In vitro culture of bovine fibroblasts using select serum-free media supplemented with *Chlorella vulgaris* extract

Authors

Galileo Defendi-Cho (ORCID: 0000-0001-8747-8143), g_defendicho@coloradocollege.edu

*Timothy M. Gould (ORCID: 0000-0002-6858-1265), tgould@coloradocollege.edu

*Corresponding author

Affiliation

Colorado College, Department of Chemistry and Biochemistry, Colorado Springs, CO, USA.

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.

Keywords

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

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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.

Statements and Declarations

There are no competing interests.

44 **Introduction**

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

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

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

73 We were intrigued by a recent study in which the authors reported growth effects of
74 algae extract on mammalian cells (Ng et al. 2020). Ng and colleagues report that extract
75 derived from *Chlorella vulgaris* promoted growth of Chinese hamster ovary (CHO) and
76 mesenchymal stem cells (MSC) under certain conditions, some of which did not contain any
77 serum. In another study, researchers reported high levels of glucose and amino acids present
78 in various microalgae extracts, with particularly high levels of aspartate and glutamate detected
79 in extract from *C. vulgaris* (Okamoto et al. 2020); these authors proceeded to show that they
80 could use such algae extracts as a substitute to culture cells in media deficient in glucose and
81 amino acids. More recently, this same group showed that *C. vulgaris* extract can be used as a
82 replacement for DMEM in the culture of primary bovine myoblasts (Okamoto et al. 2022).

83 Here we tested the efficacy of culturing cells by replacing serum in the media with algae
84 extract and found that we could achieve appreciable growth and viability of bovine fibroblasts
85 using select serum-free media supplemented with *C. vulgaris* extract (CVE). We characterized
86 molecular features of algae extracts, compared them to that of FBS, and found evidence
87 suggesting that NAD(H)/NADP(H) are common and relatively abundant components. This study

88 establishes how algae extract may be used to culture cells in serum-free conditions while

89 providing further insight into essential components required for cell growth.

90

91 **Materials and Methods**

92 Chemicals and Reagents

93 Liquid chlorella growth factor (SKU 504) and *Chlorella vulgaris* extract capsules (SKU 583) were
94 purchased from BioPure® Healing Products (Woodinville, WA). Pierce™ universal nuclease for
95 cell lysis was purchased from Thermo Fisher Scientific Corporation (Waltham, MA).
96 Recombinant human fibroblast growth factor (FGF-2, catalog no. 100-18B) and transforming
97 growth factor beta (TGF-β, catalog no. 100-21) were purchased from PeproTech, Inc. (Cranbury,
98 NJ). Bovine insulin, sodium L-ascorbate and selenium dioxide were from Sigma-Aldrich. Cell
99 titer 96® aqueous one solution cell proliferation (MTS) assay (catalog no. G3580) was purchased
100 from Promega Corporation (Madison, WI).

101 Fractionation of Algae Extracts

102 Algae extracts termed *Chlorella vulgaris* extract (CVE) and chlorella growth factor (CGF) were
103 derived from two different strains, *Chlorella vulgaris* (CVE) and *Chlorella pyrenoidosa* (CGF).
104 CVE was obtained from capsules in dry powder form and dissolved directly in serum-free
105 DMEM. CGF stock solution was purchased as a concentrated liquid hot-water extract from
106 which all solutions were made on a percent volume basis by diluting CGF directly in serum-free
107 DMEM. For fractionation experiments, crude solutions of 10 g/L CVE and 10% CGF were first
108 prepared in serum-free DMEM. Crude CVE and CGF were then either sterile-filtered through
109 0.22 μm nylon filters or centrifuged at 10,000 x *g* for 10 minutes in a benchtop microcentrifuge.
110 After centrifugation, supernatants were removed, and the pellets were resuspended directly in
111 serum-free DMEM.

112 LC-MS

113 Sample separation was performed by liquid chromatography (LC) using a UPLC BEH C18 column
114 (2.1 x 50 mm, 1.7 μm) fitted to an Acquity UPLC system with PDA detector (Waters).
115 Chromatographic profiles were acquired at wavelengths 200-400 nm. Injection volume was 7.5
116 μL after equilibration, and the column temperature was held at 30°C. Samples were eluted
117 from the column using a gradient mobile phase that consisted of phase A (0.1% formic acid in
118 water) and phase B (0.1% formic acid in acetonitrile). The linear gradient elution procedure
119 was as follows: (step 1) 100% A from 0-3 minutes, (step 2) 100% to 60% A from 3-20 minutes,
120 (step 3) 60% to 0% A from 20-21 minutes, (step 4) 0% A from 21-24 minutes, (step 5) 0% to
121 100% A from 24-25 minutes. The column flow rate was set at 0.2 mL/min from 0-21 minutes,
122 increased to 0.6 mL/min from 21-24 minutes, and then returned to the initial rate from 24-25
123 minutes. Electrospray ionization mass spectrometry (ESI-MS) was performed using an Acquity
124 QDa detector (Waters). Capillary and cone voltages were 0.8 kV and 10 V, respectively. MS
125 measurements were collected in positive ion mode with a mass scan range from 100-1200 *m*/*z*.
126 Cell Culture
127 The EBTr (NBL-4) cell line was purchased from ATCC (CCL-44™). Cells were routinely cultured at
128 37°C with 5% CO_2 in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented
129 with 10% FBS (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher
130 Scientific, catalog no. 15140148); serum-free DMEM included antibiotics but no FBS. Passaging
131 was routinely done as follows: cells were first washed with 1X phosphate-buffered saline (PBS),
132 then placed in an incubator with 0.25% Trypsin-EDTA solution (Gibco) for approximately 2
133 minutes immediately after which complete DMEM was added to quench trypsin activity; cells
134 were collected and centrifuged at 300 x *g* for 5 minutes, and then the cell pellet was

