Title

The amount range of membrane cholesterol required for robust cell adhesion and proliferation in serum-free condition.

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Abstract:

Serum-containing medium is widely used to support cell attachment, stable growth and serial passaging of various cancer cell lines. However, the presence of cholesterols and lipids in serum greatly hinders the analysis of the effects of cholesterol depletion on cells in culture. In this study, we develop a defined serum-free culture condition accessible to a variety of different types of adherent cancer cells. We tested different factors that are considered essential for cell culture and various extracellular matrix for plate coating, and found cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) basal media supplemented with Albumin (BSA) and insulin-transferrin-selenium-ethanolamine (ITS-X) on fibronectin-precoated well (called as "DA-X condition") showed comparable proliferation and survival to those in a serum-containing medium. Interestingly, we observed that DA-X condition could be adapted to a wide variety of adherent cancer cell lines, which enabled the analysis of how cholesterol depletion affected cancer cells in culture. Mechanistically, we found the beneficial effects of the DA-X condition in part can be attributed to the appropriate level of membrane cholesterol, and fibronectin-mediated signaling plays an important role in the suppression of cholesterol production.

Keywords:

Cholesterol, Serum-free medium, Membrane, Cell adhesion, Proliferation, Pseudopodia

Introduction:

Modern advances in medical and biological sciences have largely relied on the development of cell culture technology(1). The ability and quality of culture medium to support cell survival, proliferation, and function in vitro have a direct impact on research outcomes. Thus, it is essential to select the appropriate medium when conducting cell culture experiments. It is well-established that serum-containing medium provides an optimal culture condition, which is widely used to support attachment, stable growth and serial passaging of various cancer cell lines in culture. However, as cell culture research progressed, the need for serum-free culture media, which are expected to help overcome various ethical and scientific issues, became apparent(2). Compared to serum-containing media, serum-free media have advantages such as less variability between lots, more consistent, and in many cases lower cost (unless expensive growth factors and cytokines are used)(3).

Cholesterol, an essential component of mammalian cell membranes, not only maintains cell structure, but also plays an important role in other cellular functions such as biosynthesis of bile acids and hormones, embryonic development, and cell proliferation(4). Most of the cellular cholesterol enriches in the plasma membrane after transportation from endoplasmic reticulum, which regulates cellular proliferation, differentiation, and survival. Cholesterol metabolism is known to critically

52 contributes to cancer cell proliferation, migration and invasion, and accumulation of cholesterol in
53 solid tumors is considered as the hallmark for aggressive cancers(5–9). However, given that serum
54 contains numerous cholesterols and lipids, serum-containing media are therefore not ideal for studying
55 effects of cholesterols on cancer cells in culture. Although serum-free media with optimized
56 compositions for each adherent cancer cell line are available, few of these cultures can support the
57 growth of other cancer cell types, and there is a lack of universal serum-free medium applicable to a
58 wide range of adherent cell lines. In contrast, in the field of pluripotent stem cells, the use of serum-
59 free and/or xeno-free medium has become standard(10).

60 In this study, we aimed to develop a universal serum-free culture medium broadly applicable to any
61 adherent cancer cell types. While testing various essential culture parameters including extracellular
62 matrix e, we found that growing cells in Dulbecco's Modified Eagle's Medium (DMEM) basal medium
63 containing Albumin (BSA) and insulin-transferrin-selenium-ethanolamine (ITS-X) on culture plates
64 pre-coated with fibronectin (called as “DA-X condition”) showed robust cell proliferation and attached
65 cells exhibited elongated pseudopodia, which were comparable to cells grown in a serum-containing
66 medium. DA-X condition also facilitated the study of the effects of cholesterol and lipid on cancer
67 cells in vitro in a wide variety of adherent cancer cell lines. Importantly, we found that cells grown in
68 the DA-X condition maintained the right amount of membrane cholesterol important for robust cell

attachment, pseudopodia elongation and survival in serum free condition, and fibronectin-mediated signaling plays important roles in suppressing excess cholesterol production.

Materials and Methods:

Cell lines and culture

Cell lines using in this research were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Japan) and maintained in 10% Fetal Bovine Serum (Gibco, 10270)-contained DMEM medium (nacalai tesque, 08458-45) which is supplemented with 1x Penicillin-Streptomycin Mixed Solution (nacalai tesque, 26253-84), and passaged using TrypLE (Gibco, 12604013) every 4-5 days. Briefly for in RPMI-G, $2x10^4$ cells were seeded into one well of a 4-well plate without pre-coating in RPMI1640 medium (nacalai tesque, 3026485) supplemented with 1x ITS-G: Insulin-Transferrin-Selenium (Gibco, 41400045), 1x L-Glutamine (nacalai tesque, 16948-04), 1x Penicillin-Streptomycin Mixed Solution. In DA-X condition (shown as ITS-X/ DMEM/ BSA (FN) in Fig. 1a), $2x10^4$ cells were seeded into one well of a 4-well plate pre-coated with Fibronectin in DMEM medium (High Glucose) (nacalai tesque, 08458-45) supplemented with 1x ITS-X: Insulin-Transferrin-Selenium-Ethanolamine (Gibco, 51500056), 5 mg/mL BSA: Bovine Serum Albumin (Sigma, A3059), 1x L-Glutamine, 1x Penicillin-Streptomycin

