltrex or Matrigel [18] (Fig. 1H, Fig. S5). The apical–basal polarity was partly compromised in reduced concentrations of gel and fully inverted in the LA717-containing medium without gel (Fig. 1I,J, Fig. S5). Addition of an increasing amount of gel to the LA717-containing medium restored apical-in polarity (Fig. 1K–P; Fig. S5), demonstrating that LA717 may be used in combination with gel matrices. It was noted that 40–60% of Geltrex showed a higher incidence of pERM-expressing plasma membrane on the spheroid surface compared with Matrigel (Fig. S5). There were no significant differences between 80% and 100% of gels, in both Matrigel and Geltrex.

**LA717-grown spheroids do not show pERM foci during the first cell division**

The initial acquisition of apical–basal polarity in 3D has been reported in MDCK cells grown in Matrigel [13,19–22]. When cells are at a single-cell stage in Matrigel, apical markers are presented on the cell membrane. Immediately after the first cell division (i.e. at the two-cell stage), the basal markers are found at the cell–cell boundary, while apical markers remain on the out-facing membrane [19,21]. Apical proteins such as ezrin are then endocytosed from the cell membrane and translocated to the cell boundary, where the vesicular membrane fuses and forms a lumen [19,21,22]. On the remaining outer membrane, the interaction of integrins with ECM consolidates basal features; thus, the polarity is inverted and apical-in/basal-out polarity is established [13,20]. As such, the dynamics of ezrin translocation from the out-facing plasma membrane to the cell boundary via cytosol is the crucial step for the acquisition of apical-in/basal-out polarity in MDCK cells.

To examine the polarity acquisition of MCF7 cells in the absence or presence of gel, subcellular localisation of pERM and β1-integrin was analysed at the two-cell stage using Geltrex (Fig. 2). Various patterns were observed, reflecting the aforementioned multiple
**Fig. 2.** The polarity of MCF7 cells at 1- to 2-cell stages in gel and liquid. (A–C) MCF7 cells grown for 2 days either in 100% Geltrex (A) or in the medium with LA717 (B) and stained for pERM (green), β1-integrin (red) and nuclei (blue). Only 2-cell stage cells were analysed and categorised as shown in the schematic drawing. The number of observed cases is also shown at the bottom right of the scheme. (C) The number of cases shown in (A, B). Phenotype 1 (β1-integrin at the cleavage furrow) is seen in all (a–j) of Geltrex and (k–m, p–t) of LA717; phenotype 2 (e–g) and (p, t); phenotype 3 (e–g) and (m, p, t); phenotype 4 (a, d, h) and (k, q, s); phenotype 5 (b–f) and (p, q, s, t); phenotype 6 (g–j) and (p–r). Statistical analysis was performed by chi-square test. (D–F) MCF7 cells grown for 2 days either in 100% Geltrex (D) or in the medium with LA717 (E, F), stained for pERM (green), f-actin (red) and nuclei (blue). Only single cells were analysed and categorised into 3 groups: both pERM and f-actin were localised to one side (top in D, n = 20/50), only f-actin was localised to one side and no pERM expression (middle in D, n = 12/50) and evenly distributed f-actin and no pERM expression (bottom in D, n = 18/50). In the medium with LA717 (E), all single cells examined were both pERM and f-actin negative. A multicell spheroid in the same batch of LA717 culture is shown in (F) as a staining control. Scale bars in (B, D, E), 5 μm; (F), 10 μm.
steps of polarity rearrangement at the two-cell stage [19,21]. Nonetheless, one of the significant differences was that pERM was clearly seen on the outfacing plasma membrane when grown in Geltrex (n = 68/94, 72%) mostly as focal condensation, whereas with LA717, only a small number of cases showed pERM very weakly on the surface if any (6/74, 8%; Fig. 2A–C). pERM expression was also seen on the cleavage furrow in Geltrex-grown cells (n = 12/94, 13%), strongly in some cases (Fig. 2A), whereas with LA717, only weakly in lesser cases (n = 5/74, 7%; Fig. 2B), although the case numbers were not statistically different. In both the outfacing plasma membrane and cleavage furrow, the pERM staining was much weaker in cells grown with LA717. These results suggest that translocation of ezrin from the outfacing plasma membrane to the cell boundary at the two-cell stage is likely affected in the liquid culture.

It has been shown in single carcinoma cells that pERM is progressively localised to a single domain in a cap shape when grown in Matrigel [23] and liquid medium [24]. pERM is associated with f-actin underlying the cell membrane, and the localisation is crucial for the subsequent cell cleavage orientation and polarity acquisition [23,24]. Failure of pERM localisation results in disoriented cell division and following multiple-lumen formation in the gel [23], which was also observed in MCF7 cells in our study (Fig. 1A–D). We therefore examined the polarity acquisition in single MCF7 cells in the gel and liquid with LA717 (Fig. 2D–F). In 40% of cells grown in Geltrex (n = 20/50), pERM was localised to one side, accompanied by subcortical co-localisation of f-actin, and further, 24% (n = 12/50) of cells showed biased f-actin distribution without pERM (Fig. 2D). The variability might be due to the heterogeneity of MCF7 cell population as suggested by other groups [25,26]. In cells grown with LA717, however, neither pERM nor f-actin was detected in all cases examined (Fig. 2E, n = 24). This result suggests that MCF7 cells grown in the liquid culture fail to polarise at the one-cell stage and pERM assembly is impaired on the membrane, hence, little or no pERM-positive plasma membrane available for translocation to the cleavage furrow during the first cell division, thus failing polarity inversion. It is speculated that the apical-out feature is consolidated further as the spheroid grows, through the continuous contact of the outfacing plasma membrane to the surrounding fluid.

