In Vitro Culture of Bovine Fibroblasts using Select Serum-Free Media Supplemented with *Chlorella vulgaris* Extract

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Serum replacement, serum-free media, algae extract, bovine fibroblast, cell cultured meat

Competing Interests

There are no competing interests.

Abstract

Standard cell culture practices require addition of animal-derived serum to culture media to achieve adequate cell growth. Typically, 5-10% by volume of fetal bovine serum (FBS) is used, which accounts for a vast majority of the cost of media while also imposing environmental and ethical concerns associated with the use of animal serum. Here we tested the efficacy of culturing cells by replacing serum in the media with algae extract and select additives. Using LC-MS, we compared molecular signatures of FBS to *Chlorella* algae extracts and identified NAD(H)/NADP(H) as common and relatively abundant features in their characteristic profiles. Bovine fibroblasts, cultured in serum-free media supplemented with *C. vulgaris* extract and just two growth factors plus insulin, showed significant growth with enhanced viability compared to control cells cultured without serum, albeit still lower than that of controls cultured with 10% FBS. Moreover, *C. vulgaris* extract enhanced cell viability beyond that of cells cultured with the two growth factors and insulin alone. These results suggest that key components in serum which are essential for cell growth may also be present in *C. vulgaris* extract, demonstrating that it may be used at least as a partial alternative to serum for cell culture applications.

35 **Introduction**

36 In 2013 it was estimated that raising livestock contributed roughly 14% to total
37 greenhouse gas emissions, more than half of which was attributable to meat production
38 (Gerber et al., 2013). Cell cultured meat has the potential to offer substantial environmental
39 and social advantages over traditional agriculture practices using livestock (Ladak and Anthis,
40 2021; Tuomisto and Teixeira De Mattos, 2011), yet analysis on the economics of cultured versus
41 conventional meat production shows high cost barriers, particularly those associated with the
42 nutrients required (Humbird, 2021). Cell culture media is commonly supplemented with fetal
43 bovine serum (FBS) because animal serum promotes cell growth (Penttinen and Saxén, 1959).
44 Based on current commercial prices, the cost of culture-grade FBS is estimated at around $1.20
45 per mL and that of other media components at $0.04 per mL; thus, for typical complete growth
46 media containing 10% FBS, most of its total cost is attributed to FBS, by roughly three-fold.
47 Therefore, low cost and environmentally favorable alternatives to serum are attractive for cell
48 culture applications, particularly for the cultured meat industry.

49 Because animal-derived serums like FBS are very complex, including thousands of
50 different molecules that can be highly varied in their relative abundance due to many dynamic
51 physiological factors, it is unlikely that synthetic approaches to serum alternatives will prove
52 cost effective if technically feasible. Researchers have studied the culture of cells in serum-free
53 conditions for many decades with varying degrees of success, yet most cell culture practices
54 continue using FBS. Following studies which identified key components necessary to culture
55 cells in the absence of serum (Chen et al., 2011; Murakami and Masui, 1980), there are now
56 chemically-defined serum-free media formulations that are commercially available (e.g., E8^{TM}).

57 However, the cost of these serum-free formulations is still quite high, primarily due to the
58 protein growth factors required. Such formulations were developed mainly for the purpose of
59 consistently culturing human induced pluripotent stem cells (iPSCs); they were not originally
60 intended for cultured meat applications although this does typically involve culturing stem cells.
61 Nevertheless, determining the minimal components and concentrations at which they are
62 needed to achieve adequate and consistent cell growth remains an important undertaking for
63 the cultured meat industry (Specht, 2020).

64 We were intrigued by a recent study in which the authors reported growth effects of
65 algae extract on mammalian cells (Ng et al., 2020). Ng and colleagues report that extract
66 derived from *Chlorella vulgaris* promoted growth of Chinese hamster ovary (CHO) and
67 mesenchymal stem cells (MSC) under certain conditions, some of which did not contain any
68 serum. In another study, researchers reported high levels of glucose and amino acids present
69 in various microalgae extracts, with particularly high levels of aspartate and glutamate detected
70 in extract from *C. vulgaris* (Okamoto et al., 2020); these authors proceeded to show that they
71 could use such algae extracts as a substitute to culture cells in media deficient in glucose and
72 amino acids. Here we tested the efficacy of culturing cells by replacing serum in the media with
73 algae extract and found that we could achieve appreciable growth and viability of bovine
74 fibroblasts using select serum-free media supplemented with *C. vulgaris* extract (CVE). We
75 characterized molecular features of algae extracts, compared them to that of FBS, and found
76 evidence suggesting that NAD(H)/NADP(H) are common and relatively abundant components.
77 This study establishes how algae extract may be used to culture cells in serum-free conditions
78 while providing further insight into essential components required for cell growth.

79 **Materials and Methods**

80 Chemicals and Reagents

81 Liquid chlorella growth factor (SKU 504) and *Chlorella vulgaris* extract capsules (SKU 583) were
82 purchased from BioPure® Healing Products (Woodinville, WA). Concentrated living *Chlorella*
83 *vulgaris* (item # 152068) and Alga-Gro® freshwater medium (item # 153752) were purchased
84 from Carolina Biological Supply Company (Burlington, NC). Pierce™ universal nuclease for cell
85 lysis was purchased from Thermo Fisher Scientific Corporation (Waltham, MA). Recombinant
86 human fibroblast growth factor (FGF-2, catalog # 100-18B) and transforming growth factor beta
87 (TGF-β, catalog # 100-21) were purchased from PeproTech, Inc. (Cranbury, NJ). Bovine insulin,
88 sodium L-ascorbate and selenium dioxide were from Sigma-Aldrich. Cell titer 96® aqueous one
89 solution cell proliferation (MTS) assay (catalog # G3580) was purchased from Promega
90 Corporation (Madison, WI).

91 Fractionation of Algae Extracts

92 Algae extracts termed *Chlorella vulgaris* extract (CVE) and chlorella growth factor (GCF) were
93 derived from two different strains, *Chlorella vulgaris* (CVE) and *Chlorella pyrenoidosa* (CGF).
94 CVE was obtained from capsules in dry powder form and dissolved directly in serum-free
95 DMEM. CGF stock solution was a liquid from which all solutions were made on a percent
96 volume basis by diluting CGF directly in serum-free DMEM. For fractionation experiments,
97 crude solutions of 10 g/L CVE and 10% CGF were first prepared in serum-free DMEM. Crude
98 CVE and CGF were then either sterile-filtered through 0.22 μm nylon filters or centrifuged at
99 10,000 x *g* for 10 minutes in a benchtop microcentrifuge. After centrifugation, supernatants
100 were removed, and the pellets were resuspended directly in serum-free DMEM.

101 LC-MS

102 Sample separation was performed by liquid chromatography (LC) using a UPLC BEH C18 column
103 (2.1 x 50 mm, 1.7 μm) fitted to an Acquity UPLC system with PDA detector (Waters).
104 Chromatographic profiles were acquired at wavelengths 200-400 nm. Injection volume was 7.5
105 μL after equilibration, and the column temperature was held at 30°C. Samples were eluted
106 from the column using a gradient mobile phase that consisted of phase A (0.1% formic acid in
107 water) and phase B (0.1% formic acid in acetonitrile). The linear gradient elution procedure
108 was as follows: (step 1) 100% A from 0-3 minutes, (step 2) 100% to 60% A from 3-20 minutes,
109 (step 3) 60% to 0% A from 20-21 minutes, (step 4) 0% A from 21-24 minutes, (step 5) 0% to
110 100% A from 24-25 minutes. The column flow rate was set at 0.2 mL/min from 0-21 minutes,
111 increased to 0.6 mL/min from 21-24 minutes, and then returned to the initial rate from 24-25
112 minutes. Electrospray ionization mass spectrometry (ESI-MS) was performed using an Acquity
113 QDa detector (Waters). Capillary and cone voltages were 0.8 kV and 10 V, respectively. MS
114 measurements were collected in positive ion mode with a mass scan range from 100-1200 *m*/*z*.