Mixed Solution. DMEM/Ham's F-12 (nacalai tesque, 11581-15) was used for ITS-X/ DMEM/F-12/ BSA (FN) (shown in Fig. 1b). Extracellular matrix was coated on a well of 4-well cell culture plate (SPL, 30004) and incubated for 1 hour at 37˚C at the desired concentration, 1.25 μg/cm^2 for Fibronectin (FUJIFILM, 063-05591), 1.25 μg/cm^2 for Vitronectin (FUJIFILM, 220-02041), 0.5 μg/cm^2 for Laminin511-E8 fragments: iMatrix-511 (MAX, 892011), 2% for Matrigel (Corning, 354234), 0.2% for Gelatin (Sigma, G1890). For optimization of serum-free culture condition for stable adhesion, the combination of basal medium, supplements and extracellular matrix shown in the notation in Fig. 1a at a concentration mentioned above were attempted. For inhibition of fibronectin-binding and -mediated signaling, RGDS peptide (Cayman, 15359-5) as fibronectin Inhibitor was added in culture at 50 μg/mL (the solvent, DMSO, was added for control). The images of cells were collected by a microscopy in phase-contrast (Keyence, BZ-X710).

Modulation of cholesterol

For modulating of membrane cholesterol contents in culture, Methyl-β-cyclodextrin (MβCD) (Sigma, 332615) for depletion of membrane cholesterol and soluble cholesterol (Sigma, C8667) (complex of MβCD and cholesterol) were added according to each purpose at 0.2 mM, 1mM and 30 μM respectively. 2x10^4 cells maintained in 10% FBS/ DMEM were seeded onto non-coat in RPMI-G or

fibronectin-coated in DA-X condition well of 4-well cell culture plate. At 1 day after seeded, MβCD or soluble cholesterol was added (the solvent, sterile water or EtOH, was added for control respectively). For exploring of functions of cholesterol biosynthesis depending on culture medium, Ro48-8071 (Cayman, 10006415), a selective inhibitor of Oxidosqualen cyclase, known as Cholesterol biosynthesis inhibitor was used. $2x10^4$ cells maintained in 10% FBS/ DMEM were seeded onto non-coat in 10% FBS/ DMEM or fibronectin-coated in DA-X condition well of 4-well cell culture plate. At 1 day after seeded, Ro48-8071 was added at 1 μM (the solvent, EtOH, was added for control).

Qualitative estimation of membrane cholesterol

For labeling of membrane cholesterol, cells grown on 4-well plate were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The cells were washed three times in PBS, and then incubate with 1.5 mg/ml glycine in PBS for 10 min at room temperature to quench the paraformaldehyde. Cells were stained with filipin working solution (0.05 mg/ml filipin III (Cayman, 70440) in 10% FBS-contained PBS) for 2 hrs at room temperature, and washed three times in PBS, and then the images were collected by fluorescent microscopy in PBS (Keyence, BZ-X710).

RNA preparation and real-time PCR

Total RNAs were extracted by using Sepasol-RNA I Super G (nacalai tesque, 09379) to the manufacturer's instructions. RNAs were reverse-transcribed using ReverTra Ace qPCR RT Master Mix (TOYOBO, FSQ-201), and real-time PCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, QPS-201) in MIC qPCR (bio molecular systems). Expression levels of each gene were normalized to β-ACTIN (human) expression and calculated using comparative CT method. The primer sequences are shown in below: β-ACTIN-F (CTGGCACCACACCTTCTACAATG), β-ACTIN-R (AATGTCACGCACGATTTCCCGC), SREBF1-F (ACAGTGACTTCCCTGGCCTAT), SREBF1-R (GCATGGACGGGTACATCTTCAA), SREBF2-F (AACGGTCATTCACCCAGGTC), SREBF2-R (GGCTGAAGAATAGGAGTTGCC), ACSS2-F (AAAGGAGCAACTACCAACATCTG), ACSS2-R (GCTGAACTGACACACTTGGAC), HMGCR-F (TGATTGACCTTTCCAGAGCAAG), HMGCR-R (CTAAAATTGCCATTCCACGAGC), HM-GCS1-F (GATGTGGGAATTGTTGCCCTT), HMGCS1-R (ATTGTCTCTGTTCCAACTTCCAG), LDLR-F (ACCAACGAATGCTTGGACAAC), LDLR-R (ACAGGCACTCGTAGCCGAT), ACLY-F (TCGGCCAAGGCAATTTCAGAG), ACLY-R (CGAGCATACTTGAACCGATTCT).

Statistical analysis

Statistical analysis was performed using the Student's t-test. P values < 0.05 were considered to be statistically significant.