Liquid-grown MCF7 spheroids show low Akt pathway activities

Our previous study demonstrated that cancer spheroids grown in 3D with LA717 are more sensitised to an Akt inhibitor anticancer drug MK-2206 than those cultured in 2D, while the response to other anticancer drugs such as antimicrotubule agent and MEK1/2 inhibitor is similar between 3D and 2D or with a modest difference if any [11]. The high sensitivity of LA717-grown spheroids to MK-2206 was further confirmed with MCF7 cells in this study by direct comparison between 2D, 3D with LA717 and 3D with Matrigel, where IC50 was 89.7, 48.4 and 110.9 nm, respectively (Fig. 3A,B). With regard to the proliferation speed, the fastest was the 2D culture, followed by the 3D with Matrigel and the slowest was the 3D with LA717 (Fig. 3C).

As cells in 3D liquid culture with LA717 exhibited the slowest proliferation rate and were susceptible to MK-2206, it was suspected that endogenous Akt activity may be affected in cells grown with LA717. Western blot analysis revealed that MCF7 spheroids grown with LA717 for 2 or 10 days consistently exhibited lower levels of phosphorylated Akt (pAkt) than 2D-grown and Geltrex-grown cells (Fig. 3D). To confirm the effect of 3D liquid cultures on endogenous pAkt levels, spheroids were collected 6 days after the culture and compared with those collected on the day of the 3D culture setup, that is 6 h after the disassociation of 2D-grown cells. It was evident that pAkt decreased rapidly by 3D liquid cultures (Fig. 3E,F). It was noted that, on the day of dissociation, 6 h of incubation was sufficient to make cells stuck to each other in the absence of LA717. In contrast, in the presence of LA717, cells remained mostly as single cells and were prevented from sticking as expected [11] (Fig. 3F). As such, spheroids cultured without LA717 exhibited more advanced morphology and size than those with LA717, on both day 0 and day 6. It appeared that the reduction in pAkt correlated with the length of time the cells had experienced the apical-out/basal-in state.

These results suggest that MCF7 spheroids cultured in 3D without gel have lower Akt pathway activities compared to 2D or 3D with gel, hence relying on the low level of Akt activity for their viability, which likely makes them susceptible to Akt inhibitor anticancer drug MK-2206.

The PI3K-Akt pathway promotes the presentation of apical markers on the spheroid surface