115 Cell Culture

116 The EBTr (NBL-4) cell line (bovine fibroblasts derived from embryonic tracheal tissue) was
117 purchased from ATCC (CCL-44™). HEK-293 (human embryonic kidney) cells were a gift from Dr.
118 Jörg Goronzy. Both cell types were routinely cultured in an incubator at 37°C with 5% CO_2 in
119 complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Gibco),
120 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific, cat no. 15140148);
121 serum-free DMEM included antibiotics but no FBS. Passaging was routinely done as follows:
122 cells were first washed with 1X phosphate-buffered saline (PBS), then placed in an incubator

123 with 0.25% Trypsin-EDTA solution (Gibco) for approximately 2 minutes immediately after which
124 complete DMEM was added to quench trypsin activity; cells were collected and centrifuged at
125 300 x *g* for 5 minutes, and then the cell pellet was resuspended in fresh media. Both cell types
126 were passaged every 3-4 days at a 1:3 (EBTr) or 1:10 (HEK-293) passage ratio. Excess cells were
127 stored in liquid nitrogen at 1-2x10^6 cells/mL in freezing media containing 90:10% FBS:DMSO.
128 The number of passages for experiments was limited to less than twenty.

129 Viability (MTS) Assays

130 EBTr and HEK-293 cells were first harvested, counted, and resuspended at a density of 2.5x10^5
131 cells/mL in serum-free DMEM. Cells were then seeded in clear, flat-bottom 96-well plates to
132 achieve a target density of approximately 2x10^4 cells (80 μL) per well. Seeded cells were
133 incubated for 2-4 hours to allow sufficient time for cells to adhere to plates prior to
134 experiments. For all viability experiments/assays, each condition corresponded to a single
135 column in a 96-well plate. Test media conditions were achieved by delivering 20 μL of 5X test
136 solutions (prepared in serum-free DMEM) to the appropriate column wells, bringing total well
137 volumes to 100 μL. Columns 1 and 12 as well as the top and bottom rows of all 96-well plates
138 were treated as mock wells and excluded from data analysis to control for plate edge effects
139 (Burt et al., 1979). Immediately after adding 5X test solutions to plates, cells were returned to
140 the incubator for the experiment duration (typically 3 days or approximately 72 hours) after
141 which 20 μL of MTS reagent was added to each plate well. Plates were then incubated for
142 another 4 hours. (For the experiment shown in Figure 4c, only 10 μL of MTS reagent was added
143 to plate wells because there was not enough reagent to add 20 μL to all 96 wells.) End-point
144 absorbance measurements were taken with a plate reader at 490 nm (BioTek).

145 Cell Counting and Imaging

146 Cells were harvested, pelleted, and resuspended in serum-free DMEM. Two replicate 50 μL
147 samples of cell suspensions were each diluted 1:1 in trypan blue dye, from which 10 μL was
148 sampled and pipetted onto a hemocytometer (improved Neubauer ruling pattern). Live and
149 dead/blue (non-intact) cell counts were performed in duplicate (i.e., one count per sample).
150 Brightfield images were obtained using an inverted tissue culture microscope (Hund Wetzlar)
151 fitted with a fixed microscope adapter connected to a 5.1-megapixel digital camera (AmScope).
152 Original grayscale image tiles were merged into a single multi-panel image representing each
153 experiment. Multi-panel images were processed with Fiji/ImageJ2 open-source software (NIH).
154 Image processing involved pseudo flat field correction to balance uneven illumination across
155 images, Fast Fourier Transform (FFT) bandpass filtering to reduce image scan lines, and
156 brightness/contrast adjustments.

157 Data Analyses and Statistics

158 LC-MS datasets, including both matrix LC and MS data from 3 replicate injections of the same
159 sample type, were first averaged and normalized; mean data matrices were then visualized
160 using R software (v4.1.0) with viridis and ggplot2 packages. Principal component analysis (PCA)
161 on averaged MS data was performed in R using the prcomp function. Potential parent species
162 were identified in a mass spectral database (MassBank.eu) by searching the *m*/*z* value and
163 average relative intensity of peaks shared between samples. All other data were analyzed
164 and/or visualized using Prism software (v9.2.0, GraphPad), in which *t*-tests with Welch's
165 correction were used to determine statistical significance between experimental groups.

166 **Results**

167 Molecular features of FBS and algae extracts show both distinct and common components.

168 Given their potential growth effects, we sought to compare algae extracts (CVE and CGF,
169 each of which was derived from a different *Chlorella* strain) to each other and FBS using LC-MS.
170 Due to limited scope and the anticipated molecular complexity of these samples, our aim was
171 not to look for specific compounds but rather to gain a qualitative profile for each sample type.
172 Figure 1a shows LC chromatograms of FBS compared to CVE and CGF samples. Large broad
173 peaks around 260 nm for species that eluted off the column within the first few minutes were
174 observed in both CVE and CGF but not FBS samples; these large peaks did not correspond to
175 any appreciable signal(s) detected by the mass spectrometer at such early elution times (Figure
176 1b). All samples showed both substantial absorbance (between 200-250 nm) and mass signals
177 at retention times from approximately 18-21 minutes; the relative magnitude of this cluster of
178 signals was comparable for FBS and CVE samples, whereas that of CGF samples was lower.
179 Figure 1c shows the results of a principal component analysis (PCA) comparing total ion
180 chromatograms (TICs) of all FBS, CVE and CGF samples (i.e., replicates averaged in Figure 1b).
181 Replicate injections of the same sample type clustered together and separated in PC1 but not
182 PC2 values; moreover, CVE and FBS samples were more similar in PC4 values than CGF samples.
183 We also visualized how signals at each *m*/*z* value and retention time compared across samples
184 (Figure 1d). Each sample type showed a common pattern with a cluster of relatively high signal
185 peaks corresponding to *m*/*z* values ranging from roughly 800-1200 and retention times from
186 about 15-21 minutes. Both CVE and CGF samples also shared signal features at lower *m*/*z*
187 values (mainly over the 400-800 range) and retention times from about 5-15 minutes; this

188 broad cluster of signals was less apparent if not absent in FBS samples. We further examined
189 the most striking MS feature found in all samples, i.e., the cluster of signals at *m/z* values from
190 about 800-1200 and retention times from 15-21 minutes. Figure 1e highlights five relatively
191 abundant and shared peaks within this cluster, noting their *m/z* whole integer value, relative
192 abundance, and corresponding potential parent ion identity(s). The top candidate identified by
193 a mass spectral database search (MassBank.eu) for the most abundant shared peak (no. 3 in
194 Figure 1e) was nicotinamide adenine dinucleotide (NAD^+) and/or NAD phosphate ($NADP^+$).

195 <u>Algae extracts alone in the absence of FBS slightly improve cell viability but not growth.</u>

196 We initially set out to test if algae extracts have growth effects on cells cultured in the
197 absence of any FBS, using MTS assays as an initial readout and proxy for potential cell growth.
198 Figure 2 shows the relative viability of HEK-293 and EBTr cells cultured over a 10,000-fold range
199 of concentrations of CVE or CGF. Media supplemented with CVE and CGF alone significantly
200 increased HEK-293 and EBTr cell viability compared to serum-free controls; such effects were
201 more pronounced for CVE than CGF and for relatively high concentrations of CVE. However,
202 increases in relative cell viability due to CVE supplementation alone did not exceed beyond
203 roughly 25% in any condition; moreover, and importantly, upon examining CVE-treated cells
204 under the microscope immediately prior to performing MTS assays, we did not observe any
205 convincing level of growth or cell doubling(s).