Results:

| Development of a novel serum-free culture condition for HeLa cells

ITS-G/ RPMI1640 (referred to as "RPMI-G" hereafter), a defined serum-free cell culture medium reported previously(11), contains ITS-G supplement composed of Insulin-Transferrin-Selenium, known to support cell proliferation in reduced-serum medium. RPMI-G supported robust cell adhesion and proliferation of several melanoma cell lines. We tested culturing HeLa cells in RPMI-G medium. Interestingly, however, HeLa cells loosely attached to the culture plate 2~3 days after seeding, and consequently could not be maintained in RPMI-G medium (Fig. 1a). To establish a simpler and more reliable serum-free condition that can facilitate the functional analysis of cholesterol and lipid metabolism of a number of adherent cancer cell lines including HeLa cells, we optimized the RPMI-G medium taking consideration of several culture parameters: (1) Cell adhesion to culture plates, (2) Cell morphology (with or without pseudopodia), and (3) Cell proliferation. We didn't consider the long-term cultivability. After testing the effects of various supplements, base media and extracellular matrix in serum-free conditions, We found ethanolamine in ITS-X, fibronectin-precoating and DMEM

base medium had positive effects on cell adhesion and extension of pseudopodia, and the supplementation of BSA improved cell proliferation (Fig. 1a). Interestingly, we found although DMEM/Ham's F-12 is widely used as a basal media for serum-free cultures(1), cell attachment was markedly attenuated with the retraction of pseudopodia on day 5 while initial cell attachment and growth were not affected (Fig. 1b).We also tested the effects of several extracellular matrix proteins, which are widely used in pluripotent stem cells including human iPS cells (Fig. 1c)(1,2). Taken together, ITS-X/ DMEM/ BSA on fibronectin pre-coated culture plates, referred to as the "DA-X condition", was determined to be the optimal serum-free culture condition that supports stable adhesion, extended pseudopodia and robust cell proliferation, which is comparable to 10% FBS/ DMEM medium condition (Fig. 1).

| The utility of DA-X condition for studying cholesterol function

It is difficult to see the early effects of cholesterol biosynthesis inhibition or depletion of membrane cholesterol (e.g. treatment with Methyl-β-cyclodextrin) in adherent cultures, since serum even at reduced levels contains large amounts of lipids and cholesterols. In fact, Ro48-8071, a selective inhibitor of Oxidosqualen cyclase, had no effects on adherent HeLa cells cultured in 10% FBS/ DMEM medium (Fig. 2a, two images at top). In sharp contrast, addition of Ro48-8071 in DA-X condition had

a dramatic effect, and almost all cells detached and underwent apoptosis within 48 hours (Fig. 2a, two images at bottom). These results demonstrate the potential of the serum-free DA-X condition to provide an appropriate culture environment for analyzing the role of lipids and cholesterols in adherent cancer cell lines.

| DA-X condition as universal serum-free condition for cancer cell lines

The development of serum-free media that can be broadly applied to any cancer cell line is important for studying the role of lipids and cholesterols in the survival and proliferation of cancer cells. Next, we performed side-by-side comparison of RPMI-G medium and DA-X condition in terms of cell adhesion, pseudopodia and proliferation using representative human cancer cell lines derived from different organs and tissues (Fig. 2b and 2c). Cancer cells maintained in 10% FBS/ DMEM medium were switched to culture in serum-free conditions RPMI-G and DA-X, and phase contrast images were recorded on day 5 to monitor cell viability, attachment, morphology, and proliferation. In general, we found DA-X condition was more reliable than RPMI-G to support cell survival and proliferation with pronounced elongated pseudopodia in tested cell lines (Fig. 2b and 2c). Cell proliferation and attachment of SH-SY5Y (neuroblastoma) and PC-3 (prostatic cancer) in DA-X condition was comparable to those in RPMI-G. Although HepG2 (hepatoma) and SW620 (colon cancer) in DA-X

188 condition showed comparable proliferation when compared to that in RPMI-G, they exhibited more
189 evident adhesion and pseudopodia in cells than those in RPMI-G. In the other lines including A549
190 (lung cancer), Panc-1 (pancreatic cancer), HeLa (cervical cancer), MCF-7 (breast cancer), cell survival
191 and proliferation were observed only under DA-X condition but not in RPMI-G. Taken together, we
192 demonstrate the efficacy in culturing (at least for 5 days without passaging) a number of adherent
193 cancer cell lines in the serum-free DA-X condition, which holds great potential for analyzing role of
194 lipids and cholesterol in these cells (Fig. 2).
195
196 | **Varied cholesterol content in serum-free culture medium condition**
197 RPMI-G, a defined serum free cell culture medium, supports de novo fatty acid and -cholesterol
198 biosynthesis in several adherent melanoma cell lines, which enabled unperturbed cell adhesion and
199 proliferation (11). While testing the hypothesis that cells grown in the DA-X condition have increased
200 fatty acid and cholesterol production through upregulation of biosynthesis-related genes when
201 compared to the RPMI-G culture medium, we surprisingly found that the amount of cholesterol in the
202 membrane of HeLa cells in DA-X medium was greatly reduced in comparison to that in RPMI-G (Fig.
203 3a). Consistently, significant transcriptional down-regulation of cholesterol biosynthesis-related genes
204 was also observed in cells cultured in the DA-X condition. In contrast, these genes were up-regulated

in RPMI-G when compared with 10% FBS/ DMEM as previously reported (Fig. 3b)(11). Together, these results lead us to hypothesize that cells grown in RPMI-G medium are prone to detach due to the excess of membrane cholesterol, DA-X condition provides benefit for stable cell adhesion in part due to lowered membrane cholesterol content.

