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**ABSTRACT**

***Aim:***Primary biliary cirrhosis (PBC) is characterized by antimitochondrial antibody (AMA) against the pyruvate dehydrogenase complex (PDC) and chronic nonsuppurative destructive cholangitis (CNSDC). Pyruvate oxidation to acetyl-CoA by PDC is a key step in the glycolytic system. Estrogen-related receptor-α (ERRα) is functionally activated by inducible coactivators such as peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and Bcl-3. Moreover, the PGC-1α/ERRα axis interrupts glycolytic metabolism through the upregulation of pyruvate dehydrogenase kinase, isozyme 4 (PDK4), which functionally inhibits PDC-E1α and stimulates fatty acid oxidation. In this study, we investigated the PGC-1α/ERRα axis to clarify PDC dysfunction in CNSDC of PBC. ***Methods***:The expression of PGC-1α, Bcl-3, ERRα, PDK4, and PDC-E1α was examined by immunohistochemistry in liver sections from patients with PBC and controls. The expression of these molecules, the activity of mitochondrial dehydrogenase and PDC, and their alterations by starvation, a treatment used to induce PGC-1α expression, were examined in cultured human biliary epithelial cells (BECs). ***Results***:The nuclear expression of PGC-1α, Bcl-3, and ERRα was exclusively observed in CNSDC of PBC. Moreover, the expression of PDK4 and PDC-E1α was enhanced in CNSDC of PBC. In cultured BECs, the amplification of Bcl-3 and PDK4 mRNAs by RT-PCR and mitochondrial dehydrogenase activity were markedly increased but PDC activity was decreased according to the upregulation of PGC-1α. ***Conclusion***:In CNSDC of PBC, the activation of the ERRα/PGC-1α axis was exclusively observed, suggesting the interference of PDC-related glycolytic function and the induction of the fatty acid degradation system. The switching of the cellular energy system is possibly associated with the pathogenesis of CNSDC in PBC.

**INTRODUCTION**

Primary biliary cirrhosis (PBC) is a chronic autoimmune liver disease characterized by progressive inflammatory destruction and the disappearance of small intrahepatic bile ducts.[1,2] The most important serological feature of PBC is the presence of antimitochondrial antibodies (AMAs), which are detected in more than 95% of the PBC patients.[2] The mitochondrial components recognized by AMAs have been identified as distinct subunits of the mitochondrial 2-oxoacid dehydrogenase complexes (2-OADCs) consisting of the pyruvate dehydrogenase (PDH) complex (PDC), oxoglutaric dehydrogenase complex (OGDC), and branched-chain ketoacid dehydrogenase complex (BCKD). These complexes loosely adhere to the inner mitochondrial membrane and consist of multiple copies of the E1, E2, and E3 subunits. Moreover, the E1 subunit of PDC exists as 2 forms (E1α and E1β). The dominant reactivity of AMA is against the dihydrolipoamide acetyltransferase component (E2) of PDC.[3] PDH catalyzes the conversion of pyruvate to acetyl-CoA, and it is an important control point in glucose and pyruvate metabolism in glycolytic metabolism.

PBC primarily affects middle-aged women, and the interlobular bile ducts are selectively damaged.[1,2] Although bile ducts have not been identified as a target organ of hormone regulation, several hormonal factors have been suggested to play important roles in the pathogenesis of PBC.[4] The estrogen-related receptor α (ERRα) is the constitutively active nuclear hormone receptor that inhibits estrogen receptor (ER)-dependent effects through competition with ERα.[5] ERRα is associated with mitochondrial fatty acid oxidation (β-oxidation), which includes electron transport, oxidative phosphorylation, and mitochondrial biogenesis, and it plays critical roles in the regulation of cellular energy metabolism.[6] Moreover, ERRα is functionally modulated by inducible coactivators such as the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and Bcl-3, a cytokine-stimulated transcriptional regulator that synergizes with PGC-1α to coactivate ERRα.[7,8] Therefore, the PGC-1α/ERRα axis induces fatty acid oxidation but simultaneously interrupts the glycolytic system through the upregulation of pyruvate dehydrogenase kinase, isozyme 4 (PDK4), which binds to the E2 inner lipoyl domain (corresponding to the minimal T-cell epitope) for its

catalytic function and functionally inhibits PDC-E1α by phosphorylation.[9-12]

Although PBC is characterized by the presence of AMA against major autoantigens such as PDC, cellular energy metabolism that involves PDH, such as the conversion of pyruvate to acetyl-CoA in the glycolytic system, has not been observed in the bile ducts of PBC. In this study, we investigated the PGC-1α/ERRα axis to clarify the association of cellular energy metabolism in the pathogenesis of cholangiopathy in PBC.