206 Next, we decided to test similar conditions to the experiment described in Figure 2 using
207 MTS assays to assess viability but adding 5% FBS to our test conditions (Figure S1). For HEK-293
208 cells, 5% FBS alone provided about 90% viability compared to that of 10% FBS controls after 3
209 days. The addition of 0.01-1 g/L CVE very slightly increased viability (compared to 5% FBS

210 controls), yet such viability decreased with 10 g/L CVE. Compared to 5% FBS controls, the
211 viability of EBTr cells cultured with 5% FBS plus 0.01-1 g/L CVE showed a similar pattern as that
212 of HEK-293 cells (i.e., not markedly different viabilities compared to 5% FBS controls).
213 However, 10 g/L CVE with 5% FBS increased viability in EBTr cells relative to 5% FBS controls.
214 Given the partial but limited growth shown in Figure 2, and the slight decrease in
215 viability at higher concentrations of CVE for HEK-293 cells shown in Figure S1, we wondered if
216 there may be components in the CVE which were acting to inhibit cell growth. We decided to
217 separate the different components of CVE through filtration, centrifugation, and treatment
218 with nuclease, using MTS assays to compare head-to-head the extract in its crude, filtered,
219 supernatant, and pellet forms, each with and without nuclease treatment. Figure 3 shows the
220 relative viability of EBTr and HEK-293 cells cultured with these different CVE fractions. No
221 obvious differences were seen between crude, filtered and supernatant forms of extract, with
222 viabilities of such fractions all falling near the range of 15-20%. The viability of cells treated
223 with the resuspended pellet was notably lower than that of all other fractions, falling to nearly
224 0%, similar to that of no serum controls. Further, CVE fractions treated with nuclease showed
225 mostly reduced viability compared to the corresponding fractions not treated with nuclease.
226 Figure S2 shows the LC-MS characterization of the supernatant and pellet forms of CVE used in
227 the experiment described in Figure 3. UV absorbance data shows sharp peaks around 20
228 minutes in the 200-250 nm range for both the pellet and the supernatant. The supernatant
229 also shows a pair of broader peaks at around 200 and 260 nm that appear to have eluted within
230 the first minute, neither of which was present in the pellet form. TICs of mass spectrometry
231 data for *m*/*z* values between 400 and 1200 for these two forms of extract show similar

232 footprints. Both show similarly the highest intensity spectra between 15 and 20 minutes, with
233 relatively smaller spectra between 0 and 15 minutes. The spectra of the pellet form show
234 slightly greater relative signals in the 0-6 minute range. Notably, the large UV peaks in both
235 sample types correspond to increased signals in their respective mass spectra at around 20-21
236 minutes, however the large UV signal eluting early and only in the supernatant did not
237 correspond to any appreciably large signal(s) in its mass spectra.

238 Select serum-free media supplemented with *C. vulgaris* extract enhances cell viability.

239 After not seeing many promising results from algae extract alone, we decided to move
240 our research in a different direction, focusing on growth factors, which have previously been
241 used to successfully culture stem cells in an "Essential 8" (E8^{TM}) serum-free medium (Chen et
242 al., 2011). Figure 4a shows percent viability of both EBTr and HEK-293 cells treated with
243 different concentrations of basic fibroblast growth factor (FGF) and transforming growth factor
244 β1 (TGF). Neither growth factor alone improved viability beyond 5-10% relative to serum-free
245 controls, yet viability increased to around 15% for EBTr cells treated with a combination of 10
246 ng/mL FGF and 10 ng/mL TGF. Thus, we decided to use a combination of FGF and TGF for our
247 next experiment in which we tested these growth factors together with bovine insulin, vitamin
248 C (in the form of L-ascorbate), and selenium (in the form of selenium dioxide), all of which are
249 listed as ingredients of the E8^{TM} media. Figure 4b shows insulin as a critical factor in real cell
250 growth, in conjunction with growth factors; treatments with both insulin and growth factors
251 improved EBTr cell viability to around 35% relative to controls cultured with FBS. (Note that
252 since we used bovine insulin, these effects were only apparent for bovine EBTr and not human
253 HEK-293 cells.) Despite their inclusion in E8^{TM} media, adding L-ascorbate, selenium, or both,

254 slightly reduced EBTr cell viability compared to that of cells cultured with growth factors and
255 insulin alone. We decided next to bring algae extracts back into our experiments, combining
256 them with insulin and growth factors and/or L-ascorbate and selenium. Figure 4c shows
257 perhaps the most promising results thus far; EBTr cell viability reached approximately 75%
258 (relative to FBS controls) when treated with a combination of CVE, growth factors and insulin.
259 On the other hand, the viability of cells treated with CGF, growth factors and insulin was
260 actually lower than that of cells treated with growth factors and insulin alone. Once again, the
261 addition of L-ascorbate and selenium had little if any beneficial effect on cell viability.

262 Select serum-free media supplemented with *C. vulgaris* extract promotes cell growth and
263 expansion.

264 After seeing promising viability results (i.e., Figure 4c), we decided the next step was to
265 test if EBTr cells would expand when treated with insulin, growth factors and CVE in a cell count
266 experiment. Figure 5a shows cell counts of CVE and CGF with supplements (i.e., growth factors,
267 insulin, L-ascorbate, and selenium) compared to controls with and without serum (10% FBS).
268 The number of EBTr cells increased in cultures with CVE or CGF plus supplements by day 2,
269 showing definitive growth, yet still not as much growth as control cells cultured with 10% FBS.
270 By day 4, the number of cells cultured with CGF plus supplements fell to a level near that of no
271 serum controls, but cells cultured with CVE plus supplements continued growth to around half
272 that of controls cultured with 10% FBS. On day 8, the number of cells cultured with CVE plus
273 supplements was comparable to that of controls cultured with 10% FBS, the latter of which had
274 decreased since day 4. Figure 5b shows the corresponding cell death percentages for the
275 experiment described in Figure 5a. Cell death percentages were largely in the 10-15% range,

276 slightly increasing over time, with the most death occurring for cells cultured with CGF plus
277 supplements on day 4 jumping to about 40%, corroborating the loss of growth shown for such
278 cultures between day 2 and day 4 in Figure 5a. Figure 5c shows images captured of the
279 experiment described in Figures 5a and 5b. EBTr cells cultured with 10% FBS show sustained
280 growth through day 4 until reaching confluency, at which point growth plateaued between days
281 4 and 8. Cells cultured with CVE plus supplements also appeared to grow through day 4,
282 reaching a relatively high level of confluency; on day 8 the level of confluency appears about
283 the same as day 4 (perhaps slightly more) with more dead cells in the image. Cells cultured
284 with CGF plus supplements appear to show growth until day 2, though notably less than cells
285 cultured with CVE plus supplements; however, by day 4 most if not all remaining cells appear
286 dead, and by day 8, only dead cells remain in the image. (Note that EBTr cells in wells treated
287 with algae extract appear larger than cells in wells where algae extract was not present.) The
288 no serum control shows some cells with normal morphology on day 2, with many dead cells in
289 the image as well; by days 4 and 8, hardly any cells remain besides a few dead cells.
290 Given that EBTr cells showed growth and markedly improved viability when cultured
291 with CVE and select supplements, we decided next to attempt to expand EBTr cells from a 1 mL
292 culture (12-well plate) to a 2 mL culture (6-well plate) under these conditions (Figure S3). On
293 day 1, cells cultured with 10% FBS or with CVE plus supplements attached effectively, while the
294 no serum control already shows many dead cells. By day 4, the no serum control well looks
295 nearly identical to that on day 1, with some cells still attached but many dead with no visible
296 growth. By day 4, cells cultured with 10% FBS or CVE plus supplements show obvious growth,
297 nearing confluency. As observed in Figure 5c, cells cultured with CVE plus supplements appear

298 slightly bigger than cells not cultured with CVE, meaning that fewer cells were necessary to
299 reach similar levels of confluency. After the day 4 pictures were taken, cells were detached and
300 removed from the 1 mL cultures, then pelleted and resuspended into fresh 2 mL cultures. The
301 following day, after cells attached to the plate (day 5), the relative amount of cells in the 10%
302 FBS control well was notably greater than that in the well with CVE plus supplements. By day 8,
303 cells cultured with 10% FBS reached near-confluency, while cells cultured with CVE plus
304 supplements remained at nearly 50% confluency. On both days 5 and 8, the no serum control
305 wells were essentially devoid of live cells, with only a few dead cells remaining.

