Hypoxia impacts small intestinal organoid stemness and differentiation

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Summary

Comprised of several cell types functioning differently, the small intestinal epithelial cells perform their functions and synergistically maintain homeostasis in the first part of the mammalian intestine. Due to the uneven distribution of the vessel, the oxygen level exhibits a gradient decreasing pattern in the normal intestine and becomes aberrant in some intestinal diseases. In this work, we find certain levels of hypoxia simulated by cobaltous chloride ($CoCl_2$) caused an increase in the secretive cell types and a decrease in the absorptive cell types *in vitro* cultured mouse small intestinal organoids. Importantly, the intestinal stem cell amount is impacted which leads to attenuated epithelial regeneration. Our study highlights the cell-type-specific alterations under the hypoxia insult, which gives possible therapeutic hints for hypoxia-relevant gastrointestinal diseases.

Keywords

Hypoxia, intestinal stem cell, differentiation, organoid

Introduction

The human small intestinal epithelium is the fastest self-renewing tissue, which renews every 3 to 5 days, with the structure of bending cells forming the crypts and villi (1). Intestinal stem cells (ISCs) reside at the crypt base and differentiate into the transit-amplifying (TA) compartment which divides 4-5 times within 12 hours (2). After that, the TA cells move upwards and differentiate into multiple cell types including enterocytes, goblet cells and enteroendocrine cells. During normal homeostasis or some intestinal injuries, ISCs can undergo self-renewal and proliferate to replenish cells that are lost to shedding (3, 4). The self-renewal and lineage differentiation into mature enterocytes, goblet cells and enteroendocrine cells are under the control of Wnt/β-catenin, Notch and bone morphogenetic protein (BMP) pathway signalings (5-7). Among them, Wnt/β-catenin signaling is of critical significance, and its downstream regulated gene Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) is identified as the marker for proliferative ISCs (8, 9).

Mutations that activate the Wnt/β-catenin pathway can result in human colorectal cancer (10, 11). Studies suggested Wnt as an essential growth factor for the maintenance of intestinal proliferation and homeostasis (12, 13), and ectopic Wnt signaling has been

implicated in the initiation and progression of colorectal cancers (14, 15).

In a healthy human intestine, due to the counter-current blood and the existence of a large number of gut bacteria, the oxygen pressure (pO2) level is lower than the normal oxygen pressure (160 mmHg), therefore, it's described as "physiologic hypoxia" (16). The local pO2 level decreases along the crypt-villus axis and the oxygen gradient becomes the lowest at the villus tip (17). This unique gradient physiologic oxygen pressure results in the stabilization of the transcription factor hypoxia-inducible factor (HIF) and the activation of hypoxia-relative signaling, which plays multiple roles in metabolism, mitochondrial autophagy and inflammation (18). However, in pathological conditions, profound hypoxic or anoxic are usually found in mucosal lesions (19). Hypoxia is also a feature of inflammatory bowel disease (IBD), and it affects inflammation in some intestinal diseases (16, 20). Studies have demonstrated the protective role of HIF on some intestinal diseases such as IBD and necrotizing enterocolitis (NEC) upon *in vivo* hypoxia injury (21-23), and the effect of hypoxia on intestinal cell response in intestinal cancer cells or upon radiotherapy and chemotherapy *in vitro* (24-26). In both scenarios, the intestinal stem cells (ISCs) have been shown to react by promoting intestinal recovery from injuries (27, 28). However, the mechanism of how ectopic hypoxia affects ISC function and how it impacts intestinal cell lineage differentiation is less clarified. Mazumdar et al. mentioned that HIF-1α regulates Wnt/β-catenin signaling in hypoxic embryonic stem (ES) cells and neural stem cells (29). Rohwer et al. demonstrated the role of HIF-1α in intestinal epithelial cells (IECs) during intestinal tumorgenesis, controlling Wnt/β-catenin activity and regulating metabolic reprogramming and inflammation (30). However, it remains to be elucidated how hypoxia impacts different cell types in intestine with or without affecting Wnt signaling.

Intestinal organoids isolated from adult stem cells have been established as an ingenious craft to study the human intestine (8). It's a 3D model of intestinal tissue embedded in 3D matrices such as Matrigel and can be passaged and cultured for a long time. The organoids display the characteristic villi-crypt organization by heterogeneous epithelial cell monolayer, allowing it to recapitulate many features of normal gut epithelium with IECs comprising all types of epithelial cells (31). In addition, the organoid system is applied to estimate IEC functions *in vivo* such as barrier function and stem cell function (32, 33). Compared with the *in vivo* mouse model and 2D cell lines (26, 34) to study the influence of hypoxia on intestinal functions or tumorigenesis, intestinal organoids can be easily established from different staging of endoscopic biopsies in patients, making it a better model that can closely recapitulate the hypoxic intestine and a faster model to come up with therapeutic regimens.

Hypoxia exists in intestinal diseases such as IBD and NEC, wherein the mechanism by which hypoxia impacts ISC stemness and differentiation remains poorly understood. In the present study, we examined obvious changes of different cell lineages upon hypoxia treatment to intestinal organoids, as well as the upstream transcription factors regulating the ISC stemness. Our study further confirms organoids as the model to study intestinal diseases that are concurrent with hypoxia. This cobaltous chloride ($CoCl_2$)-caused hypoxia-treated organoid model makes it a good method to study HIF-1α regulated signaling in intestine, helping the translation of basic science studies into IBD treatment.

Result

1. Hypoxia treatment affects intestinal organoid stem cell renewal function

It's known that the ability of IECs to form organoids and organoid regeneration after being passaged depends mainly on ISC function (4, 8). To investigate the effect of hypoxia exposure on intestine organoid stem cell regeneration capacity, we passaged the ISC-containing organoid as an assay of intestinal regenerative potential (8). Increasing dosages of $CoCl_2$ (0, 10, 50, 100, 200 μM) were used to induce cellular hypoxia (35, 36), and did not impair the budding and morphology of organoids (Fig. 1A). The protein level of HIF-1α was upregulated 24 hours after hypoxia exposure (Fig. 1B). The organoid regeneration, which indicates stem cell function and generated from single intestinal organoid cells engendered fewer progenies in a gradient decreasing manner, as showed in the clonogenicity assay (Fig 1C).

We further explored whether the reduction of the organoid regeneration function was preceded by the reduction of stem cell trait. Indeed, the mRNA levels of Lgr5 which mark the intestinal stem cells, were also down-regulated (Fig. 2A). Therefore, these data suggest that hypoxia exposure in intestine organoids impairs ISC regeneration by reducing ISC numbers and organoid-forming capacity.

2. Wnt signaling was down-regulated upon hypoxia treatment in intestinal organoids

Wnt signals play a crucial role in the regulation of intestinal stem cell proliferation (11, 37, 38). As the key stem cell regulator in intestine stem cells, Lgr5 is controlled by Wnt/β-catenin signaling (4, 39) and exhibits a reduced mRNA level after 200 μM $CoCl_2$ treated hypoxia treatment (Fig. 2A). To further clarify the effect of hypoxia on Wnt signaling in intestinal organoid, multiple Wnt target genes were tested in hypoxia-exposed intestine organoids. As the hypoxic condition gets severe, the protein level of β-catenin was down-regulated in a dosage-dependent manner (Fig 2B). Also, the β-catenin target Cyclin-D1, which is the gene involved in cell survival (40), exhibited significant protein level reduction. Expression of another Wnt target, the receptor tyrosine kinase EphB2, which reduces from the crypt base to the differentiated cells (41) and is highly enriched in ISCs (42), also shows a reduced protein level after 24 hrs hypoxia treatment. Consistent with this, protein expression of another Wnt/β-catenin target gene Axin2 (43) was also decreased. These demonstrate that hypoxia treatment dampens the activity of Wnt/β-catenin signaling in intestinal organoids.

3. Hypoxia treatment affects intestine differentiation and increases secretory cell types

Pathological conditions such as inflammation insults are usually accompanied by cellular hypoxia, further disrupting intestinal epithelial barrier function and metabolism (18). The barrier function of intestinal epithelium is largely composed of enterocytes to maintain intact epithelium construction, and goblet cell secreted mucus helps to form the healthy mucosa layer, making the epithelium devoid of bacteria (44).

Then we deciphered whether hypoxia drives different intestine cell lineage cell differentiation, and if it impairs certain cell types executing their functions. As shown in the mRNA levels, hypoxia exposure led to the increase of Lysozyme (Lyz), Chromogranin A (ChrA) and Mucin 2 (Muc2), which mark Paneth cell, enteroendocrine cell and goblet cell, respectively (Fig. 3A). At the same time, the protein expressions showed by the immunofluorescence (IFC) signal indicated a greater number of Lysozyme positive Paneth cells, Chromogranin A positive enteroendocrine cells, and Mucin2 positive goblet cells in hypoxia treated intestinal organoids (Fig 3B). Paneth cells act as the source of Wnt signaling

136 and secrete Wnt legends. However, our data suggest that this increase in Paneth cells per
137 buds, though, does not compensate for the lower level of Wnt signaling upon hypoxia
138 treatment.

139 During active inflammation in Crohn's disease (CD) and ulcerative colitis, pro-inflammatory
140 signals like cytokines promote the stabilization of HIF proteins (45). Hypoxia contributes to
141 inflammation, and Paneth cells are considered to be the origin of intestinal inflammation
142 (46). We then test whether hypoxia induces the inflammatory response in intestinal
143 organoids. Our data showed that hypoxia-induced stabilization of HIF-1α promotes the
144 transcription of proinflammatory cytokines tumor necrosis factor alpha (TNF-α) in intestinal
145 organoids (Fig. 4A, left). This increased TNF-α is likely secreted by the Paneth cells and we
146 wonder if TNF-α would boost this cell lineage. Single intestinal organoid cells treated with a
147 gradient concentration of TNF-α (5-50 ng/ml) for 48 hrs showed increased clonogenic ability,
148 as well as organoid swelling phenotype, especially in the higher TNF-α concentration
149 treatment group (Fig. 4B, C). Interestingly, we notice atypical cell morphology at the bottom
150 of the organoid-Matrigel culture plate in the TNF-α treated cells, which show elongated cell
151 morphology with protrusions (Fig. 4B, red arrows). This boosted growth of organoids
152 probably leads to an adaptation behavior under inflammation assault.

