

1        Comparison of the effects of metformin on MDA-MB-231 breast  
2        cancer cells in a monolayer culture and in tumour spheroids as a  
3        function of nutrient concentrations

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26

## 27 **Abstract**

28 Metabolic pathways of cancer cells depend on the concentrations of nutrients in their micro-  
29 environment. However, they can vary also between monolayer cultures of cancer cells and  
30 tumour spheroids. Here we examined whether the absence of glucose, pyruvate and glutamine  
31 increases the sensitivity of MDA-MB-231 cells to metabolic drug metformin using two *in*  
32 *vitro* cell models (monolayer culture and tumour spheroids). To evaluate the effects of  
33 nutrient depletion in more detail, we tested the effects of metformin in commonly used media  
34 (DMEM, MEM and RPMI-1640) that differ mainly in the concentrations of amino acids. We  
35 used MTS, Hoechst and propidium iodide assay to determine cell number, viability and  
36 survival, respectively. We evaluated the effects of metformin on the size of tumour spheroids  
37 and determined cell survival by calcein and propidium iodide staining. Finally, we observed  
38 the effects of metformin in nutrient depleted conditions on the phosphorylation of AMP-  
39 activated protein kinase using Western blotting. Our main finding is that the effects of  
40 metformin on MDA-MB-231 cells depend on *in vitro* cell model used (monolayer culture vs.  
41 tumour spheroids). While metformin did not have any major effect on proliferation of MDA-  
42 MB-231 cells grown in complete cell culture media in a monolayer culture, it disintegrated  
43 tumour spheroids in MEM and RPMI-1640 medium. The effects of metformin on tumour  
44 spheroids were most pronounced in MEM, which is deficient of several non-essential amino  
45 acids. Glutamine depletion had no effect on the sensitivity of MDA-MB-231 cells to  
46 metformin in all tested conditions, whereas pyruvate depletion sensitized MDA-MB-231 cells  
47 to metformin in a monolayer culture only in MEM. Taken together, our results show that  
48 media formulation as well as *in vitro* cell model (monolayer culture vs. tumour spheroids)  
49 must be considered, when we evaluate the effects of metformin on MDA-MB-231 cells as a  
50 function of nutrient availability.

51

## 52 **Introduction**

53 Triple negative breast cancer, which lacks estrogen and progesterone receptors and is  
54 negative for human epidermal growth factor receptor 2 (HER-2), is highly aggressive form of  
55 breast cancer with limited treatment options [1]. Cancer cells have altered metabolic pathways  
56 in comparison to normal cells. The first metabolic alteration that was described in cancer cells  
57 is their increased consumption of glucose under aerobic conditions, which is also known as  
58 the Warburg effect [2]. Moreover, cancer cells differ from normal cells in consumption of  
59 other nutrients [3] as well as in intrinsic characteristics that affect metabolic pathways [4,5].  
60 Metabolic phenotype of cancer cells at least partially depends on their micro-environment,  
61 which is often depleted of some nutrients [6–8]. Furthermore, under *in vitro* conditions  
62 metabolic pathways differ between 2D monolayer cultures of cancer cells and the  
63 physiologically more relevant 3D tumour spheroids [9–12]. Importantly, the type of *in vitro*  
64 cell model (monolayer culture vs. tumour spheroids) and media formulation might also alter  
65 the sensitivity of breast cancer cells to pharmaceutical compounds that target cell metabolism  
66 and has a potential to treat breast cancer. One of such compounds is metformin.

67 Metformin is the most commonly used oral drug to treat type 2 diabetes and has  
68 potential anti-cancer effects in patients with breast cancer [13–15]. However, its mechanism  
69 of action is still not completely understood. In type 2 diabetes, metformin ameliorates glucose  
70 homeostasis and alleviates hyperinsulinemia, thus reducing the risk factors for development  
71 of insulin-sensitive cancers [16]. Besides its systemic action, metformin can also target cancer  
72 cells directly. Its main direct mechanism of action is probably suppression of cellular  
73 oxidative phosphorylation via inhibition of complex I in mitochondrial respiratory chain [17–  
74 20]. Another important mechanism is inhibition of mitochondrial glycerophosphate  
75 dehydrogenase, which reduces gluconeogenesis in hepatocytes [21]. Metformin-mediated  
76 inhibition of mitochondrial oxidative phosphorylation reduces cellular NAD<sup>+</sup>/NADH ratio

77 [22,23], attenuates mitochondrial anaplerotic reactions [24,25], in particular aspartate  
78 biosynthesis [22,23], and induces reductive metabolism of glutamine-derived carbon in  
79 tricarboxylic acid (TCA) cycle [24,26,27]. Furthermore, metformin activates AMP-activated  
80 protein kinase (AMPK), which is the main regulator of cellular energy homeostasis [28].  
81 Once activated, AMPK accelerates catabolic and inhibits anabolic processes in cancer cells,  
82 which enables survival of cancer cells in energetic crisis [29,30]. Metformin-stimulated  
83 AMPK activation in breast cancer cells is more pronounced in glucose depleted conditions  
84 [30,31], while AMPK does not mediate the effects of metformin under serine depletion [33].  
85 The effects of depletion of other nutrients on metformin-stimulated AMPK activation are still  
86 largely unknown.

87 Cancer cell sensitivity to metformin *in vitro* is not merely an intrinsic property of  
88 cancer cells, but can be modulated by nutrient concentrations in cell culture media  
89 [17,22,23,31–36] and consequently also by medium renewal protocols [31]. Metformin  
90 suppresses proliferation of various cancer cells more effectively in medium without glucose  
91 [31,36–39] or pyruvate [22,23,33,34], while the absence of glutamine increases metformin's  
92 effects on liver cancer cells [26]. Studies imply that the absence of several amino acids might  
93 increase the effects of metformin on cancer cells synergistically with the absence of other  
94 nutrients [22,33,35]. However, the effects of depletion of nutrient combinations on the  
95 sensitivity of the most widely used triple negative breast cancer cells, MDA-MB-231 cells  
96 [40], to metformin were not examined in detail. Besides, although it is well-known that  
97 metformin affects cancer cells grown in various 3D cell culture models [41–45], the role of  
98 nutrient availability on the effects of metformin on tumour spheroids remains unknown.

99 To our knowledge, the effects of metformin on MDA-MB-231 cells as a function of  
100 nutrient concentrations have never been directly compared between a 2D monolayer cell  
101 culture and 3D tumour spheroids. Here we examined whether the absence of three major

102 nutrients, glucose, pyruvate and glutamine, increases the sensitivity of MDA-MB-231 cells to  
103 metformin in a monolayer culture and in tumour spheroids. We tested effects of nutrients and  
104 metformin using three widely used media (DMEM, MEM and RPMI-1640) that differ mainly  
105 in the concentrations of amino acids. Our main finding is that sensitivity of MDA-MB-231  
106 cells to metformin varies between a monolayer culture and the more physiologically relevant  
107 tumour spheroids. Furthermore, usage of MEM increased the effects of metformin on tumour  
108 spheroids and sensitized MDA-MB-231 cells in a monolayer culture to metformin in  
109 pyruvate-depleted condition.

110

## 111 **Materials & Methods**

### 112 **Antibodies and reagents**

113 Antibodies against phospho-ACC (Ser<sup>79</sup>) (CST3661), and phospho-AMPK $\alpha$  (Thr<sup>172</sup>)  
114 (CST2535) were from Cell Signaling Technology. Antibodies against actin were from Cell  
115 Signaling Technology. Bis-Tris 4–20% polyacrylamide gels were from Sigma-Aldrich.  
116 TruPAGE™ TEA-Tricine SDS Running Buffer was from Sigma-Aldrich and horseradish  
117 peroxidase secondary antibody conjugate (170–6515) were from Bio-Rad. Polyvinylidene  
118 Fluoride (PVDF) Immobilon-P membrane (IPVH00010) was from Merck and protein  
119 molecular weight marker (P7712S) from New England BioLabs. Enhanced  
120 chemiluminescence (ECL) reagent was from Life Technologies (Thermo Fisher Scientific).  
121 Metformin was from Calbiochem (Merck Millipore). All other reagents, unless otherwise  
122 specified, were from Sigma-Aldrich.

123

### 124 **MDA-MB-231 cell culture**

125 MDA-MB-231 cells were from ATCC (USA). MDA-MB-231 cells were grown in RPMI-  
126 1640 medium (Genaxxon bioscience, Germany) supplemented with 4.5 g/l of glucose, 2 mM  
127 L-glutamine, 1 mM pyruvate and 10 % fetal bovine serum (FBS; Sigma-Aldrich). They were  
128 maintained at 37 °C in a humidified atmosphere with 5% (v/v) CO<sub>2</sub>. Experiments were  
129 performed in RPMI-1640 (Genaxxon, custom made medium) or DMEM (Gibco, A14430)  
130 with or without 4.5 g/l of glucose, 2 mM glutamine and 1 mM pyruvate. Alternatively, some  
131 experiments were performed in MEM medium (Sigma, M5775). MEM medium was supplied  
132 with 1 g/l of glucose so we increased glucose concentration of MEM in all the experiments to  
133 match RPMI and DMEM medium (4.5 g/l). Furthermore, MEM medium was also

134 supplemented with 2 mM glutamine and/or 1 mM pyruvate. Cell culture media used differ  
 135 mainly in the concentrations of amino acids (Table 1).