306 **Discussion**

307 Regarding our characterization of algae extracts compared to FBS, the most striking and
308 obvious feature of the CVE and CGF samples that was not present in FBS samples were large
309 absorbance peaks around 260 nm, which eluted off the column with the aqueous mobile phase
310 within the first couple minutes. Such an absorbance profile is a characteristic property of
311 nucleic acid (Setlow and Boyce, 1960), and not surprising as a relatively abundant component
312 present in crude cellular extracts. Moreover, since no appreciable mass signal(s) at this
313 retention period was/were observed, it supports the notion that the species producing this
314 absorbance spectra are likely nucleic acid species (e.g., DNA/RNA fragments containing at least
315 4 bases) with *m*/*z* values greater than that which could be detected (i.e., >1200). However,
316 because the sensing of foreign nucleic acid often triggers cell death responses (Schlee and
317 Hartmann, 2016), we did not expect that foreign nucleic acid would be beneficial to cell
318 growth/viability, rather on the contrary. Yet our results suggest that this putative nucleic acid
319 at least did not harm cells and may have even been beneficial because nuclease-treated
320 extracts, as well as the insoluble pellet with presumably very little nucleic acid (as evidenced by
321 loss of the characteristic absorbance peak at 260 nm in Figure S2a), both decreased cell viability
322 as compared to cells not treated with nuclease or cells treated with soluble supernatant.

323 Despite an increase in HEK-293 and EBTr cell viability observed for cells cultured with
324 different concentrations of CVE and GCF alone, when such plates were observed under the
325 microscope, no convincing amount of cell division was visible. When algae extract (CVE) was
326 combined with growth factors, insulin and other additives, a similar marginal increase in cell
327 viability of around 20% was observed in an environment where cells were already dividing.

328 These observations suggest that algae extract improves cell viability without stimulating
329 proliferation. The possibility that algae extract enhances the cellular metabolic potential
330 without promoting cell division requires further investigation. Cell viability assays using
331 tetrazolium salts like MTS are often used as a proxy for cell growth, yet what such assays
332 actually measure is the activity of mitochondrial NADH and NADPH-dependent dehydrogenase
333 enzymes which reduce the reagent to a formazan product (Dunigan et al., 1995). In most cases,
334 the more cells there are the more mitochondria are available to reduce the MTS reagent, but it
335 is possible that the algae extracts have a positive effect on such metabolic activity without
336 promoting cell growth. Given that a common and relatively abundant signal feature found in
337 the mass spectra of algae extract samples mapped to NAD^+/$NADP^+$ as the parent species, it is
338 possible that enriched levels of NAD(H)/NADP(H) present in the algae extracts may have caused
339 enhanced activity of the dehydrogenase enzymes in the cells thus explaining the observed
340 increase in viability. Further, it was previously shown that CVE also contains high levels of
341 glutamate (Okamoto et al., 2020), as *Chlorella* algae use NADPH-dependent glutamate
342 dehydrogenase enzymes to control their cell cycle (Talley et al., 1972). Glutamate has been
343 shown to increase oxidation of NADH (Kannurpatti and Joshi, 1999; Maalouf et al., 2007), and
344 increase cancer cell viability and growth (Gelb et al., 2015; Reiman et al., 1981; Takano et al.,
345 2001; Yamaguchi et al., 2020). Moreover, in fibroblasts, glutamate reduced toxicity induced by
346 high levels of cysteine (Bannai and Ishii, 1982), and glutamate dehydrogenase deficiency
347 reduced cell viability (Tatsumi et al., 1989). Therefore, it is possible that enriched levels of
348 glutamate in algae extracts may also be responsible for the increased viability observed.

349 When examined alone, there were cases in which cells cultured with 5% FBS and algae
350 extract showed viability nearly matching 10% FBS controls. In one case, viability was greater
351 than that of 10% FBS controls, however we are cautious not to overinterpret this data. It is
352 important to note that any gains in cell viability were marginal compared to 5% FBS controls.
353 Often, cells can be cultured in 5% FBS nearly as effectively as 10% FBS (Talbot et al., 2004).
354 Thus, the reason that addition of CVE increased cell viability could also be attributed to the
355 same phenomenon discussed above, i.e., by enhancing metabolism without promoting growth.
356 Our experiments with components of the E8^{TM} media clearly showed that both insulin,
357 basic-FGF and TGF-β are necessary for cell growth, and that these components are roughly
358 additive in terms of their benefits to cell viability. Early studies examining serum-free culture
359 conditions showed FGF and insulin as key determinants for growth (Mcfarland et al., 1991).
360 Insulin causes cells to import glucose, providing energy and nucleotide building blocks needed
361 for replication. In viability assays, addition of CVE also proved to be roughly additive, with CVE
362 in addition to basic-FGF, TGF-β, insulin, and other additives combined yielding the highest
363 viability reading of any serum-free condition tested. We demonstrated that EBTr cells can grow
364 and expand into a new culture vessel when cultured in serum-free media that included at
365 minimum basic-FGF, TGF-β, insulin and algae extract in the form of CVE, although such growth
366 was not as robust as that of cells cultured with 10% FBS. Recent studies have shown that
367 mitochondrial NADP(H) levels are essential for cell growth (Tran et al., 2021), without which
368 cells cannot make proline required to support nucleotide and protein synthesis. Thus, it is
369 plausible that enriched levels of NAD(H)/NADP(H) in CVE extract may provide a true benefit to
370 cell growth; however, whether the additive increase in viability from CVE translates to any real

371 benefit in growth rate beyond that from basic-FGF, TGF-β and insulin alone remains unclear.
372 Regardless of whether algae extract is truly beneficial to cell growth, it is important to note that
373 bovine fibroblasts could be cultured with only a subset of the E8™ components present. This
374 may at least suggest some potential economic benefit due to lower cost serum-free
375 formulations requiring fewer components. Since growth factors and insulin directly interact
376 with their respective receptors through a relatively small range of residues across the entire
377 protein, further experiments could test whether smaller peptide fragments mapping to such
378 regions within these proteins remain actionable enough to sustain cell growth. Such
379 substitutes may simplify and further lower the cost of serum-free formulations in which cells
380 can be effectively cultured. Similarly, small molecules that act as agonists on basic-FGF and/or
381 TGF-β receptors could also be tested for the same purpose.
382 Other limitations of our studies include aspects of the growth experiments in which we
383 did not anticipate how differences in cell size could impact the results. In these experiments, it
384 appears from the images as though cells cultured with algae extract were larger in size as
385 compared to those not cultured with algae extract. Notably, increases in cell viability correlate
386 with increased cell size (Dreesen and Fussenegger, 2011), which could at least partially explain
387 this observation since we also found that algae extract typically increased cell viability.
388 However, differences in cell size observed with and without algae extract in the culture make
389 the growth and expansion experiments difficult to interpret. For instance, during the expansion
390 experiment, it appears that more cells were transferred from the 1 mL culture with 10% FBS
391 compared to the culture with CVE plus supplements despite these two groups appearing to
392 have a similar level of confluency on day 4. While both conditions promoted growth, the cells

393 cultured in 10% FBS not only grow faster but require less space per cell. As a result, in the 2 mL
394 cultures, the cells cultured in 10% FBS had a higher base count from which to start growing and
395 were thus much more confluent by day 8. Furthermore, it is important to note limitations in
396 counting the fraction of dead cells recorded in our cell count experiments. As noted in the
397 methods section, we first removed the old media and washed the cells briefly, which likely
398 removed a large amount of previously dead and floating cells. Thus, the dead cell count more
399 accurately represents the percentage of cells which were nearly dead, or dead, but not yet
400 detached and floating.

401 Taken together, our studies show that algae extract derived from *Chlorella vulgaris*
402 (CVE) enhances viability of bovine fibroblasts, possibly due at least in part to its enriched levels
403 of NAD(H)/NADP(H) and/or glutamate, and that CVE can be used together with other
404 supplements (basic-FGF, TGF-β, and insulin) to grow cells in the absence of serum. Further
405 studies are needed to determine the extent to which CVE promotes cell growth and whether
406 the supplementary components can be further modified to reduce their cost.
407

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Author Contributions

G.D.C. and T.M.G. designed the study, performed experiments, analyzed data, and wrote the paper, with both authors contributing equally.