153 Paneth cells have active endoplasmic reticulum (ER) functions, and Paneth cell
154 differentiation depends on unfolded protein response (UPR) signaling (47). Unresolved ER
155 stress is common in the IBD epithelial cells (48). Importantly, unresolved ER stress and UPR
156 activation within Paneth cells can be treated as the nidus for intestinal inflammation (46).
157 We detected that mRNA levels of UPR proteins Atf4 and Chop are elevated upon hypoxia
158 exposure (Fig. 4A, right). While X-box binding protein 1 (Xbp1), Grp78 and Perk are not
159 changed significantly (data not shown).

160 Accordingly, hypoxia treatment increased the secretory lineage cell differentiation in
161 intestinal organoids, further causing the Paneth cell-relative ER stress response.
162

163 4. Hypoxia treatment reduces the absorptive cell type in intestinal organoids

164 Enterocytes constitute the most abundant cells and the only absorptive lineage cells in of
165 intestine. In addition to being an element of the epithelial lining, the differentiated mature
166 enterocytes at the upper villi in small intestine play a critical role in digestive, metabolic, and
167 barrier physical integrity maintenance functions (49). They are the cells most susceptible to
168 hypoxia (50). We found that mRNA levels of alkaline phosphatase (Alpi) and fatty acid
169 binding proteins (Fabp1/2) which hallmark enterocytes decreased after the gradient of
170 $CoCl_2$ treatment (Fig. 5A). IFC staining also showed a reduced amount of aldolase B (Aldob,
171 marker of enterocyte) positive cells (Fig. 5B), suggesting that enterocyte differentiation is
172 specifically impaired.

173 Enterocytes are involved in nutrient metabolism including glycolysis, fatty acid and lipid
174 metabolism (51, 52). The adipogenic gene Peroxisome proliferator-activated receptor γ
175 (PPARγ) has a beneficial metabolic and anti-inflammatory effect in the GI tract (53). We
176 found that PPARγ, although expressed at a low level in small intestine, was down-regulated
177 after hypoxia treatment (Fig. 5C).

178 Cells that are exposed to inflammatory cytokines manifest increased glycolysis, which is
179 reminiscent of those observed in hypoxic cells (54, 55). Glucose is also a relevant energy

source for enterocytes, and pyruvate kinases are responsible for catalyzing the final step in glycolysis (56). We detected the protein level of glycolysis gene pyruvate kinases M (PKM) was reduced after hypoxia exposure (Fig. 5C).

In addition to the general structure of intestinal organoids as the barrier, which we detected no change after hypoxia treatment (Fig. 1A), we wondered whether the intrinsic elements of the epithelium barrier were affected. We detected hypoxia caused a gradient down-regulation of the epithelial barrier protein E-cadherin (Fig. 5C), which might indicate higher permeability in the gastrointestinal epithelium

Therefore, hypoxia exposure decreased the absorptive enterocytes, with reduced metabolic-related proteins and might dampened intestinal barrier function.

Discussion

Hypoxia occurs in intestinal diseases such as IBD and NEC (22, 23). It is well established that hypoxia-induced intestinal injuries and low oxygen-relative physiological responses (23). Studies using murine model of colitis indicated that HIF-1α plays a protective role in intestinal inflammatory mucosal disease, most of which suggested a barrier-protective function of HIF-1α (16, 20, 22). As different intestinal lineage cells executive specific functions, however, the mechanism of hypoxia caused various cellular or metabolic responses failed to be elucidated at the cell-type specific level in IEC. In this study, we showed that hypoxia exposure by hydroxylase inhibitor $CoCl_2$ significantly changes the small intestinal organoid cell lineage differentiation and reduces the ISC function by down-regulating Wnt signaling.

It's known that hypoxia contributes to colorectal cancer cell (CRC) and activates Wnt signaling (57, 58). However, in normal intestine epithelium under homeostasis, it's less clarified whether hypoxia impacts ISC function and alters Wnt signaling. Consistent with Liu and Zu et al. showing that Wnt signaling is reduced in the hypoxia-induced NEC and ischemia-reperfusion (I/R) murine model (59, 60), our results further demonstrate that hypoxia-caused impaired ISC function was accompanied by reduced Wnt signaling.
This intestinal organoid-based hypoxia exposure model provides an opportunity with the cell type-specific model which correlates HIF-1 and the target genes in inflammation or metabolism dysfunctions.

Notch signaling plays a crucial role in regulating cell fate decisions in intestinal homeostasis (61, 62). Upon binding to their ligands, Jagged (Jag) and Delta-like (Dll) proteins, the Notch receptor was cleaved and the intracellular domain translocates to the nucleus, where it activates the transcription of the target genes, such as hairy and enhancer split-1 (Hes1), regulating enterocyte differentiation (63). In reverse, Hes1 represses the expression of atonal homolog 1 (Atoh1), which regulates secretory cell lineage differentiation.

Studies have implied that hypoxia increases the expression of Notch direct downstream genes in neuronal and myogenic cells (64). Enterocyte differentiation is impaired as a consequence of reduced Notch signaling pathway in patients with CD which is accompanied by hypoxia-featured inflammation (65). While Notch1 signaling is upregulated in colorectal hypoxic Caco-2 cells (66). It is still controversial regarding the role of Notch signaling in regulating hypoxia-relevant intestinal diseases. Yeung et al. reported that hypoxia increases

223 clonogenicity and maintains the stem-like phenotype of colorectal cell line-derived cancer
224 stem cells, preventing differentiation of enterocytes and goblet cells by activating Notch1
225 (67). Of note, it remains less reported about the relationship between hypoxia and Notch
226 signaling in mediating stemness or barrier function in intestine under homeostasis. Although
227 we found the absorptive enterocytes decrease after hypoxia treatment, its regulatory Notch
228 signal transcription factors such as Hes1 and Notch intracellular domain (NICD) should be
229 tested in the future to answer if hypoxia affects the Notch signal directly.

230 Cell cultures have been used to study intestinal epithelial permeability after
231 repair/restitution (68, 69), while intestinal organoids provide us better *in vitro* model to
232 study the mechanism of injuries such as hypoxia on intestinal epithelial barrier function
233 which can best mimic the *in vivo* intestine. In intestinal cell lines or *in vivo* hypoxia-exposed
234 models (70, 71), HIF-1α was reported to influence intestinal epithelial permeability (19, 72).
235 Liu et al. suggested that Notch1 signaling plays a role in maintaining the intestinal barrier
236 function by regulating TJ proteins (66). More experiments are expected in the future such as
237 testing the expression of TJs (Zo-1 and occludin) and intestinal mucosal permeability
238 alterations, to prove the regulation of Notch to TJ proteins and its role in maintaining barrier
239 function.

240 On the other hand, we detected a higher number of goblet cells with increasing Muc2 level
241 after hypoxia exposure, consistent with D. Ortiz-Masiá et al.'s finding with increased Muc2
242 in chronic CD patients (65). Goblet cells secret the mucins that form the physical epithelial
243 barrier, and Paneth cells secrete antimicrobial and some signaling factors to regulate the ISC
244 stemness (73). The differentiation of IECs from the ISCs into the terminal differentiated cells
245 is under the regulation of master transcription factors (74). The progenitor cells committed
246 to secretory lineage have high expression of Atoh1. GFI1 and Spdef specify goblet and
247 Paneth cells, Neurogenin3 and NeuroD1 mark enteroendocrine cell differentiation, and Elf3
248 and Klf4 regulate goblet cell differentiation. More investigation is expected to delineate
249 whether hypoxia shifts early differentiation toward the secretory lineage via changing the
250 upstream secretory transcription factors.

251 Cells undergo stress and increase the production of certain proteins in their endoplasmic
252 reticulum (ER) when they experience stressful conditions like hypoxia (75), which is known
253 as the unfolded protein response (UPR). Heijmans et al. showed ER stress plays an
254 important role in regulating normal intestinal epithelial stem cell differentiation (47). We
255 detected the UPR targets Chop and Atf4 are up-regulated after hypoxia exposure. As XBP1 is
256 a regulator of the unfolded protein response, it's required for ER expansion (Shaffer et al.,
257 2004) and adaptation of tumor cells to hypoxic conditions (48). XBP1 deletion induces
258 inflammation in intestine (48). Although XBP1 was reported to drive the triple-negative
259 breast cancer (TNBC) tumorigenicity by controlling the HIF1α pathway (76), however, we
260 observed the level of Xbp1 did not change obviously in hypoxia-exposes normal intestinal
261 organoids.

262 As hypoxia treatment dampens intestinal organoid clonogenicity, while TNF-α treatment
263 boosts it, they likely target different cell types in the intestinal organoids. Compared with
264 the absorptive cell types (i.e., enterocytes) as the most vulnerable cells under hypoxia threat,
265 Paneth cells being the major warriors in front of TNF-α might explain the opposite growth
266 trend under these two different insults. Because TNF-α treatment 2 days post single

267 organoid seeding has the opposite barrier function protein alteration compared with those
268 single-cell formed organoids (data not shown), further enhancing the possibility that TNF-α
269 might boost Paneth cells in the first two days of organoid growth (wherein stem cells are
270 the majority cells) while mainly kill the enterocyte cell at later days. These two different
271 insults targeting different intestinal cell types help to explain that each cell type performs its
272 function under homeostasis and pathologic conditions. Besides, as we also notice atypical
273 cell protrusions in TNF-α treated cells, further experiments such as hypoxia and TNF-α co-
274 treatment can be done to elucidate whether this inflammation-fueled cell type-specific
275 outcome helps intestinal cancer cell metastasis, and whether hypoxia enhanced the
276 secretive cell number can further promote this phenomenon.

