Bile acids and bilirubin effects on osteoblastic gene profile.

Implications in the pathogenesis of osteoporosis in liver diseases.

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List of abbreviations:

ALPL, gene encoding the tissue-nonspecific alkaline phosphatase; ATCC, American Type Culture Collection; BGLAP, osteocalcin or bone gamma-carboxyglutamic acid-containing protein; BMPs, Bone Morphogenetic Proteins; CALCR, calcitonin receptor; CASP1, Caspase 1; CASP9, Caspase 9; COL10A1, Collagen X alpha-1; COL15A1, collagen XV alpha-1; COL19A1, Collagen XIX Alpha-1; COL7A1, collagen VII alpha-1; CSF2, colony stimulating factor 2; CYP24A1, 1,25-dihydroxyvitamin D3 24-hydroxylase; DKK1, dickkopf-related protein 1; DMEM, Dubelcco's modified Eagle medium; DSPP, dentin sialophosphoprotein; FBS, fetal bovine serum; HBSS, Hanks Balanced Salt Solution; IGF1, Insulin-like growth factor 1; LCA, lithocholic acid; MGP, matrix Gla protein ; PBC, primary biliary cholangitis; RANKL, receptor activator of nuclear factor-kappaB ligand; RIN, RNA integrity number; RUNX2, runt-related transcription factor 2; Saos-2, human osteosarcoma cells; SD, standard deviation;

SMAD 6, decapentaplegic homolog 6; SP7, osterix transcription factor; SPOCK3, osteonectin; SPP1, osteopontin; TGFB1, Transforming growth factor beta-1; UDCA, Ursodeoxycholic acid.

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Abstract

Osteoporosis in advanced cholestatic and end-stage liver disease is related to low bone formation. Previous studies have demonstrated the deleterious consequences of lithocholic acid (LCA) and bilirubin on osteoblastic cells. These effects are partially or completely neutralized by ursodeoxycholic acid (UDCA). We have assessed the differential gene expression of osteoblastic cells under different culture conditions. The experiments were performed in human osteosarcoma cells (Saos-2) cultured with LCA (10 μM), bilirubin (50 μM) or UDCA (10 and 100 μM) at 2 and 24 hours. Expression of 87 genes related to bone metabolism and other signalling pathways were assessed by TaqMan micro fluidic cards. Several genes were up-regulated by LCA, most of them pro-apoptotic (*BAX*, *BCL10*, *BCL2L13*, *BCL2L14*), but also *MGP* (matrix Gla protein), B*GLAP* (osteocalcin), *SPP1* (osteopontin) and *CYP24A1*, and down-regulated bone morphogenic protein genes (*BMP3* and *BMP4*) and *DKK1* (Dickkopf-related protein 1). Parallel effects were observed with bilirubin, which up-regulated apoptotic genes and *CSF2* (colony-stimulating factor 2) and down-regulated antiapoptotic genes (*BCL2* and *BCL2L1)*, *BMP3*, *BMP4* and RUNX2. UDCA 100 μM had specific consequences since differential expression was observed, up-regulating *BMP2*, *BMP4*, *BMP7*, *CALCR* (calcitonin receptor), *SPOCK3* (osteonectin), *BGLAP* (osteocalcin) and *SPP1* (osteopontin), and down-regulating pro-apoptotic genes. Furthermore, most of the differential expression changes induced by both LCA and bilirubin were partially or completely neutralized by UDCA. *Conclusion*: Our observations reveal novel target genes, whose regulation by retained substances of cholestasis may provide additional insights into the pathogenesis of osteoporosis in cholestatic and end-stage liver diseases.

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Key words: Osteoporosis; Bone turnover; Cholestasis; Bile acids; Primary biliary cholangitis; Osteoblasts.

Introduction

Osteoporosis is a skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to an increased fragility and susceptibility to fracture. It is a common complication of liver diseases, particularly in chronic cholestasis and especially in those patients with primary biliary cholangitis (PBC) [1-4]. Low bone formation as a consequence of a deficient osteoblast activity is the main cause for bone loss [1], but an increased resorption has been described as well [5].

Different studies have found that high concentrations of bilirubin and bile acid can contribute to the abnormal osteoblast function [6], as both bilirubin and lithocholic acid (LCA) in addition to serum from jaundiced patients have detrimental effects on these bone-forming cells [7].

Ursodeoxycholic acid (UDCA), the standard treatment for patients with PBC has greatly changed the natural history of the disease [8-11]. Moreover, in bone cells UDCA increases survival and improves differentiation of human osteoblasts, neutralizing the detrimental effects of LCA and bilirubin on osteoblast survival, differentiation and mineralization [12]. Lastly, while bilirubin and LCA act as pro-apoptotic agents in human osteoblasts, UDCA has anti-apoptotic effects and neutralizes the apoptosis induced by LCA and bilirubin in osteoblastic cells [12].