| Range of optimal membrane cholesterol for stable cell adhesion

To determine whether there is an appropriate range of membrane cholesterol for stable cell adhesion in serum-free medium condition, we modulate the membrane cholesterol levels in both RPMI-G and DA-X conditions to see their effects on cell adhesion. Augmentation and depletion of membrane cholesterol were achieved by supplementation with soluble cholesterol and Methyl-β-cyclodextrin (referred to as "MβCD" below), respectively (Fig. 3c, d). Depletion of cellular membrane cholesterol by MβCD in RPMI-G (Fig. 3a, b) resulted in a significant improvement in cell adhesion (Fig. 3c). In contrast, under DA-X conditions, both increase and depletion of membrane cholesterol led to the attenuation of cell adhesion accompanied by the retraction of elongated pseudopodia (Fig. 3d). These results suggest that the excess of membrane cholesterol in RPMI 1640 medium may have caused cell death due to compromised cell adhesion, and the beneficial effects of the DA-X condition may due to the appropriate amount of membrane cholesterol achieved by the suppression of cholesterol

222 biosynthesis-related gene expression (Fig. 3)(12).

223

224 **| Involvement of fibronectin in the suppressing genes needed for cholesterol biosynthesis**

225 To determine the molecular mechanisms by which expression of cholesterol biosynthesis-related
226 genes were suppressed in DA-X condition, we inhibited fibronectin-binding and -mediated signaling
227 by the addition of RGDS peptide(2,13,14), and quantified the expression levels of cholesterol
228 biosynthesis-related genes by quantitative PCR analysis (Fig. 4a, b). Inhibition of fibronectin with
229 RGDS peptide in DA-X condition resulted in not only different cell morphology (Fig. 4a) but also
230 significant up-regulation of cholesterol biosynthesis-related genes (Fig. 4b). Therefore, fibronectin-
231 binding or -mediated signaling play important roles in the suppression of cholesterol production in
232 DA-X condition.

233

234 **Discussion:**

235 Here, we show that the amount of cholesterol in the cell membrane greatly affects cell attachment
236 and pseudopodia formation, and eventually the survival of cells under serum-free conditions.
237 Furthermore, functional analysis by modulating membrane cholesterol content in RPMI-G and DA-X
238 conditions revealed that there is the appropriate range of membrane cholesterol for stable cell

239 attachment, pseudopodia formation, and proliferation in serum-free medium condition (Fig. 3c, d and
240 4c). We demonstrated that cells grown in the DA-X culture condition maintained the right amount of
241 cholesterol in cell membrane in part through extracellular matrix (fibronectin) signaling. The positive
242 results seen using eight representative human cancer cell lines suggest that the DA-X condition serves
243 as a universal serum-free culture condition that support pseudopodia formation, robust cell attachment
244 and proliferation prior to cell passaging. This contrasts with conventional serum-free media, which are
245 typically developed and optimized for each cell types. We therefore believe the DA-X culture
246 condition can serve as a powerful platform for clarifying the role of cholesterol in different cancer
247 cells(1).

248 We also demonstrated that supplementation of Ro48-8071 in DA-X condition had profound effects
249 on cell detachment and viability (Fig. 2a). Statin, inhibitors of HMG-CoA reductase, has also shown
250 to inhibit cancer cell proliferation and increase apoptosis(8,15,16), which seems dependent on cell
251 attachment since similar effects were not observed in acute lymphoid leukemia grown in suspension
252 (7). Future studies are warrantied to further examine the mechanism(s) of cell death by cholesterol
253 biosynthesis inhibition on cancer cells.

254 We observed that marked increase of membrane cholesterol content in RPMI-G cultured HeLa
255 cells (Fig. 3a, b), which is consistent with what was seen in other type of serum-free conditions (17).

Cells in these conditions are prone to detachment and cell apoptosis. Enrichment of cholesterol in the lipid raft of membrane in cancer cells make them more sensitive to cholesterol depletion and induces anoikis like cell death(18), suggesting that there is a close relationship between the amount of membrane cholesterol in cancer cells and cell survival in serum-free culture condition.

Finally, from the perspective of decarbonization, recently research on cultured meat is rapidly progressing but most, if not all, cultures still use serum-supplemented medium. Serum-free medium will be essential for the establishment of cultured meat in the future in order to reduce cost, achieve consistency and minimize the impact of carbonization(19). Our study revealed that keeping the right amount of membrane cholesterol is essential for cell survival and proliferation in serum-free culture conditions, and we believe these results will facilitate the development of serum-free medium for cultured meat.