277 **Method**

278 **Animals and crypt isolation organoid culture**

279 Small intestines were removed from sacrificed 4-16-week-old C57BL/6 mice, flushed in cold
280 PBS and cut open. Mesentery was removed and the tissue was washed three times before
281 being cut into pieces. Then the tissues were digested with 2 mM EDTA and stayed on ice for
282 45 min. The digested intestines were transferred into a tube with cold PBS and shaken
283 around 3 times to detach the villi and remove residual EDTA. The suspension was filtered
284 through 70-µm meshes (BD Falcon) to enrich crypts. The crypt-containing solution was
285 centrifuged at 150 g for 3 min. The crypt pellet was then resuspended with DMEM and
286 embedded in Matrigel (Corning, #356231) for solidification for 15 min in a 37° C incubator.
287 Then culture medium was added above the crypt-Matrigel mix with DMEM/F12 (Gibco)
288 medium supplemented with EGF (50 ng/ml, Invitrogen), Noggin (50 ng/ml, R&D SYSTEMS),
289 R-spondin 500 ng/ml (R&D SYSTEMS), 1 × N-2 (Invitrogen), 1 × B-27 (Invitrogen), HEPES (10
290 mM), 1 x GlutaMax (Gibco), penicillin and streptomycin (100 U/ml), N-Acetyl Cysteine (1 µM,
291 Sigma). Y27632 (10 µM, Selleck) was included in the culture medium during the first 2 days
292 after being passaged. The medium was changed every other day.

293 **Hypoxia treatment**

294 Organoids were treated with gradient concentrations of $CoCl_2$ (Sigma, #V900059) by 0, 10,
295 50, 100, and 200 µM for 24 hours. DMSO (concentration less than 0.1%) was used as the
296 solvent and control reagent.

297 **Clonogenicity assay**

298 The colony-forming experiment was measured by seeding 200–300 single-cell organoids in
299 Matrigel and calculating organoid number after 2 days. The organoids were cultured for five
300 to nine days, then the crypt number was collected each day. Single organoid cells were
301 obtained by TrypLE (ThermoFisher) treatment for 10 min at 37°C, followed by gentle
302 pipetting if cell clusters remained.

303 **RNA Isolation and Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

304 Total cellular RNA was extracted using EZ-10 RNA Mini-Preps Kits Handbook, (#BS88583,
305 Markham ON, Canada). Around 250 ng RNA was used to do reverse transcription by
306 RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo, Waltham, MA, USA). Primers are
307 shown in Table 1. RT-qPCR was performed using an SYBR qPCR Master Mix (Q321-03,
308 Vazyme, Nanjing, China) by the following conditions: pre-heat at 95°C for 10 s, then

309 followed with 39 cycles at 95°C for 5/10 s and 60°C for 30 s (72°C for 30 sec). The relative
310 mRNA levels were analyzed using the $2^{-\Delta\Delta Ct}$ method. Experiments were repeated at least
311 three times. Then the mean value was used to calculate the significant alterations in
312 statistical analysis.

313 **Table 1.** Primers used for RT-qPCR.

Gene		Primer (5′–3′)
ACTB	Forward	GGCTGTATTCCCCTCCATCG
	Reverse	CCAGTTGGTAACAATGCCATGT
HIF-1α	Forward	TCTCGGCGAAGCAAAGAGTC
	Reverse	AGCCATCTAGGGCTTTCAGATAA
Lgr5	Forward	CCTACTCGAAGACTTACCCAGT
	Reverse	GCATTGGGGTGAATGATAGCA
Lyz	Forward	GAGACCGAAGCACCGACTATG
	Reverse	CGGTTTTGACATTGTGTTCGC
ChrA	Forward	ATCCTCTCTATCCTGCGACAC
	Reverse	GGGCTCTGGTTCTCAAACACT
Muc2	Forward	GAAGCCAGATCCCGAAACCA
	Reverse	GAATCGGTAGACATCGCCGT
Tnf-α	Forward	GCACAGAAAGCATGATCCGC
	Reverse	CCCCATCTTTTGGGGGAGTG
Alf4	Forward	GGACAGATTGGATGTTGGAGAAAATG
	Reverse	GGAGATGGCCAATTGGGTTCAC
Chop	Forward	CTCGCTCTCCAGATTCCAGTC
	Reverse	CTTCATGCGTTGCTTCCCA
Alpi	Forward	CCAGCAGTAACTCACCTCATGG
	Reverse	GAAGCCTTGTGGATTCCTGCTG
Fabp1	Forward	ATGAACTTCTCCGGCAAGTACC
	Reverse	CTGACACCCCCTTGATGTCC
Fabp2	Forward	TCCCTACAGTCTAGCAGACGGA
	Reverse	CCAGAAACCTCTCGGACAGCAA

314

315 **Whole mount staining and Immunofluorescence analysis**

316 Organoids were cultured in 6-well plate containing 2 ml medium. Four days after passaging
317 and then treated with $CoCl_2$ for 24h, organoids were collected in ice-cold culture medium
318 and Cell Recovery Solution (BD Biosciences) was added and incubated for 1 hour to remove
319 the Matrigel. After being washed 3 times by medium, the organoids were fixed by 2%
320 formaldehyde overnight at 4°C. On the second day, organoids were permeabilized in 0.1%
321 Tween 20/PBS for 30 min at room temperature. Blocking was done by 0.2% Bovine serum
322 albumin (BSA) for one hour at 4°C. Then organoids were incubated in 1st antibody at 4°C
323 overnight. On the third day, three times of washing were performed with ice-cold 0.1%
324 Tween 20/PBS, followed by organoid sedimentation at room temperature. Then secondary

rabbit or mouse anti-rat IgG (H+L) reagent was added to incubate for 1 hour, followed by three times washing with 0.1% Triton X-100. Finally, the pelleted organoids were mounted on the glass slides. Images were taken by Spinning Disk Confocal Microscopy and five random fields were chosen to observe the 1st antibody (Lyz, ChrA, Muc2, and Aldob) positive cells and measure their percentage. The experiment was repeated for at least three times.

Western blotting

Organoids cultured in the 24-well plate were washed by cold PBS and moved into 1.5 ml tubes. Supernatant with Matrigel was removed and 80 µl 4X loading buffer (with DTT) was added and pipetted for mixing. Samples were denatured at 100°C for 10 min. Protein samples were stored at -80°C if not used immediately. Protein samples (around 50 µg) were loaded and for electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels, followed by transferring to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, CA, USA). Then the blocking was carried in non-fat dry milk at 37°C for 1 h. First antibody incubation was done at 4 degrees with the following antibodies (1:1000). The rabbit monoclonal anti-HIF-1α (#36169T), anti-β-Catenin (#8480) and anti-Cyclin-D1 (#2978) were purchased from Cell Signaling Technology (Devers, MA, USA). The rabbit monoclonal anti-Axin2 (#2151) was obtained from Abcam (Cambridge, UK). The mouse monoclonal anti-E-cadherin was purchased from BD Biosciences (610181, San Jose, CA, USA). The mouse monoclonal, anti-Ephb2 (sc-130752), anti-PKM (sc-365684) and anti-PPARγ (sc-7273) were purchased from Santa Cruz (Dallas, TX, US). After 1st antibody incubation, membranes were washed by Tris-Buffered Saline Tween (TBST)-20 for 3 times. Incubation with the secondary goat anti-rabbit or goat anti-mouse IgG (1:1000, ZSGB-BIO, Beijing, China) were performed at 37°C for 1 h. Then the PVDF membranes were washed three times for 10 min each time. The blots were developed using an enhanced chemiluminescence detection kit (Millipore, MA, USA). Image J was used to do the densitometric analysis.

Statistical analysis

GraphPad Prism 9.0 was used to perform statistical analysis. one-way ANOVA was used to test the differences among groups, which is followed by Tukey's multiple comparisons test. Each experiment was repeated at least 3 times. The results were deemed significantly different at $P < 0.05$.

Author contributions

Xi Lan designed the paper, Xi Lan, Qiu Ping and Mou Chunfeng did the experiments, and Mou Chunfeng approved the version to be published. All authors approved this version for publication.

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May this tiny work find the sincerest passion and effort of those unrooted scientists, in honor of their unseen sweat and industrious missing contribution.

Conflict of interest

The authors declare no conflict of interest.