136 **Table 1. The concentrations of amino acids in cell culture media.**

Amino acids	RPMI-1640	DMEM	MEM	RPMI-1640	DMEM	MEM
	mM			Relative to RPMI-1640		
L-Arginine hydrochloride	1.15	0.398	0.597	1.00	0.346	0.519
L-Asparagine × H <sub>2</sub> O	0.333	0	0	1.00	0	0
L-Aspartic acid	0.150	0	0	1.00	0	0
L-Cystine	0.208	0.201	0.0999	1.00	0.967	0.480
L-Glutamic acid	0.136	0	0	1.00	0	0
Glycine	0.133	0.400	0	1.00	3.00	0
L-Histidine hydrochloride-H <sub>2</sub> O	0.0967	0.200	0.200	1.00	2.07	2.07
L-Hydroxyproline	0.153	0	0	1.00	0	0
L-Isoleucine	0.382	0.801	0.397	1.00	2.10	1.04
L-Leucine	0.382	0.801	0.397	1.00	2.10	1.04
L-Lysine hydrochloride	0.219	0.798	0.396	1.00	3.65	1.81
L-Methionine	0.101	0.201	0.101	1.00	2.00	1.00
L-Phenylalanine	0.0909	0.400	0.194	1.00	4.40	2.13
Proline	0.174	0	0	1.00	0	0
L-Serine	0.286	0.400	0	1.00	1.40	0
L-Threonine	0.168	0.798	0.403	1.00	4.75	2.40
L-Tryptophan	0.0245	0.0784	0.049	1.00	3.20	2.00
Tyrosine	0.110	0.398	0.230	1.00	3.61	2.09
L-Valine	0.171	0.803	0.393	1.00	4.70	2.30
Total amino acids	4.47	6.68	3.46	1.00	1.49	0.774

137

### 138 **MTS cell viability assay**

139 MTS assay (Promega Corp, Fitchburg, WI, USA) was performed as described previously[31].

140 Briefly, upon completion of the experiment, MDA-MB-231 cells were washed with phosphate

141 saline buffer (PBS) and placed in a serum-free RPMI-1640 medium with 4.5 g/l of glucose, 2  
142 mM glutamine and 1 mM pyruvate. Then, Cell Titer®96AQueous One (MTS) solution  
143 (Promega Corp, Fitchburg, WI, USA) was added to each well and cells were incubated for  
144 about 1-hour at 37 °C, 5 % CO<sub>2</sub>. Absorbance of supernatant was measured at 490 nm using  
145 the Tecan Infinite 200 (Tecan Group Ltd, Männedorf, Switzerland).

146

## 147 **Cell number**

148 The number of MDA-MB-231 cells was determined as described previously[31,36]. Briefly,  
149 medium was removed from each well and plates were frozen at -20 °C. On the day of the  
150 analysis, cells were thawed and lysed with a 0.04% SDS solution at room temperature for 30  
151 minutes. Then, buffer containing 50 mM TRIS-HCl, 100 mM NaCl (pH = 8.25) and 5 µg/ml  
152 Hoechst 33342 stain (Thermo Fisher Scientific) was added to each well. Fluorescence  
153 intensity was determined at 350 nm excitation and 461 nm emission using Tecan Infinite 200  
154 (Tecan, Männedorf, Switzerland).

155

## 156 **Propidium iodide staining**

157 MDA-MB-231 cells were seeded in 12-well plates. Next day, they were placed in a fresh cell  
158 culture medium and treated with 5 mM metformin. At the end of treatment, MDA-MB-231  
159 cells were collected, pelleted and resuspended in PBS. Propidium iodide was added to a final  
160 concentration of 0.15 mM and cell suspension was analysed by Attune™ NxT flow cytometer  
161 (Thermo Fisher Scientific, Waltham, USA). Propidium iodide signal of at least  $2 \times 10^4$  events  
162 per sample was collected using the BL-2 filter (574/26). Final analysis was performed with  
163 Attune® Cytometric Software (Thermo Fisher Scientific, Waltham, USA).



164

## 165 **Tumour spheroids**

166 For formation of 3D cellular spheroids, MDA-MB-231 cells were seeded in various cell  
167 culture media in U-shaped low adherent 96 well cell culture plates (Corning, New York,  
168 USA). Following seeding, plates were centrifuge for 2 minutes at 300 rcf. After 72 hours,  
169 tumour spheroids were stained with calcein (1  $\mu$ M final concentration, Life Technology) and  
170 propidium iodide (0.15 mM final concentration) for 10 and 5 minutes, respectively. Images  
171 were acquired using fluorescent Leica DM IL LED microscope (Leica Microsystem) and  
172 analyzed by ImageJ Software (National Institute of Helath, USA).

173

## 174 **Western blotting**

175 MDA-MB-231 cells were treated with 5 mM metformin in complete RPMI-1640 medium or  
176 in RPMI-1640 medium without glutamine, pyruvate or glucose for 24 hours. Then cells were  
177 washed twice with ice-cold PBS and harvested in Laemmli buffer (62.5 mM Tris-HCl, pH  
178 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 5% 2-mercaptoethanol,  
179 0.002% bromophenol blue). Total protein concentration was measured by Pierce 660  
180 (Thermofisher). Samples, containing equivalent amount of proteins, were loaded on a 4–20%  
181 polyacrylamide gel (TruPAGE™ Precast Gels, Sigma) and separated using electrophoresis  
182 (Mini-protean tetra cell system, Bio Rad). Subsequently, proteins were transferred to PVDF  
183 membrane. Ponceau S (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) was used to evaluate  
184 the efficiency of the protein transfer and sample loading. Membranes were blocked in 5%  
185 (w/v) skimmed milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.02% (v/v) Tween-20, pH 7.5),  
186 which was followed by overnight incubation in primary antibodies at 4°C. After washing,  
187 membranes were incubated with the appropriate secondary horseradish peroxidase-conjugated

188 antibody. Enhanced chemiluminescence using Agfa X-ray film was used to detect immuno-  
189 reactive. ImageJ was used for densitometric analysis.

190

## 191 **Statistical analysis**

192 Statistical analysis was performed with GraphPad Prism (v6; GraphPad Software, Inc., La  
193 Jolla, CA, USA) using one-way ANOVA or two-way ANOVA, followed by Bonferroni,  
194 Tukey or Sidak test. Statistically significant results are displayed as follows: \* $P \leq 0.05$ ;  
195 \*\* $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ .

## 196 **Results**

### 197 **The effect of nutrient availability on proliferation of MDA-MB-** 198 **231 cells in RPMI-1640 medium**

199 Nutrient availability in the microenvironment of cancer cells modulates their  
200 metabolism [3], while intrinsic properties of cancer cells determine their dependency on  
201 specific nutrients for proliferation and survival [4,5]. To assess nutrient requirements of  
202 MDA-MB-231 cells, we grew them in the complete RPMI-1640 medium or in the nutrient-  
203 deficient RPMI-1640 medium without pyruvate, glucose or glutamine for 72 hours (Fig. 1). In  
204 all our experiments complete medium contained 2 mM glutamine, 1 mM pyruvate and 4.5 g/l  
205 of glucose. The complete and nutrient-deficient media, lacking glucose, glutamine and/or  
206 pyruvate, were supplemented with 10% FBS. The depletion of glutamine and pyruvate did not  
207 affect MDA-MB-231 cell proliferation, whereas depletion of glucose slightly suppressed  
208 proliferation of cells after 72 hours (Fig. 1A). As a positive control for suppression of  
209 proliferation, we used serum-free RPMI-1640 medium, which blocked MDA-MB-231 cell  
210 proliferation (Fig. 1B). Thus, unlike depletion of serum growth factors, removal of glutamine  
211 and pyruvate does not have any major effect on proliferation of MDA-MB-231 cells.

212

### 213 **Figure 1: The effect of nutrient availability on proliferation of MDA-MB-231 cells in RPMI-1640** 214 **medium**

215 MDA-MB-231 cells were grown for 72 hours in complete RPMI-1640 medium or in RPMI-1640  
216 medium without glucose, glutamine or pyruvate (A). Alternatively MDA-MB-231 cells were grown  
217 for 72 hours in complete RPMI-1640 medium or in serum-free RPMI-1640 medium (B). Relative  
218 number of cells was determined by Hoechst staining. Results are means $\pm$ SEM (n = 3-4).

219

## 220 **Nutrient availability determines the sensitivity of MDA-MB-231** 221 **cells to metformin**

222 Limited availability of glucose and/or other nutrients increases the sensitivity of  
223 cancer cells to metformin [17,22,23,26,31–39,46,47]. However, excepting glucose, the role of  
224 other nutrients and their combinations on the sensitivity of MDA-MB-231 cells to metformin  
225 was not examined in detail. To determine whether depletion of pyruvate and glutamine affects  
226 sensitivity to metformin, we grew MDA-MB-231 cells in RPMI-1640 medium in the absence  
227 of glucose, pyruvate or glutamine and treated them with 5 mM metformin for 72 hours (Fig.  
228 2). We determined the number and viability of MDA-MB-2321 cells using Hoechst staining  
229 and MTS assay [31], respectively (Fig. 2A-D). Metformin did not affect the number and  
230 viability of MDA-MB-231 cells grown in the complete RPMI-1640 medium or in RPMI-1640  
231 medium lacking glutamine or pyruvate, while it significantly reduced the number and viability  
232 of MDA-MB-231 cells in RPMI-1640 medium without glucose (Fig. 2A, B).

233

### 234 **Figure 2: Nutrient availability determines the sensitivity of MDA-MB-231 cells to metformin**

235 (A, B) MDA-MB-231 cells were grown in complete RPMI-1640 medium or in RPMI-1640 medium  
236 without glucose, glutamine or pyruvate and treated with 5 mM metformin. After 72 hours, cell number  
237 (A) and viability (B) were determined by Hoechst and MTS staining, respectively. Results are  
238 means±SEM (n = 2-4). (C, D) MDA-MB-231 cells were grown in complete RPMI-1640 medium,  
239 serum-free RPMI-1640 or in RPMI-1640 medium lacking glucose, glutamine and pyruvate (with  
240 serum) and treated with 5 mM metformin. After 72 hours, cell number (C) and viability (D) were  
241 determined by Hoechst and MTS staining, respectively. Results are means±SEM (n = 3-4). (E) MDA-  
242 MB-231 cells were cultured for 72 hours in serum-free RPMI-1640 medium without glutamine,  
243 glucose and/or pyruvate and treated with 5 mM metformin. Cell survival was determined by  
244 propidium iodide assay using flow cytometry. Results are means±SEM (n = 3-4).