## MATERIALS and METHODS

### *Patients and preparation of liver tissue*

All tissue specimens were collected from the hepatobiliary file of our department. A total of 69 needle liver specimens were obtained from 26 patients with PBC (histological stage[13] I/II/III, 12/10/4; mean age, 57 years; male/female, 3/23) and controls, which included 16 patients with hepatitis C virus-related chronic hepatitis (CH-C), 5 patients with primary sclerosing cholangitis (PSC), and 22 patients with autoimmune hepatitis (AIH). The histopathological diagnoses were established by at least 2 pathologists who considered the clinical and laboratory data. All PBC patients were examined prior to ursodeoxycholic acid therapy, and AMA and/or M2 were detected in all PBC cases. All liver specimens consisted of neutral formalin-fixed paraffin-embedded tissues; 4-μm-thick sections were prepared for routine histological examinations and immunohistochemistry. This study was approved by the Kanazawa University Ethics Committee.

### *Immunohistochemistry*

After deparaffinization, the sections were pretreated in Target Retrieval Solution (Dako Japan Co., Ltd., Tokyo, Japan) in a microwave oven or water bath at 95°C for 20 min to improve the antigenicity of the tissue prior to immunostaining.[14,15] Following endogenous peroxidase blocking in methanolic hydrogen peroxide for 20 min and incubation in normal goat serum (diluted 1:10; Vector Laboratories, Inc., Burlingame, CA) for 20 min, the sections were incubated at 4°C overnight with

primary antibodies against PGC-1α (rabbit polyclonal, diluted 1:100, Bethyl Laboratories, Inc., Montgomery, TX), Bcl-3 (mouse monoclonal, clone 1E8, diluted 1:50, Abcam Japan, Tokyo, Japan), ERRα (mouse monoclonal, clone 1ERR87, 1 μg/mL, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), PDK4 (mouse monoclonal, clone 1A10, 1 μg/mL, Abnova Corporation, Taipei City, Taiwan), and PDC-E1α (mouse monoclonal, clone 9H9AF5, 5 μg/mL, Abcam Japan). The CSA system (Dako Japan Co., Ltd.) was used for ERRα, and the Envision-HRP system (Dako Japan Co., Ltd.) was used for the others. After the benzidine reaction, the sections were weakly counterstained with hematoxylin. No positive staining was obtained when the primary antibodies were replaced with an isotype-matched, nonimmunized immunoglobulin, which was used as a negative control for the staining procedures.

***Histological examination***

We primarily examined the interlobular bile ducts in these patients, including those with chronic nonsuppurative destructive cholangitis (CNSDC), in this study because they are selectively affected in PBC.[1] For the PBC patients, in addition to evaluating their histological stages, the histological activity for chronic cholangitis was evaluated according to Nakanuma's system.[13] In brief, chronic cholangitis activity (CA) was categorized into 4 grades (CA0–3) according to the degree and distribution of chronic cholangitis. CA0 (no activity) was defined as absent or ambiguous bile duct damage. In CA1 (mild activity), 1 bile duct showed evident chronic cholangitis. In CA2 (moderate activity), 2 or more bile ducts showed evident chronic cholangitis. In CA3 (marked activity), at least 1 damaged bile duct showed CNSDC and/or granulomatous cholangitis. For the semiquantitative evaluations of the immunohistochemistry, representative portal tracts that contained the interlobular bile ducts, including those with CNSDC, were chosen in each section for assessment. The immunoreactivity in the bile ducts was semiquantitatively graded as follows: negative (−), weakly positive (+), or strongly/enhanced positive (++).