It has been shown that polarised distribution of phosphatidylinositol phosphates (PIPs) and their kinases is crucial in specialising apical and basal plasma membranes [22,27,28]. Ezrin binds to 4,5-bisphosphate (PI (4,5)P2), which is enriched on the apical plasma
Fig. 3. Low pAkt in liquid culture spheroids. (A) Effect of MK-2206 on cell viability of MCF7 cells cultured in 2D, 3D with LA717 or 3D with Matrigel for 8 days. Y-axis shows the ratio of the number of viable cells compared with the vehicle control, shown in percentage. Data represent means ± SD of three independent experiments. (B) IC\textsubscript{50} of MK-2206. Values in parentheses represent 95% confidence interval. (C) Average doubling time between day 0 and 8 calculated using the relative light unit data of vehicle control in (A). Statistical significance was analysed by Dunnett’s test. Data represent means ± SD of three independent experiments. (D) Western blots of MCF7 cells grown in 2D, 3D with LA717 (2 or 10 days) and 3D with Geltrex (10 days), detecting endogenous phosphorylated Akt (pAkt) and total Akt proteins. Akt activator SC-79 and inhibitor MK-2206 were used to treat in 2D cells for 2 days as controls for pAkt detection. Molecular markers (kDa) are indicated on the right side of the blot. Below bar charts are quantification of pAkt/total Akt and total Akt/GAPDH ratios, normalised against the first lane of 2D without drugs. Tukey’s test following ANOVA showed that pAkt/Akt ratio was significantly different between 2D and 3D in LA (P < 0.01) and 3D in LA and gel (P < 0.01), whereas the total Akt/GAPDH ratio was significantly different between 2D and 3D in gel (P < 0.05) and 3D in LA and gel (P < 0.01). There was no statistical difference in the total Akt/GAPDH ratio between 2D and 3D in LA. (E) Western blots of MCF7 cells grown in 3D with or without LA717 for 6 h (day 0) or 6 days (day 6). Molecular markers (kDa) are indicated on the right side of the blot. (F) Light microscopic images of each sample used in (E). The cell density does not reflect the Western sample collection, as the total protein amount was adjusted prior to loading in (E). Without LA717, cells tend to stick together and gather in the dish. Scale bar, 100 µm.
membrane [29–31], and then, it is phosphorylated and recruits F-actin to subcortical apical membrane, thus enforcing the apical subcellular structure [30]. In agreement with this, PI3K blockers interfere with the establishment of apical–basal polarity and subsequent acinus formation in MDCK cells, especially PI3Kδ blockers inducing apical-out MDCK spheroids [27]. To examine the role of the PI3K-Akt pathway in the apical-out polarity in LA717-grown MCF7 spheroids, the pathway inhibitors and activators were applied to the spheroids and cultured for 7–8 days with LA717. Both PI3Kδ inhibitor CAL-101 and Akt inhibitor MK-2206 reduced pERM staining on the surface of the spheroids (Fig. 4A–H). On the other hand, activators of PI3K and Akt, 740Y-P [32] and SC-79, respectively, enhanced the pERM expression (Fig. 4A–H). In addition, cells on the spheroid surface tended to protrude and showed convex appearance, where pERM was particularly strongly stained (Fig. 4C,G; Fig. S1 for quantification). This was reminiscent of ‘apical budding’ observed in tumour spheroids with inverted (apical-out) polarity spread to the peritoneum [10]. It was also reminiscent of blebbing, which is seen when contractile actin cortex below the cell membrane undergoes assembly dynamics [33]. The protrusion was also positive for another apical marker podocalyxin [14] and was found from day 5 onwards (Fig. S6). The change in pERM staining by PI3K-Akt modulators was not accompanied by reciprocal changes of β1-integrin (Fig. 4A–H). Similar changes in pERM presentation by CAL-101 and 740Y-P were also observed in HCT116 and SW480 cells, in which focal pERM staining on the spheroid surface was reduced by CAL-101 and increased by 740Y-P (Fig. S7). However, given the
function of the PI3K/Akt pathway in promoting cell viability and low endogenous pAkt in the LA717-grown spheroids (Fig. 3D). Further inhibition of the pathway by CAL-101 and MK-2206 may have a cytotoxic effect. To evaluate the effect of drug treatment, MCF7 cells were transfected with Akt1 constructs, a constitutively active form (T308D;S473D) and a dominant-negative form (T308A;S473A) [34,35], before the spheroid formation in LA717. While the dominant-negative form did not show a difference from a control DNA transfected group, the active form of Akt1 mimicked the result of 740Y-P and SC-79 (Fig. 4I–L). As suggested in Fig. 3D, the endogenous pAkt was already at a minimal level in the LA717-grown spheroids; hence, the dominant-negative Akt may not have exerted its function. It was considered that the effect of inhibitor drugs was more thorough and enduring than DNA transfection, and yet, the cytotoxic effect of Akt inhibition remained possible. Taken together, it was suggested that the presentation of apical markers on the surface of LA717-grown spheroids is maintained by a low endogenous level of Akt activity, and hence, additional PI3K/Akt activation facilitates the apical marker presentation.

**Low engraftment efficacy of liquid culture spheroids in subcutaneous xenograft**

The engraftment efficacy of spheroids grown by liquid culture with LA717 was compared with 2D-cultured cells and 3D Matrigel-grown spheroids using nude mice. 3D-cultured MCF7 spheroids or 2D-cultured cells were subcutaneously injected, one site on the left shoulder per mouse, and the tumour volume was assessed for 28 days, after which the tumours were dissected and weighed (Fig. 5A). While all grafts were successfully implanted and palpable on the first week (n = 5 for each group), all five LA717-grown grafts became disintegrated and were palpable on day 7 (n = 1), day 14 (n = 3) and day 20 (n = 1) onwards, and not detected in the dissection on day 28. Two out of five 2D-grown grafts also became palpable on days 17 and 20 and not detected in the dissection. All five gel-grown spheroids were detected on the day of dissection. They were relatively small compared with those originated from 2D, although they were not statistically significant (Fig. 5B). Overall, MCF7 xenografts of LA717-grown spheroids showed statistically smaller sizes compared with other groups on day 10 and day 14 (Fig. 5A); however, the statistical difference was not detected on following days due to the disintegration issue. Because of this, the same type of xenograft was conducted using HCT116, another cancer cell line of epithelial origin. HCT116 cell grafts prepared in 2D, 3D with Matrigel and 3D with LA717, all grew without disintegration, and those from LA717 developed to significantly smaller tumours compared to 2D and 3D with gel spheroids, while the latter two groups did not show a significant difference (Fig. 5C,D). For further histological analysis, MCF7 xenografts were harvested on day 7 before disintegration. Pan-cytokeratin antibodies, detecting the grafted MCF7 cells, clearly showed abundant tumour cells originated from 2D and 3D with Matrigel conditions, whereas those from LA717-grown spheroids showed only scattered, small sizes of cell clusters with necrotic foci surrounded by inflammatory cells (Fig. 5E–J). HCT116 xenografts harvested on day 21 did not show a clear difference in histological sections, although the gross size was clearly different, reflecting the subcutaneous measurement (Fig. 5K–P). These results suggest that in the subcutaneous condition with rich ECM, LA717-grown spheroids are more disadvantaged in the settling capability compared with 3D gel-grown spheroids and 2D-grown cells.