In previous studies a down-regulation of RUNX2 gene by bilirubin and an up-regulation of the RANKL/OPG expression ratio by jaundiced sera in osteoblastic cells were demonstrated [7]. However, more data on the influence of the retained substances of cholestasis in osteoblastic cell gene expression is lacking. To gain new insights into cholestatic-induced osteoporosis, we have assessed whether the damaging effects of the

retained substances such as bilirubin and bile acids can modify the gene expression profiling of osteoblastic cells.

Materials and methods

Materials

Dubelcco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Hanks Balanced Salt Solution (HBSS), L-glutamine and trypsin were purchased from Invitrogen (Grand Island, NY, USA); LCA, UDCA and bilirubin were from Sigma Chemical Co. (St. Louis, MO, USA); Penicillin-streptomicin was from LabClinics (Barcelona, Spain).

Cell culture and incubation

The experiments were performed with human osteosarcoma cell line Saos-2. The cell line Saos-2 was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) (HTB85; ATCC) and cultured as a monolayer in DMEM containing 10% FCS, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO^2 in air.

Administrated treatments

Saos-2 cells were cultured for 2 and 24 h in different conditions: (a) LCA (10 μM), bilirubin (50 μM) and UDCA 10 μM and 100 μM. (b) To analyze the interaction of UDCA with LCA and bilirubin, cells were incubated with a steady concentration of LCA (10 μM) or bilirubin (50 μM) and two concentrations of UDCA (10 μM and 100 μM).

All treatment concentrations used were selected based on the cytotoxicity assays carried out in previous studies [7,13].

Experimental bilirubin solution preparation

Bilirubin (Sigma) stock solution of 1600 μM was prepared just before use by dissolving bilirubin in 10 ml 0.01N NaOH under dim light as previously described [12,15-18]. It was filtered through a sterile filter (0.22 μm pore size) and adjusted to pH 7.2-7.4 with 0.1N HCl, if necessary. The bilirubin stock solution was added to a final concentration of 50 μM in the culture medium. The cell cultures were kept in dark conditions to prevent bilirubin light degradation. Control cells were treated with vehicle (NaOH 0.1N).

RNA isolation and quantification

Total cellular RNA was extracted from cultured cells using an acid guanidinium-phenol-chloroform method (Trizol reagent; Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocols. RNA integrity was determined by a microfluidics-based electrophoresis system using a 2100 Bioanalyzer (Agilent Techologies, Palo Alto, CA). RNA integrity number (RIN) from automated analysis software allows classification of RNA in a numeric system with one for complete degradation and ten for optimal intactness. Both analyses displayed highly intact RNA, with RIN values of 9.8–10.

Expression analysis with TaqMan microfluidic cards

cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with a Master Mix containing 2.5 U/μl of MultiScribe Reverse Transcriptase and 1 μg of total RNA. The reaction mixture was incubated at 25˚C for 10 min, followed by 120 min at 37˚C and then by heat inactivation

of the enzyme at 85˚C for 5 sec. Next, we mixed 2 µl of single-stranded cDNA (equivalent to around 100 ng of total RNA) with 48 µl of nuclease-free water and 50 µl of TaqMan Universal PCR Master Mix. After loading 100 µl of the sample-specific PCR mixture into one sample port of the microfluidic cards (Human ABC Transporter Panel; Applied Biosystems), the cards were centrifuged twice for 1 min at 280g and sealed to prevent well-to-well contamination. The cards were placed in the microfluidic card Sample Block of an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The thermal cycling conditions were 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles of 30 sec at 97˚C and 1 min at 59.7˚C. The assay for each gene on the microfluidic card was carried out in triplicate, due to the design of this specific panel. The calculation of the threshold cycle (Ct) values were performed using the SDS 2.2 software (Applied Biosystems), after automatically setting the baseline and the threshold.

Gene selection

A total of eighty-seven genes were selected to investigate the profile gene expression under bilirubin, LCA and UDCA treatment. Genes were chosen according to their relevant function in cellular processes and signaling pathways related to bone metabolism (Table 1).

Statistics

Significant differences between two groups were determined by Student's t-test or Mann–Whitney U-test. When multiple groups were compared, ANOVA was utilized, followed by a Tukey's multiple contrast test, when applicable. A p-value ≤ 0.05 was considered significant. All analyses were performed using the PASW Statistics 20 (SPSS, Chicago, IL, USA).

Results

Effect of LCA and bilirubin on gene profiles

As compared with controls the effects of LCA at 10µM and bilirubin at 50µM were observed after 2 hours of treatment, but frequently were more apparent after 24 hours. The most relevant results are shown in figures 1A and 1B.