***Cultured human biliary epithelial cells (BECs) and the induction of PGC-1α***

Two cultured human BEC lines were isolated from the explanted liver of 2 PBC patients. Informed consents to conduct research were obtained from both the patients. These cells were grown as monolayers in a standard medium containing 20% fetal calf serum (Life Technologies Japan, Ltd., Tokyo, Japan) in a 5% $CO_2$-humidified incubator at 37°C.[16] The cell lines were confirmed to be biliary epithelial cells by the expression of the biliary-type cytokeratins CK7 and CK19 (>99%) and also aquaporin 1 (>90%). BECs were used between passages 6 and 10 for this study. To induce the expression of PGC-1α, the cultured BECs were treated by starvation (1% fetal calf serum),[17,18] and the following examinations were performed.

***Assessment of cell viability and mitochondrial dehydrogenase activity***

Approximately $1 \times 10^4$ cells/well placed into 96-well plates were treated by starvation to induce PGC-1α. Cell counts and mitochondrial dehydrogenase activity were measured 24 h later with a microplate reader using the DNA-IdU Labeling and Detection Kit (Takara Bio Inc., Otsu, Japan) and the tetrazolium salt WST-1 assay (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. Mitochondrial dehydrogenase activity per cell was presented as a relative ratio of the optical density (OD) in WST-1/DNA-IdU labeling assays.

***Isolation of RNA and real-time reverse-transcription polymerase chain reaction (RT-PCR)***

The basal levels of expression of ERRα, PGC-1α, Bcl-3, PDK4, and PDC-E1α mRNAs and their alterations by starvation were examined by RT-PCR and real-time PCR, respectively. Total RNA was extracted from cultured BECs using the RNeasy Total RNA System (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Following this, 1 μg of total RNA was reverse transcribed with an oligo-(dT) primer and ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) to synthesize a cDNA template for PCR. For relative quantification, real-time quantitative PCR was performed according to a standard protocol using the Brilliant II SYBR Green QPCR Reagents and Mx300P QPCR System (Agilent Technologies, Tokyo, Japan), and the relative levels of gene expression were calculated using the comparative cycle threshold method. The specific

primers were as follows: PGC-1α: forward, 5′-TTGGTAACCGAACTGGTGCT-3′ and reverse, 5′-GTGCAAAGTTCCCTCTCTGC-3′; Bcl-3: forward, 5′-CCCTATACCCCATGATGTGC-3′ and reverse, 5′-GGTGTCTGCCGTAGGTTGTT-3′; ERRα: forward, 5′-TGGCTACCCTCTGTGACCTC-3′ and reverse, 5′-CTCATCCTGCAGTGGCAGT-3′; PDK4: forward, 5′-GGCCTAGTGTTGTGGTGCTT-3′ and reverse, 5′-GAGCTGGACTCCCACCATTA-3′; PDC-E1α: forward, 5′-GTCAGTGCTTCAAGCCAACA-3′ and reverse, 5′-TTAAACTGCAGCCTGCCTTC-3′; and glyceraldehyde 3 phosphate dehydrogenase (GAPDH, internal positive control): forward, 5′-GGCCTCCAAGGAGTAAGACC-3′, and reverse, 5′-AGGGGTCTACATGGCAACTG-3′. The results were obtained from 2 independent experiments and are presented as the relative levels of mRNA expression compared with the levels without any treatments. Negative controls were obtained by replacing the reverse transcriptase or cDNA samples with RNase- and DNase-free water.

***Quantitative and functional assessments of PDK4 expression and PDH activity***

The levels of expression of PDK4 and PDH activity and the alterations in them resulting from the induction of PGC-1α by starvation in cultured BECs were measured using the PDK4 Human Enzyme-Linked Immunosorbent Assay (ELISA) Kit and the PDH Enzyme Activity Microplate Assay Kit, respectively, according to the manufacturer's instructions.

***Statistical analysis***

The data were analyzed using Mann–Whitney U test, paired *t* test, Wilcoxon signed-ranks test, and Spearman's correlation coefficient by rank test. P values $<0.05$ were considered statistically significant.