135 resuspended in fresh media. EBTr cells were passaged every 3-4 days at a 1:3 passage ratio.
136 Excess cells were stored in liquid nitrogen at 1-2x10^6 cells/mL in freezing media containing
137 90:10% FBS:DMSO. The number of passages for experiments was limited to less than twenty.

138 Viability (MTS) Assays

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

154 Cell Counting and Imaging

155 Cells were harvested, pelleted, and resuspended in serum-free DMEM. Two replicate 50 μL
156 samples of cell suspensions were each diluted 1:1 in trypan blue dye, from which 10 μL was

157 sampled and pipetted onto a hemocytometer (improved Neubauer ruling pattern). Live and
158 dead/blue (non-intact) cell counts were performed in duplicate (i.e., one count per sample).
159 Brightfield images were obtained using an inverted tissue culture microscope (Hund Wetzlar)
160 fitted with a fixed microscope adapter connected to a 5.1-megapixel digital camera (AmScope).
161 Original grayscale image tiles were merged into a single multi-panel image representing each
162 experiment. Multi-panel images were processed with Fiji/ImageJ2 open-source software (NIH).
163 Image processing involved pseudo flat field correction to balance uneven illumination across
164 images, Fast Fourier Transform (FFT) bandpass filtering to reduce image scan lines, and
165 brightness/contrast adjustments.

166 Data Analyses and Statistics

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

175 **Results**

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

177 Given their potential growth effects, we sought to compare algae extracts (CVE and CGF,
178 each of which was derived from a different *Chlorella* strain) to each other and FBS using LC-MS.
179 Due to limited scope and the molecular complexity of these samples, our aim was not to look
180 for specific compounds but rather to gain a qualitative profile for each sample type. Fig. 1a
181 shows LC chromatograms of FBS compared to CVE and CGF samples. Large broad peaks around
182 260 nm for species that eluted off the column within the first few minutes were observed in
183 both CVE and CGF but not FBS samples; these large peaks did not correspond to any
184 appreciable signal(s) detected by the mass spectrometer at such early elution times (Fig. 1b).
185 All samples showed both substantial absorbance (between 200-250 nm) and mass signals at
186 retention times from approximately 18-21 minutes; the relative magnitude of this cluster of
187 signals was comparable for FBS and CVE samples, whereas that of CGF samples was lower. Fig.
188 1c shows the results of a principal component analysis (PCA) comparing total ion
189 chromatograms (TICs) of all FBS, CVE and CGF samples (i.e., replicates averaged in Fig. 1b).
190 Replicate injections of the same sample type clustered together and separated in PC1 but not
191 PC2 values; moreover, CVE and FBS samples were more similar in PC4 values than CGF samples.
192 We also visualized how signals at each *m*/*z* value and retention time compared across samples
193 (Fig. 1d). Each sample type showed a common pattern with a cluster of relatively high signal
194 peaks corresponding to *m*/*z* values ranging from roughly 800-1200 and retention times from
195 about 15-21 minutes. Both CVE and CGF samples also shared signal features at lower *m*/*z*
196 values (mainly over the 400-800 range) and retention times from about 5-15 minutes; this

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

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

205 We initially set out to test if algae extracts have growth effects on cells cultured in the
206 absence of any FBS, using MTS assays as an initial readout and proxy for potential cell growth.
207 Fig. 2 shows the relative viability of EBTr cells cultured over a 10,000-fold range of
208 concentrations of CVE or CGF. Media supplemented with CVE and CGF alone significantly
209 increased EBTr cell viability compared to serum-free controls; such effects were more
210 pronounced for CVE than CGF and for relatively high concentrations of CVE. However,
211 increases in relative cell viability due to CVE supplementation alone did not exceed beyond
212 roughly 15% in any condition; moreover, and importantly, upon examining CVE-treated cells
213 under the microscope immediately prior to performing MTS assays, we did not observe any
214 convincing level of growth or cell doubling(s).