245

246 Serum starvation causes changes in AMPK activation [48], indicating it might also  
247 affect the sensitivity of MDA-MB-231 cells to metformin, which is an indirect AMPK  
248 activator. To evaluate whether serum starvation or deficiency of all three analyzed nutrients  
249 (glutamine, glucose and pyruvate) affect sensitivity to metformin, we grew MDA-MB-231  
250 cells for 72 hours in the complete RPMI-1640 medium, in serum-free RPMI-1640 medium  
251 (with glucose, glutamine and pyruvate) or in a nutrient-deficient RPMI-1640 medium with  
252 serum, but without glutamine, glucose and pyruvate (Fig. 2C, D). Serum-free RPMI-1640  
253 medium and nutrient-deficient RPMI-1640 medium with serum markedly reduced the number  
254 of cells and their viability. Metformin did not further decrease the number or viability of  
255 MDA-MB-231 cells.

256 To determine whether nutrients and/or metformin affects survival of MDA-MB-231 in  
257 the absence of serum and specific nutrients, we cultured them in serum-free RPMI-1640  
258 medium without glutamine, glucose and/or pyruvate (Fig. 2E). After 72-hour treatment with 5  
259 mM metformin, we determined the survival of MDA-MB-231 cells using propidium iodide  
260 assay and flow cytometry. Compared with control, cell survival was reduced in RPMI-1640  
261 medium lacking glucose and pyruvate and in RPMI-1640 medium lacking all three nutrients,  
262 glucose, pyruvate and glutamine. Metformin did not further reduce survival of cells grown  
263 under these two conditions. In comparison to non-treated cells, metformin reduced survival of  
264 cells grown in RPMI-1640 medium without glucose or in RPMI-1640 medium lacking  
265 glucose and glutamine.

266

267 **The size of tumour spheroids is increased by metformin and**  
268 **depends on nutrient availability**

269 Metabolic phenotype of cancer cells depends on whether they are grown in 2D  
270 monolayer cultures or in 3D tumour spheroids [9–12]. We therefore evaluated if sensitivity to  
271 metformin differs between MDA-MB-231 cells grown in a monolayer culture and those  
272 grown in tumour spheroids. To this end, tumour spheroids were treated with 5 mM metformin  
273 in complete RPMI-1640 medium without glucose, glutamine or pyruvate for 72 hours (Fig.  
274 3). We determined the size of each tumour spheroid and survival of MDA-MB-231 cells  
275 composing it, using calcein and propidium iodide staining. Metformin slightly increased the  
276 size of tumour spheroids in all tested conditions and spheroids became less compact. The  
277 effect was most pronounced in RPMI-1640 medium without glucose (Fig. 3A, B). Survival of  
278 metformin-treated MDA-MB-231 cells was decreased only in the absence of glucose (Fig  
279 3C).

280

281 **Figure 3: The size of tumour spheroids is increased by metformin and depends on nutrient**  
282 **availability**

283 (A, B, C) Tumour spheroids were grown in complete RPMI-1640 medium or in RPMI-1640 medium  
284 without glucose, glutamine or pyruvate and treated with 5 mM metformin for 72 hours. Tumour  
285 spheroids were double stained by propidium iodide and calcein and observed using fluorescence  
286 microscopy (A) The size of tumour spheroids was determined by ImageJ programme. Results are  
287 means±SEM (n = 5). (B). Fluorescence intensity of propidium iodide positive MDA-MB-231 cells in  
288 each tumour spheroid was determined by ImageJ programme. Results are means±SEM (n = 3) (C). (D,  
289 E, F) Tumour spheroids were grown in complete RPMI-1640 medium, in serum-free RPMI-1640  
290 medium or in RPMI-1640 medium lacking glucose, glutamine and pyruvate (with serum). Tumour  
291 spheroids in all conditions were treated with 5 mM metformin for 72 hours. Tumour spheroids were  
292 double stained by propidium iodide and calcein and observed using fluorescence microscopy (D). Size

293 of tumour spheroids was determined by ImageJ programme. Results are means $\pm$ SEM (n = 3-5) (E).  
294 Fluorescence intensity of propidium iodide positive MDA-MB-231 cells in each tumour spheroid was  
295 determined by ImageJ programme. Results are means $\pm$ SEM (n = 2-3) (F).

296

297 We also investigated the effects of serum-free RPMI-1640 medium and the absence of  
298 all three nutrients, glucose, glutamine and pyruvate, on the size and survival of tumour  
299 spheroids treated with 5 mM metformin for 72 hours. Tumour spheroids grown in serum-free  
300 RPMI-1640 medium with or without 5 mM metformin were completely disintegrated (Fig.  
301 3D, E). Metformin did not significantly affect the percentage of dead cells composing tumour  
302 spheroids in this condition. On the other hand, 5 mM metformin increased the size of tumour  
303 spheroids and the percentage of dead cells composing them in RPMI-1640 medium without  
304 glucose, glutamine and pyruvate (Fig. 3D, F).

305

## 306 **Metformin disintegrates tumour spheroids grown in MEM**

307 Formulation of cell culture medium affects the sensitivity of cancer cells to metformin  
308 in a monolayer culture [22,35], while the effects of nutrient availability on the sensitivity of  
309 MDA-MB-231 cells in tumour spheroids remain largely unknown. We therefore compared  
310 the effects of metformin on MDA-MB-231 cells grown in tumour spheroids in RPMI-1640  
311 medium with its effects in DMEM and MEM that are also commonly used in cancer cell  
312 culturing. DMEM has higher concentrations of the majority of amino acids than RPMI-1640  
313 medium, but does not contain aspartate. MEM does not contain non-essential amino acids:  
314 alanine, glycine, glutamate, proline, serine, asparagine and aspartate (Table 1).

315 Tumour spheroids were grown in complete DMEM or MEM and treated with 5 mM  
316 metformin for 72 hours. Cell survival was determined by propidium iodide and calcein

317 staining (Fig. 4). Untreated spheroids had similar size independent from cell culture medium.  
318 Metformin disintegrated tumour spheroids grown in MEM but not in DMEM (Fig. 4A, B).  
319 Cell survival was lower in untreated tumour spheroids grown in MEM than in DMEM.  
320 Metformin did not further reduce cell survival in tumour spheroids grown in MEM or DMEM  
321 (Fig. 4C).

322

#### 323 **Figure 4: Metformin disintegrates tumour spheroids grown in MEM**

324 Tumour spheroids were grown in complete DMEM or MEM and treated with 5 mM metformin for 72  
325 hours. Tumour spheroids were double stained by calcein and propidium iodide and observed by  
326 fluorescence microscopy (A). Relative size of tumour spheroids was determined by ImageJ  
327 programme. Results are means±SEM (n = 3) (B). Fluorescence intensity of propidium iodide positive  
328 cells was determined by ImageJ programme Results are means±SEM (n = 3) (C).

329

330

#### 331 **Effects of nutrient availability on AMPK activation**

332 Glucose depletion increases AMPK activation by metformin [31,32]. Whether  
333 depletion of other nutrients has similar effects on the sensitivity of MDA-MB-231 cells to  
334 metformin has not been examined in detail. Thus, we compared the effects of deficiency of  
335 pyruvate, glutamine and/or glucose on metformin-stimulated AMPK activation in MDA-MB-  
336 231 cells (Fig. 5). Activation of AMPK was estimated by measuring phosphorylation of  
337 AMPK (Thr<sup>172</sup>) and its downstream target acetyl-CoA carboxylase (ACC). We treated MDA-  
338 MB-231 cells with 5 mM metformin in DMEM without glutamine, pyruvate and/or glucose  
339 for 24 hours. Metformin increased phosphorylation of AMPK in DMEM without glucose and  
340 in DMEM without glucose and pyruvate (Fig. 5A, B). Metformin increased phosphorylation



341 of ACC in the absence of pyruvate, glucose and their combination (Fig. 5A, C). Deficiency of  
342 glutamine did not enhance metformin-stimulated AMPK or ACC phosphorylation.

343

#### 344 **Figure 5: Effects of nutrient availability on AMPK activation**

345 MDA-MB-231 cells were grown in DMEM without glutamine, glucose and/or pyruvate and treated  
346 with 5 mM metformin for 24 hours. Phosphorylation of AMPK (Thr<sup>172</sup>) (A, B) and phosphorylation of  
347 ACC (Ser<sup>79</sup>) (C) was measured by Western blot. Results are means±SEM (n = 3).

348

#### 349 **Metformin reduces the number of MDA-MB-231 cells grown in** 350 **MEM without pyruvate**

351 Cell culture media contain various concentrations of amino acids (Table 1), which  
352 might alter the sensitivity of MDA-MB-231 cells to metformin in the absence of glutamine,  
353 pyruvate and/or glucose. We tested how depletion of glucose, glutamine and pyruvate affects  
354 proliferation and survival of MDA-MB-231 cells grown in DMEM and MEM (Fig. 6). MDA-  
355 MB-231 cells were grown in a complete DMEM lacking glutamine, pyruvate and/or glucose  
356 and treated with 5 mM metformin for 96 hours. Metformin did not affect proliferation and  
357 survival of MDA-MB-231 cells grown in the complete DMEM and DMEM without  
358 glutamine or pyruvate (Fig. 6A, B). However, it suppressed proliferation of MDA-MB-231  
359 cells and reduced their survival in DMEM medium without glucose. The effect of metformin  
360 on cell survival was even more profound when DMEM was without both glucose and  
361 pyruvate (Fig. 6B).