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Figure captions

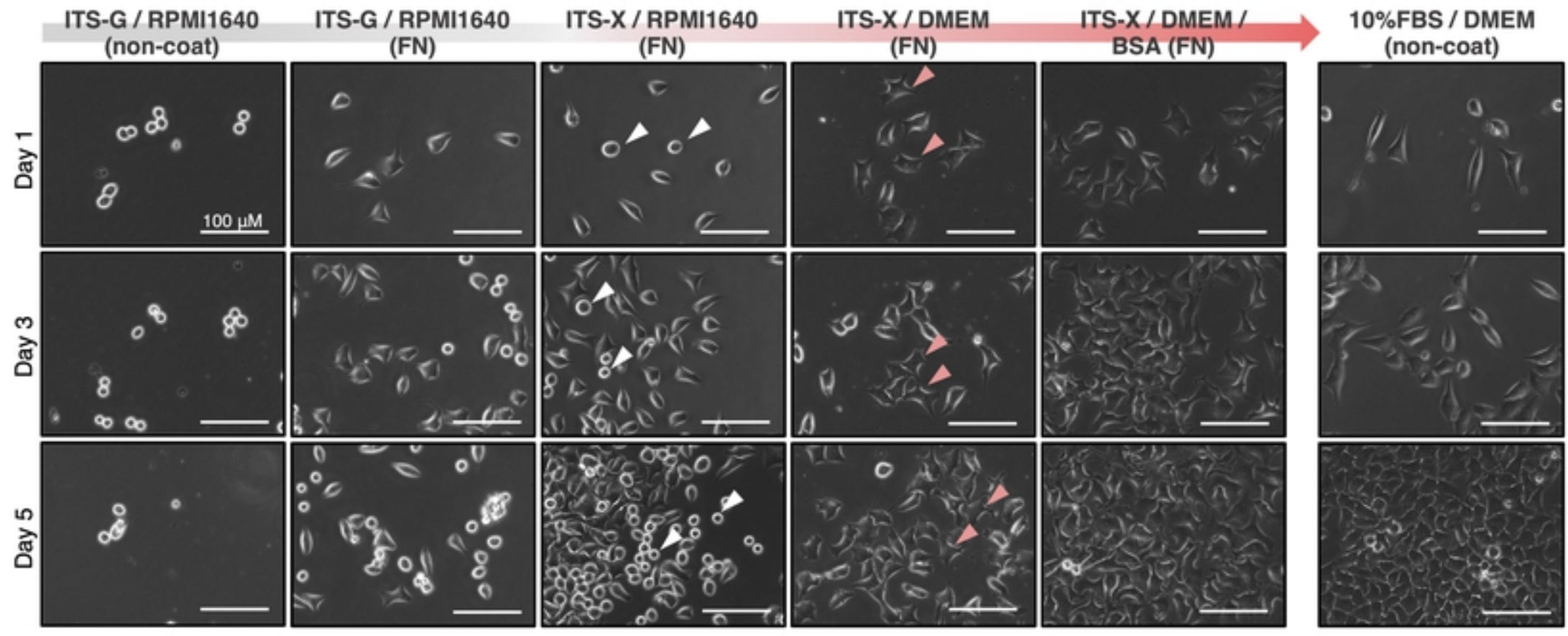
Figure 1 | Optimization of novel serum-free condition on HeLa cells. a, Representative images of cells in each culture condition. In screening for novel serum-free condition, $2x10^4$ cells of HeLa cell (human cervical cancer cell line) maintained in 10% FBS/ DMEM were seeded onto each serum-free media condition. Combinations of media composition and extra-cellular matrix are shown. Improving stepwisely from RPMI-G with replacing the media composition resulted in establishment of the ITS-X/ DMEM/ BSA on fibronectin-precoated well (called as DA-X condition), which significantly improved cell adhesion, pseudopodia elongation, or proliferation. Non-coat, without any pre-coating. White and pink arrowheads indicate cells with losing attachment and cells with firm elongated pseudopodia, respectively. FN, fibronectin-coated. Scale bar, 100 μm. **b,** Representative images of cells cultured in DMEM/ Ham's F-12-based medium with ITS-X/ BSA on fibronectin-precoated well. **c,** Representative images of cells in comparison of effect of extracellular matrix. $2x10^4$ cells of HeLa cell maintained in 10% FBS/ DMEM were seeded onto a pre-coated well with each extracellular matrix. Regarding to cell adhesion, pseudopodia elongation, or proliferation, fibronectin was shown as much better than the other extracellular matrix. The effect of vitronectin was shown as comparable to that of

341 fibronectin in cell adhesion and cell proliferation, but not in pseudopodia elongation. Scale bar, 100
342 μm.
343
344 **Figure 2 | Availability of DA-X condition on analysis for cholesterol function, and in several**
345 **cancer lines. a,** Effect of cholesterol biosynthesis inhibitor, Ro48-8071, on HeLa cells in serum-
346 containing and -free culture condition for 48 hrs. In cells grown in 10% FBS/ DMEM, Ro48-8071
347 was shown to have no effect on cell survival and even their proliferation, while almost cells cultured
348 in DA-X condition were shown to be dead. Scale bar, 100 μm. **b** and **c,** Comparison of the effects of
349 RPMI-G medium and DA-X condition on cell adhesion, pseudopodia and proliferation in
350 representative human cancer cell lines of each organ and tissue. Images of cells cultured in RPMI-G
351 (b) and DA-X condition (c) at day 5 are shown. The number of cells at the start of culture was
352 optimized for each cell type to reach about 80% confluency at day 5 in 10% FBS/ DMEM medium
353 (the using cancer cell lines and their number of cells at the start of cultures in one well of a 4-well
354 plate are shown below). HepG2 (hepatoma, $4x10^4$), A549 (lung cancer, $2x10^4$), Panc-1 (pancreatic
355 cancer, $4x10^4$), HeLa (cervical cancer, $2x10^4$), SH-SY5Y (neuroblastoma, $4x10^4$), SW620 (colon
356 cancer, $4x10^4$), PC-3 (prostatic cancer, $3x10^4$), MCF-7 (breast cancer, $3x10^4$). Scale bar, 100 μm.
357