215 Given the partial but limited growth shown in Fig. 2a, we wondered if there may be
216 components in the CVE which were acting to inhibit cell growth. We decided to separate the
217 different components of CVE through filtration, centrifugation, and treatment with nuclease,
218 using MTS assays to compare head-to-head the extract in its crude, filtered, supernatant, and

219 pellet forms, each with and without nuclease treatment. Fig. S1a shows the relative viability of
220 EBTr cells cultured with these different CVE fractions. No obvious differences were seen
221 between crude, filtered and supernatant forms of extract, with viabilities of such fractions all
222 falling near the range of 15-20%. The viability of cells treated with the resuspended pellet was
223 notably lower than that of all other fractions, falling to nearly 0%, similar to that of no serum
224 controls. Further, CVE fractions treated with nuclease showed mostly reduced viability
225 compared to the corresponding fractions not treated with nuclease.

226 Fig. S1 (panels b and c) show the LC-MS characterization of the supernatant and pellet
227 forms of CVE used in the experiment described in Fig. S1a. UV absorbance data shows sharp
228 peaks around 20 minutes in the 200-250 nm range for both the pellet and the supernatant. The
229 supernatant also shows a pair of broader peaks at around 200 and 260 nm that appear to have
230 eluted within the first minute, neither of which was present in the pellet form. TICs of mass
231 spectrometry data for *m*/*z* values between 400 and 1200 for these two forms of extract show
232 similar footprints. Both show similarly the highest intensity spectra between 15 and 20
233 minutes, with relatively smaller spectra between 0 and 15 minutes. The spectra of the pellet
234 form show slightly greater relative signals in the 0-6-minute range. Notably, the large UV peaks
235 in both sample types correspond to increased signals in their respective mass spectra at around
236 20-21 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 <u>Select serum-free media supplemented with *C. vulgaris* extract enhances cell viability.</u>

239 After seeing limited results from algae extract alone, we decided to move our research
240 in a different direction, focusing on growth factors, which have previously been used to

241 successfully culture stem cells in an "Essential 8" (E8^{TM}) serum-free medium (Chen et al. 2011).
242 Fig. 2b shows percent viability of EBTr cells treated with different concentrations of basic
243 fibroblast growth factor (FGF) and transforming growth factor β1 (TGF). Neither growth factor
244 alone improved viability beyond 5-10% relative to serum-free controls, yet viability increased to
245 around 15% for EBTr cells treated with a combination of 10 ng/mL FGF and 10 ng/mL TGF.
246 Thus, we decided to use a combination of FGF and TGF for our next experiment in which we
247 tested these growth factors together with bovine insulin, vitamin C (in the form of L-ascorbate),
248 and selenium (in the form of selenium dioxide), all of which are listed as ingredients of the E8^{TM}
249 media. Fig. 2c shows insulin as a critical factor in real cell growth, in conjunction with growth
250 factors; treatments with both insulin and growth factors improved EBTr cell viability to around
251 35% relative to controls cultured with FBS. Despite their inclusion in E8^{TM} media, adding L-
252 ascorbate, selenium, or both, slightly reduced EBTr cell viability compared to that of cells
253 cultured with growth factors and insulin alone. We decided next to bring algae extracts back
254 into our experiments, combining them with insulin and growth factors and/or L-ascorbate and
255 selenium. Fig. 2d shows that EBTr cell viability reached approximately 75% (relative to FBS
256 controls) when treated with a combination of CVE, growth factors and insulin. On the other
257 hand, the viability of cells treated with CGF, growth factors and insulin, was actually lower than
258 that of cells treated with growth factors and insulin alone. Once again, the addition of L-
259 ascorbate and selenium had little if any beneficial effect on cell viability.
260 <u>Select serum-free media supplemented with *C. vulgaris* extract promotes cell growth and</u>
261 <u>expansion.</u>

262 After seeing promising viability results (i.e., Fig. 2d), we decided the next step was to
263 test if EBTr cells would expand when treated with insulin, growth factors and CVE in a cell count
264 experiment. Fig. 3a shows cell counts of CVE and CGF with supplements (i.e., growth factors,
265 insulin, L-ascorbate, and selenium) compared to controls with and without serum (10% FBS).
266 The number of EBTr cells increased in cultures with CVE or CGF plus supplements by day 2,
267 showing definitive growth, yet still not as much growth as control cells cultured with 10% FBS.
268 By day 4, the number of cells cultured with CGF plus supplements fell to a level near that of no
269 serum controls, but cells cultured with CVE plus supplements continued growth to around half
270 that of controls cultured with 10% FBS. On day 8, the number of cells cultured with CVE plus
271 supplements was comparable to that of controls cultured with 10% FBS, the latter of which had
272 decreased since day 4. Fig. 3b shows the corresponding cell death percentages for the
273 experiment described in Fig. 3a. Cell death percentages were largely in the 10-15% range,
274 slightly increasing over time, with the most death occurring for cells cultured with CGF plus
275 supplements on day 4 jumping to about 40%, corroborating the loss of growth shown for such
276 cultures between day 2 and day 4 shown in Fig. 3a. Fig. 3c shows images captured of the
277 experiment described in Fig. 3a and 3b. EBTr cells cultured with 10% FBS show sustained
278 growth through day 4 until reaching confluency, at which point growth plateaued between days
279 4 and 8. Cells cultured with CVE plus supplements also appeared to grow through day 4,
280 reaching a relatively high level of confluency; on day 8 the level of confluency appears about
281 the same as day 4 (perhaps slightly more) with more dead cells in the image. Cells cultured
282 with CGF plus supplements appear to show growth until day 2, though notably less than cells
283 cultured with CVE plus supplements; however, by day 4 most if not all remaining cells appear