362

#### 363 **Figure 6: Metformin reduces the number of MDA-MB-231 cells grown in MEM without** 364 **pyruvate**

365 (A, B) MDA-MB-231 cells were grown in complete DMEM or in DMEM without glutamine,  
366 pyruvate and/or glucose and treated with 5 mM metformin for 96 hours. Relative cell number (A) and  
367 propidium iodide positive cells (B) were determined by Hoechst and propidium iodide staining,  
368 respectively. Results are means $\pm$ SEM (n = 3). (C, D) MDA-MB-231 cells were grown in complete  
369 MEM or in MEM without glutamine or pyruvate and treated with 5 mM metformin for 96 hours.  
370 Relative cell number (C) and propidium iodide positive cells (D) were determined by Hoechst and  
371 propidium iodide staining, respectively. Results are means $\pm$ SEM (n = 2-3).

372

373 In the absence of metformin, depletion of glutamine or pyruvate did not affect survival  
374 and the number of MDA-MB-231 cells in MEM (Fig. 6C, D). Metformin treatment did not  
375 significantly reduce the number and survival of cells grown in the complete MEM or in MEM  
376 without glutamine. In contrast, metformin reduced the number of MDA-MB-231 cells grown  
377 in MEM without pyruvate to about 60% (Fig. 6C), but it did not have any direct effect on  
378 their survival (<4% propidium iodide positive MDA-MB-231 cells) (Fig. 6D).

379

## 380 Discussion

381 The metabolic phenotype of cancer cells is partially determined by nutrient  
382 concentrations in their microenvironment [3]. Metabolic pathways of cancer cells *in vitro* vary  
383 also between monolayer cultures and physiologically more relevant tumour spheroids [9–12].  
384 Nutrient availability and metabolic phenotype can modify the sensitivity of cancer cells to  
385 pharmacological compounds that target cancer cell metabolism, such as metformin  
386 [17,22,23,26,31–39,46,47]. Recently, we have shown that maintaining constant glucose  
387 concentrations by daily medium renewal blocks the effects of metformin on MDA-MB-231  
388 cells [31]. In contrast, MDA-MB-231 cells are sensitive to metformin in glucose-depleted  
389 conditions [31,32,36–39,47]. In the present study, we compared the effects of three major  
390 nutrients, glucose, glutamine and pyruvate, on the sensitivity of MDA-MB-231 cells to  
391 metformin in a monolayer culture and in tumour spheroids. Previous studies have shown that  
392 the sensitivity of cancer cells to metformin in a monolayer culture depends on media  
393 formulation [22,35]. Importantly, the formulations of commonly used media vary greatly in  
394 the concentrations of several nutrients. Here we show that the effects of metformin on MDA-  
395 MB-231 cells as a function of nutrient concentrations depend on *in vitro* cell model used  
396 (monolayer culture vs. tumour spheroids). Furthermore, we show that media formulation  
397 modulates the sensitivity of MDA-MB-231 cells to metformin upon pyruvate depletion.

398 Although 5 mM metformin did not have any major effect on proliferation of MDA-  
399 MB-231 cells grown in complete cell culture media in a monolayer culture, it disintegrated  
400 tumour spheroids in MEM and RPMI-1640 medium. This is broadly consistent with other  
401 studies that observed the effects of metformin on various 3D cell culture models [41–45]. The  
402 effect of metformin on disintegration of tumour spheroids was greatest in MEM, which lacks  
403 alanine, glycine, glutamate, proline, serine, asparagine and aspartate (Table 1). Thus, the  
404 effect of metformin on cell-cell interactions in tumour spheroids is enhanced if medium is

405 deficient in non-essential amino acids. Furthermore, poor penetration of amino acids, which  
406 are present in lower concentrations in MEM, might contribute to reduced survival of MDA-  
407 MB-231 cells in tumour spheroids. On the other hand, MDA-MB-231 cells in tumour  
408 spheroids grown in MEM might have reduced survival upon treatment with metformin,  
409 because they were unable to adapt to metabolic stress. For instance, they were unable to cope  
410 with increased glycolytic rate that was partially required as a response to inhibition of  
411 oxidative phosphorylation [39] and partially to restore energy homeostasis upon detachment  
412 and to resist anoikis, a cell death mechanism induced by a loss of cell-cell or cell-matrix  
413 interactions [49]. Taken together, in order to better predict effects of metformin in tissues, it is  
414 useful to evaluate its effects in nutrient limited conditions in tumour spheroids, which better  
415 mimic micro-environment of cancer cells in tumors.

416 Combined treatment with metformin and inhibitors of glutaminase synergistically  
417 reduce survival of prostate cancer cells [26]. In addition, glutamine depletion increases  
418 sensitivity of Huh7 liver cancer cells to metformin [26], which indicates that the absence of  
419 glutamine might also sensitize MDA-MB-231 cells to metformin. However, we show that  
420 media lacking glutamine did not enhance effects of metformin on proliferation and survival of  
421 MDA-MB-231 cells and did not have any major effects on tumour spheroids in comparison to  
422 the effects of metformin in complete medium. Glutamine is an important source of nitrogen  
423 that is required for protein and nucleotide biosynthesis. Besides, several studies show that  
424 cells treated with metformin rely on reductive metabolism of glutamine to support TCA  
425 cycle [24,26,27]. Since metformin failed to reduce viability of MDA-MB-231 cells in media  
426 lacking glutamine, we may speculate that these cells produce sufficient amount of  $\alpha$ -  
427 ketoglutarate and obtain enough nitrogen from other nutrient sources [50]. Metformin  
428 attenuates anaplerotic flux of glutamine-derived carbon into the TCA cycle [24], which  
429 together with our results indicates that glucose is probably the major carbon source to sustain

430 proliferation of metformin-treated MDA-MB-231 cells [46]. Furthermore, upon metformin-  
431 mediated inhibition of oxidative phosphorylation [18,19], MDA-MB-231 cells might produce  
432 the vast majority of energy from glycolysis. Taken together, our results suggest that media  
433 replete with glutamine do not block action of metformin on MDA-MB-231 cells in tumour  
434 spheroids or in a monolayer cell culture.

435 Metformin suppressed proliferation of MDA-MB-231 cells and reduced their survival  
436 under glucose-depleted conditions. Metformin attenuates mitochondrial anaplerotic reactions  
437 [24] and concomitantly augments glucose consumption of cancer cells [24,46,51]. MDA-MB-  
438 231 cells treated with metformin in the absence of glucose are thus unable to cope with  
439 energetic stress, which leads to suppressed cell proliferation and ultimately to cell death  
440 [17,31,39]. Energetic stress[52] or glucose depletion [53] activates AMPK, which upregulates  
441 catabolic processes in the cell and is promoting cell survival [29,30]. Cancer cells with  
442 impaired AMPK activation pathway are more sensitive to phenformin, an analogue of  
443 metformin [54]. In addition, metformin activates metabolic pathways that suppress  
444 proliferation of cancer cells also in an AMPK-independent manner [24,55]. The impairment  
445 of AMPK activation pathway increases the sensitivity of lung and colon cancer cells to  
446 metformin in glucose-depleted condition [56], which suggests that metformin-stimulated  
447 AMPK activation in medium without glucose that was observed in our study has a pro-  
448 survival role. We used 5 mM metformin, which is more than 100-times higher concentration  
449 than the one observed in plasma of diabetic patients [57]. However, *in vitro* studies using  
450 millimolar concentrations of metformin can predict the effects of metformin on tumour tissue  
451 in a murine model that has similar plasma concentrations of metformin than diabetic patients  
452 [20,34,58,59]. Therefore, since metformin in the absence of glucose reduced survival of  
453 MDA-MB-231 cells grown in both *in vitro* cell models (monolayer culture and tumour

454 spheroids) it would probably also reduce survival of poorly perfused cancer cells *in vivo*, such  
455 as those in the center of the tumour.

456 Pyruvate depletion attenuates proliferation of cells with impaired oxidative  
457 phosphorylation [60–62], which was recently demonstrated also for cancer cells treated with  
458 metformin [22,23,34]. However, we observed that metformin suppressed proliferation of  
459 MDA-MB-231 cells only in MEM without pyruvate, but not in pyruvate-depleted DMEM or  
460 RPMI-1640 medium. On the other hand, depletion of pyruvate and glucose synergistically  
461 reduced survival of metformin-treated MDA-MB-231 cells grown in DMEM. Combined  
462 depletion of both nutrients increased metformin-stimulated AMPK activation, while pyruvate  
463 depletion alone enhanced metformin-stimulated phosphorylation of ACC, a downstream  
464 target of AMPK. Recent studies indicate that metformin suppresses proliferation of cancer  
465 cells in an AMPK-independent manner [22,24], which suggests that AMPK activation in the  
466 absence of glucose and pyruvate is probably a pro-survival response of MDA-MB-231 cells to  
467 energetic stress [29,30]. Pyruvate as well as glucose can be metabolized to acetyl-CoA and  
468 oxidized in the TCA cycle, thus driving ATP production. However, since metformin  
469 attenuates mitochondrial anaplerotic reactions [24,25] and suppresses oxidative  
470 phosphorylation [18,19], pyruvate probably blocks the effects of metformin through  
471 mechanisms independent from ATP production. Metformin decreases the NAD<sup>+</sup>/NADH ratio  
472 in cancer cells [22,33]. Thus, one of the main roles of exogenous pyruvate in cells with  
473 impaired oxidative phosphorylation is to support NAD<sup>+</sup> production via its conversion into  
474 lactate [22,62]. NAD<sup>+</sup> drives glycolysis, which may explain the synergistic effects of pyruvate  
475 and glucose depletion on survival of metformin-treated MDA-MB-231 cells.