The apoptosis-related genes were the ones most affected after treatments with LCA and bilirubin, resulting in severe gene expression changes. LCA and bilirubin significantly up-regulated the expression of some pro-apoptotic genes and down-regulated some anti-apoptotic genes ($p < 0.05$). Caspase 1 (*CASP1*) was overexpressed under LCA treatment and bilirubin decreased the expression of caspase 9 (CASP9).

When assessing bone morphogenetic proteins (BMPs), both LCA and bilirubin diminished the expression of some of these genes, mainly *BMP3* and *BMP4* (Figures 1A and 1B). These effects were observed after just two hours of treatment and were more evident under LCA treatment (figure 2).

The expression of collagen X alpha-1 (*COL10A1*), collagen XIX alpha-1 (*COL19A1*) and collagen VII alpha-1 (*COL7A1*) was down-regulated by bilirubin after 2 hours of treatment. However, collagen XV alpha-1 (*COL15A1*) was up-regulated after 24 hours. No significant changes were observed with LCA on the different evaluated collagen genes.

The analysis of selected osteogenic genes displayed substantial overexpression of osteocalcin or bone gamma-carboxyglutamic acid-containing protein (*BGLAP)* and osteopontin (SPP1) ($p<0.001$) under LCA treatment. Moreover, a significant decrease of

alkaline phosphatase expression *(ALPL)* was found under LCA treatment ($p<0.001$) and under bilirubin treatment at 24 hours ($p<0.05$).

Regarding the specific transcription factors, SP7 (osterix transcription factor) expression was down-regulated after 24 hours under LCA treatment, while it was up-regulated after 24 hours with bilirubin treatment. The runt-related transcription factor 2 (RUNX2) was down-regulated by bilirubin. This effect was observed at 2 and 24 hours.

With respect to genes involved in osteoclast differentiation and bone resorption, LCA up-regulated the receptor activator of nuclear factor-kappaB ligand (*RANKL*) and the calcitonin receptor (*CALCR)* after 2 hours. This effect was not evident at 24 hours. Similarly, LCA induced a constant overexpression of the matrix Gla protein (*MGP*). No significant effects of bilirubin were observed in the expression of these genes. Conversely, the colony stimulating factor 2 (*CSF2*) ($p<0.001$) was significantly down-regulated after 2 hours under LCA, but upregulated by bilirubin at 24 hours.

Within the growth factor family, LCA increased Insulin-like growth factor 1 (*IGF1*) expression levels after 2 hours, and bilirubin significantly increased transforming growth factor beta-1 (*TGFB1*) after 24 hours ($p=0.03$).

LCA significantly up-regulated 1,25-dihydroxyvitamin D_3 24-hydroxylase (*CYP24A1)* gene expression, an effect which was observed from the first two hours and sustained throughout of the experiment ($p<0.001$)

Finally, expression levels of both dentin sialophosphoprotein (*DSPP*) ($p<0.01$) and decapentaplegic homolog 6 (*SMAD 6*) ($p<0.02$) were down-regulated after 2 hours under bilirubin treatment, whereas LCA produced the same effect on the Wnt signaling pathway by down-regulating the dickkopf-related protein 1 (*DKK1*) gene expression. This effect was observed after 2 hours of treatment and was much higher at 24 hours ($p=0.01$).

Effect of UDCA and interaction with LCA and bilirubin on gene profiles

As compared with controls, UDCA has a significant effect, mainly on three big family genes: apoptosis (figure 3A), bone morphogenetic proteins (BMPs) (figures 2 and 3B) and bone specific genes (osteoblastic transcription factors and specific osteogenic factors) (figure 3C). UDCA resulted in specific gene expression changes by itself or by modifying the effects of LCA and bilirubin. Accordingly, UDCA 10μM and 100μM diminished the gene expression of pro-apoptotic genes *BAX*, *BCL10*, *BCL2L13*, *BCL2L14* and *BCL3*, and counteracted both the LCA and bilirubin effects. UDCA increased the expression of the anti-apoptotic genes *BCL2*, *BCL2A1*, *BCL2L1* and *BCL2L2*, being significant for *BCL2A1* ($p=0.03$). Moreover, this UDCA anti-apoptotic function neutralized the LCA and bilirubin effects by abolishing the significant decrease produced by them on these anti-apoptotic genes ($p<0.03$). No significant changes were found in the expression of caspase family genes under UDCA (figure 3A).

UDCA significantly increased *BMP2*, *BMP3*, *BMP4* and *BMP7* gene expression from 2 hours of treatment at 10μM and 100μM and neutralized the down-regulation induced by LCA and bilirubin on BMPs expression ($p<0.001$) (Figures 2 and 3B).

Furthermore, UDCA significantly increased gene expression of the bone resorption marker CALCR and of the osteoblastic specific bone markers *BGLAP*, *SPOCK3* and *SPP1*, when cells were treated with UDCA (10μM or 100μM) or when cells were treated at the same time with 10μM LCA or 50μM bilirubin (Figure 3C).