419 **References**

420 Bannai, S. and Ishii, T. (1982). Transport of Cystine and Cysteine and Cell Growth in Cultured
421 Human Diploid Fibroblasts: Effect of Glutamate and Homocysteate. *Journal of Cellular*
422 *Physiology* 112, 265–272.

423 Burt, S. M., Carter, T. J. N. and Kricka, L. J. (1979). Thermal characteristics of microtitre plates
424 used in immunological assays. *Journal of Immunological Methods* 31, 231-236.

425 Chen, G., Gulbranson, D. R., Hou, Z., Bolin, J. M., Ruotti, V., Probasco, M. D., Smuga-Otto, K.,
426 Howden, S. E., Diol, N. R., Propson, N. E., et al. (2011). Chemically defined conditions for
427 human iPSC derivation and culture. *Nature Methods* 8, 424–429.

428 Dreesen, I. A. J. and Fussenegger, M. (2011). Ectopic expression of human mTOR increases
429 viability, robustness, cell size, proliferation, and antibody production of chinese hamster
430 ovary cells. *Biotechnology and Bioengineering* 108, 853–866.

431 Dunigan, D., Waters, S. and Owen, T. (1995). Aqueous soluble tetrazolium/formazan MTS as an
432 indicator of NADH- and NADPH-dependent dehydrogenase activity. *Biotechniques* 19, 640–
433 649.

434 Gelb, T., Pshenichkin, S., Rodriguez, O. C., Hathaway, H. A., Grajkowska, E., Diraddo, J. O.,
435 Wroblewska, B., Yasuda, R. P., Albanese, C., Wolfe, B. B., et al. (2015). Metabotropic
436 glutamate receptor 1 acts as a dependence receptor creating a requirement for glutamate
437 to sustain the viability and growth of human melanomas. *Oncogene* 34, 2711–2720.

438 Gerber, P. J., Steinfield, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A. and
439 Tempio, G. (2013). Tackling climate change through livestock – a global assessment of

440 emissions and mitigation opportunities. Food and Agriculture Organization of the United
441 Nations (FAO), Rome.
442 Humbird, D. (2021). Scale-up economics for cultured meat. *Biotechnology and Bioengineering*
443 118, 3239–3250.
444 Kannurpatti, S. S. and Joshi, N. B. (1999). Energy metabolism and NAD-NADH redox state in
445 brain slices in response to glutamate exposure and ischemia. *Metabolic Brain Disease* 14,
446 33-43.
447 Ladak, A. and Anthis, J. R. (2021). Animals, Food, and Technology (AFT) Survey: 2020 Update.
448 Maalouf, M., Sullivan, P. G., Davis, L., Kim, D. Y. and Rho, J. M. (2007). Ketones inhibit
449 mitochondrial production of reactive oxygen species production following glutamate
450 excitotoxicity by increasing NADH oxidation. *Neuroscience* 145, 256–264.
451 Mcfarland, D. C., Ane, J., Pesall, E., Norberg, J. M. and De, M. A. D. (1991). Proliferation of the
452 turkey myogenic satellite cell in a serum-free medium. *Comp. Biochem. Physiol.* 99A, 163–
453 167.
454 Murakami, H. and Masui, H. (1980). Hormonal control of human colon carcinoma cell growth in
455 serum-free medium. *Proc. Natl. Acad. Sci. USA* 77, 3464–3468.
456 Ng, J. Y., Chua, M. L., Zhang, C., Hong, S., Kumar, Y., Gokhale, R. and Ee, P. L. R. (2020). Chlorella
457 vulgaris Extract as a Serum Replacement That Enhances Mammalian Cell Growth and
458 Protein Expression. *Frontiers in Bioengineering and Biotechnology* 8, 564667.
459 Okamoto, Y., Haraguchi, Y., Sawamura, N., Asahi, T. and Shimizu, T. (2020). Mammalian cell
460 cultivation using nutrients extracted from microalgae. *Biotechnology Progress* 36, e2941.

461 Penttinen, K. and Saxén, E. (1959). Growth-Controlling Action of Human Serum in Cell Culture.
462 *Nature* 184, 1570–1571.
463 Reiman, R. E., Huvos, A. G., Benua, R. S., Rosen, G., Gelbard, A. S. and Laughlin, J. S. (1981).
464 Quotient Imaging with N-13 L-Glutamate in Osteogenic Sarcoma: Correlation with Tumor
465 Viability. *Cancer* 48, 1976–1981.
466 Schlee, M. and Hartmann, G. (2016). Discriminating self from non-self in nucleic acid sensing.
467 *Nature Reviews Immunology* 16, 566–580.
468 Setlow, R. and Boyce, R. (1960). The ultraviolet light inactivation of phiX174 bacteriophage at
469 different wave lengths and pH's. *Biophysical Journal* 1, 29–41.
470 Specht, L. (2020). An analysis of culture medium costs and production volumes for cultivated
471 meat. The Good Food Institute.
472 Takano, T., Lin, J. H. C., Arcuinoo, G., Gao, Q., Yang, J. and Nedergaard, M. (2001). Glutamate
473 release promootes growth of malignant gliomas. *Nature Medicine* 7, 1010–1015.
474 Talbot, N. C., Powell, A. M. and Caperna, T. J. (2004). Comparison of Colony-Formation
475 Efficiency of Bovine Fetal Fibroblast Cell Lines Cultured with Low Oxygen, Hydrocortisone,
476 L-Carnosine, bFGF, or Different Levels of FBS. *Cloning and Stem Cells* 6, 37–47.
477 Talley, D. J., White, L. H. and Schmidt, R. R. (1972). Evidence for NADH-and NADPH-specific
478 isozymes of glutamate dehydrogenase and the continuous inducibility of the NADPH-
479 specific isozyme throughout the cell cycle of the Eucaryote Chlorella*. *Journal of Biological*
480 *Chemistry* 247, 7927–7935.
481 Tatsumi, C., Yorifuji, S., Takahashi, M. and Tarui, S. (1989). Decreased viability of skin fibroblasts
482 from patients with glutamate dehydrogenase deficiency. *Neurology* 39, 451-452.

483 Tran, D. H., Kesavan, R., Rion, H., Soflaee, M. H., Solmonson, A., Bezwada, D., Vu, H. S., Cai, F.,
484 Phillips, J. A., DeBerardinis, R. J., et al. (2021). Mitochondrial NADP+ is essential for proline
485 biosynthesis during cell growth. *Nature Metabolism* 3, 571–585.
486 Tuomisto, H. L. and Teixeira De Mattos, M. J. (2011). Environmental impacts of cultured meat
487 production. *Environmental Science and Technology* 45, 6117–6123.
488 Yamaguchi, I., Yoshimura, S. H. and Katoh, H. (2020). High cell density increases glioblastoma
489 cell viability under glucose deprivation via degradation of the cystine/glutamate
490 transporter xCT (SLC7A11). *Journal of Biological Chemistry* 295, 6936–6945.
491
492

493 **Figure Legends**

494 **Figure 1. Comparative characterization of *Chlorella* extracts versus FBS by LC-MS**

495 (**a**) Perspective 3D surface plots are shown comparing UV chromatograms of FBS, CVE and CGF
496 samples (wavelengths 200-400 nm); each plot shows the mean normalized absorbance spectra
497 of three replicate injections of the same sample type. Arrows indicate large, broad peaks
498 around 260 nm present in CVE and CGF samples. (**b**) Normalized total ion chromatograms
499 (TICs) are shown as the sum of signals of all *m*/*z* values from 400-1200 for FBS, CVE and CGF
500 samples; representative data shown are averaged spectra from three injections of the same
501 sample type. (**c**) Scatter plot shows results of principal component analysis (PCA) on panel b MS
502 TIC data; sample types are indicated by different colors with PC2 (circles) and PC4 (diamonds)
503 values plotted on the same y-axis scale. (**d**) Perspective 3D surface plots show normalized mass
504 spectra of FBS, CVE and CGF samples; data shown are averages of three replicate injections.
505 Dashed box indicates region of interest where signal location/intensity appears similar across
506 all three sample types (i.e., retention times from 15-21 minutes and *m*/*z* values from 800-1200).
507 (**e**) Aligned mass spectra show the sum of all signals for retention times from 15-21 minutes at
508 each whole integer *m*/*z* value from 800-1200; data are shown as mean normalized spectra of
509 three replicate injections of the same sample. Red vertical lines indicate signals of relatively
510 high abundance shared across all three sample types; table lists potential parent species for
511 corresponding signals based on top candidates identified by database (MassBank.eu) search.