284 dead, and by day 8, only dead cells remain in the image. (Note that EBTr cells in wells treated
285 with algae extract appear larger than cells in wells where algae extract was not present.) The
286 no serum control shows some cells with normal morphology on day 2, with many dead cells in
287 the image as well; by days 4 and 8, hardly any cells remain besides a few dead cells.

288 Given that EBTr cells showed growth and markedly improved viability when cultured
289 with CVE and select supplements, we decided next to attempt to expand EBTr cells from a 1 mL
290 culture (12-well plate) to a 2 mL culture (6-well plate) under these conditions (Fig. S2). On day
291 1, cells cultured with 10% FBS or with CVE plus supplements attached effectively, while the no
292 serum control already shows many dead cells. By day 4, the no serum control well looks nearly
293 identical to that on day 1, with some cells still attached but many dead with no visible growth.
294 By day 4, cells cultured with 10% FBS or CVE plus supplements show obvious growth, nearing
295 confluency. As observed in Fig. 3c, cells cultured with CVE plus supplements appear slightly
296 bigger than cells not cultured with CVE, meaning that fewer cells were necessary to reach
297 similar levels of confluency. After the day 4 pictures were taken, cells were detached and
298 removed from the 1 mL cultures, then pelleted and resuspended into fresh 2 mL cultures. The
299 following day, after cells attached to the plate (day 5), the relative amount of cells in the 10%
300 FBS control well was notably greater than that in the well with CVE plus supplements. By day 8,
301 cells cultured with 10% FBS reached near-confluency, while cells cultured with CVE plus
302 supplements remained at nearly 50% confluency. On both days 5 and 8, the no serum control
303 wells were essentially devoid of live cells, with only a few dead cells remaining.

304 **Discussion**

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

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

326 20% was observed in an environment where cells were already dividing. These observations of
327 an increase in viability caused by CVE in both diving and non-dividing environments are
328 consistent with other studies (Okamoto et al. 2022), suggesting that CVE increases cell viability
329 independent of effects on proliferation. The possibility that algae extract enhances the cellular
330 metabolic potential without promoting cell division requires further investigation. Cell viability
331 assays using tetrazolium salts like MTS are often used as a proxy for cell growth, yet what such
332 assays actually measure is the activity of mitochondrial NADH and NADPH-dependent
333 dehydrogenase enzymes which reduce the reagent to a formazan product (Dunigan et al. 1995).
334 In most cases, the more cells there are the more mitochondria are available to reduce the MTS
335 reagent, but it is possible that the algae extracts have a positive effect on such metabolic
336 activity without promoting cell growth. Given that a common and relatively abundant signal
337 feature found in the mass spectra of algae extract samples mapped to NAD^+/$NADP^+$ as the
338 parent species, it is possible that enriched levels of NAD(H)/NADP(H) present in the algae
339 extracts may have caused enhanced activity of the dehydrogenase enzymes in the cells thus
340 explaining the observed increase in viability. Further, it was previously shown that CVE also
341 contains high levels of glutamate (Okamoto et al. 2020), as *Chlorella* algae use NADPH-
342 dependent glutamate dehydrogenase enzymes to control their cell cycle (Talley et al. 1972).
343 Glutamate has been shown to increase oxidation of NADH (Kannurpatti and Joshi 1999;
344 Maalouf et al. 2007), and increase cancer cell viability and growth (Reiman et al. 1981; Takano
345 et al. 2001; Gelb et al. 2015; Yamaguchi et al. 2020). Moreover, in fibroblasts, glutamate
346 reduced toxicity induced by high levels of cysteine (Bannai and Ishii 1982), and glutamate
347 dehydrogenase deficiency reduced cell viability (Tatsumi et al. 1989). Therefore, it is possible

348 that enriched levels of glutamate in algae extracts may also be responsible for the increased
349 viability observed.