**RESULTS**

***Exclusive expression of PGC-1a, Bcl-3, and ERRa in the damaged bile ducts of PBC***

The expression of PGC-1α and ERRα was detected in the cytoplasm and nucleus of positive cells, and the nuclear expression indicated the activated forms. This was considered to be functional positivity. The expression of Bcl-3 was limited to the nucleus of positive cells. In the controls, strong nuclear expression of PGC-1α, Bcl-3, and ERRα was not detected in any interlobular bile ducts, including mildly injured bile ducts (hepatic bile duct injury) in CH-C and AIH. The bile ducts that exhibited almost normal or mild cholangitis also lacked these molecules in PBC; however, the damaged bile ducts that showed moderate to severe cholangitis, including CNSDC in PBC, preferentially expressed PGC-1α, Bcl-3, and ERRα in a nuclear pattern. As shown in Fig. 1, the nuclear expression of PGC-1α, Bcl-3, and ERRα in the bile ducts was exclusive in PBC, and the levels of expression of PGC-1α and Bcl-3 correlated well with the degree of CA activity in PBC.

***Expression of PDK4 and PDC-E1α in bile ducts***

The expression of PDK4 was basically negative or weakly positive in the interlobular bile ducts, and bile ducts exhibiting strong positivity were not observed (Fig. 2). However, the frequency of weakly positive bile ducts was higher in PBC compared with that in controls (Fig. 2). In contrast, PDC-E1α was consistently expressed in all interlobular bile ducts (Fig. 2). Strong expression was more frequent in the bile ducts of PBC patients (Fig. 2).

***Effects of the induction of PGC-1α in cultured BECs on mitochondrial dehydrogenase activity***

To investigate the alterations in mitochondrial dehydrogenase activity by the induction of PGC-1α in cultured BECs, we analyzed the relative cell counts and mitochondrial dehydrogenase activities in starved BECs using WST-1 and DNA-IdU labeling assays, respectively. As shown in Fig. 3, the induction of PGC-1α by starvation downregulated the cell number; however, it did not affect the degree of total mitochondrial dehydrogenase activity. Therefore, the mitochondrial dehydrogenase activity per cell that was presented as the relative ratio of OD values was increased in the starved BECs by approximately 2-fold compared with that in nonstarved (static) BECs (Fig. 3).

***Expression of Bcl-3, ERRα, PDK4, and PDC-E1α and their alterations by the induction of PGC-1α in cultured BECs***

RT-PCR analysis revealed that the amplification of all mRNAs of PGC-1α, ERRα, PDK4, and PDC-E1α in cultured human BECs (Fig. 4A). Moreover, real-time PCR analysis demonstrated that the levels of amplification of mRNAs of PGC-1α, ERRα, and PDK4 were statistically increased according to the upregulation of PGC-1α by starvation, although the degrees of increase varied (paired *t* test or Wilcoxon signed-ranks test) (Fig. 4B).

***Assessment of PDK4 expression and PDH activity***

PDK4 ELISA demonstrated that the levels of expression of PDK4 were significantly upregulated by the induction of PGC-1α by starvation in cultured BECs. In contrast, PDH enzyme activity was decreased in the starved state (Fig. 4C).

**DISCUSSION**

In contrast to ER, ERRα and its related family members, ERRβ and ERRγ, do not have known ligands; therefore, they are called orphan nuclear receptors. ERRα plays key roles in the gene regulatory control of the mitochondrial energetic systems. ERRα has been identified as a novel PGC-1α-binding partner, and the ERR family members must interact with PGC-1 coactivators to be transcriptionally active.[19] Bcl-3 synergizes with PGC-1α to coactivate ERRα, and the complex of ERRα, PGC-1α, and Bcl-3 is found on an ERRα-responsive element within the PDK4 gene promoter.[8] In mitochondrial energy systems, ERRα regulates fatty acid degradation (β-oxidation), which is an aerobic and more effective energy metabolic system compared with the anaerobic glycolytic system. Therefore, the PGC-1α/ERRα axis plays a key role in various aspects of cellular energy homeostasis, including mitochondrial biogenesis, thermal regulation, and glucose metabolism.[10,20] Consistent with this function, ERRα is prominently expressed in tissues that have a high capacity for the β-oxidation of fatty acids, such as the heart, brown fat, and skeletal muscle.[21] In