**Low attachment efficacy of liquid culture spheroids on ECM**

To examine the attachment capacity to ECM, spheroids were placed on gelatine- or collagen I-coated plates and examined over time. Whereas Matrigel-grown spheroids were attached within 2.5 h with all cells fully flattened, LA717-grown spheroids took at least 6 h and cells were flattened only at the peripheral of the spheroid (Fig. 6A,B; Fig. S8A). The result likely reflects basal-out and apical-out features, respectively. Regarding migratory and invasion capacities in vitro, there was no significant difference between LA717-grown and Matrigel-grown spheroids (Fig. 6C,D; Fig. S8B). These results suggest that disintegration of LA717-grown MCF7 spheroids in xenografting may, at least in part, be attributed to the low attachment capacity to ECM.

Further characterisation of LA717-grown and Matrigel-grown spheroids was performed in gene expression, in view of motility and cell-extracellular interaction. There are mainly two types in cell motility: one is seen in spindle-shaped mesenchymal cells such as fibroblasts extending sharply towards the migrating direction, involving expression of Snail and MMPs and cadherin switch, often observed on the rigid ECM, while the other is often seen in round cells as blebs with amoeboid movement in pliable environments, Rho/ROCK/PI3K-dependent and MMP-independent [36–39]. Both are seen not only in migrating
Comparison of cancer spheroids in liquid and ECM

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**MCF7 Xenograft**

|       | 2D          | 3D with Matrigel | 3D with LA717 |
|-------|-------------|------------------|---------------|
| Tumourigenic capacity | 3/5         | 5/5              | 0/5           |
| Tumour (g)     | 5.4 ± 6.5   | 3.1 ± 1.3        | -             |

**HCT116 Xenograft**

|       | 2D          | 3D with Matrigel | 3D with LA717 |
|-------|-------------|------------------|---------------|
| Tumourigenic capacity | 5/5         | 5/5              | 5/5           |
| Tumour (g)     | 0.739 ± 0.323 | 0.795 ± 0.373 | 0.143 ± 0.076* |
Fig. 5. Low tumorigenicity of MCF7 and HCT116 cells grown in the liquid culture in subcutaneous xenografting. (A, C) The average volume of xenografts of MCF7 (A) and HCT116 (C) originated from cells/spheroids grown in 2D, 3D with Matrigel and 3D with LA717 (n = 5 mice per group, one graft per mouse). In MCF7 xenografting, two of 2D-derived xenografts became impalpable on days 17 and 20, respectively, and not detected on dissection on day 28. Likewise, all of five 3D-LA717-derived xenografts became impalpable on day 7 (n = 1), day 14 (n = 3) and day 20 (n = 1). Plotted volumes are the average of all 5 cases, including the impalpable cases considered as 0 mm³. Statistical analysis was conducted by Bonferroni’s test, comparing the 3D with Matrigel and LA717 groups. *P < 0.05, **P < 0.01. (B, D) The weight of tumours on the day of harvest (day 28 for MCF7 cells; day 21 for HCT116 cells) after dissection. Data represent means ± SD. *P < 0.05 in Bonferroni’s test comparing the 3D with Matrigel and LA717 groups. (E–M) Histological analyses of xenografts 7 days (MCF7 cells) or 21 days (HCT116 cells) after implantation. Paraffin sections were stained with haematoxylin and eosin (H&E) (E–G, K–M) or immunohistochemically using pan-cytokeratin (CK) antibodies (brown) to detect implanted MCF7 xenografts along with haematoxylin (blue) (H–J). Scale bars, 50 μm. (N–P) HCT116 tumours dissected on day 21. Scale bars, 1 cm.

Discussion

The 3D culture method has been employed in various contexts. One of the major applications has been to MDCK cells using Matrigel, which has served as the benchmark model system of epithelial cells, whereas, in cancer cell studies, both gel-based and liquid-based cultures may be required to reflect the metastatic property, which potentially locate cells in both fluidal and matrix environment in vivo. In this study, using LA717 in contrast to and in combination with gel matrices, we have revealed the dynamics of apical–basal polarity and Akt pathway activities as well as the interactive features of the spheroids with ECM. We demonstrated that LA717-grown spheroids present the apical plasma membrane on the out-facing surface and the feature changes in response to varying concentrations of gel matrices. Mixing LA717 with varying concentrations of Matrigel or Geltrex has also opened a range of applications in studying diverse cancer microenvironment. We also found that the apical-out spheroids are the dynamic assembly of cells compared with gel-grown, basal-out spheroids. It is based on our observation of (a) the bleb-like structure (Fig. 1D), which shows ECM-independent motility of the cells [38], (b) the increased E-cadherin internalisation (Fig. 1E–G), which is a sign of unstable cell–cell contacts [16], and (c) transformation of spheroids from apical-out to apical-in structure by transferring them from the liquid culture condition with LA717 to gel matrices (Fig. S4). This offers further experimental applications to reflect dynamic changes in the cancer microenvironment. LA717 also allowed us to analyse the initial polarisation of cells. Comparing to the MDCK’s apical-in/basal-out polarity-establishing process [13,19–22], our study showed that MCF7 cells can undergo a similar process to MDCKs when grown in gel, whereas, in the absence of gel, pERK is expressed only weakly at one- to two-cell stages, if any, and fail to gather on the cell membrane, which seems the first indication of the failure of apical-in/basal-out polarisation. LA717 has facilitated to reveal such crucial steps as it maintained clonal growths and prevented cell attachment during the culture.