476 Second role of pyruvate in cells with impaired oxidative phosphorylation is to support  
477 aspartate synthesis [22,60,62]. The addition of aspartate prevents metformin-mediated  
478 suppression of proliferation of some cancer cells [22,23,34], indicating that this effect

479 depends on their intrinsic properties and/or the availability of other nutrients. In our study  
480 metformin tended, but did not significantly suppress proliferation of MDA-MB-231 cells  
481 grown in DMEM without pyruvate, which did not contain aspartate (Table 1). Breast cancer  
482 cells can produce pyruvate from glucose as well as from serine and glycine [63]. Besides, the  
483 absence of serine suppresses glycolysis [64] and thus prevents an adaptive response to  
484 inhibition of oxidative phosphorylation [33]. DMEM without pyruvate contains serine and  
485 glycine, which can block the effects of metformin. Indeed, metformin suppressed proliferation  
486 of MDA-MB-231 cells in MEM without pyruvate, which does not contain several non-  
487 essential amino acids, including aspartate, serine and glycine (Table 1). Taken together, our  
488 results show that metformin-treated MDA-MB-231 cells require pyruvate, which can be  
489 acquired per se or derived from glucose, serine and glycine, for their optimal proliferation.  
490 Thus, medium formulation must be considered, when we evaluate the effects of pyruvate-  
491 depletion on MDA-MB-231 cells.

492

## 493 **Conclusions**

494 Here we show that the effects of metformin on MDA-MB-231 cells depend on *in vitro*  
495 cell model used (monolayer culture vs. tumour spheroids). While metformin had no effects on  
496 MDA-MB-231 cells in a monolayer culture in MEM medium, it disintegrated tumour  
497 spheroids in the same cell culture medium. Secondly, we show that media formulation  
498 modulates the sensitivity of MDA-MB-231 cells to metformin in the absence of pyruvate.  
499 Based on interaction between pyruvate depletion with depletion of glucose and non-essential  
500 amino acids, our results suggest that pyruvate, which can be obtained directly from cell  
501 culture media or derived from glucose, serine and glycine, blocks the effects of metformin on  
502 MDA-MB-231 cells. Therefore, media formulation as well as cell culture model (monolayer

503 culture vs. tumour spheroids) must be considered, when we evaluate the effects of metformin  
504 on MDA-MB-231 cells as a function of nutrient availability.

505

506



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510

511

## 512 **Author Contributions**

513

514 Conceived and designed the experiments: MB PM MP SP. Performed the experiments: MB  
515 PM. Analyzed the data: MB PM MP SP. Wrote the paper: MB MP SP.