Figure 3 | Required cholesterol level on membrane optimized by DA-X condition in HeLa cells. **a,** Cholesterol labeling with filipin fluorescence staining in each culture condition. Cells were cultured in 10% FBS/ DMEM, RPMI-G, DA-X condition for 2 days, and then applied for the staining. Signals of fluorescence were enriched in membrane of cells regardless of culture condition, and the intensity was strong in 10% FBS/ DMEM and RPMI-G, but quite faint in DA-X condition. Images for filipin staining were obtained using the same exposure time. Scale bar, 50 μm. **b,** Quantitative PCR analysis of expression of de novo cholesterol biosynthesis-related genes. Cells cultured in each condition for 2 days, and collected for further gene expression analysis. Error bars indicate s.d. (n=3, biological replicates). t-test, $^{**}p < 0.01$ and $^{*}p < 0.05$. **c,** Enhancement of cell adhesion and proliferation by depletion of membrane cholesterol with Methyl-β-cyclodextrin (MβCD) in RPMI-G culture medium. Pink arrowheads indicate cells with firm elongated pseudopodia. **d,** Effects of excess and depletion of membrane cholesterol on cell adhesion by addition of soluble cholesterol and MβCD in DA-X condition. At day 4 and 5, attenuated cell adhesion with the retraction of pseudopodia was widely observed not only in addition of cholesterol, but also in depletion of membrane cholesterol with MβCD. Scale bar, 100 μm. White arrowheads indicate cells with losing attachment.

Figure 4 | Involving fibronectin in down-regulation of cholesterol biosynthesis-related genes

375 **expression in DA-X condition. a,** Representative images of HeLa cells under RGDS peptide
376 treatment in DA-X culture condition. After exposed to RGDS peptide for 3 days, retracting the
377 pseudopodia of cells was observed accompanied by tightly association between neighbor cells. Scale
378 bar, 100 μm. White arrowheads indicate cells with the retraction of pseudopodia. **b,** Quantitative PCR
379 analysis of expression of de novo cholesterol biosynthesis-related genes. HeLa cells exposed to RGDS
380 peptide for 4 days under DA-X condition were collected for further gene expression analysis. Error
381 bars indicate s.d. (n=3, biological replicates). t-test, **p < 0.01 and *p < 0.05. **c,** Schematic
382 representation summarizing the appropriate range of cholesterol contents on cell membrane in serum-
383 free medium condition for cell adhesion and proliferation.
384