One feature of LA717-grown MCF7 spheroids we found in this study is that the endogenous Akt activity decreases from 2D cultures, while the Akt protein per se is maintained (Fig. 3). The amount of pAkt reflects cells’ response to growth factors via receptor tyrosine kinase [41] and PI(3,4,5)P3 [28], both of which are preferably located at the basolateral membrane...
Fig. 6. Comparison between gel-grown and liquid-grown spheroids in adhesion, migration and invasion, and on the gene expression profile. (A) Representative images of LA717-grown or Matrigel-grown spheroids adhered to gelatine-coated plates. Scale bar, 100 µm. (B) Time course of spheroid adhesion to gelatine-coated plates. The degree of adhesion was defined as shown in the panel: full adhesion (fully flattened), partial adhesion (adhered and not fully flattened) and nonadhesion (remained as a clump), and indicated in the graph in dark blue, light blue and white, respectively. Scale bar, 100 µm. Most Matrigel-grown spheroids have adhered or at least the peripheral cells have flattened within 2.5 h, while the majority of LA717-grown spheroids remained unadhered. Six hours later, adhesion was advanced in both groups of spheroids, with more full-adhered cases in Matrigel-grown spheroids. Following three independent experiments of analysing 10–13 spheroids, statistical significance was detected by Student’s t-test as follows: at 2.5 h, full adhesion ($P < 0.05$) and nonadhesion ($P < 0.05$); at 6 h, full adhesion ($P < 0.001$) and nonadhesion ($P < 0.05$), when compared to the Matrigel and LA717 groups. A similar result was obtained using collagen I plates, as shown in Fig. S8A. (C) Migration capacity examined on gelatine-coated plates, showing no statistically significant difference between the cells from Matrigel-grown and LA717-grown spheroids. A similar result was obtained using collagen I plates, as shown in Fig. S8B. (D) Invasion capacity examined using ECM-coated polycarbonate Transwell filter, showing no significant difference between the cells from Matrigel-grown and LA717-grown spheroids. (E) qPCR analysis of the indicated gene expression. Red columns are spheroids grown in the LA717-containing medium, whereas blue columns indicate spheroids grown in Matrigel. Spheroid samples were collected directly from the 3D culture. The relative expression levels on the y-axis are the ratio to GAPDH. All data in (B–E) are shown as means ± SD of three independent experiments. Statistical analyses were performed using Student’s t-test.
In liquid-grown spheroids, the outer surface is mainly covered by apical plasma membrane, whereas inward cell-cell boundaries are E-cadherin-positive 'lateral' membrane (Fig. 1); therefore, the spheroids are likely in short of 'basal' plasma membrane. This might be a possible explanation as to why pAkt is reduced in the spheroids grown in liquid. One of the main functions of the PI3K/Akt pathway is to aid cell survival and proliferation. In agreement with this, our result showed that cell proliferation is slow in 3D liquid cultures compared to 2D or 3D with Matrigel (Fig. 3C), which may be explained by the lack of physical anchorage leading cells to high RhoA/ROCK/PTEN activity and low Akt pathway activity [44]. However, regarding cell proliferation, activation of the Akt pathway by SC-79 did not rescue the cell proliferation rate in 3D with LA717 (data not shown), suggesting that other mechanisms than the Akt pathway are responsible for the slow proliferation speed in the liquid culture. Rather, our results demonstrated that liquid-grown spheroids maintain the apical-out features by relatively low levels of endogenous Akt activities. The low endogenous level might have facilitated to reveal the effect of both PI3K/Akt activators and inhibitors. LA717-grown spheroids were very responsive to PI3K/Akt activators 740Y-P and SC-79, whereby spheroids presented enhanced apical-out phenotype (Fig. 4). The high sensitivity to inhibitors, on the other hand, is a reflection of high susceptibility to Akt inhibitor anticancer drug (Fig. 3) [11]. Another finding in this study in relation to PI3K is the role of PI3K in apical–basal polarity. It was demonstrated that PI3K blockers inhibit apical-in/basal-out polarity in MDCK cells, that is MDCK cells treated with the blockers formed the inverted apical-out/basal-in polarity [27]. In contrast, our finding is that PI3K/Akt blockers compromised apical–basal polarisation, that is neither apical nor basal markers were presented on the spheroid surface in MCF7 (Fig. 4). This has led us to an understanding that the role of PI3K is not merely to promote the apical-in/basal-out polarity as shown in MDCK cells but also to promote the apical–basal polarisation in MCF7 cells.