the liver, ERRα and PGC-1α are expressed at low levels; however, they are induced in fasting animals.[18] Although these metabolic studies have been conducted in liver hepatocytes,[18,22] there have been no reports regarding the metabolic state of BECs and the dysregulations of BECs in human biliary diseases, including PBC. The present study revealed that the activated expression of ERRα, PGC-1α, and Bcl-3 in a nuclear pattern was exclusively observed in the CNSDC of PBC. Although bile ducts that are similarly damaged from hepatic bile duct injury or hepatitis-associated bile duct injury have been observed in AIH and CH-C, no or weak ERRα expression in the nuclei was observed in these damaged bile ducts. However, the expression of PGC-1α and Bcl-3 correlated well with the degree of chronic CA in PBC, indicating that the cooperative expression of the ERRα/PGC-1α axis was closely associated with the energy metabolic responses in the pathogenesis of CNSDC in PBC.

The PDC-E1 enzyme is a heterotetramer of 2 α and 2 β subunits. The E1α subunit, which contains the E1 active site, plays a key role in the function of PDC. PDK decreases PDH activity through the phosphorylation of PDC-E1α. Three serine phosphorylation sites on PDC-E1α are targeted by PDKs, and the phosphorylation of PDC-E1α completely inhibits the activity of PDH.[23] There is increased phosphorylation of PDC in the heart and skeletal muscle in cases of starvation and diabetes, and this allows pyruvate to be conserved while mitochondrial fatty acid oxidation is increased.[24] Four PDK isoenzymes (PDK1, -2, -3, and -4) have been identified. The expression of PDK4 is suppressed under basal conditions in most tissues; however, its expression is increased by starvation, glucocorticoids, diabetes, a high-fat diet, and extended exercise through the PGC-1α/ERRα axis, particularly in the heart, skeletal and other muscle tissues, kidney, and liver. PDK4 overexpression prevents glucose oxidation.[10,24,25] The present study revealed the expression of PDK4 and its increases according to the induction of PGC-1α by starvation in cultured human BECs. Moreover, functional analysis confirmed the decrease in PDH function. These findings suggested that the functions of PDH in human BECs are regulated by the ERRα/PGC-1α axis and that in CNSDC, the exclusive expression of the PGC-1α/ERRα axis and the enhanced expression of PDK4 result in PDH dysfunction through PDK4.

Although samples with CNSDC have reactive findings such as enlargement compared with the original size of the bile duct and increased mitochondria,[1,26,27] these damaged bile ducts finally undergo disappearance (bile duct loss),[28] mainly through biliary apoptosis. This unique finding concerning the histogenesis of CNSDC in PBC can be explained by metabolic alterations. In other words, switching from the utilization of glucose to that of fatty acids as an energy source results in a more effective energy system, indicating that metabolic switching by ERRα/PGC-1α increases the metabolic activity of CNSDC in PBC. The in vitro study of cultured BECs demonstrated that the induction of PGC-1α by starvation caused an increase in mitochondrial dehydrogenase activity per cell. Although the oxidative phosphorylation of fatty acids is a vital part of metabolism, it produces reactive oxygen species such as superoxides and hydrogen peroxide, which lead to the propagation of free radicals and result in damaged and apoptotic cells and contribute to several diseases. In fact, several reports have already demonstrated that increased oxidative stress and enhanced biliary apoptosis are observed in the bile ducts of PBC patients.[28-31] Therefore, metabolic switching from glycolytic systems to fatty acid oxidation has been speculated to cause an increased susceptibility to the apoptotic induction of CNSDC in PBC through oxidative stress.