350 Our experiments with components of the E8™ media clearly showed that insulin, basic-
351 FGF and TGF-β are necessary for cell growth, and that these components are roughly additive in
352 terms of their benefits to cell viability. Early studies examining serum-free culture conditions
353 showed FGF and insulin as key determinants for growth (Mcfarland et al. 1991). Insulin causes
354 cells to import glucose, providing energy and nucleotide building blocks needed for replication.
355 In viability assays, addition of CVE also proved to be roughly additive, with CVE in addition to
356 basic-FGF, TGF-β, insulin, and other additives combined yielding the highest viability reading of
357 any serum-free condition tested. We demonstrated that EBTr cells can grow and expand into a
358 new culture vessel when cultured in serum-free media that included at minimum basic-FGF,
359 TGF-β, insulin and algae extract in the form of CVE, although such growth was not as robust as
360 that of cells cultured with 10% FBS. Recent studies have shown that mitochondrial NADP(H)
361 levels are essential for cell growth (Tran et al. 2021), without which cells cannot make proline
362 required to support nucleotide and protein synthesis. Thus, it is plausible that enriched levels
363 of NAD(H)/NADP(H) in CVE extract may provide a true benefit to cell growth; however, whether
364 the additive increase in viability from CVE translates to any real benefit in growth rate beyond
365 that from basic-FGF, TGF-β and insulin alone remains unclear. Regardless of whether algae
366 extract is truly beneficial to cell growth, it is important to note that bovine fibroblasts could be
367 cultured with only a subset of the E8™ components present. This may at least suggest some
368 potential economic benefit due to lower cost serum-free formulations requiring fewer
369 components. Since growth factors and insulin directly interact with their respective receptors

370 through a relatively small range of residues across the entire protein, further experiments could
371 test whether smaller peptide fragments mapping to such regions within these proteins remain
372 actionable enough to sustain cell growth. Such substitutes may simplify and further lower the
373 cost of serum-free formulations in which cells can be effectively cultured. Similarly, small
374 molecules that act as agonists on basic-FGF and/or TGF-β receptors could also be tested for the
375 same purpose.

376 Some limitations of our studies include aspects of the growth experiments in which we
377 did not anticipate how differences in cell size could impact the results. In these experiments, it
378 appears from the images as though cells cultured with algae extract were larger in size as
379 compared to those not cultured with algae extract. Notably, increases in cell viability correlate
380 with increased cell size (Dreesen and Fussenegger 2011), which could at least partially explain
381 this observation since we also found that algae extract typically increased cell viability.
382 However, differences in cell size observed with and without algae extract in the culture make
383 the growth and expansion experiments difficult to interpret. For instance, during the expansion
384 experiment, it appears that more cells were transferred from the 1 mL culture with 10% FBS
385 compared to the culture with CVE plus supplements despite these two groups appearing to
386 have a similar level of confluency on day 4. While both conditions promoted growth, the cells
387 cultured in 10% FBS not only grow faster but require less space per cell. As a result, in the 2 mL
388 cultures, the cells cultured in 10% FBS had a higher base count from which to start growing and
389 were thus much more confluent by day 8. Despite the observed increase in viability, it is also a
390 possibility that this increase in cell size corresponds to replicative senescence. Cells increase in
391 size when they enter a permanent cell-cycle arrest and senescence (Biran et al. 2017), so

392 further experiments examining the extent to which proliferation is affected by CVE are
393 necessary. In a separate case, it should be noted that the day 8 FBS control result in our cell
394 count experiment (Fig. 3c) shows a sharp decrease in cell number, which can be explained by
395 over-confluence of the culture dish, resulting in cell death. As a result, the day 4 image and cell
396 count more accurately display the efficacy of CVE plus supplements, since it would be expected
397 that in a larger dish, the FBS control condition would continue to promote cell growth through
398 day 8. Furthermore, we must acknowledge limitations in counting the fraction of dead cells
399 recorded in our cell count experiments. As noted in the methods section, old media was first
400 removed and the cells were briefly washed, which likely removed a large amount of previously
401 dead and floating cells. Thus, the dead cell count more accurately represents the percentage of
402 cells which were nearly dead, or dead, but not yet detached and floating. Finally, it is important
403 to note that algae extracts may be subject to batch-to-batch variation depending on the growth
404 and extraction conditions used to generate them, thus further study of such variation in *C.*
405 *vulgaris* extract is important to confirm the reproducibility of results.
406 Taken together, our studies show that algae extract derived from *Chlorella vulgaris*
407 enhances viability of bovine fibroblasts, possibly due at least in part to its enriched levels of
408 NAD(H)/NADP(H) and/or glutamate, and that CVE can be used together with other supplements
409 (basic-FGF, TGF-β, and insulin) to grow cells in the absence of serum. Further studies are
410 needed to determine the extent to which CVE promotes cell growth and whether the
411 supplementary components can be further modified to reduce their cost.
412