512 **Figure 2. *Chlorella* extracts alone slightly improve cell viability in the absence of FBS.**

513 (**a**) Bar plots show relative viability of HEK-293 cells cultured with a range of concentrations of

514 CVE or CGF at day 1, 3 and 7, compared to 10% FBS and no serum controls; panel (**b**) shows that

515 of HEK-293 (blue) and EBTr (yellow) cells cultured with 10-fold higher CVE and CGF at day 3.

516 Statistical significance was determined by *t*-test with Welch's correction in which experimental

517 groups were compared to respective no serum control groups; significance levels are indicated

518 as *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns=not significant, n=6.

519 **Figure 3. Nuclease-treated *C. vulgaris* extract and its insoluble fraction decrease cell viability.**

520 Bar plots shows relative viability of EBTr (**a**) and HEK-293 (**b**) cells cultured with different

521 fractions of 10 g/L CVE relative to control cells cultured in 10% FBS. Statistical significance was

522 calculated by *t*-tests with Welch's correction in which experimental CVE fractionation groups

523 were compared with versus without nuclease treatment; significance levels are indicated as

524 *p<0.05, **p<0.01, ***p<0.001, ns=not significant, n=6.

525 **Figure 4. Select growth factor and insulin supplemented *C. vulgaris* extract improves cell**

526 **viability in the absence of FBS.**

527 (**a**) Box plots show relative viability of EBTr (yellow) and HEK-293 (blue) cells cultured in serum-

528 free media, supplemented with 10 or 100 ng/mL fibroblast growth factor 2 (FGF) or 1 or 10

529 ng/mL transforming growth factor beta1 (TGF), relative to controls cultured in 10% FBS.

530 Statistical comparisons were between experimental groups and respective no serum control

531 groups. Panel (**b**) shows relative viability of EBTr and HEK-293 cells cultured in serum-free

532 media, supplemented with growth factors (GFs) or 3 μM bovine insulin or 0.2 mM L-ascorbate

533 (LA) or 80 nM selenium dioxide (Se) alone, or combinations thereof, relative to controls

534 cultured in 10% FBS. Growth factors were used at 30 ng/mL (FGF) and 10 ng/mL (TGF).

535 Statistical comparisons were between experimental groups and respective no serum control

536 groups. Panel (**c**) shows relative viability of EBTr cells cultured in serum-free media,

537 supplemented with 10 g/L CVE or 10% CGF alone, or GFs and insulin with and without LA and

538 Se, or combinations thereof, compared to controls cultured in 10% FBS. Concentrations of GFs,

539 insulin, LA and Se were the same as that for panel b. Statistical comparisons were between

540 groups treated with CVE alone and CVE plus other supplements. Statistical significance was

541 determined by *t*-tests with Welch's correction; significance levels are indicated as *$p<0.05$,

542 **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns=not significant, n=6.

543 **Figure 5. Growth factor and insulin supplemented *C. vulgaris* extract stimulates cell growth in**

544 **the absence of FBS.**

545 Line plots show live (**a**) and percent dead/non-intact (**b**) EBTr cells after 0, 2, 4 and 8 days in

546 culture. Different media conditions are indicated by different colors; all counts were performed

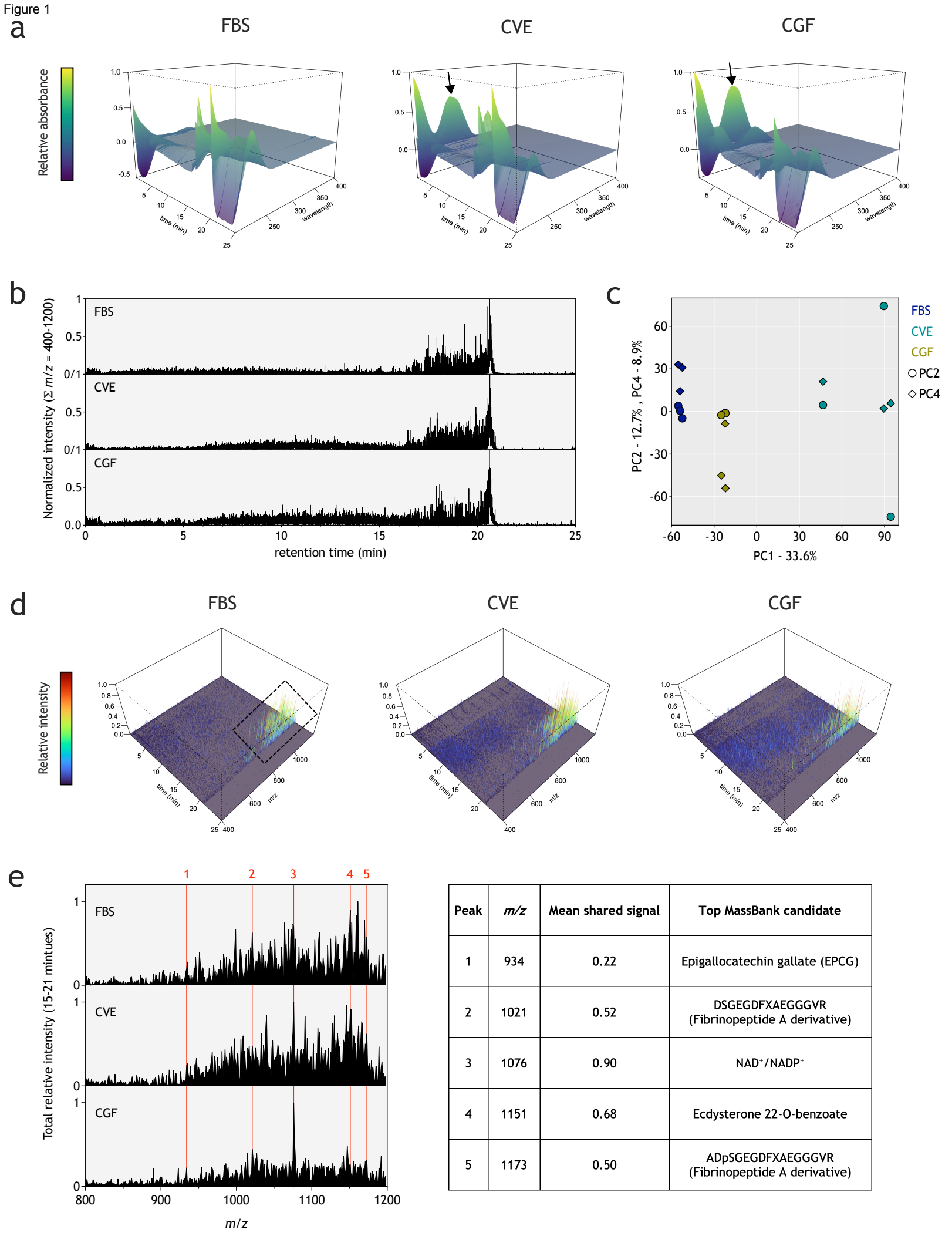
547 in duplicate. (**c**) Brightfield images show representative EBTr cell density and morphology for

548 each condition and time point; white scale bar indicates length of ~100 μm. CVE was used at 10

549 g/L and CGF at 10% by volume. Supplements include 10 ng/mL TGF, 30 ng/mL FGF, 3 μM

550 bovine insulin, 0.2 mM L-ascorbate, and 80 nM selenium.