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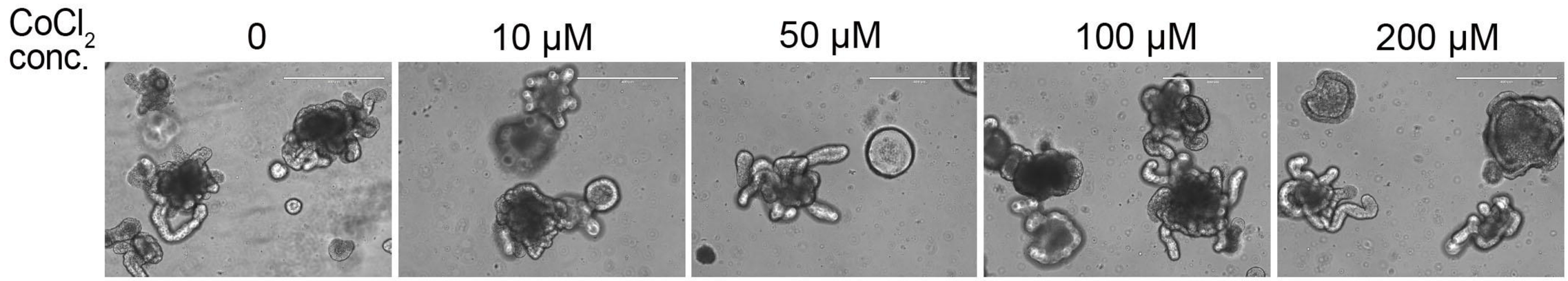
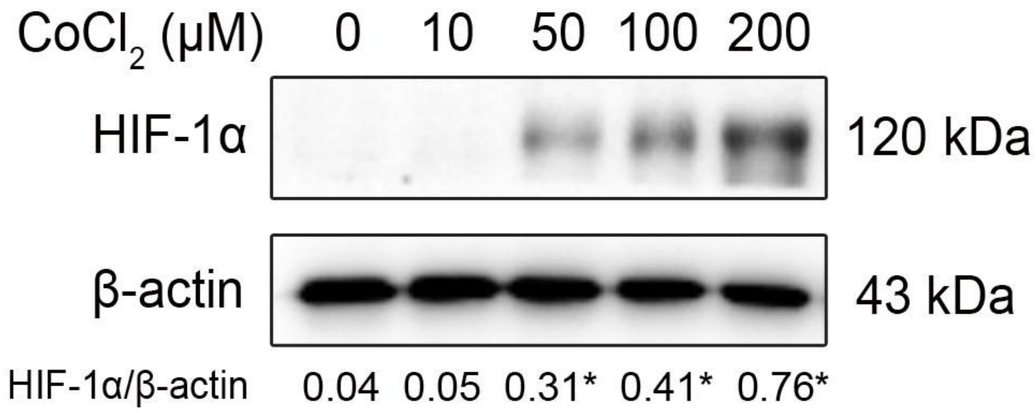
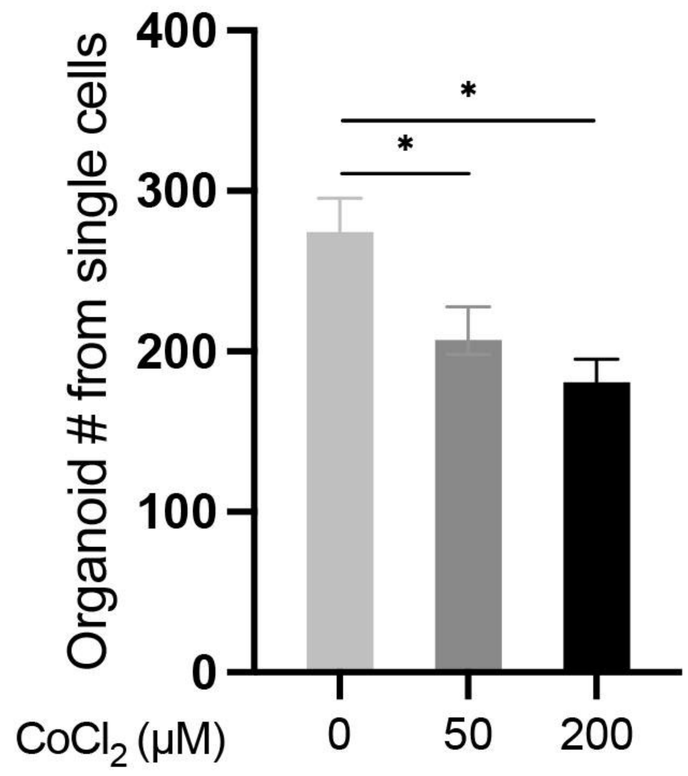
A

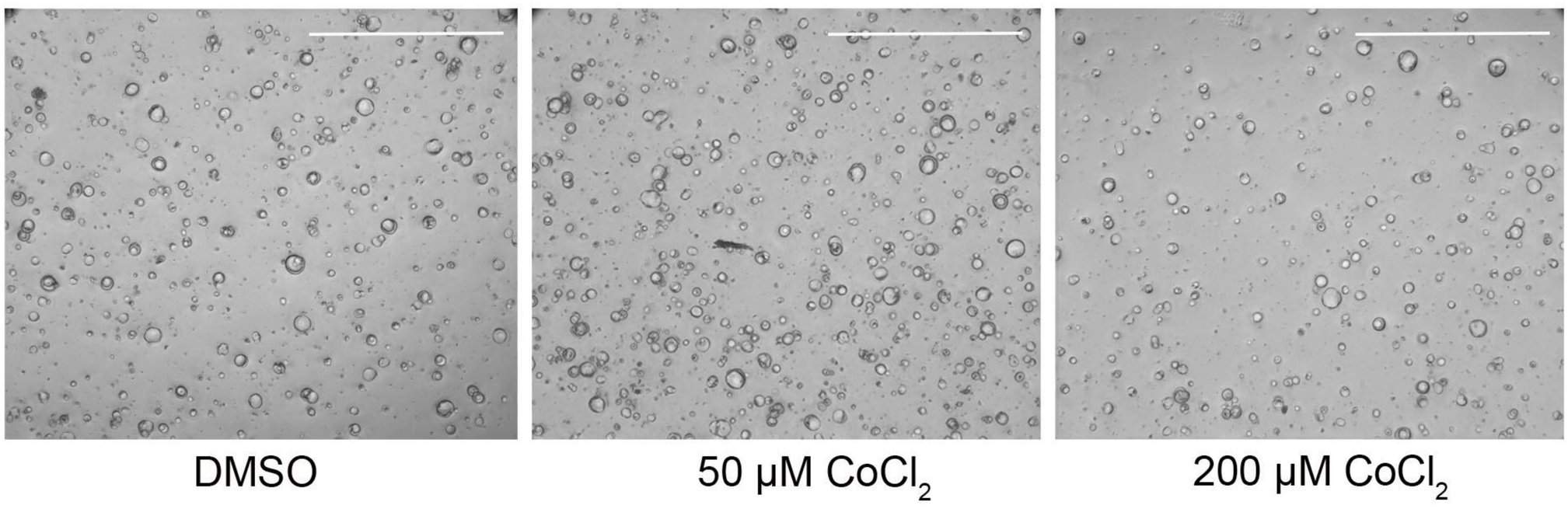

CoCl2 conc.
0
10 μM
50 μM
100 μM
200 μM

B

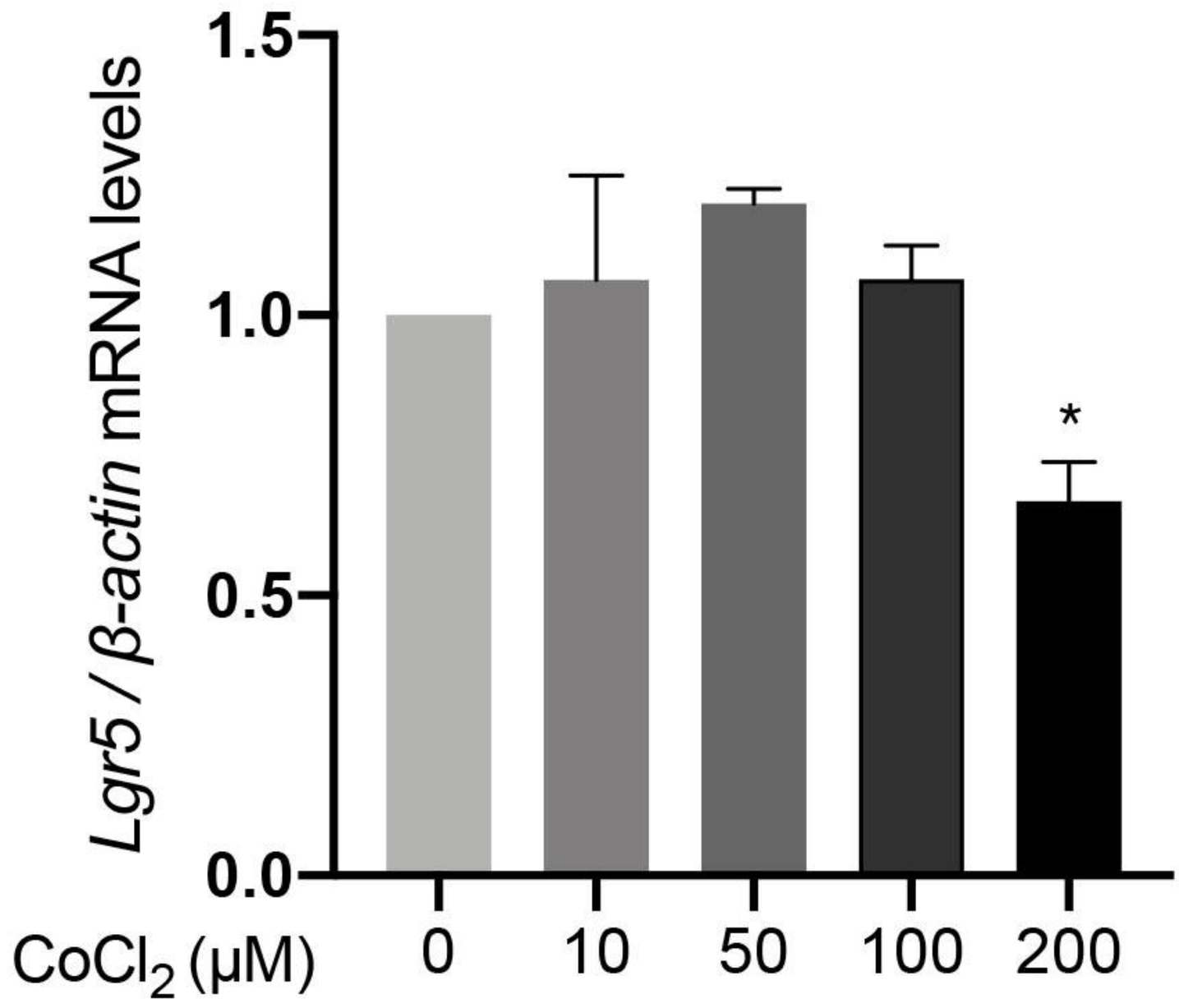

CoCl2 (μM) 0 10 50 100 200
HIF-1α
120 kDa
β-actin
43 kDa
HIF-1α/β-actin 0.04 0.05 0.31* 0.41* 0.76*