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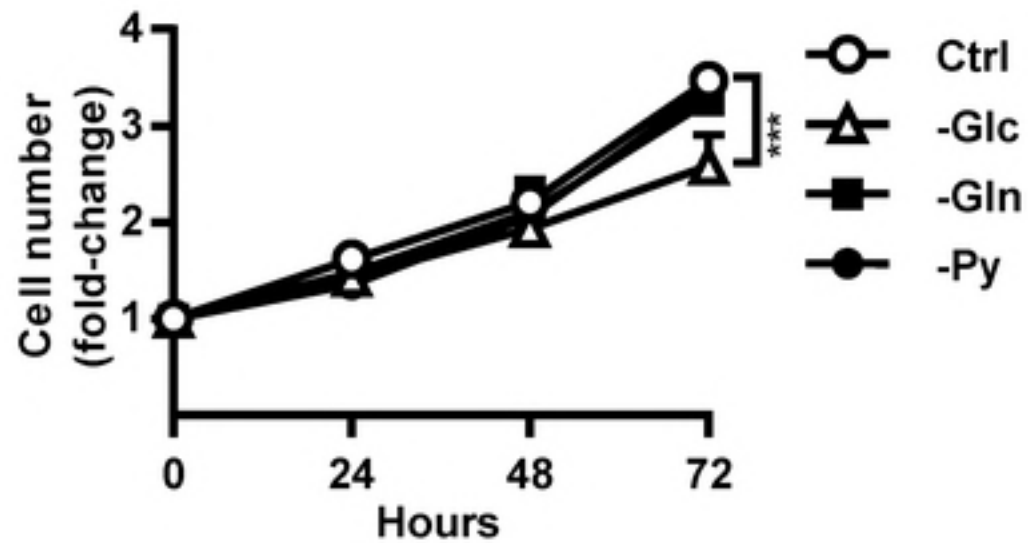
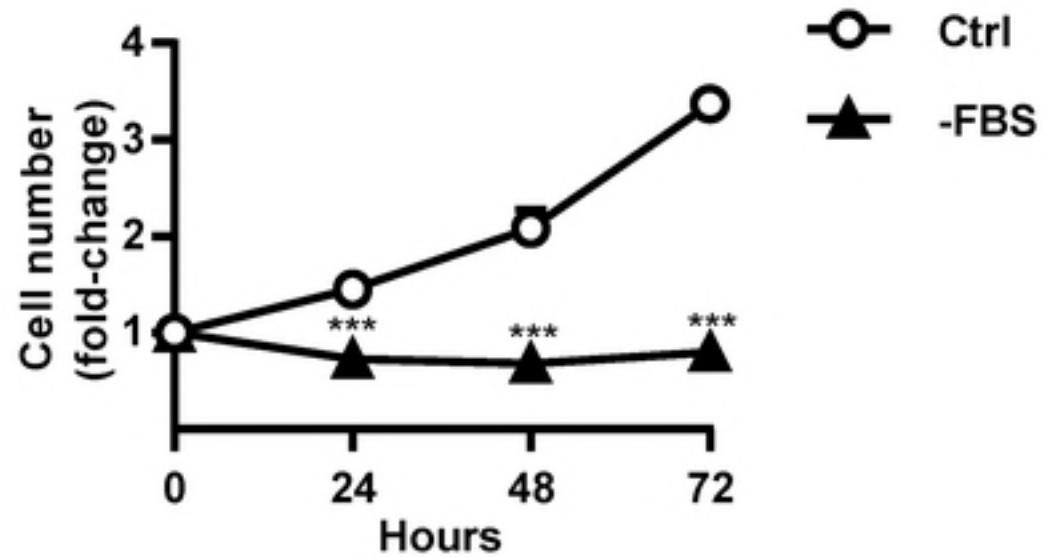
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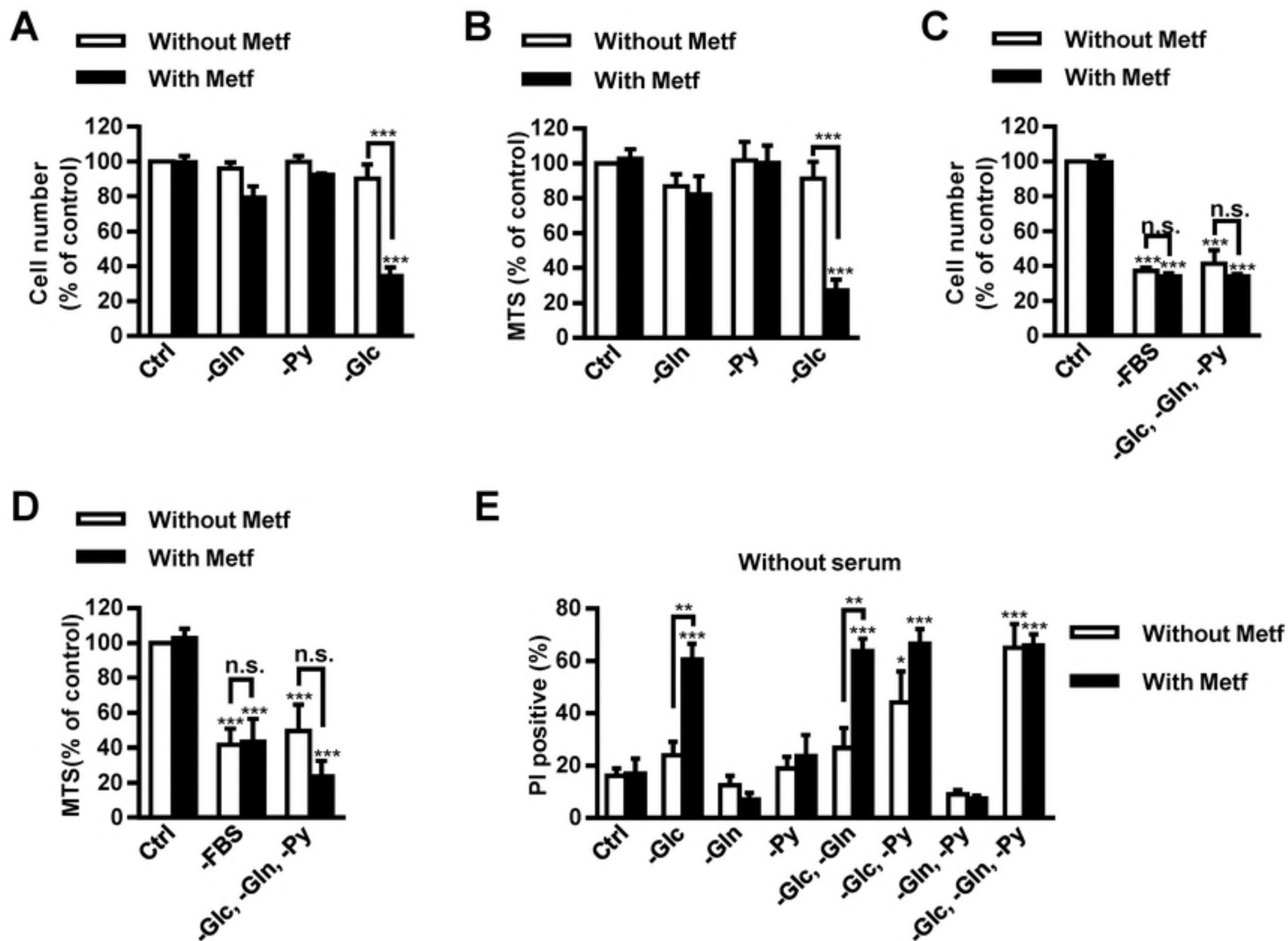
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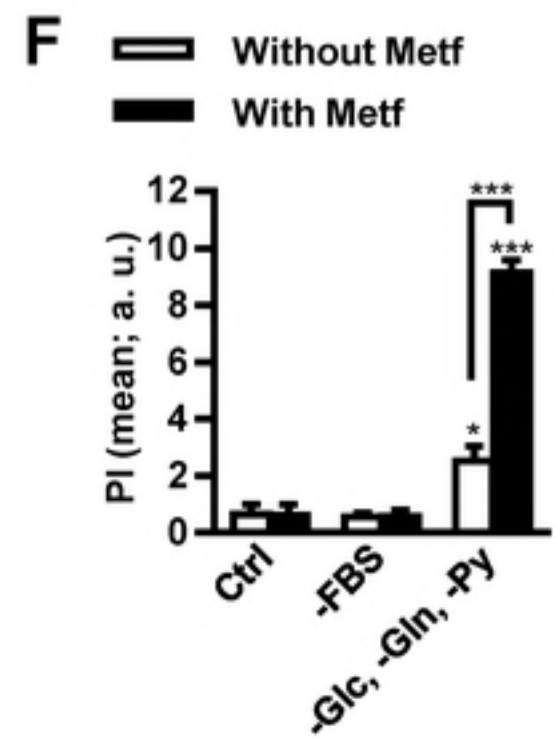
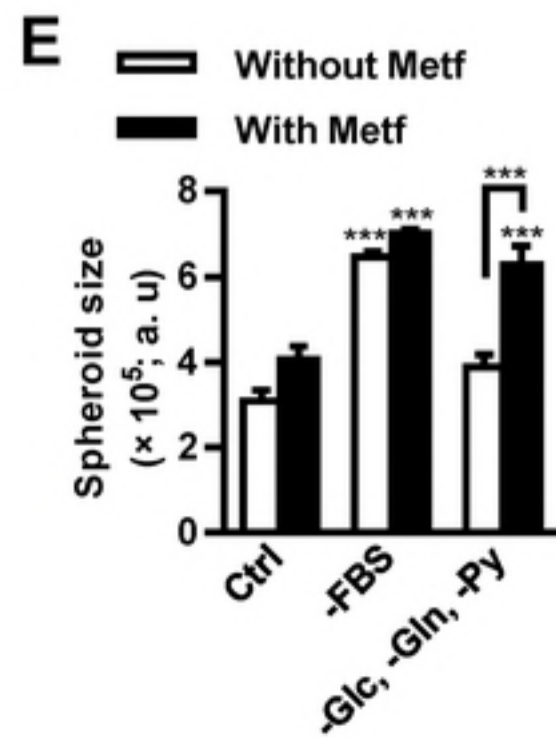
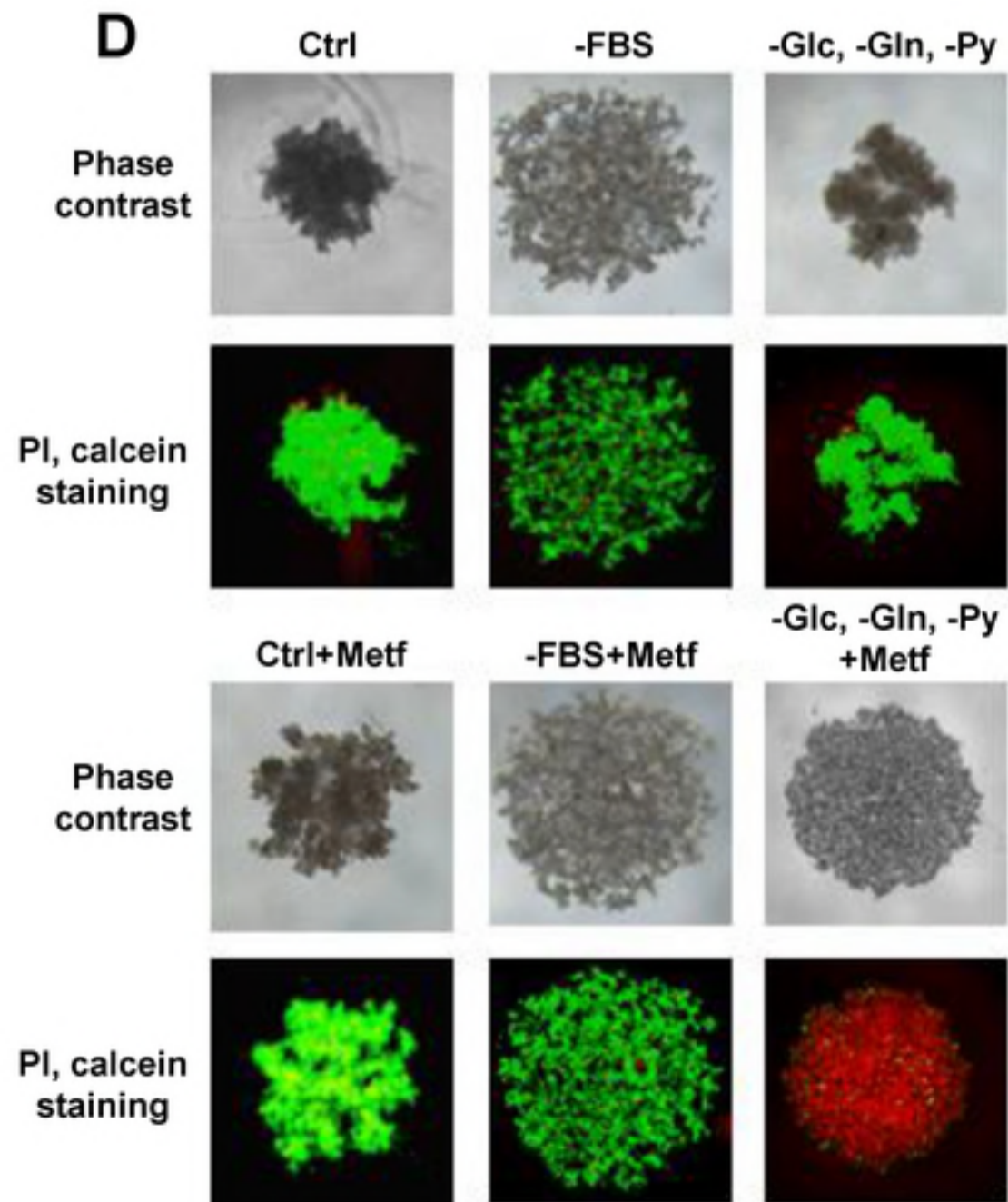
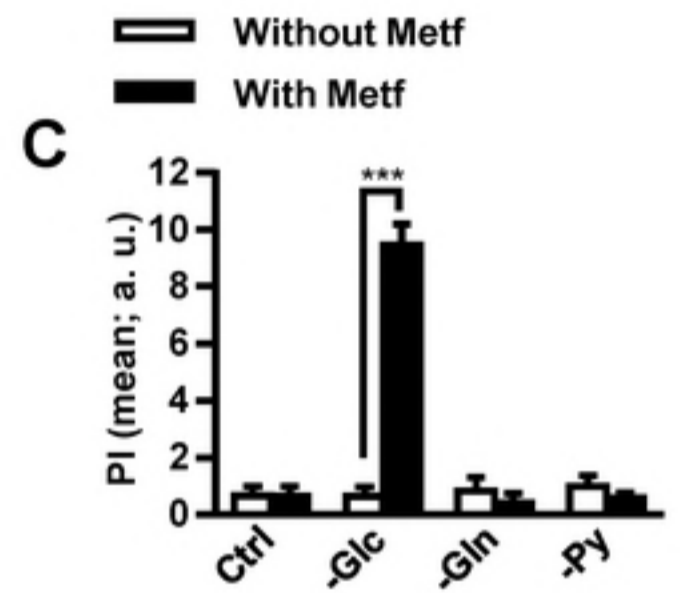
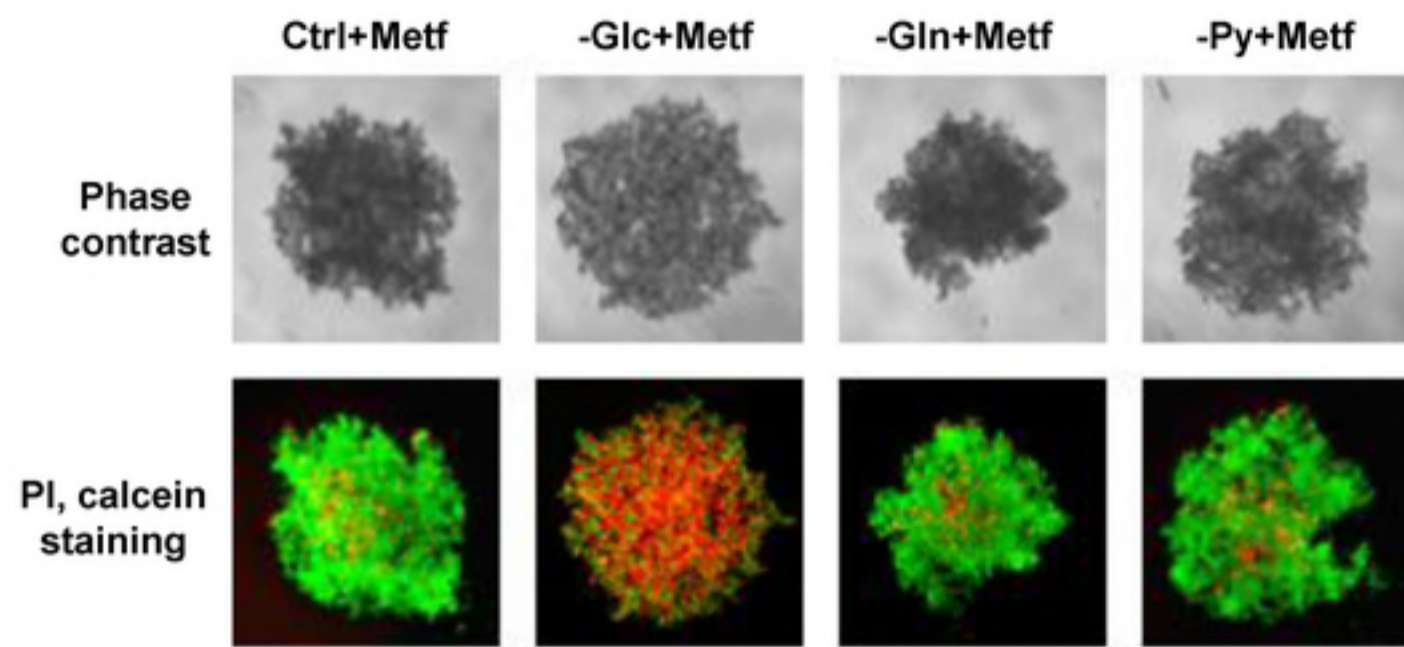
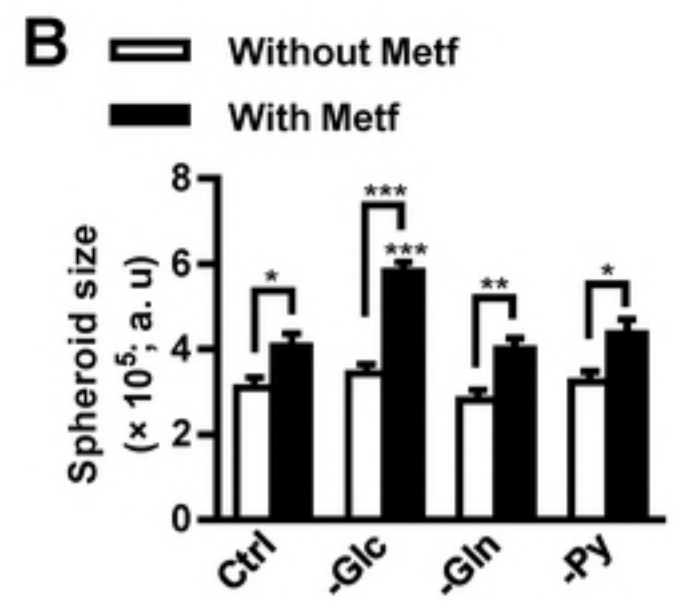
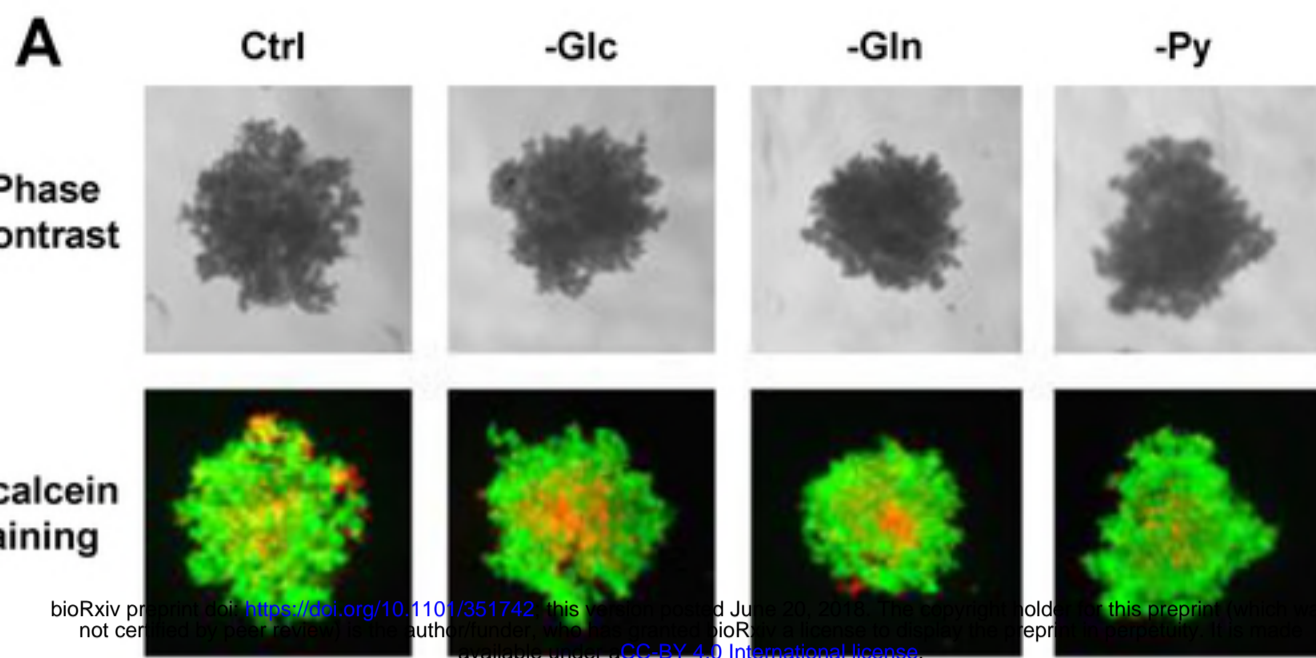
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**A****B**