Discussion

The effects of substances retained in chronic cholestasis on bone cells have been previously described, suggesting direct harmful effects of bilirubin and LCA on human osteoblastic cells. Janes at al. observed that plasma with high concentrations of bilirubin resulted in decreased human osteoblast-like cells proliferation [19]. Similarly, bilirubin induced a marked dose-dependent inhibition of avian chondrocytes proliferation [20]. Previous studies from our group have shown a decrease of human osteoblastic cells viability and differentiation when bilirubin and sera from jaundiced patients was added to the culture media [7]. Also, an increase in apoptosis was observed in both bilirubin and LCA-treated osteoblastic cells [24]. However, there are scarce data on the targets of these substances in osteoblasts [7,24]. The current study provides new insights in the effects of bilirubin and LCA on the expression of relevant groups of genes, as well as on the involved molecular pathways.

The essential impact of bilirubin on the expression of genes involved in apoptosis in human osteoblasts is confirmed in this study. It should be noted that bilirubin at 50 µM dramatically increases the pro-apoptotic related genes and decreases the anti-apoptotic genes. Both phenomena occur after only few hours of treatment, suggesting that bilirubin could play a major role in the regulation of the apoptotic related genes. This observation leads us to confirm our earlier observations about the pro-apoptotic role of bilirubin on osteoblastic cells [24] and other cell types and tissues [15-16,25,26]. Likewise, the relationship between LCA and apoptosis in different tissues and cell types has been widely demonstrated. Previous studies observed LCA-induced apoptosis mediated by the nuclear receptor Nur77 expression in both human liver and colon cancer cells as well as in mouse hepatocytes [27]. LCA has also a pro-apoptotic effect in human colon adenocarcinoma cell lines [28], in human neuroblastoma cells [29] and in cultured

274 syncytiotrophoblast cells [30]. Finally, LCA induces apoptosis in human osteoblasts,
275 increasing DNA fragmentation, caspase-3 activity and producing an up-regulation and a
276 down-regulation of *BAX* and *BCL2* respectively [24]. Accordingly, the current study
277 describes the apoptotic action of LCA through the increase of pro-apoptotic and the
278 decrease of anti-apoptotic genes.
279
280 A new finding described in this study is the relationship between BMPs gene expression
281 under the effects of LCA, bilirubin and UDCA (figures 2 and 3B). BMPs are members of
282 the transforming growth factor-β (TGF-β) superfamily and a subset of BMPs possess the
283 ability to induce bone and cartilage formation and enhance osteogenesis and fracture
284 healing [31-33]. BMPs are extracellular cytokines, originally isolated from bone extract
285 and are produced in nearly all skeletal cells [34,35]. Accordingly, *BMP2* vastly increases
286 osteocalcin, and a short-term expression of *BMP2* is necessary and sufficient to
287 irreversibly induce bone formation [36,37]. Additionally, *BMP7* accelerates calcium
288 mineralization and induces the expression of osteoblastic differentiation markers such as
289 ALP activity [38,39], and loss of both BMP2 and BMP4 results in severe impairment of
290 osteogenesis [40]. The decrease of *BMP3* and *BMP4* expression observed in our study
291 under LCA and bilirubin treatments open up new approaches to illustrate their mechanism
292 of action. On the other hand, the current study clearly demonstrates that UDCA causes an
293 important up-regulation of *BMP2*, *BMP3*, *BMP4* and *BMP7* genes, and in addition
294 counteracts the effects of LCA and bilirubin induced down-regulation of these genes.
295 The runt-related transcriptional factor 2 (RUNX2) [41] is the key transcription factor
296 involved in osteoblasts differentiation under the guidance of BMPs signaling, since its
297 expression can be induced by both *BMP2* and *BMP7* [42]. The increased expression of

298 BMPs under UDCA treatment leads us to relate it with the expression levels of the
299 transcriptional factor RUNX2, which are down-regulated after bilirubin 50μM
300 administration on cells treated for a short time and maintained steadily for 24 hours. These
301 results are consistent with those published in previous studies, in which a down-regulation
302 of RUNX2 with a consequent decrease in osteoblast differentiation was produced by the
303 cell exposure to 50μM of bilirubin [7] and under low concentrations of bilirubin (3 and
304 30μM) in rat osteoblasts primary culture with osteogenic medium for 3 or 14 days [43].
305 The potential beneficial effects of UDCA on bone cells may be partially explained the
306 BMPs up-regulation, which in turn induces RUNX2 expression. These effects were not
307 clearly observed in these experiments, although UDCA partially attenuates the RUNX2
308 down-regulation induced by bilirubin.