Figure 1



Peak	*m/z*	Mean shared signal	Top MassBank candidate
1	934	0.22	Epigallocatechin gallate (EPCG)
2	1021	0.52	DSGEGDFXAEGGGVR (Fibrinopeptide A derivative)
3	1076	0.90	$NAD^+/NADP^+$
4	1151	0.68	Ecdysterone 22-O-benzoate
5	1173	0.50	ADpSGEGDFXAEGGGVR (Fibrinopeptide A derivative)

Figure 2

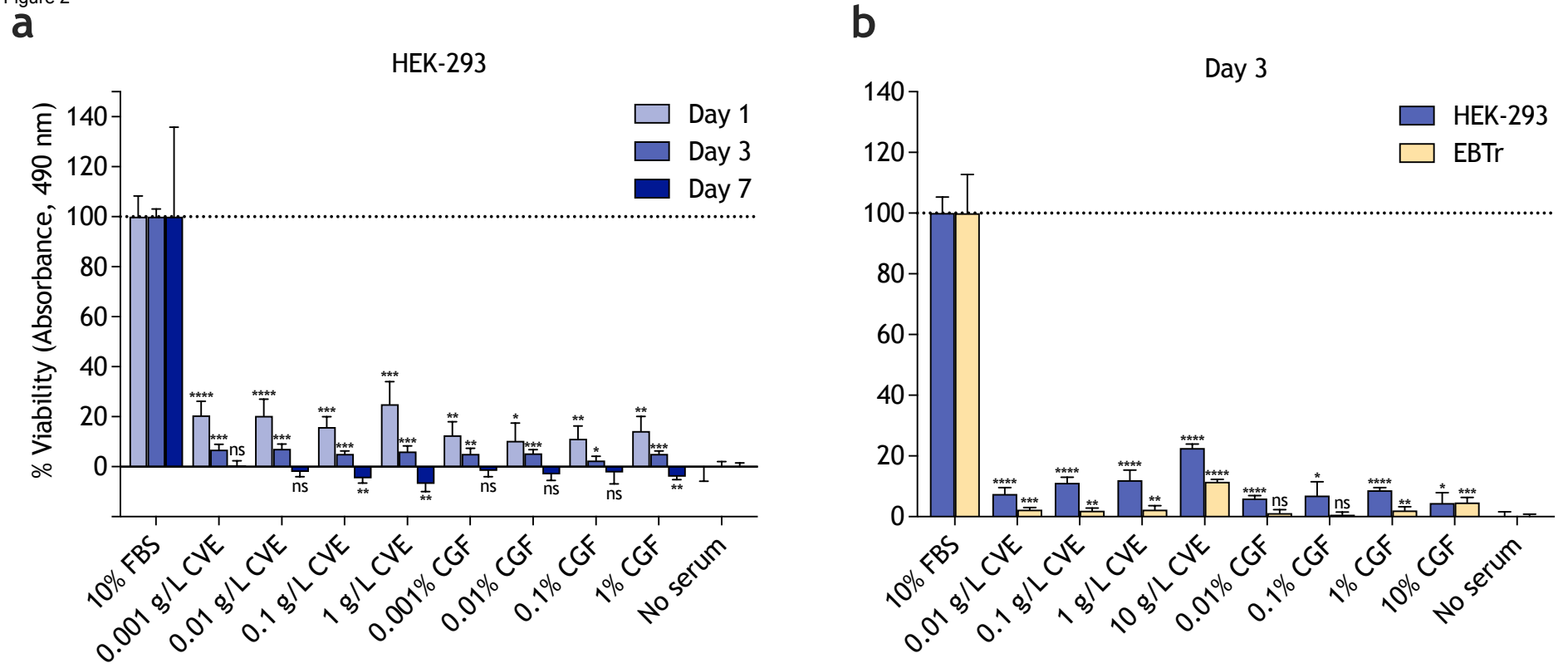

a
HEK-293
% Viability (Absorbance, 490 nm)
Day 1
Day 3
Day 7
10% FBS
0.001 g/L CVE
0.01 g/L CVE
0.1 g/L CVE
1 g/L CVE
0.001% CGF
0.01% CGF
0.1% CGF
1% CGF
No serum
b
Day 3
HEK-293
EBTr
10% FBS
0.01 g/L CVE
0.1 g/L CVE
1 g/L CVE
10 g/L CVE
0.01% CGF
0.1% CGF
1% CGF
10% CGF
No serum

Figure 3

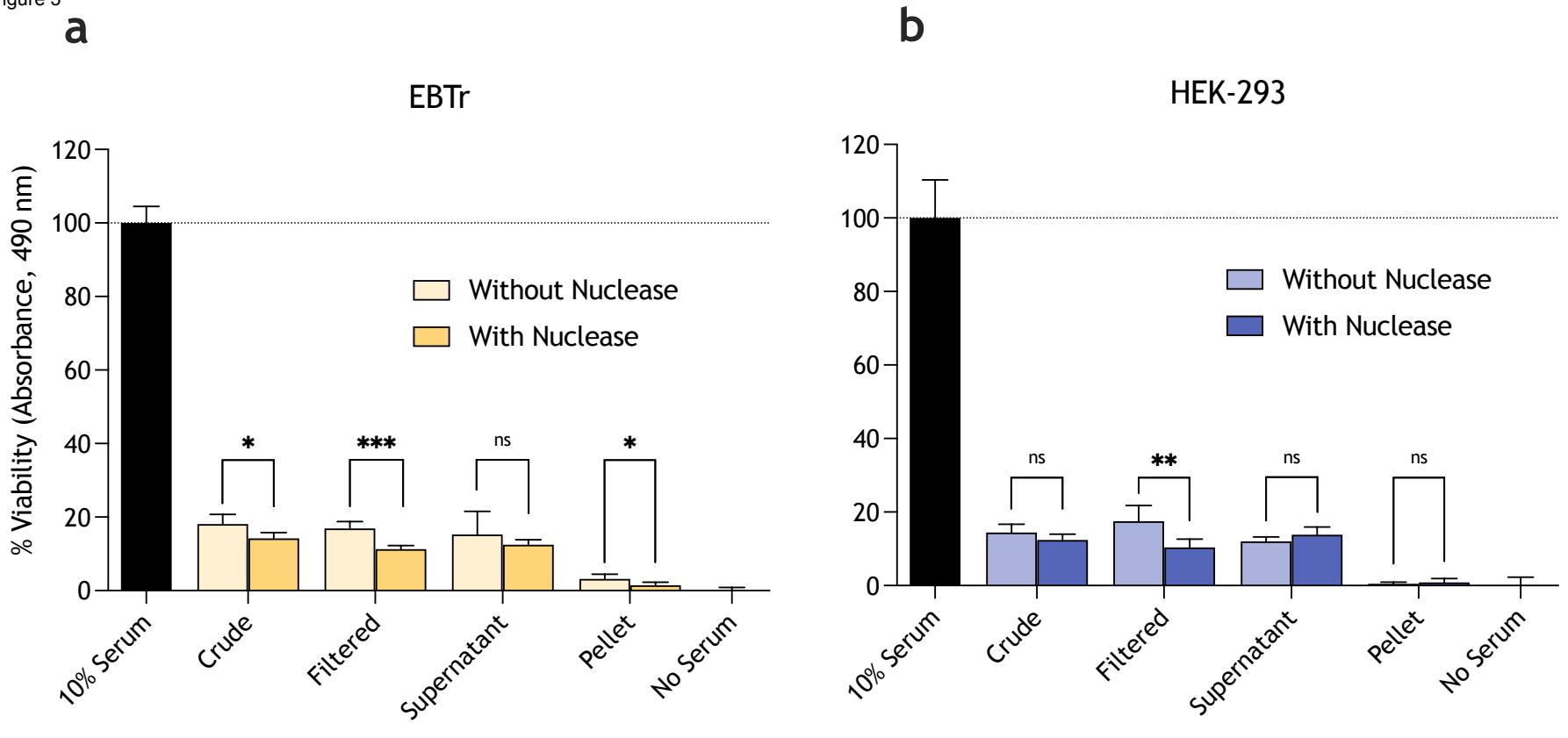

a
EBTr
% Viability (Absorbance, 490 nm)
120
100
80
60
40
20
0
Without Nuclease
With Nuclease
*
***
ns
*
10% Serum
Crude
Filtered
Supernatant
Pellet
No Serum
b
HEK-293
120
100
80
60
40
20
0
Without Nuclease
With Nuclease
ns
**
ns
ns
10% Serum
Crude
Filtered
Supernatant
Pellet
No Serum