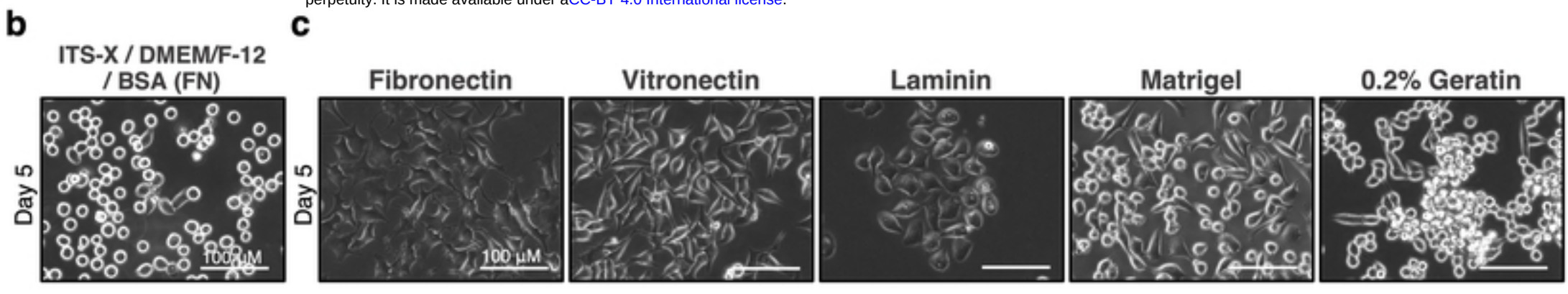


Figure 1

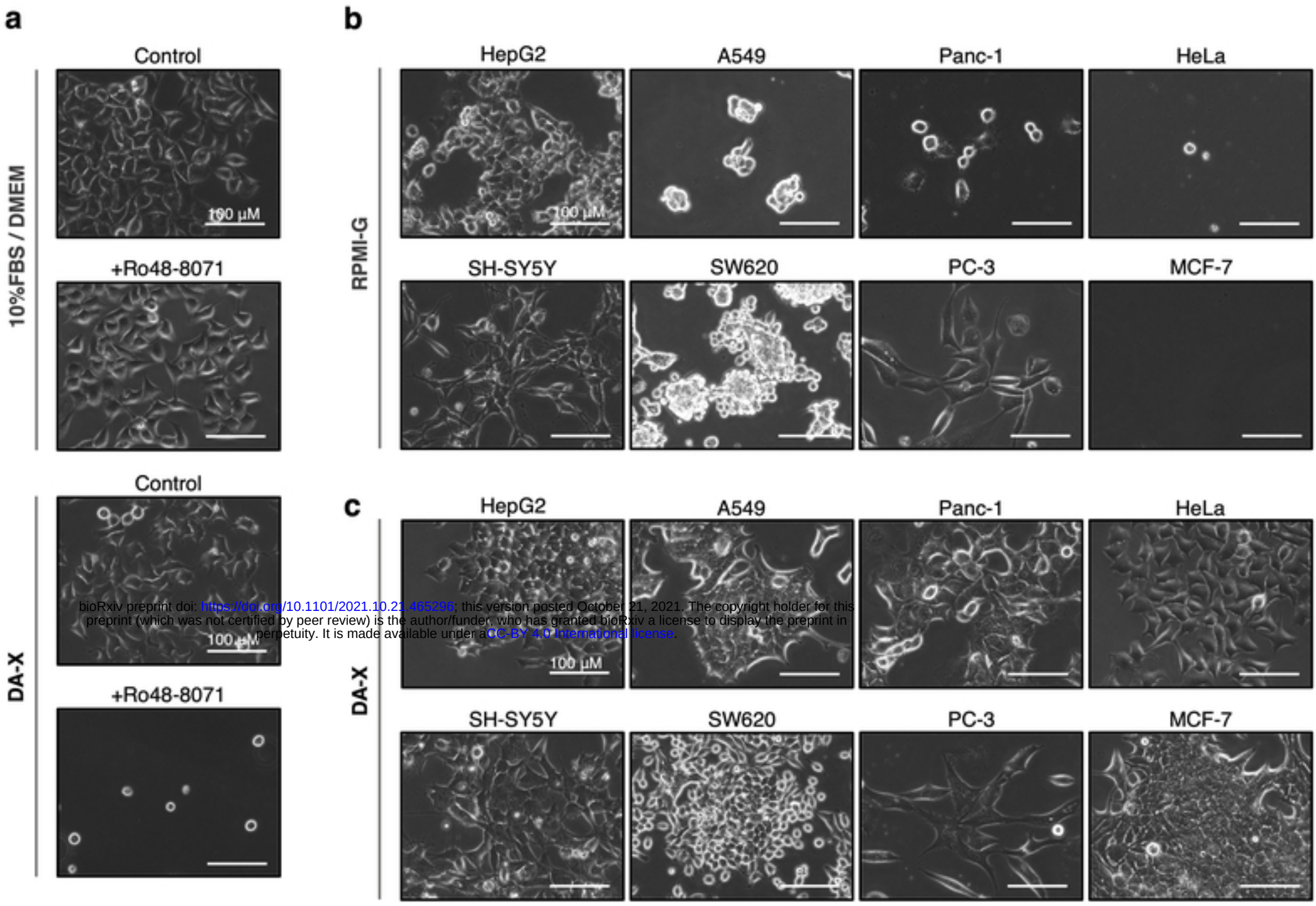
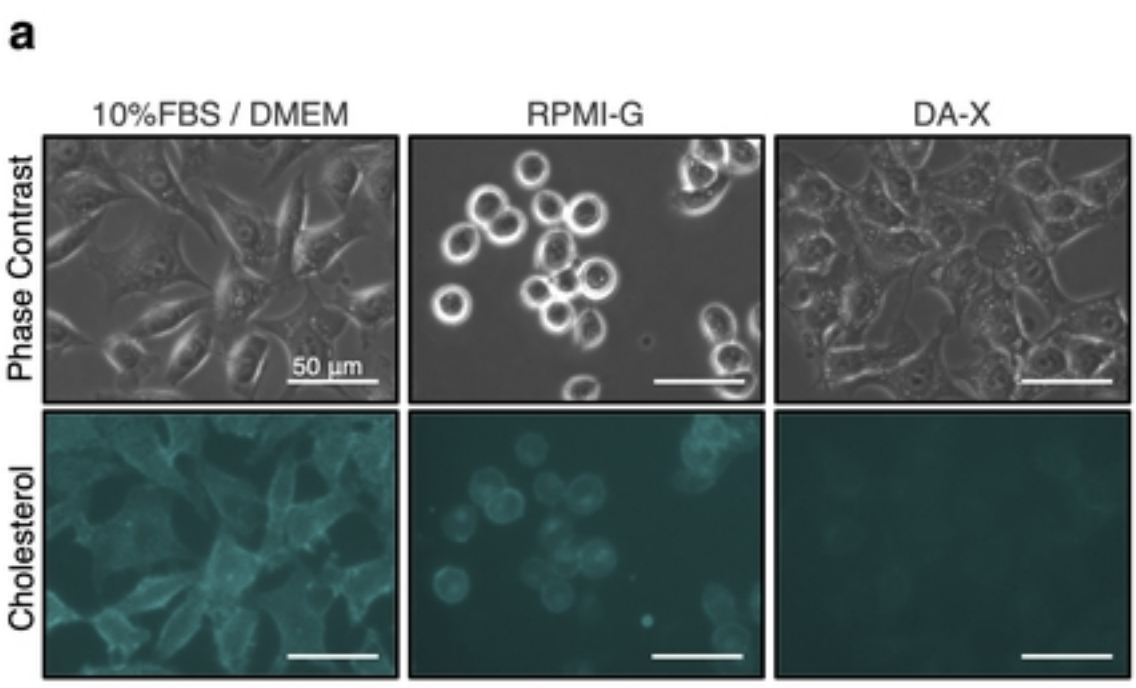
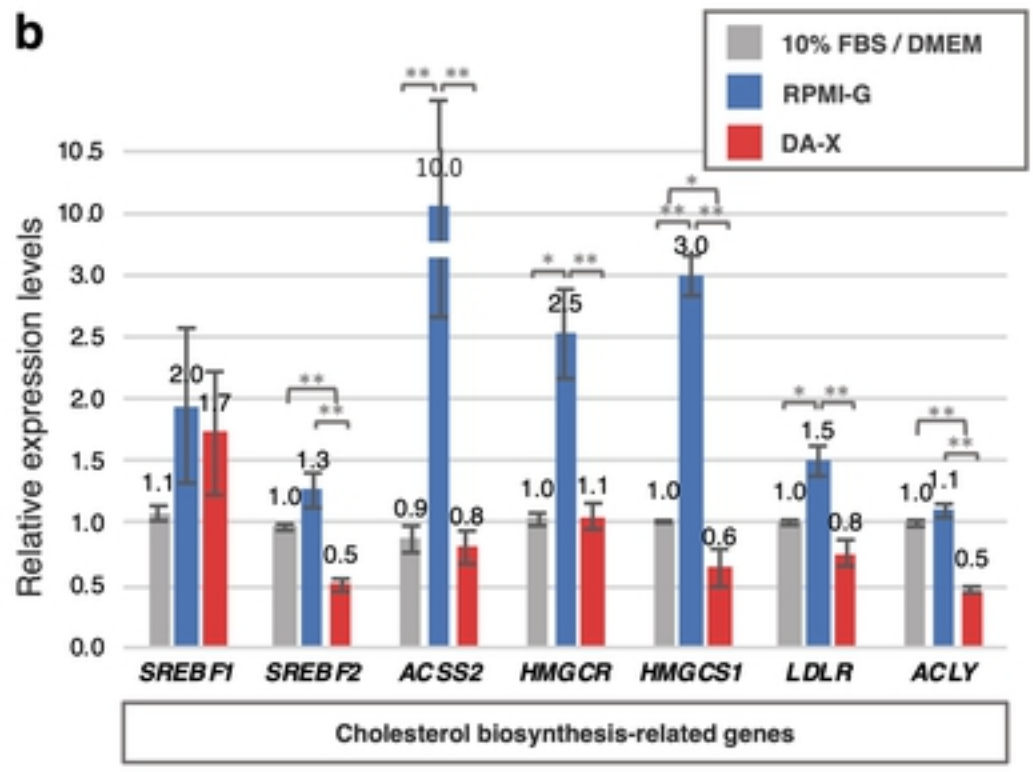