309 Despite the fact that new insights into gene profiling induced by bilirubin, LCA and
310 UDCA have been described in this study, some limitations should be taken into account.
311 The main concern is that the experiments were carried-out using the human osteosarcoma
312 cell line Saos-2, which although it is most similar cell line to human primary osteoblasts,
313 its behavior could be different. Our next approach will then be to check the gene
314 expression of the most relevant results of our current study in primary human cultures,
315 particularly those related to BMPs and some transcription factors such as RUNX2.

316 In summary, the current study shows that accumulated products of cholestasis decrease
317 the expression of some BMPs, which are mostly strong osteogenic agents and synergize
318 with osteogenic transcriptional factors such as RUNX2, that was down-expressed as well.
319 Furthermore, the addition of biliary acids and bilirubin up-regulated pro-apoptotic and
320 down-regulated anti-apoptotic genes. These changes in the apoptosis pathways involving
321 the Saos-2 cells could modify the rate of bone formation and, therefore, be considered as

322 a truthful pathogenic mechanism of osteoporosis in cholestatic and in end-stage liver
323 diseases.

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458 Table 1. Selected genes.

Apoptosis	**Proapoptotics**	
	BAX	BCL2 Associated X, Apoptosis Regulator
	BCL10	BCL10, Immune Signaling Adaptor
	BCL2L13	BCL2 Like 13
	BCL2L14	BCL2 Like 14
	BCL3	BCL3, Transcription Coactivator
	Antiapoptotics	
	BCL2	BCL2, Apoptosis Regulator
	BCL2A1	BCL2 Related Protein A1
	BCL2L1	BCL2 Like 1
	BCL2L2	BCL2 Like 2
	Caspases	
	CASP1	Caspase 1
	CASP3	Caspase 3
	CASP5	Caspase 5
	CASP6	Caspase 6
	CASP9	Caspase 9
Transcription factors	MSX1	Msh Homeobox 1
	MSX2	Msh Homeobox 2
	RUNX2	Runt Related Transcription Factor 2
	SP7	Sp7 Transcription Factor
BMPs	BMP1	Bone Morphogenetic Protein 1
	BMP2	Bone Morphogenetic Protein 2
	BMP3	Bone Morphogenetic Protein 3
	BMP4	Bone Morphogenetic Protein 4
	BMP5	Bone Morphogenetic Protein 5
	BMP7	Bone Morphogenetic Protein 7
	BMPR1A	Bone Morphogenetic Protein Receptor Type 1A
Osteogenics	ALPL	Alkaline Phosphatase, Biomineralization Associated
	BGLAP	Bone Gamma-Carboxyglutamate Protein
	CDH11	Cadherin 11
	SPOCK	SPARC (Osteonectin), Cwcv And Kazal Like Domains Proteoglycan 1
	SPP1	Secreted Phosphoprotein 1
Osteoclast differentiation	CSF	Colony Stimulating Factor 1
	CSF2	Colony Stimulating Factor 1
	CSF3	Colony Stimulating Factor 3
Bone resorption	CALCR	Calcitonin Receptor
	PTHR1	Parathyroid Hormone 1 Receptor
	OPG	TNF Receptor Superfamily Member 11b (osteoprotegerin)
	RANKL	TNF Superfamily Member 11
Mineralization	DMP1	Dentin Matrix Acidic Phosphoprotein 1
	DSPP	Dentin Sialophosphoprotein
	MGP	Matrix Gla Protein
Collagen	COL1A2	Collagen Type I Alpha 2 Chain
	COL2A1	Collagen Type II Alpha 1 Chain
	COL4A1	Collagen Type IV Alpha 1 Chain
	COL4A4	Collagen Type IV Alpha 4 Chain
	COL7A1	Collagen Type VII Alpha 1 Chain
	COL10A1	Collagen Type X Alpha 1 Chain
	COL11A1	Collagen Type XI Alpha 1 Chain
	COL15A1	Collagen Type XIV Alpha 1 Chain
	COL17A1	Collagen Type XVII Alpha 1 Chain
	COL19A1	Collagen Type XIX Alpha 1 Chain
Growth factors	EGFR	Epidermal Growth Factor Receptor
	FGF1	Fibroblast Growth Factor 1
	FGF2	Fibroblast Growth Factor 2
	FGFR2	Fibroblast Growth Factor Receptor 2
	IGF1	Insulin Like Growth Factor 1
	IGF2	Insulin Like Growth Factor 2
	IRS1	Insulin Receptor Substrate 1
	TGFB1	Transforming Growth Factor Beta 1
MAPKs	MAPK8	Mitogen-Activated Protein Kinase 8
Vitamin D metabolism	CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1