Figure 4

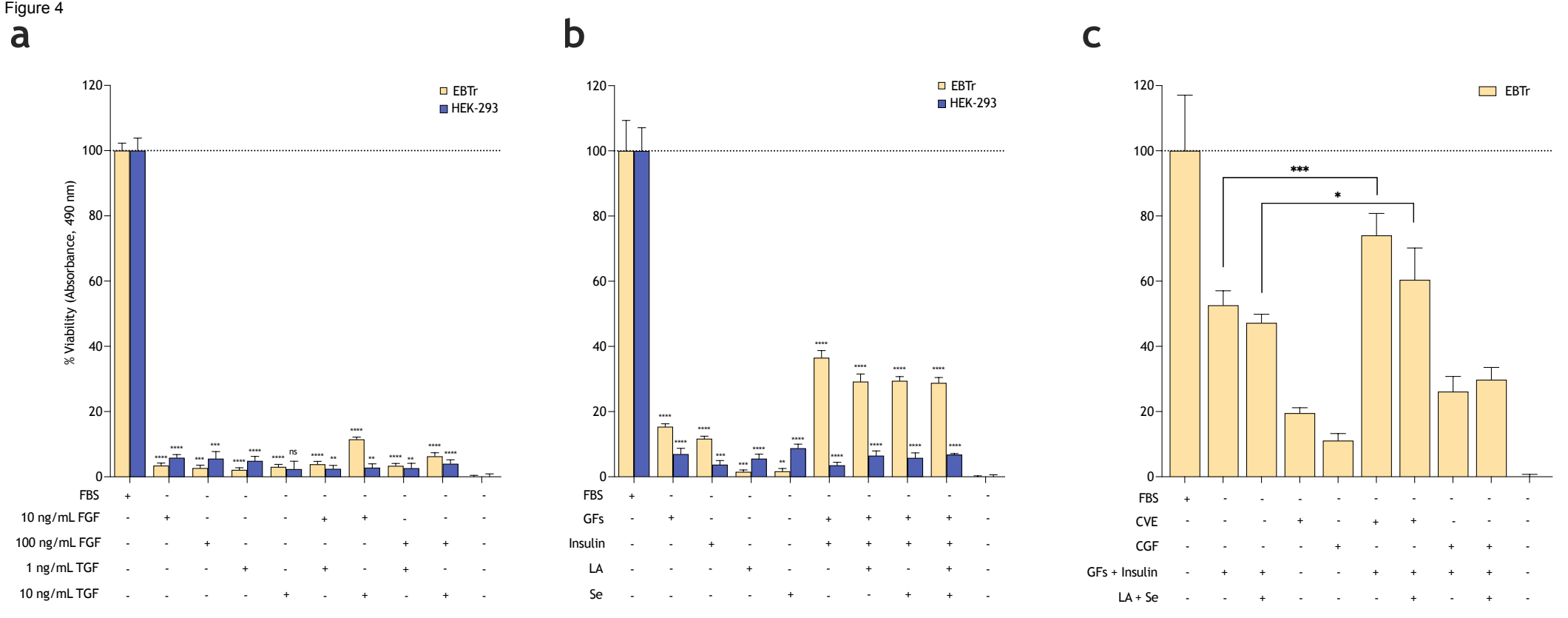
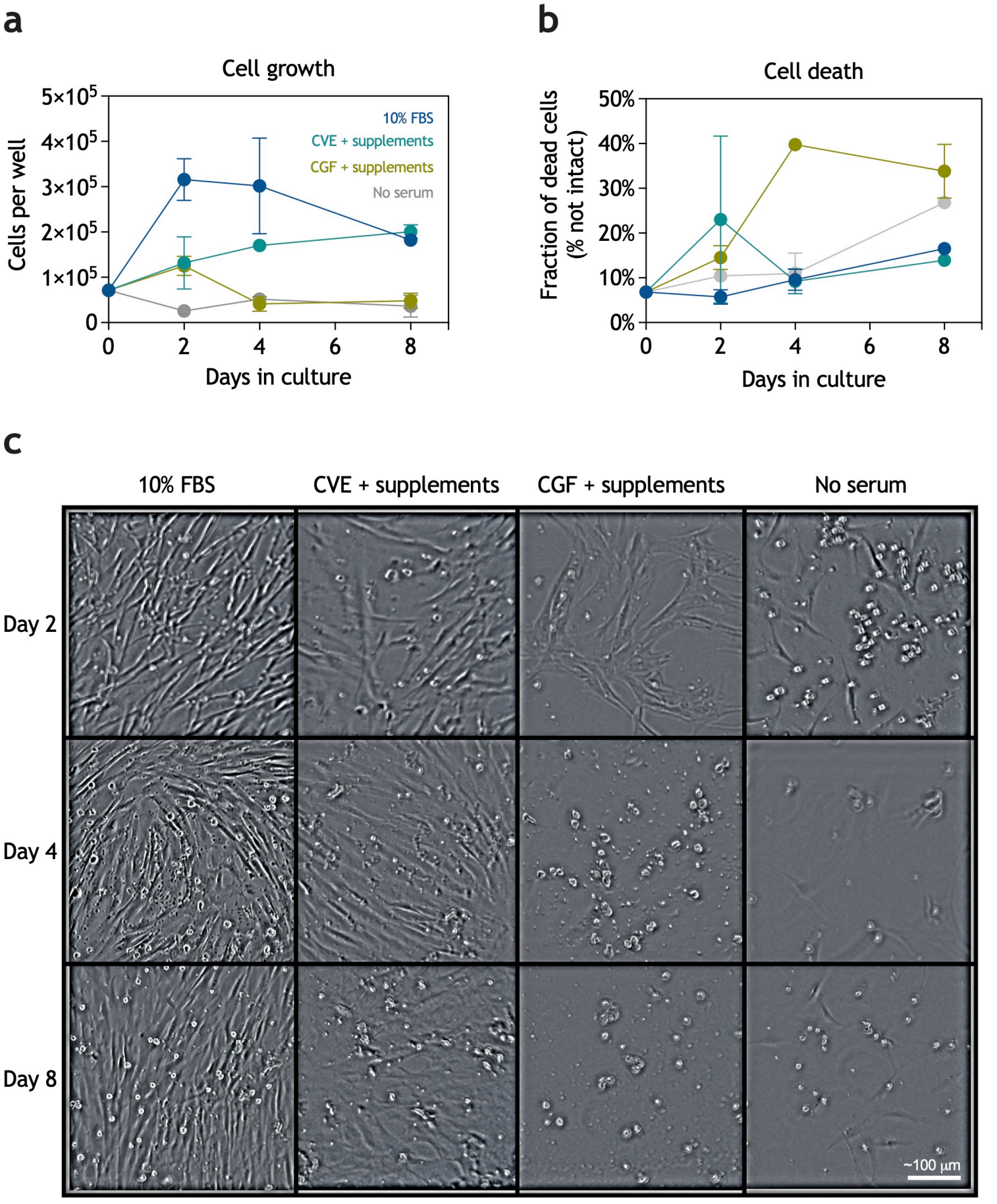

a
b
c
EBTr
HEK-293
% Viability (Absorbance, 490 nm)
120
100
80
60
40
20
0
FBS
10 ng/mL FGF
100 ng/mL FGF
1 ng/mL TGF
10 ng/mL TGF
GFs
Insulin
LA
Se
CVE
CGF
GFs + Insulin
LA + Se
***
*
ns

Figure 5


a
Cell growth
Cells per well
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
0
2
4
6
8
Days in culture
10% FBS
CVE + supplements
CGF + supplements
No serum
b
Cell death
Fraction of dead cells
(% not intact)
50%
40%
30%
20%
10%
0%
0
2
4
6
8
Days in culture
c
10% FBS
CVE + supplements
CGF + supplements
No serum
Day 2
Day 4
Day 8
~100 µm