Organoid # from single cells
400
300
200
100
0
*
*
CoCl2 (μM)
0
50
200

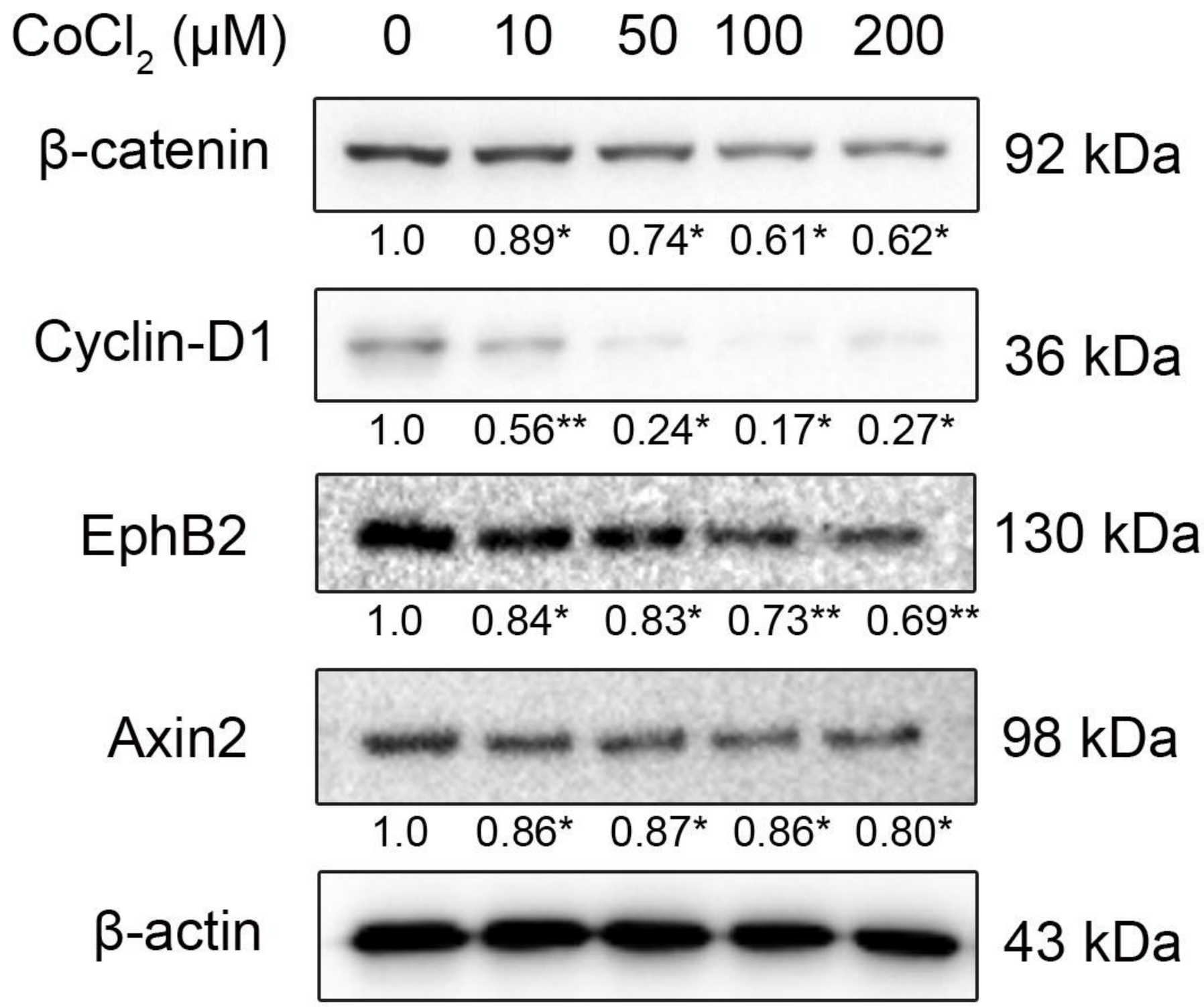
C


DMSO
50 μM CoCl2
200 μM CoCl2

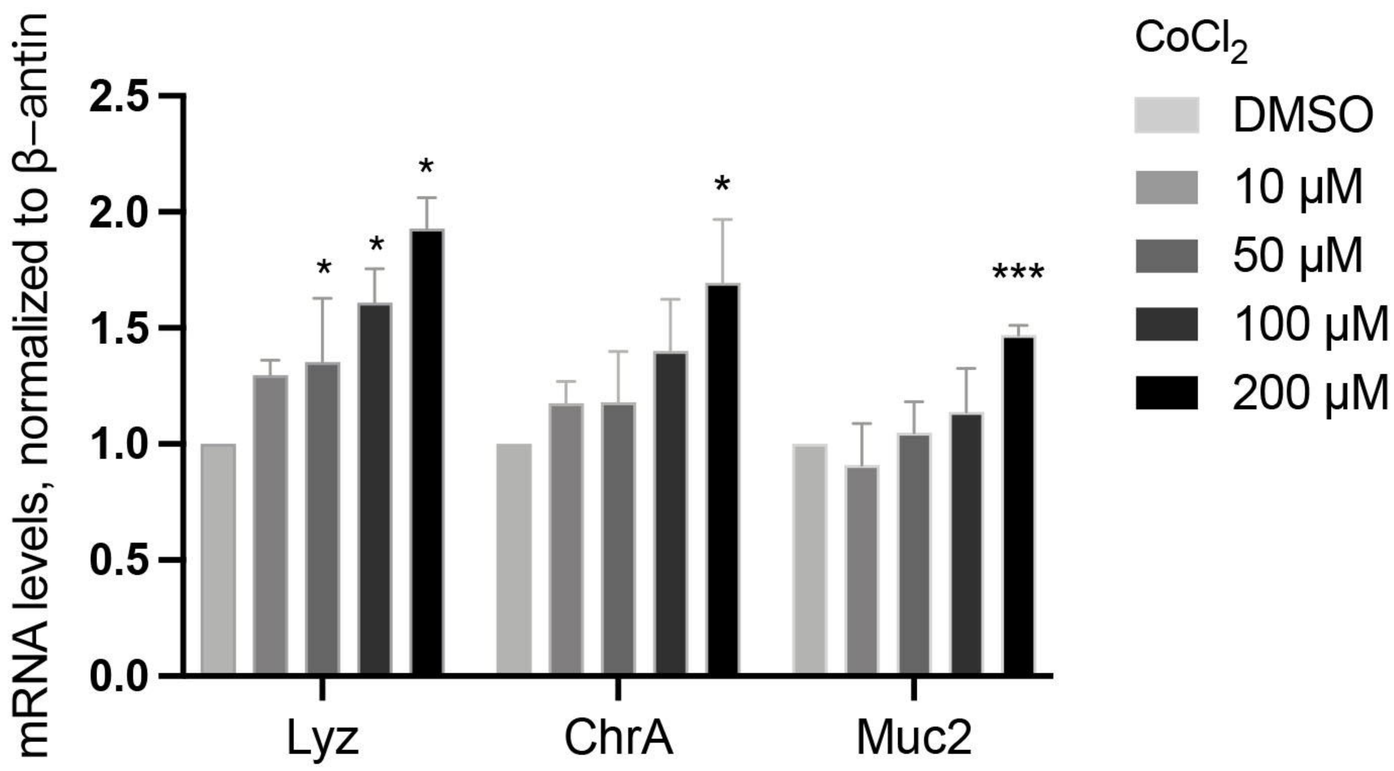
A


Lgr5 / β-actin mRNA levels
1.5
1.0
0.5
0.0
*
CoCl2 (μM)
0
10
50
100
200

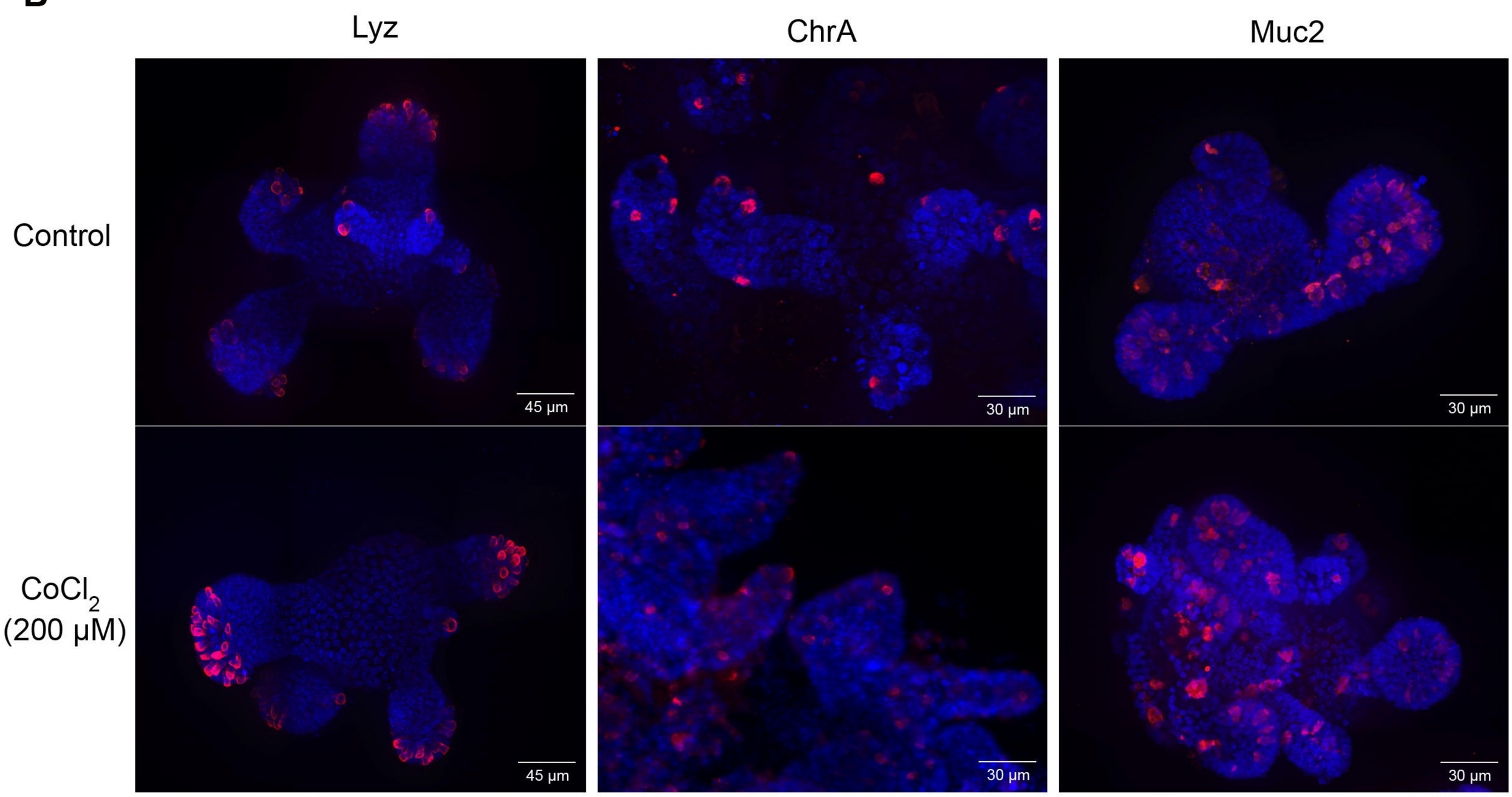
B

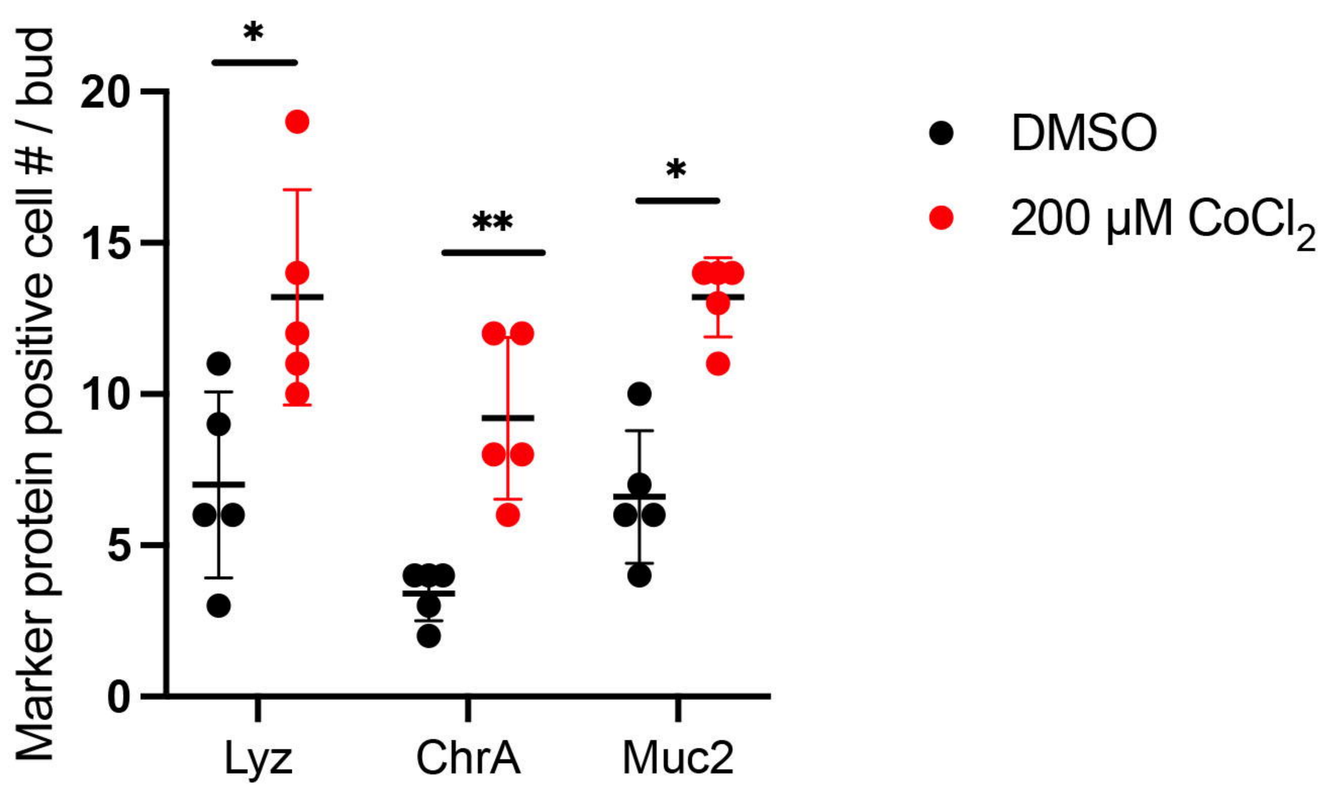

CoCl2 (μM) 0 10 50 100 200