	CYP27B1	Cytochrome P450 Family 27 Subfamily B Member 1
	VDR	Vitamin D Receptor
Wnt pathway	DKK1	Dickkopf WNT Signaling Pathway Inhibitor 1
	LRP5	LDL Receptor Related Protein 5
	SOST	Sclerostin
Metaloproteinases	MMP2	Matrix Metallopeptidase 2
	MMP8	Matrix Metallopeptidase 8
	MMP10	Matrix Metallopeptidase 10
	MMP13	Matrix Metallopeptidase 13
SMADs	SMAD1	SMAD Family Member 1
	SMAD3	SMAD Family Member 3
	SMAD6	SMAD Family Member 6
	SMAD7	SMAD Family Member 7
Coagulation	PRLR	Prolactin Receptor
Glucolysis	HK2	Hexokinase 2
Cell Cycle	CDK2	Cyclin Dependent Kinase 2
	CDK4	Cyclin Dependent Kinase 4
Oncogens	BRCA1	BRCA1, DNA Repair Associated
	CDH11	Cadherin 11
	FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
	MYC	MYC Proto-Oncogene, BHLH Transcription Factor
	TP53	Tumor Protein P53
Embrionic development	HOXA1	Homeobox A1
Vascular	VEGFA	Vascular Endothelial Growth Factor A
	VEGFB	Vascular Endothelial Growth Factor B
	VEGFC	Vascular Endothelial Growth Factor C

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Figure legends

FIG. 1. Heat map of the effects of litocholic acid (A) and bilirubin (B) on gene expression profiles in Saos-2 osteoblastic cells. The red colour corresponds to genes that are up-regulated, and green colour corresponds to genes that are down-regulated as compared to controls without LCA or bilirubin. * indicates significant differences vs non-treated cells.

FIG. 2. Percentage changes in bone morphogenetic protein (BMP3, BMP4 and BMP7) gene expression at 2 hours (dark grey bars) and 24 hours (light grey bars), in different culture conditions (LCA, Bilirubin, and UDCA) and the effect of UDCA on cells treated with LCA or bilirubin. (*$p<0.001$ vs controls; ^$p<0.01$ vs controls; ~$p<0.05$ vs controls; # $p<0.001$ vs UDCA treated cells; @ $p<0.01$ vs UDCA treated cells).

FIG. 3. Heat map of the differential gene expression induced by UDCA and neutralizing effects on osteoblastic cells cultured with lithocholic acid (LCA) and bilirubin. A) UDCA apoptotic profile; B) UDCA BMPs profile, and C) UDCA transcription factors and osteogenic markers profiles.

The red colour corresponds to genes that are up-regulated, and green colour corresponds to genes that are down-regulated as compared to controls. #indicates significant differences with respect to non-treated cells. In the experiments with lithocholic acid (LCA) or bilirubin # significant differences of UDCA treated cells with respect to LCA or bilirubin treated cells, or with respect to controls.

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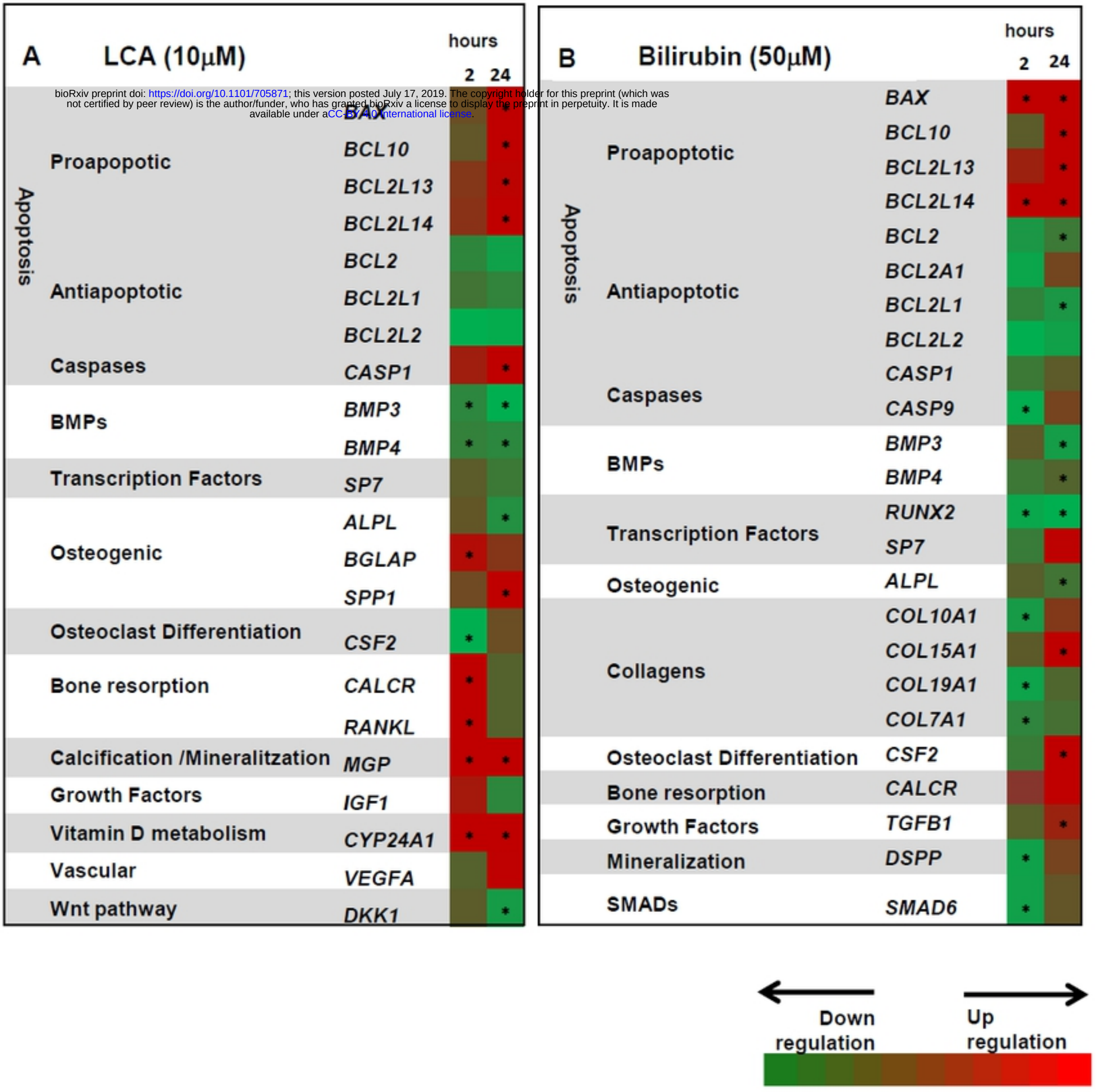