ERRs share homology with ERs; however, ERRs do not bind to estrogen or other known physiological ligands. Therefore, ERRs inhibit ER-dependent estrogen effects through competition with ERα. ERs form homo or heterodimers that consist of ERα and ERβ, which bind to an estrogen-response element (ERE) and affect cell proliferation and the promotion of apoptosis and differentiation, respectively.[32] Both ERα and ERβ are observed in bile ducts in the early stages of PBC; however, the disappearance of ERα in bile ducts during the cirrhotic stage and an estrogenic deficiency have been speculated to accompany the evolution of PBC toward ductopenia.[4] In addition to this loss of ERα, the present study revealed that the activated expression of ERRα in a nuclear pattern was exclusively found in CNSDC of PBC, indicating that the activation of ERRα inhibits the cell-proliferating function of ERα and induces the bile duct loss caused by regenerative failure in CNSDC of PBC.

In conclusion, we demonstrated the activation of the ERRα/PGC-1α axis and the upregulation

of PDK4 in CNSDC of PBC, suggesting an interference in PDH function and the switching from glycolytic to fatty acid oxidation. Moreover, the dysfunction of PDH which are major epitopes for AMA suggests any associations with the pathogenesis of AMA. Further studies are required to clarify the etiology and mechanisms underlying the activation of the ERRα/PGC-1α axis in vivo and the production mechanism of AMA. Although compensatory responses to some bile duct injuries have been suggested, metabolic switching was possibly associated with the pathogenesis of CNSDC and the consequent bile duct loss in PBC. The enzymes conducting the correction of the metabolic system in BECs may also be the target of drugs in PBC.

Competing Interest: None declared.

**Take home messages**

- In CNSDC of PBC, the activation of the ERRα/PGC-1α axis is exclusively observed.
- The interference of PDC-related glycolytic function and the induction of the fatty acid degradation system are speculated in CNSDC of PBC.
- The switching of the cellular energy system is possibly associated with the pathogenesis of CNSDC in PBC.

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**Figure legends**

Fig. 1 The expression of PGC-1α, Bcl-3, and ERRα in liver tissue and its correlation with chronic cholangitis activity (CA). A: Immunohistochemistry for PGC-1α, Bcl-3, and ERRα. Biliary epithelial cells with CNSDC of PBC expressed PGC-1α and ERRα strongly in the nucleus and weakly in the cytoplasm and expressed Bcl-3 in the nucleus (arrows). However, undamaged (almost normal) bile ducts with PBC and bile ducts with hepatitis C virus-related chronic hepatitis (CH-C) were negative for these molecules (arrowheads). B: There was good correlation between the expression of PGC-1α and Bcl-3 compared with the degree of chronic cholangitis (CA) in PBC. PGC-1α; r=0.69, p-value=0.001, Z value=3.20, Z (0.975)=1.95. Bcl-3; r=0.66, p-value=0.0009, Z value =3.11, Z (0.975)=1.95. C: Semiquantitative analyses revealed that the expression of these molecules in bile ducts was higher in PBC than that in controls. PGC-1α, p-value=0.002; Bcl-3, p-value < 0.001; ERRα, p-value < 0.001. Moreover, the statistical analysis excluding positive reactive cases (+) also revealed that the ratio of strong positive cases (++) were significantly higher in PBC than those in controls. PGC-1α, p-value = 0.004; Bcl-3, p-value < 0.001; ERRα, p-value < 0.001.

Fig. 2 The expression of PDK4 and PDC-E1α in liver tissue. A: Tissue from patients with CNSDC in PBC weakly expresses PDK4 and strongly expresses PDC-E1α in cytoplasmic patterns. B: Semiquantitative analyses revealed that the expression of PDK4 was negative or weakly positive in the interlobular bile ducts, and the frequency of weakly positive bile ducts was higher in PBC patients than that in controls. PDC-E1α was basically expressed (weakly or strongly positive) in the interlobular bile ducts, and the frequency of strongly positive bile ducts was higher in PBC patients than that in controls. PDK4, p-value=0.008; PDC-E1α, p-value=0.015.