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489
490

491 **Figure Legends**

492 **Fig. 1**

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

511 **Fig. 2**

512 **Select growth factor and insulin supplemented *C. vulgaris* extract improves cell viability in the**
513 **absence of FBS.** (**a**) Bar plots show viability of EBTr cells cultured with CVE and CGF relative to
514 controls cultured in 10% FBS at day 3. Panel (**b**) shows relative viability of EBTr cells cultured in
515 serum-free media supplemented with 10 or 100 ng/mL fibroblast growth factor 2 (FGF), or 1 or
516 10 ng/mL transforming growth factor β1 (TGF), or combinations thereof, relative to controls
517 cultured in 10% FBS after 3 days. Panel (**c**) shows relative viability of EBTr cells cultured in
518 serum-free media supplemented with growth factors (GFs), or 3 μM bovine insulin, or 0.2 mM
519 L-ascorbate (LA), or 80 nM selenium dioxide (Se), or combinations thereof, relative to controls
520 cultured in 10% FBS. Growth factors were used at 30 ng/mL (FGF) and 10 ng/mL (TGF). Panel
521 (**d**) shows relative viability of EBTr cells cultured in serum-free media supplemented with 10 g/L
522 CVE or 10% CGF alone, or GFs and insulin with and without LA and Se, or combinations thereof,
523 compared to controls cultured in 10% FBS. Concentrations of GFs, insulin, LA and Se were the
524 same as that for panel c. Statistical significance was determined by *t*-test with Welch's
525 correction; significance levels are indicated as *$p<0.05$, ***$p<0.001$, ****$p<0.0001$, ns=not
526 significant, n=6

527 **Fig. 3**

528 **Growth factor and insulin supplemented *C. vulgaris* extract stimulates cell growth in the**

529 **absence of FBS.** Line plots show live (**a**) and percent dead/non-intact (**b**) EBTr cells after 0, 2, 4

530 and 8 days in culture. Different media conditions are indicated by different colors; all counts

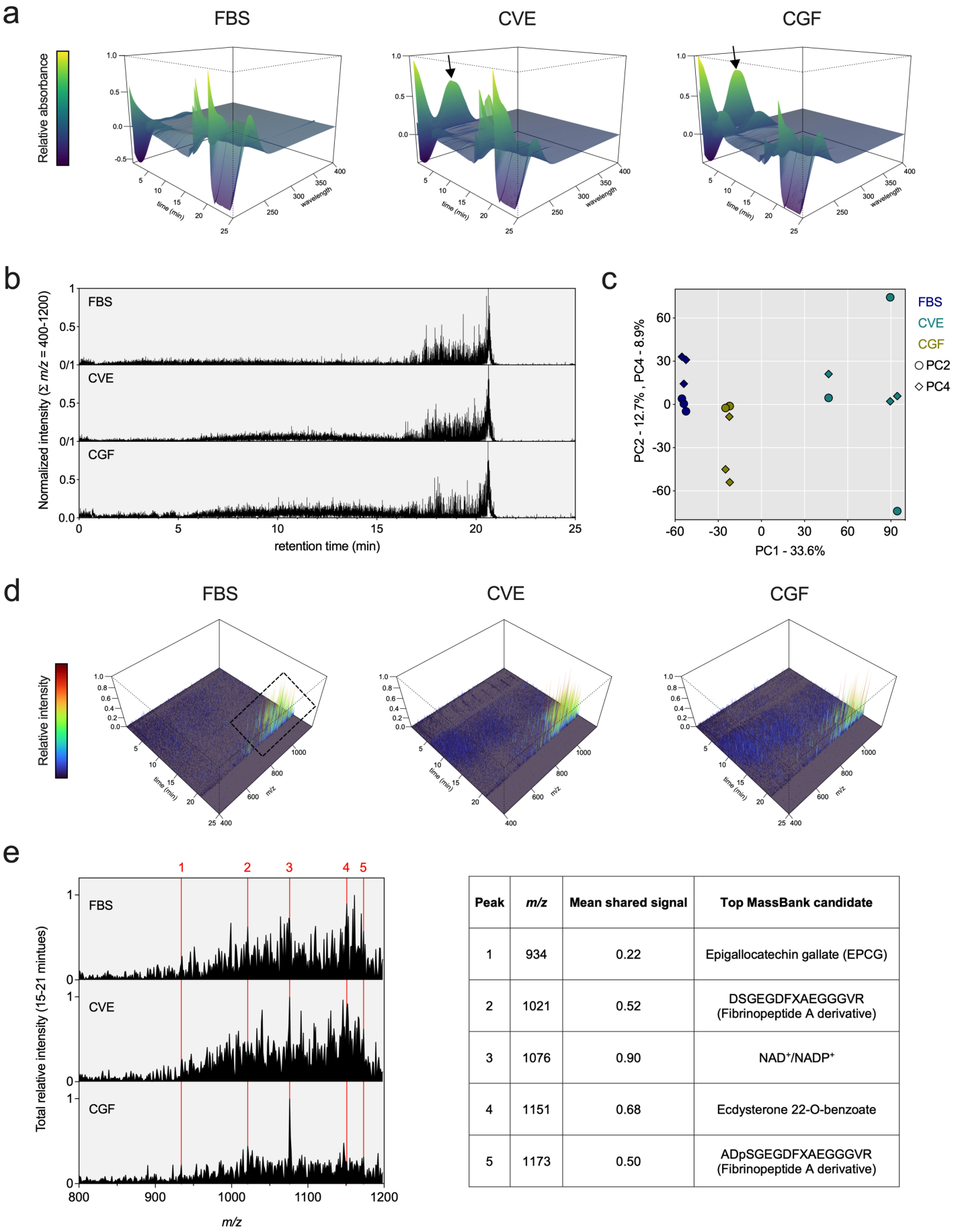
531 were performed in duplicate. (**c**) Brightfield images show representative EBTr cell density and