Figure 1

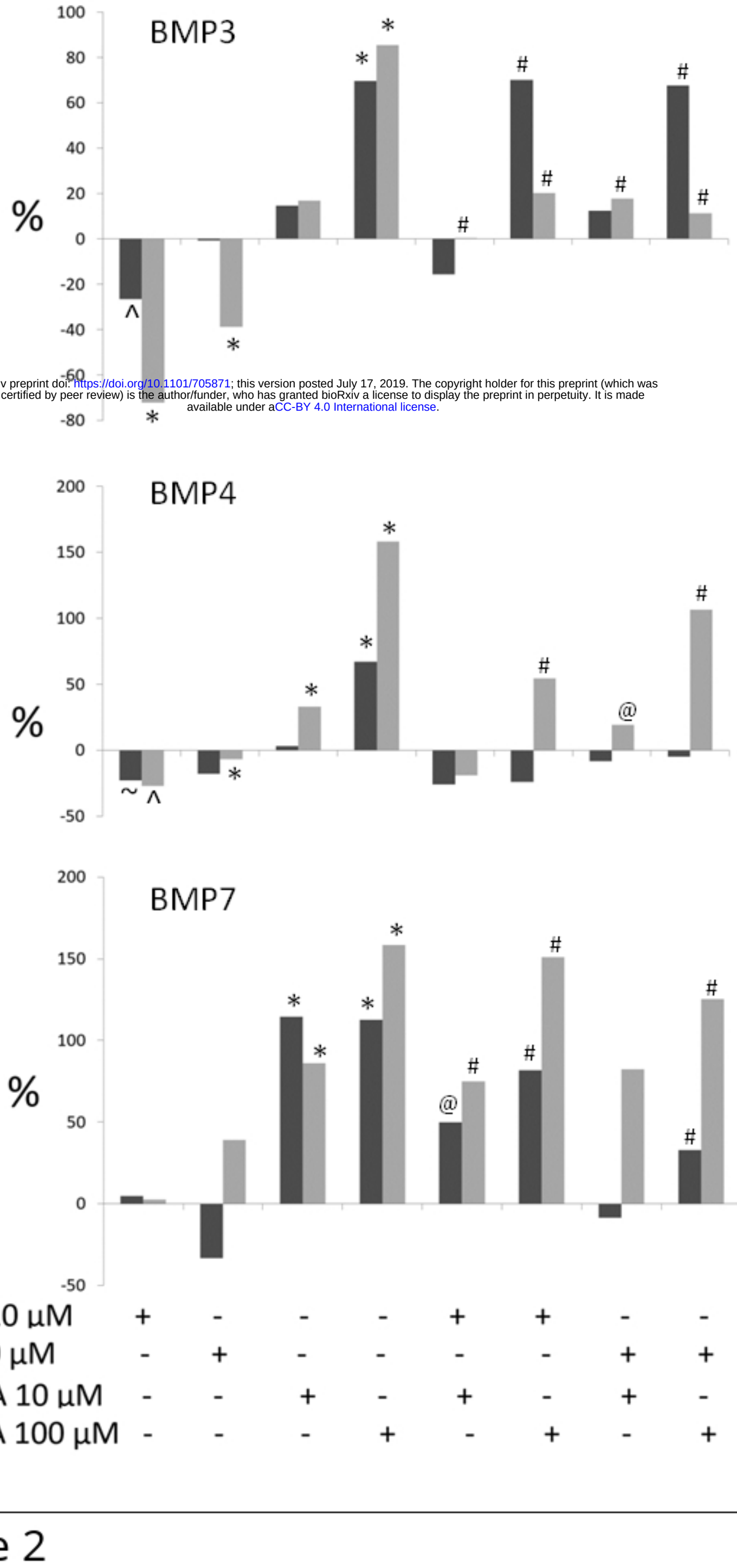