β-catenin
92 kDa
1.0 0.89* 0.74* 0.61* 0.62*
Cyclin-D1
36 kDa
1.0 0.56** 0.24* 0.17* 0.27*
EphB2
130 kDa
1.0 0.84* 0.83* 0.73** 0.69**
Axin2
98 kDa
1.0 0.86* 0.87* 0.86* 0.80*
β-actin
43 kDa

A

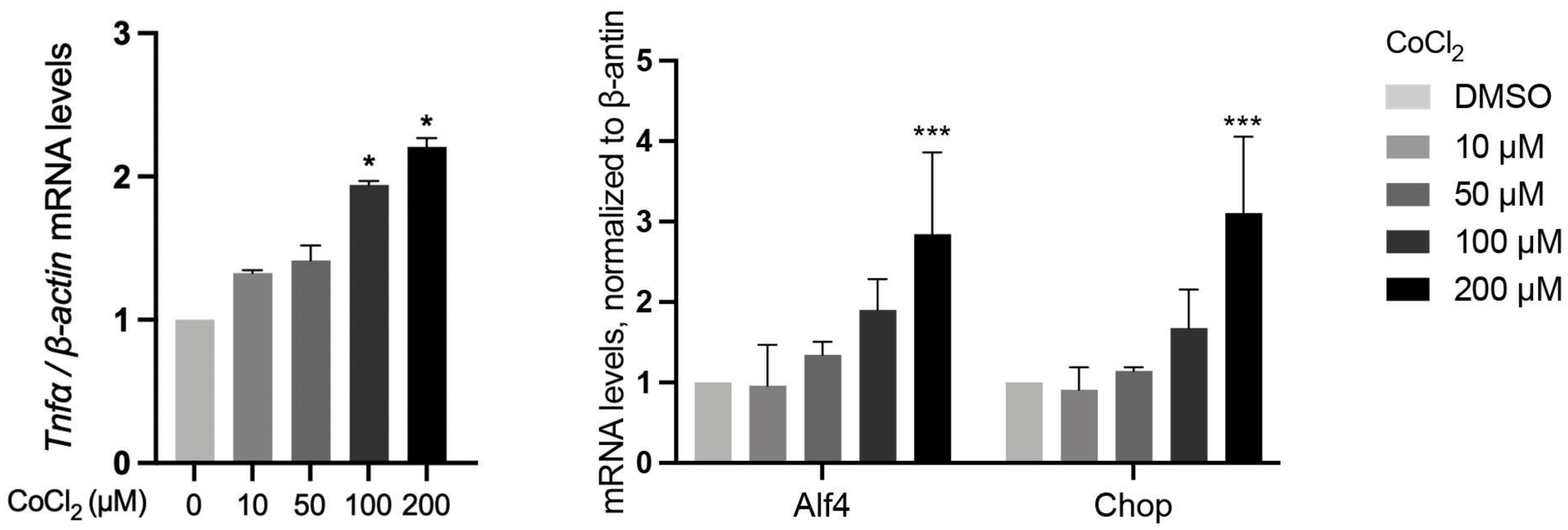

mRNA levels, normalized to β−antin
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
***
Lyz
ChrA
Muc2
CoCl2
DMSO
10 μM
50 μM
100 μM
200 μM