Figure 2





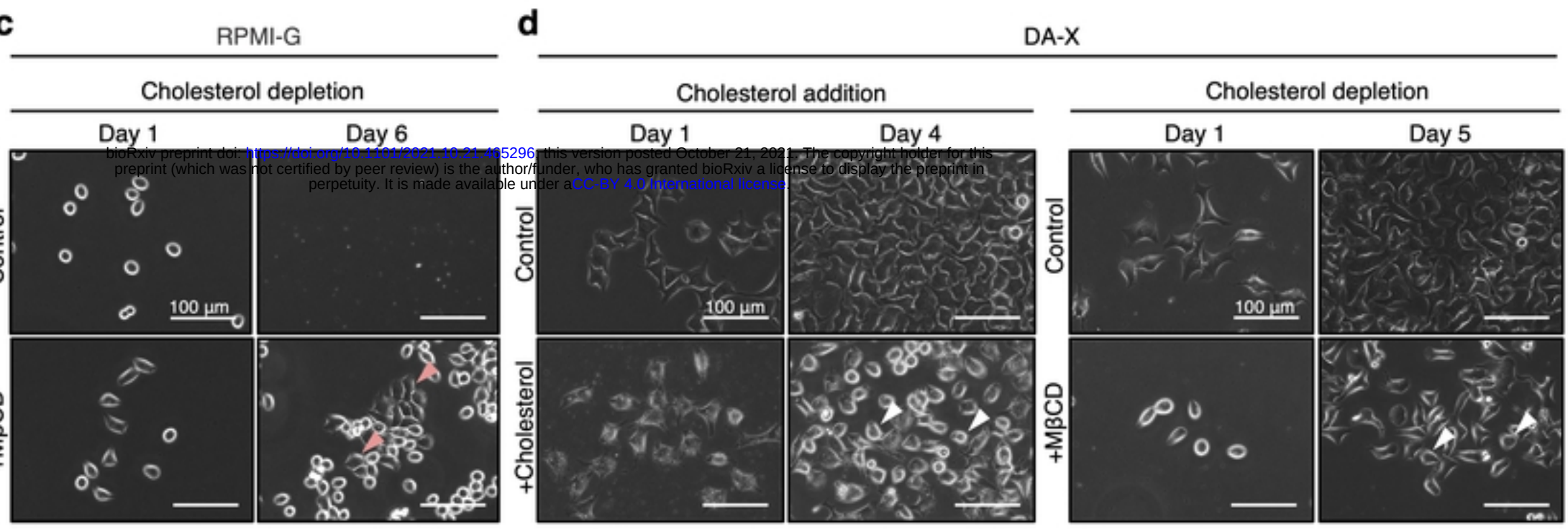


Figure 3