This study on LA717-grown spheroids revealed unique features of anchorage-independent cancer cells. One of them is protrusions resembling blebs observed on LA717-grown spheroids. Blebs are spherical protrusion of the cell membrane detached from the underlying actomyosin cortex and often seen when cells are motile in the absence of ECM [38,39]. They are first initiated as the detachment of the cell membrane from the underlying actomyosin cortex, followed by expansion as a result of actomyosin contractility powered increase in hydrostatic pressure in the cytoplasm [38]. The protruding cell membrane is initially not engaged with the actin cortex; however, as the expansion proceeds, ezrin is recruited under the bleb membrane and new actin cortex is formed [33]. Based on the dense pERM staining on many of the protruded surface in Figs 1 and 4, LA717-grown spheroids are considered to form a bleb-like protrusion. In support of this, our qPCR results showed the increase in THBS1, not EMT-related SNAIL, MMPs and cadherins (Fig. 6E) suggesting anchorage-independent cell motility. Thrombospondin 1 (TSP1), encoded by THBS1, induces focal adhesion disassembly, thus making cells motile [45]. TSP1 also supports cell survival under adhesion-independent conditions to facilitate tissue remodelling [40]. These results demonstrate that the features unique to liquid cultures were successfully presented in spheroids grown with LA717.

In this study, Matrigel-grown spheroids showed higher engraftment efficacy in subdermal implantation than LA717-grown spheroids, probably because gel-grown ones can readily interact with ECM for the settle-down process, due to the basal-out surface. Peritoneal xenografts, on the other hands, may have provided different results: the peritoneal cavity has the lining of mesothelium with microvilli [46] and offers aqueous microenvironment. In the parietal effusion, tumour spheroids were found with apical-out polarity [10]. Hence, apical-out spheroids may be favoured for peritoneal grafting. Unfortunately, successful peritoneal xenografting often requires co-injection of ECM gels, [47] hence hindering us from comparing spheroids with and without ECM. In fact, our attempt to conduct peritoneal grafting of LA717-grown spheroids without additional ECM was not successful. Intravascular space is another possible liquid environment for cancer spheroids. While in vivo circulating tumour cells present diverse features depending on the origin, size and the protein expression on the surface [48], the apical-out feature, especially the ezrin pole, plays crucial roles in attachment and extravasation [24,49]. Podocalyxin is upregulated upon attachment and the intracellular domain interacts with the cytoskeletal linker protein ezrin, inducing cortical polarisation associated with extravasation [50]. Hence, the presentation of apical features on the surface is crucial for the acquisition of invasiveness. β1-integrin is also required for extravasation of cancer cells at the initial adhesion to endothelial wall, although not required for the following protrusion step [49]. The fact that spheroids grown in diluted gel matrices or LA717 present both apical and basal membranes on the surface (Fig. 1B-D; Fig. S2) opens a possibility that in vivo tumour
spheroids may also present both features. As such, LA717-grown spheroids could well be a model for circulating tumour spheroids. Besides, given a limited size of cancer spheroids can traverse vessels (~ 20 cells per spheroid) [51], LA717 serves as an ideal method for in vitro model of cancer spheroids by preventing them from adhering to each other and growing to a large size. Furthermore, while LA717 alone serves as a method for anchorage-independent culture material, combining it with Matrigel or Geltrex allows us to control the strength of the scaffold.

Application of 3D culture is ever-expanding. An example is an organoid culture [52–54]. Intestinal organoids (enteroids) with inverted apical–basal polarity have been shown as a useful in vitro tool, where the gut ‘luminal surface’ is exposed directly to the medium in the experimental condition [54]. Another application of 3D cultures is that growing cancer spheroids in the liquid in 3D effectively increases stem cell population than in 2D [55,56]. LA717 will likely increase the efficacy of 3D cultures in these applications. In addition, the ability of LA717 to distribute cells evenly in the liquid promises to enable reliable large-scale 3D cultures.

Materials and methods

Cell culture, compounds and reagents for immunocytochemistry

For immunocytochemical analysis, MCF7, MDCK, SW480 and HCT116 cells (ATCC) were grown in either Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, Gillingham, UK, D5546) for MCF7, MDCK and SW480 or McCoy’s 5A medium (Thermo Fisher Scientific, Gloucester, UK, 16600082) for HCT116, with 10% fetal bovine serum (Sigma, F7524) in 2D prior to the 3D culture setup. Low molecular weight agar LA717 was purchased from Ina Food Industry Co., Ltd. (Nagano, Japan) and added to the growth medium at 0.03% (w/v) where necessary. For the setup of 3D cultures, 2D-grown cells were trypsinised, resuspended in the LA717-containing medium, Matrigel® (Corning, NY, USA, 356234) or Geltrex® (Thermo Fisher Scientific, A1413202), and cultured on an ultra-low attachment plate (Corning, #3473). The 3D gel culture method was described previously [57]. Unless stated otherwise, spheroids were cultured for 5–7 days for immunocytochemistry, or 7–8 days when drug treatment was involved. Chemical compounds used were as follows: dimethyl sulfoxide (DMSO) (Sigma D2650) at 0.2% (w/v); CAL-101 (GENERON, Slough, UK) at 20 μM; 740Y-P (Bio-Technne, Abingdon, UK) at 20 μg·mL⁻¹; MK-2206 (Santa Cruz, Dallas, TX, USA) at 5 μM; and SC-79 (Merck, Gillingham, UK) at 25 μM. Akt1 T308D;S473D was a gift from Julian Downward (Addgene plasmid # 39536; http://n2t.net/​addgene:39536; RRID: Addgene_39536) [43]. Akt1 T308A;S473A was a gift from William Sellers (Addgene plasmid # 9030; http://n2t.net/​addgene:9030; RRID: Addgene_9030) [58]. Akt1 constructs or control plasmid DNA were transfected to MCF7 cells using Lipofectamine 3000 (Thermo Fisher Scientific) on 2D. On the next day, the cells were trypsinised and cultured in 3D with LA717 for 6 days.