Figure 2

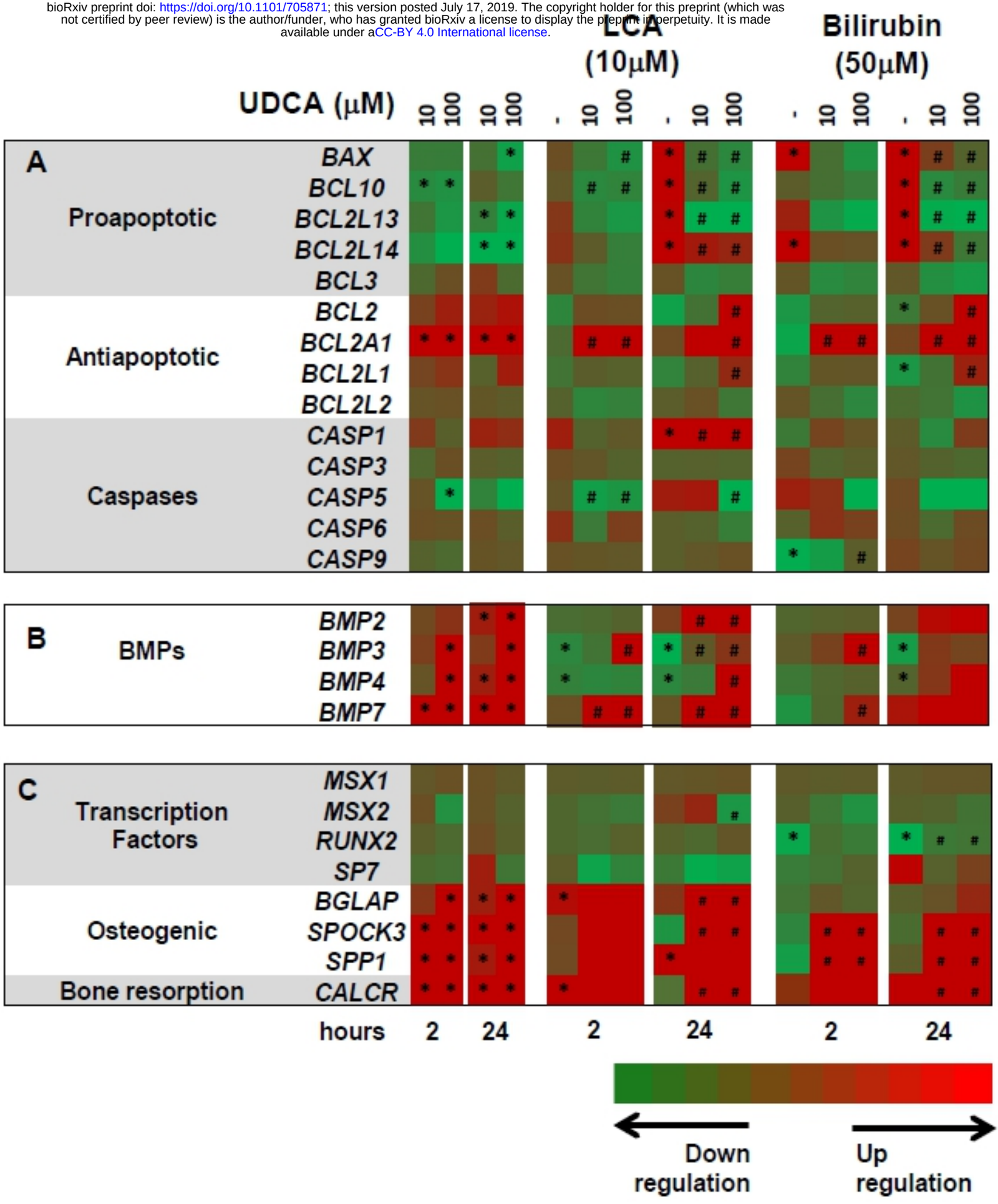

Figure 3