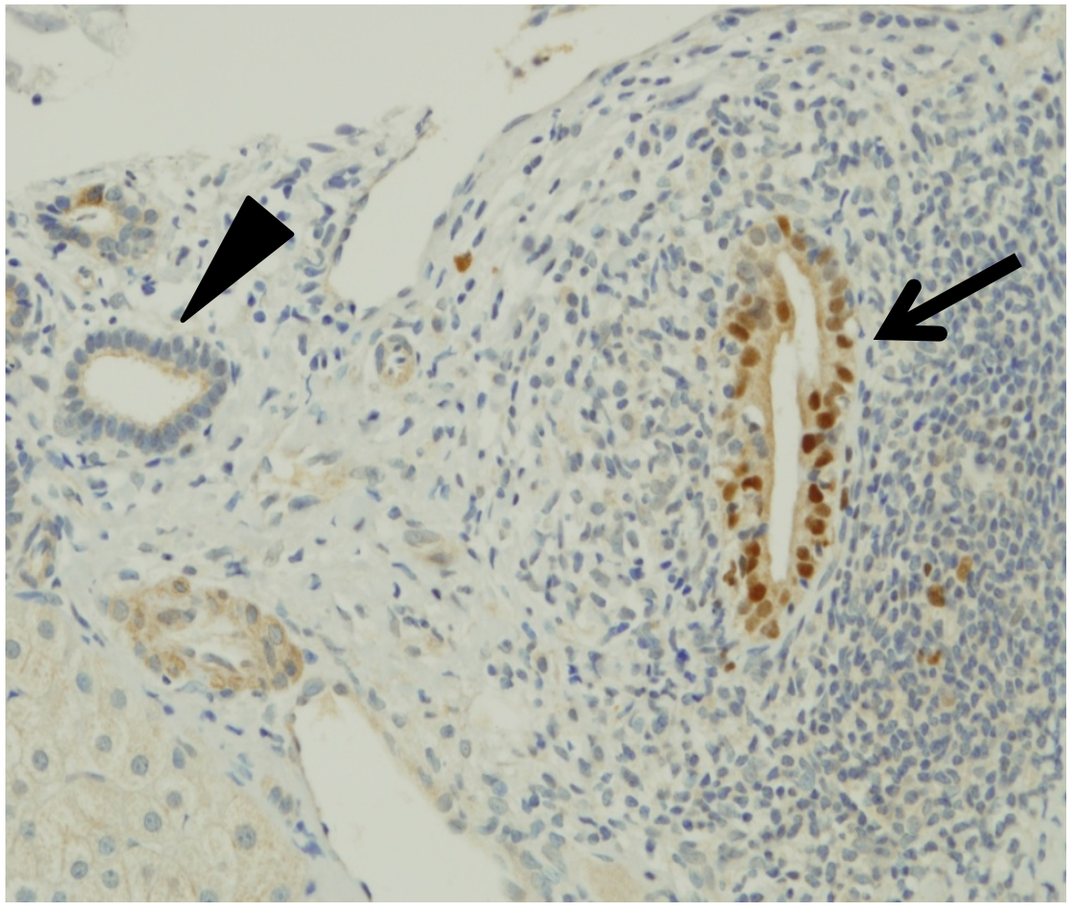
Fig. 3 Mitochondrial dehydrogenase activity in cultured BECs. The number of cells that were starved was decreased; however, the degree of mitochondrial dehydrogenase activity remained significantly unchanged in total. Mitochondrial dehydrogenase activity per cell, which is presented as the relative ratio of optical density (OD), was significantly higher in starved BECs by 0.69 ± 0.10-fold (mean ± S.E.M) than that in untreated (static) BECs (0.32 ± 0.04-fold). p-value = 0.013. Bars indicate mean and S.E.M. In the induction of PGC-1α expression by the starvation of cultured BECs, cell counts and mitochondrial dehydrogenase activity were measured with WST-1 and DNA-IdU labeling assays, respectively. The results were obtained from 2 independent experiments with 2 cell lines of cultured BECs.