For western blot analysis, Geltrex-cultured spheroids were treated with Dispase II (Sigma) at 10 units-mL⁻¹ at 37 °C for up to 12 min to remove gel prior to the cell extraction.

Antibodies used for immunocytochemistry were against phospho-ezrin (Thr567)/radixin (Thr564)/moesin (Thr558) (pERM) (Cell Signaling Technology, Danvers, MA, USA, 3726), β1-integrin (Millipore, Watford, UK, MABT409), podocalyxin (Santa Cruz, sc-23903), CK18 (Abcam, Cambridge, UK, ab668) and ZO-1 (Thermo Fisher Scientific, 339100). Antibodies used for western blots were against phospho-Akt (Ser473) (pAkt) (Cell Signaling Technology, 4060), total Akt (Cell Signaling Technology, 9272) and GAPDH (Sigma, G9295).

Fluorescence staining quantification

Fluorescent images were taken with fixed exposure time for the quantitative measurement of staining intensity on IMAGEJ (U. S. National Institutes of Health, Bethesda, MD, USA). Two thresholds of brightness were set to pick up strong and weak signals, and another threshold was chosen to detect the total surface area of the spheroid on the image. The strongly and weakly stained surface areas were normalised against the total surface area and shown in percentage. Threshold setting and surface area measurement are shown in Fig. S1.

Cell viability assay

MCF7 cells were suspended in Eagle’s minimum essential medium (EMEM) (FUJIFILM Wako Pure Chemical, Osaka, Japan, 051-07615) containing 2% fetal bovine serum (Thermo Fisher Scientific, 26140-079) and 1% nonessential amino acids (NEAA, FUJIFILM Wako Pure Chemical, 139-15651) and seeded at 500 cells in 75 L of growth medium at 0.03% (w/v) for well into 96-well flat-bottom cell-attachable plates (Corning, 3585) for 2D cultures or low attachment plates (Corning, 3474) with 0.030% (w/v) LA717 for 3D cultures with LA717. For 3D cultures with Matrigel, the same number of cells was suspended in 75 μL of 60% Matrigel® growth factor reduced (Corning, 354230) and incubated at 37 °C for 30 min, after which 25 μL of growth medium was added on top of the set gel. After one day of culture, 75 μL of the medium containing a twofold concentration of MK-
tumours were excised and weighed.

MCF7 cells (5.0 × 10⁶) in 100 µL per well. All conditions were triplicated. Following seven days of culture, the numbers of viable cells were evaluated using ATP assay as follows: an equal volume of ATP reagent ( CellTiter-Glo® Luminescent Cell Viability Assay; Promega, Tokyo, Japan) was added to the culture medium and the luminescence intensity (relative light unit value) was measured with Enspire (Perkin Elmer, Tokyo, Japan). The IC₅₀ values were calculated with EXSAS by fitting a sigmoidal dose–response regression curve.

Subcutaneous xenografting

MCF7 and HCT116 cells were cultured in 2D, 3D with LA717 and 3D with Matrigel for 7 days and prepared for grafting. Cells cultured in 2D were trypsinised and collected as single cells. Cells cultured in 3D were collected as spheroid suspension by centrifugation, with additional treatment with Dispase I (F. Hoffman-La Roche, 4942086001) for Matrigel-grown spheroids. Single cells (2D) and spheroids (3D) were suspended in serum-free DMEM, and cell numbers were counted by ATP assay (2D and 3D) and trypan blue assay (2D) and diluted to 5.0 × 10⁵ cells per mL for implantation.

BALB/c nude mice (nu/nu) were purchased fromCLEA Japan, Inc. (Tokyo, Japan). The mice were maintained under conventional conditions at a constant temperature of 25 °C with a 12-h light/dark cycle. All mice were 5–6 weeks old on the day of grafting. The protocol used in the experiment was approved by Nissan Chemical Corporation (Saitama, Japan). Mice were anaesthetised with isoflurane, and the luminescence intensity (relative light unit value) was measured with Enspire (Perkin Elmer, Tokyo, Japan). The IC₅₀ values were calculated with EXSAS by fitting a sigmoidal dose–response regression curve.

Histopathology and immunohistochemistry

The removed tumours were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm and processed for haematoxylin and eosin (H&E) or immunohistochemical staining. Immunostaining was performed with antibodies against cytokeratin (Dako, Santa Clara, CA, USA, clone; AE1/AE3) [59]. The sections were heated in 10 mM citrate buffer for 10 min using a pressure cooker and further incubated in 0.3% hydrogen peroxide for 10 min at room temperature. Following treatment with mouse IgG blocking reagent (M.O.M. Immunodetection Kit; Vector Laboratories, San Francisco, CA, USA) for 1 h at room temperature, the sections were incubated with primary antibodies for 20 min at room temperature. The sections were processed for visualisation according to the kit instruction using 3,3-diaminobenzidine.