532 morphology for each condition and time point; white scale bar indicates length of ~100 μm.

533 CVE was used at 10 g/L and CGF at 10% by volume. Supplements include 10 ng/mL TGF, 30

534 ng/mL FGF, 3 μM 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

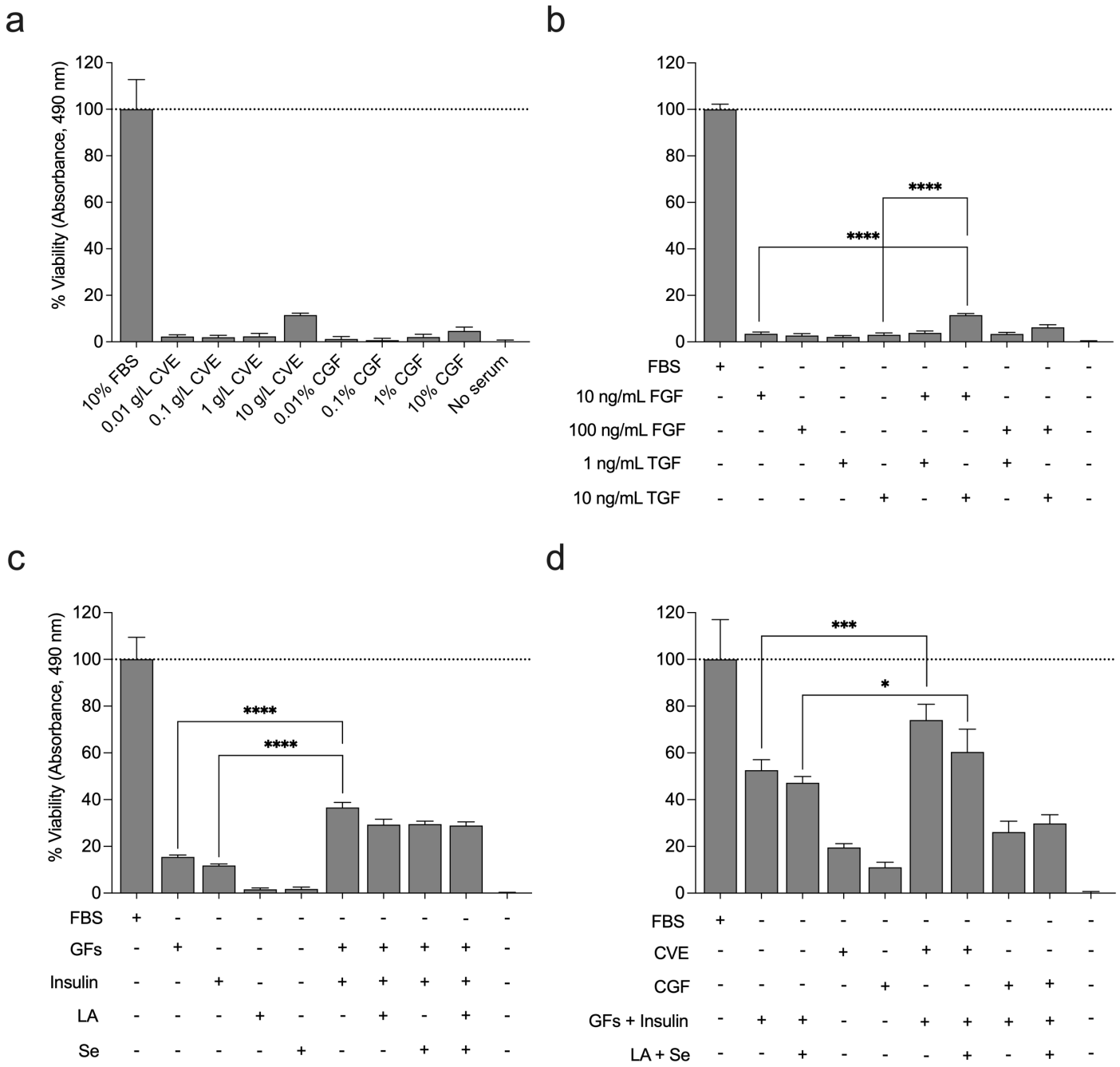
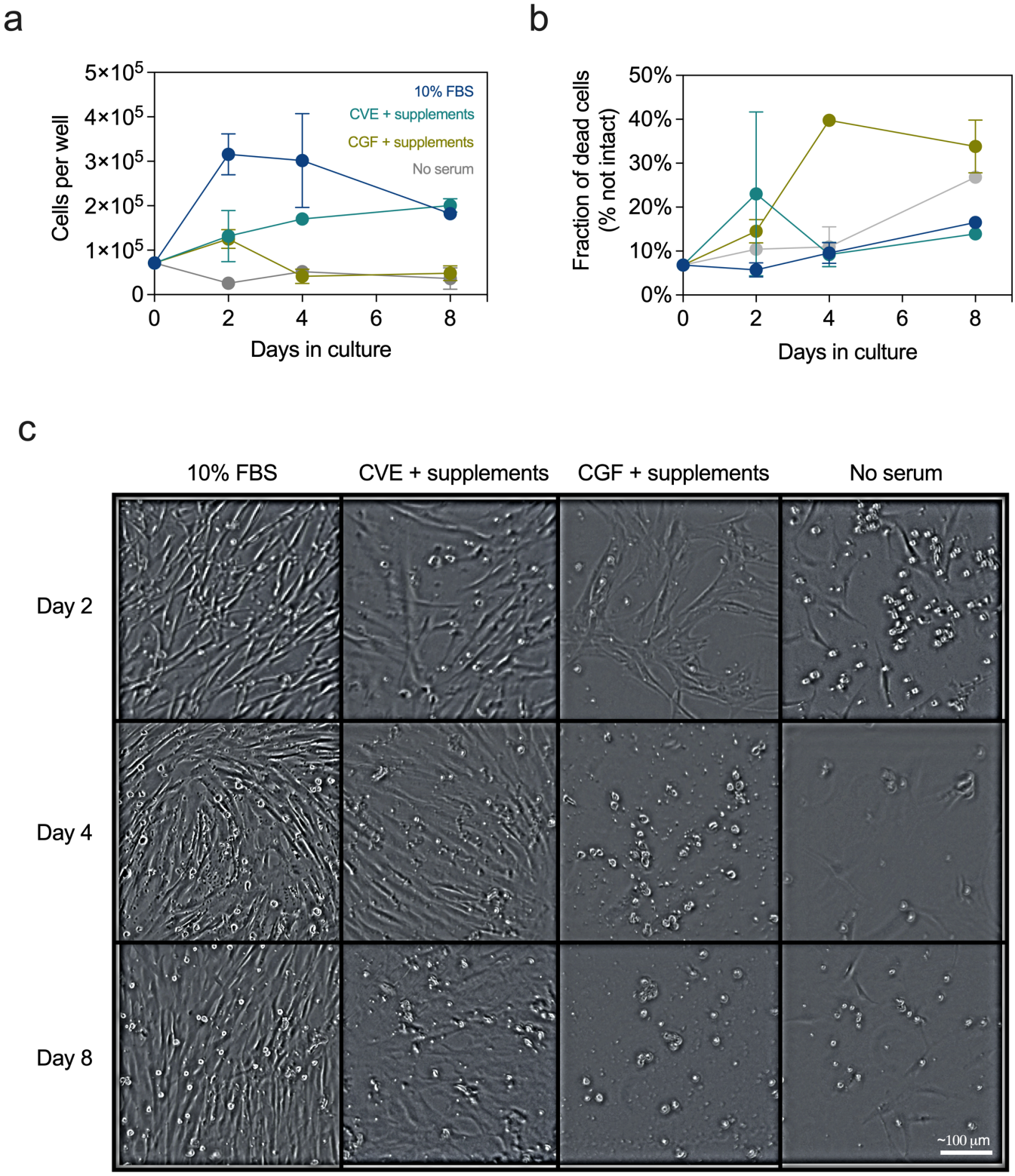

a
% Viability (Absorbance, 490 nm)
120
100
80
60
40
20
0
10% FBS
0.01 g/L CVE
0.1 g/L CVE
1 g/L CVE
10 g/L CVE
0.01% CGF
0.1% CGF
1% CGF
10% CGF
No serum
b
****
****
FBS + - - - - - - - - -
10 ng/mL FGF - + - - - + + - - -
100 ng/mL FGF - - + - - - - + + -
1 ng/mL TGF - - - + - + - + - -
10 ng/mL TGF - - - - + - + - + -
c
% Viability (Absorbance, 490 nm)
****
****
FBS + - - - - - - - - -
GFs - + - - - + + + + -
Insulin - - + - - + + + + -
LA - - - + - - + - + -
Se - - - - + - - + + -
d
***
*
FBS + - - - - - - - - -
CVE - - - + - + + - - -
CGF - - - - + - - + + -
GFs + Insulin - + + - - + + + + -
LA + Se - - + - - - + - + -

Figure 3


a
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
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