B

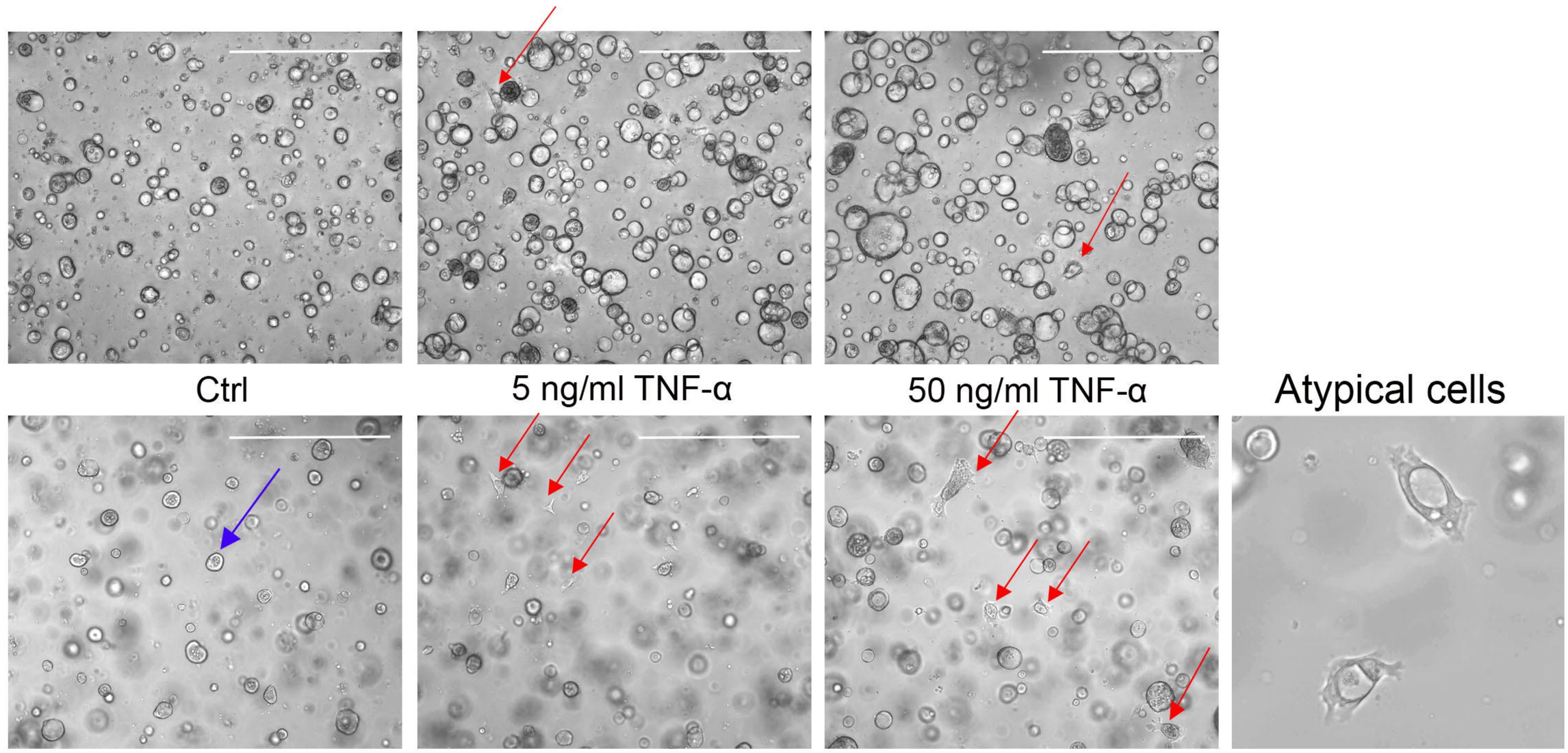

Lyz
ChrA
Muc2
Control
CoCl2
(200 μM)
45 μm
30 μm
30 μm
45 μm
30 μm
30 μm


Marker protein positive cell # / bu
20
15
10
5
0
*
**
*
Lyz
ChrA
Muc2
DMSO
200 μM CoCl2

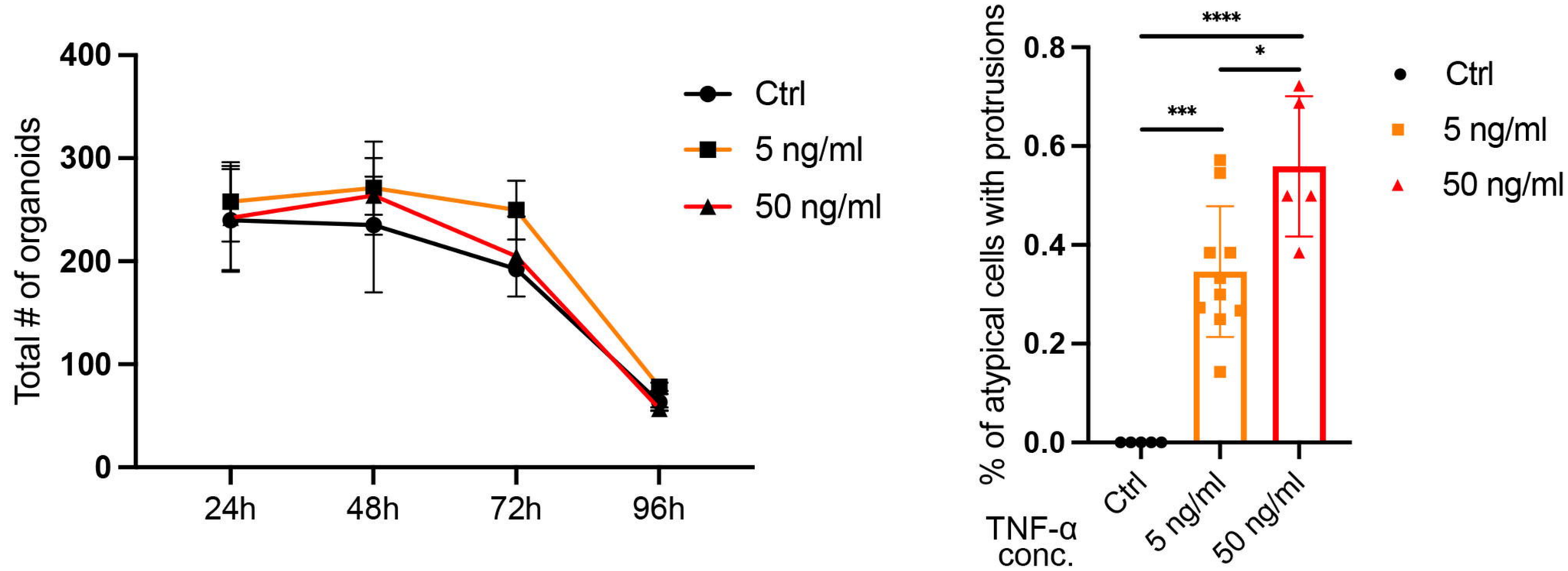
A


Tnfα / β-actin mRNA levels
CoCl2 (μM)
0
10
50
100
200
mRNA levels, normalized to β-antin
Alf4
Chop
CoCl2
DMSO
10 μM
50 μM
100 μM
200 μM