Adhesion and migration assay

96-well flat-bottom cell-attachable plates (Corning, 3585) were coated either with StemSure® 0.1 % (w/v) gelatine solution (FUJIFILM Wako Pure Chemical, 190-15805) for 1 h at 37 °C or with 50 µg·mL⁻¹ collagen stock solution (Corning, 354236) diluted in 0.02 N acetic acid for 2 h at room temperature. Spheroids grown in Matrigel and LA717 were suspended in EMEM containing 2% FBS (Thermo Fisher Scientific, 26140-079) and 1% nonessential amino acids (NEAAs, FUJIFILM Wako Pure Chemical, 139-15651) and placed on gelatine- or collagen-coated plates. Immediately after the placing, spheroids with the comparable size with the radius in the range of 26–43 µm were identified. After 2.5 and 6 h at 37 °C, spheroid adhesion was classified into full, partial and nonadhered by visual observation in the phase-contrast view using Invirotigen EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific) as shown in Fig. 6B for gelatine-coated plates and Fig. S8A for collagen plates. The images of over 36 spheroids were classified in each group. For quantification of migration, spheroids attached within 2.5 or 6 h of incubation in Matrigel or LA717-containing medium were selected, respectively. Those whose surface area had increased more than 1.2 times during the attachment were regarded as the successful attachment and used for quantification (5–6 spheroids in each group in one experiment). The cell images were captured using the Cell Imaging System immediately after the attachment and 24 h after that, and the surface area was measured using IMAGEI software. The data were presented as an average of three independent experiments (15–16 spheroids).

Transwell invasion assay

Spheroids were prepared by culturing MCF7 cells in 3D with LA717 or Matrigel for 7 days and suspended in EMEM without serum with 1% NEAA. The cell density was calculated by ATP assay as described above in the section of cell viability assay. Spheroids were then resuspended at 5 × 10⁴ cells in 150 µL of EMEM without serum and placed on ECM-coated polycarbonate membrane inserts of CytoSelect™ 24-well Cell Invasion Assay Kit with 8 µm pore size (fluorometric format) (Cell Biolabs, San Diego, CA, USA, CBA-111). The lower chamber was filled with 500 µL of EMEM with 10% FBS and 1% NEAA. After 48 h of incubation, cells in the lower chamber were collected and quantified using Enspire (Perkin Elmer) at 480 nm/520 nm. The relative fluorescence intensity, the relative fluorescence unit (RFU) value, was used to present the cell number.
qPCR

MCF7 spheroids grown in 3D with LA717 or Matrigel for 7 days were subject to mRNA extraction using RNeasy following manufacturer’s protocol (Qiagen, Hilden, Germany, 7410) followed by cDNA synthesis using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan, RR0036A). PCR was performed using Premix Ex Taq™ (Perfect Real Time) (Takara Bio, RR0039A) at 50 °C for 2 min and 95 °C for 10 min, followed by 50–60 cycles of 95 °C for 15 s and 60 °C for 1 min, with TaqMan® primers: GAPDH: Hs02786624_g1, SNAIL: Hs00195591_m1, THBS1: Hs00962908_m1, MMP2: Hs01548727_m1, MMP9: Hs00957562_m1, CDH1: Hs01023894 and CDH2: Hs00983056. The gene expression level was evaluated relative to GAPDH. The Applied Biosystems® 7500 Real Time PCR System (Thermo Fisher Scientific) was used for the reaction.

Statistical analysis

Statistical analysis methods were stated in the relevant figure legend. The analysis was done by Pharmaco Basic (Scientist Press, Tokyo, Japan) or Excel software. The level of significance was set at 0.05 unless stated otherwise.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

NA-F, TN and NI performed the experiment and analysed data; and NA-F, TN and NI wrote the paper.

Peer Review

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Quantification of pERM fluorescent staining.

**Fig. S2.** Quantification of pERM staining of spheroids grown with various concentrations of Matrigel and Geltrex in LA717-containing medium.

**Fig. S3.** Expression of apical and basal markers in MCF7 spheroids grown in Geltrex, Matrigel or LA717.

**Fig. S4.** Partial restoration of apical-in/basal-out polarity in liquid cultured spheroids following transfer to gel matrices.

**Fig. S5.** Incidence of pERM-expressing plasma membrane facing the outer surface of MDCK spheroids in 40-80% gel with LA717 and 100% gel matrices.

**Fig. S6.** The effect of LA717 and PI3K modulators on MCF7 cells.

**Fig. S7.** The effect of LA717 and PI3K modulators on HCT116, SW480 and MDCK cells.

**Fig. S8.** Comparison of Matrigel-grown- and LA717-grown-spheroids in adhesion (A) and migration (B) on collagen-coated plates. Movie S1. MCF7 cell spheroids grown in 100% Geltrex.Movie S2. MCF7 cell spheroids grown in 60% Geltrex.Movie S3. MCF7 cell spheroids grown in 25% Geltrex.Movie S4. MCF7 cell spheroids grown in 0% Geltrex.Movie S5. MDCK cell spheroids grown in 0% Geltrex.