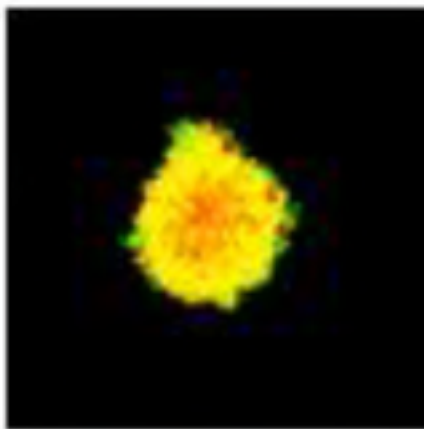
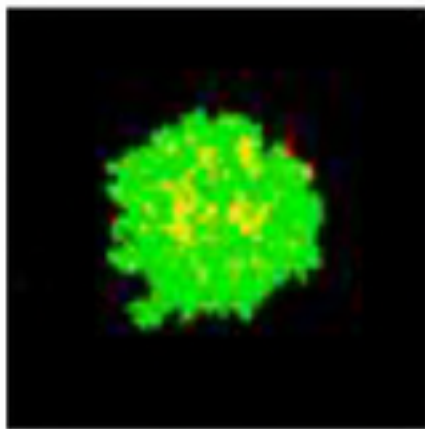
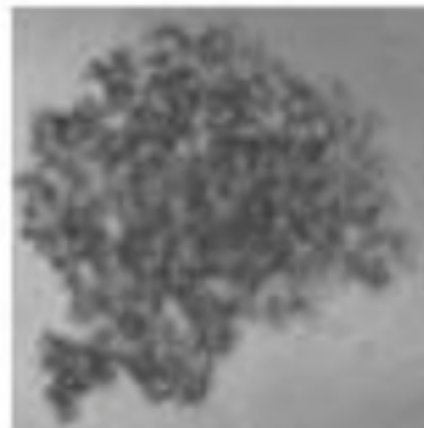
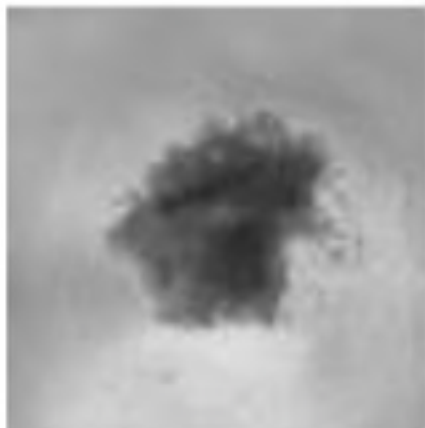
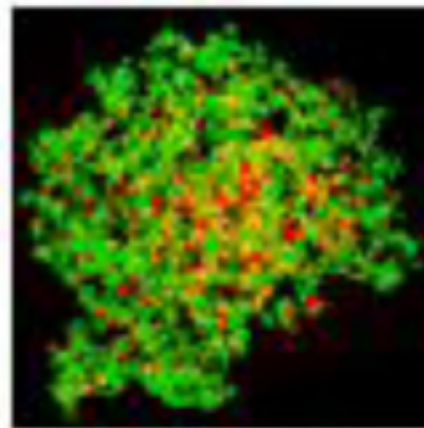
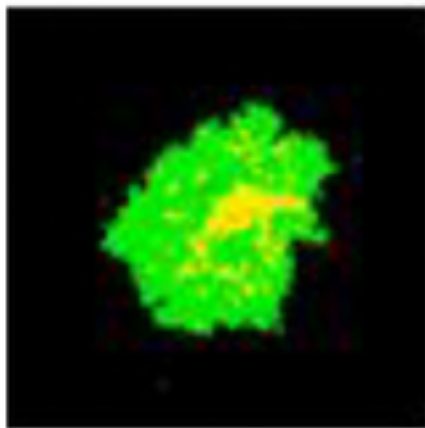