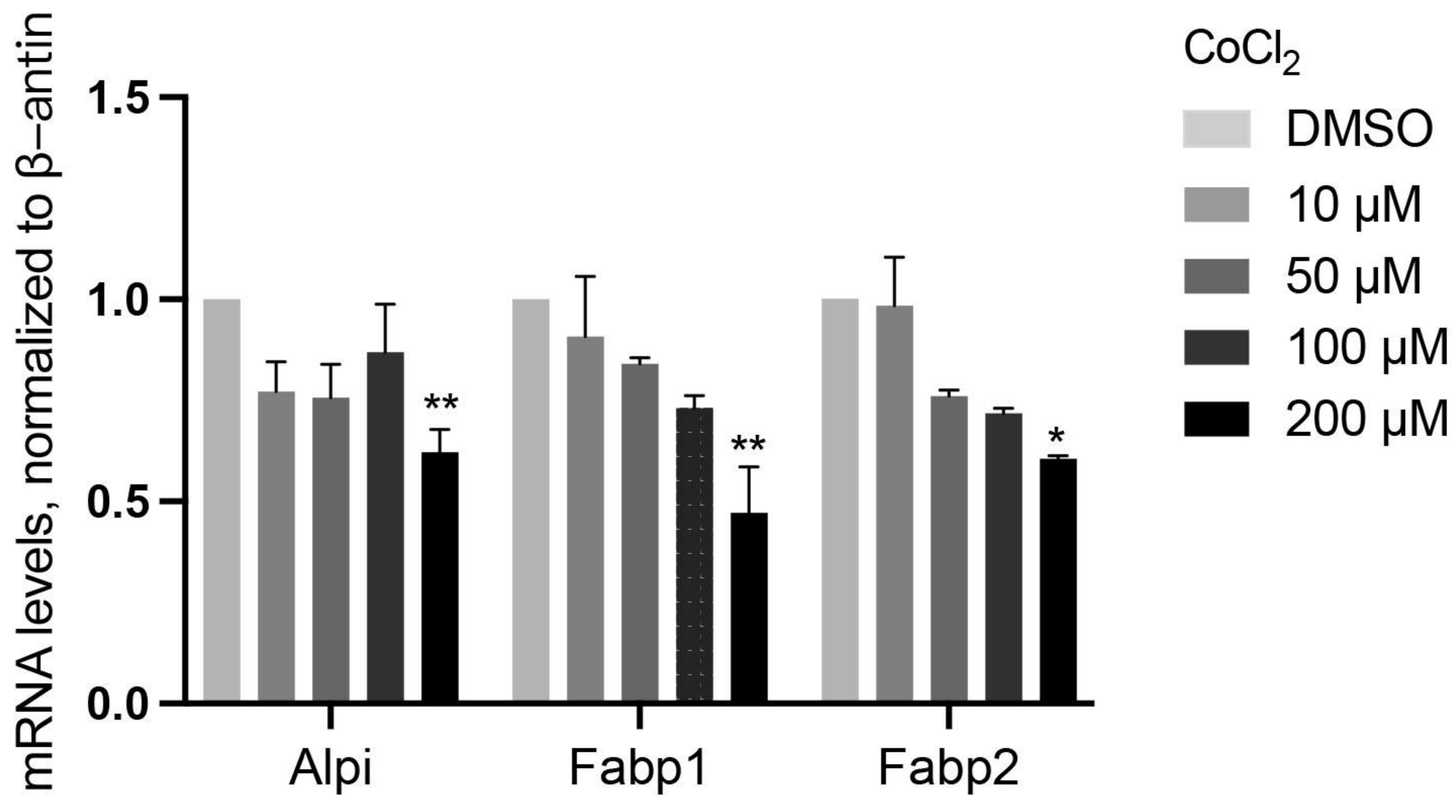
B


Ctrl
5 ng/ml TNF-α
50 ng/ml TNF-α
Atypical cells

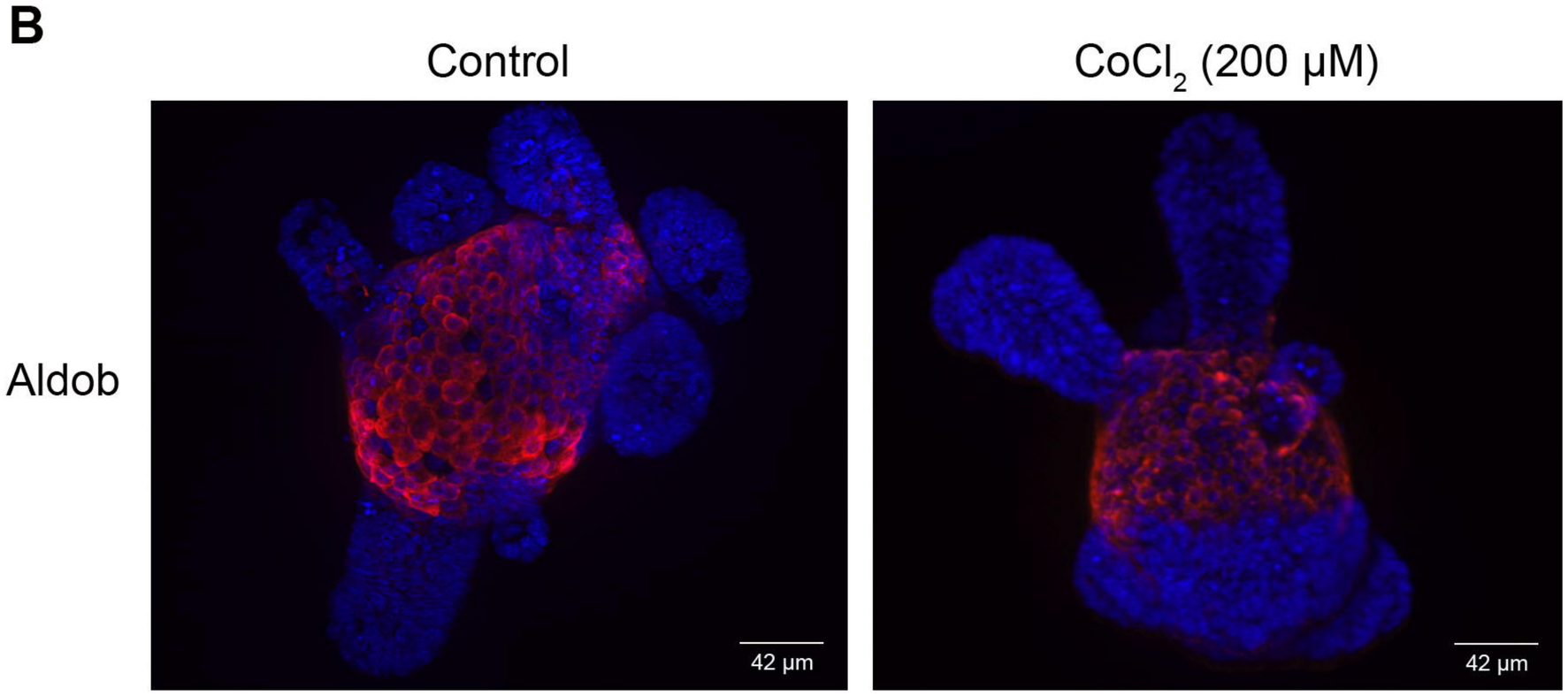
C


Total # of organoids
24h
48h
72h
96h
Ctrl
5 ng/ml
50 ng/ml
% of atypical cells with protrusions
TNF-α conc.
Ctrl
5 ng/ml
50 ng/ml

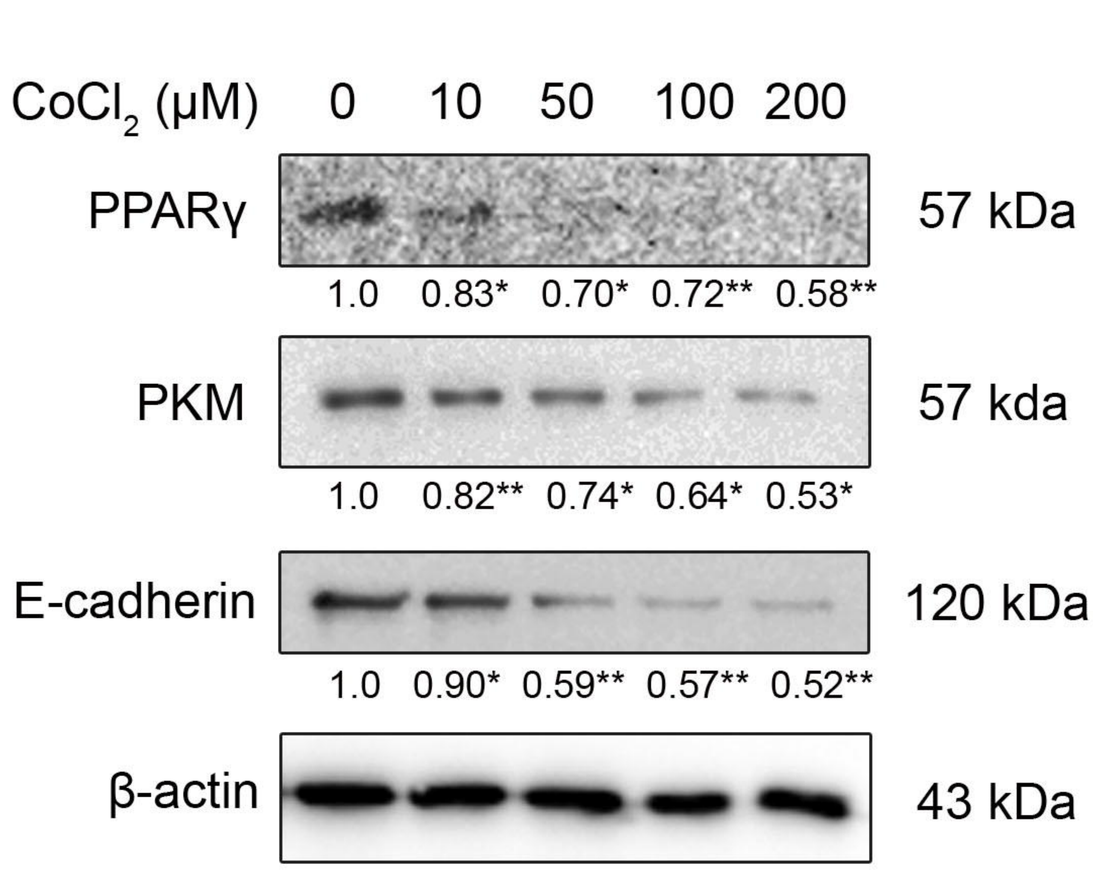
A

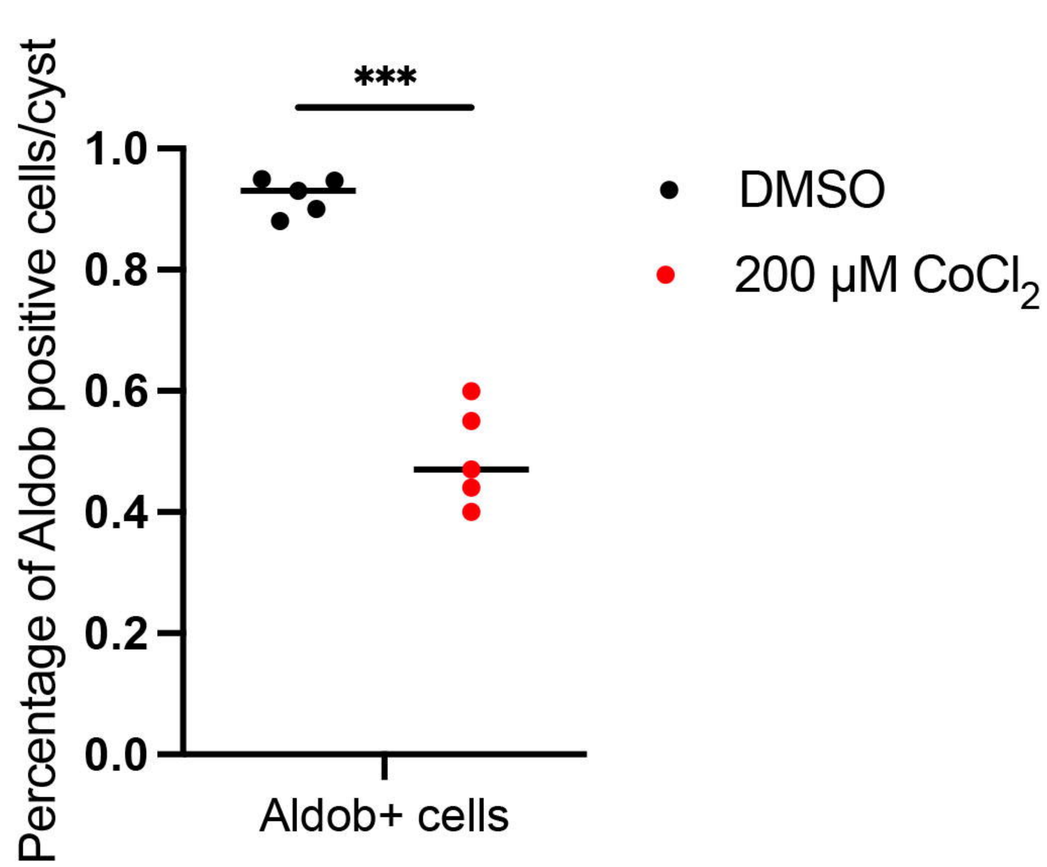

mRNA levels, normalized to β–antin
1.5
1.0
0.5
0.0
**
**
*
Alpi
Fabp1
Fabp2
CoCl2
DMSO
10 μM
50 μM
100 μM
200 μM

B


Control
CoCl2 (200 μM)
Aldob
42 μm
42 μm

C


CoCl2 (μM) 0 10 50 100 200
PPARγ
57 kDa
1.0 0.83* 0.70* 0.72** 0.58**
PKM
57 kda
1.0 0.82** 0.74* 0.64* 0.53*
E-cadherin
120 kDa
1.0 0.90* 0.59** 0.57** 0.52**
β-actin
43 kDa


Percentage of Aldob positive cells/cys
***
1.0
0.8
0.6
0.4
0.2
0.0
Aldob+ cells
DMSO
200 μM CoCl2