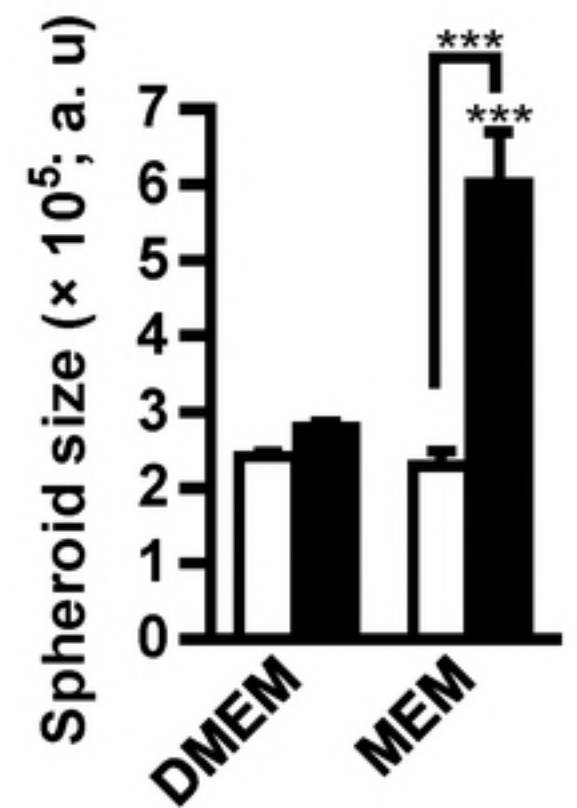


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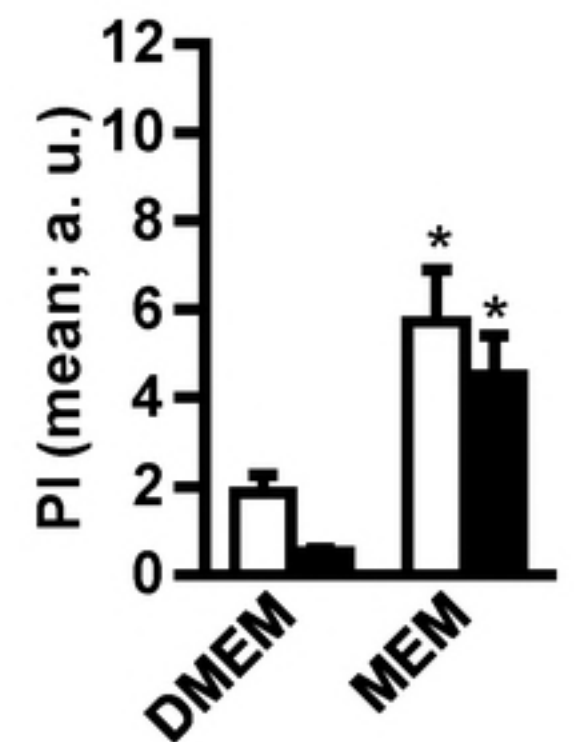
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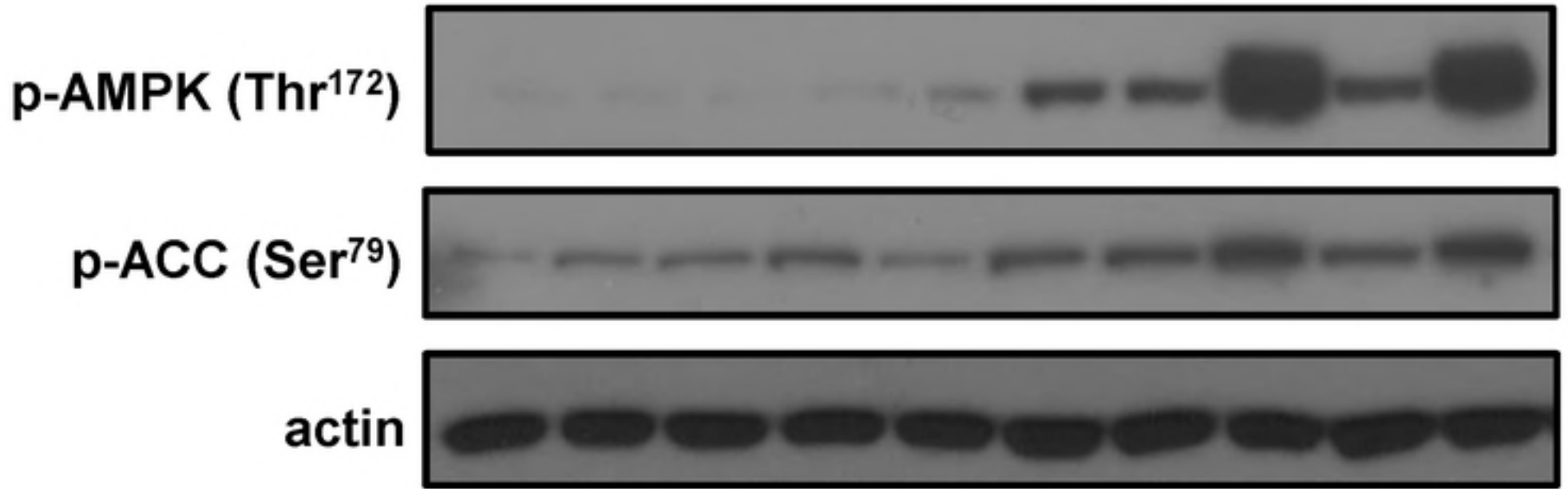
**Phase contrast****PI, calcein staining****DMEM+Metf****MEM+Metf****Phase contrast****PI, calcein staining****B**

Without Metf  
With Metf

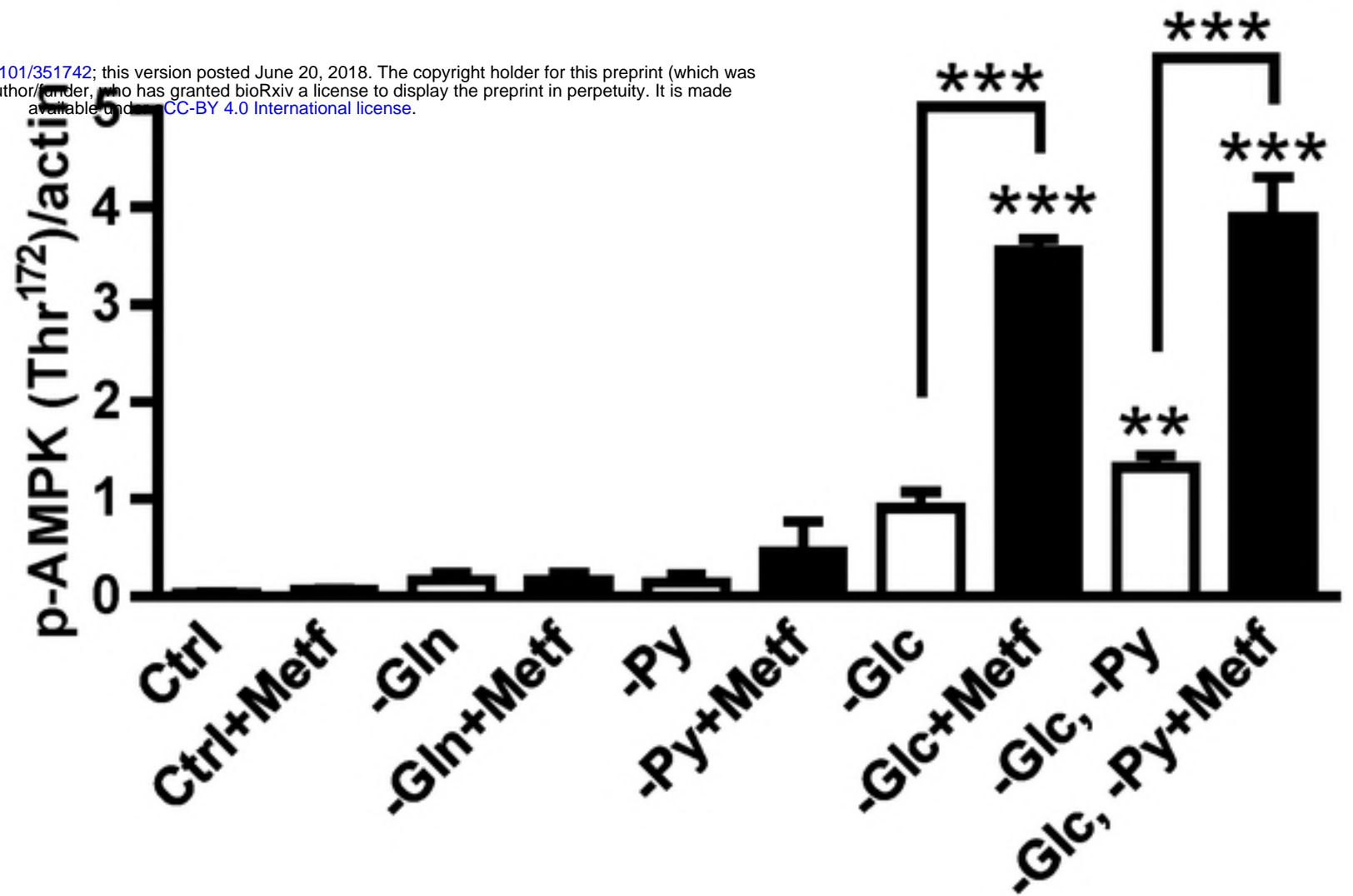
**C**

Without Metf  
With Metf



**A****B**

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**C**