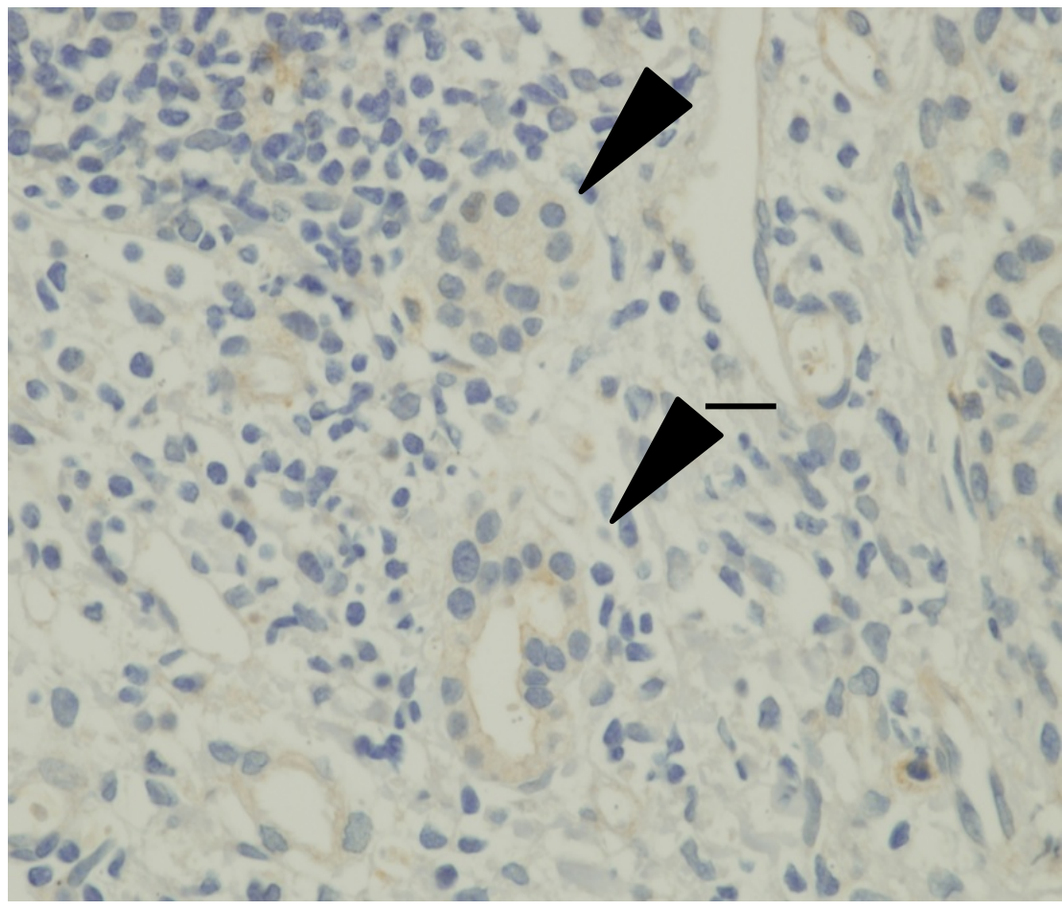
Fig. 4 The expression of Bcl-3, ERRα, PDK4, and PDC-E1α and PDH activity in cultured BECs. A: RT-PCR was performed for 40 cycles, and amplification of ERRα, PGC-1α, Bcl-3, PDK4, and PDC-E1α was detected as a single band from cultured BECs at the expected sizes. Negative controls (NCs) were obtained by replacing the reverse transcriptase with RNase- and DNase-free water for the reverse transcription. B: Real-time PCR analyses demonstrated that the fold-increase of ERRα, PGC-1α, Bcl-3, PDK4, and PDC-E1α by starvation were 1.7 ± 0.2- (mean ± S.E.M, p-value = 0.048), 16.3 ± 1.8- (p-value = 0.003), 3.5 ± 1.3- (p-value = 0.19), 4.6 ± 0.4- (p-value = 0.004), and 1.3 ± 0.1 (p-value = 0.030)-fold, respectively. C: An enzyme-linked immunosorbent assay for PDK4 demonstrated that the expression of PDK4 was significantly upregulated by 2.5 ± 0.2-fold by the induction of PGC-1α (starved state) in cultured BECs (p-value = 0.01). In contrast, PDH enzyme activity was decreased by 0.4 ± 0.01-fold in the starved state (p-value < 0.001). The results were obtained from 2 independent experiments with 2 cell lines and are shown as the relative levels of expression compared with the levels without any treatments (static state).

PBC

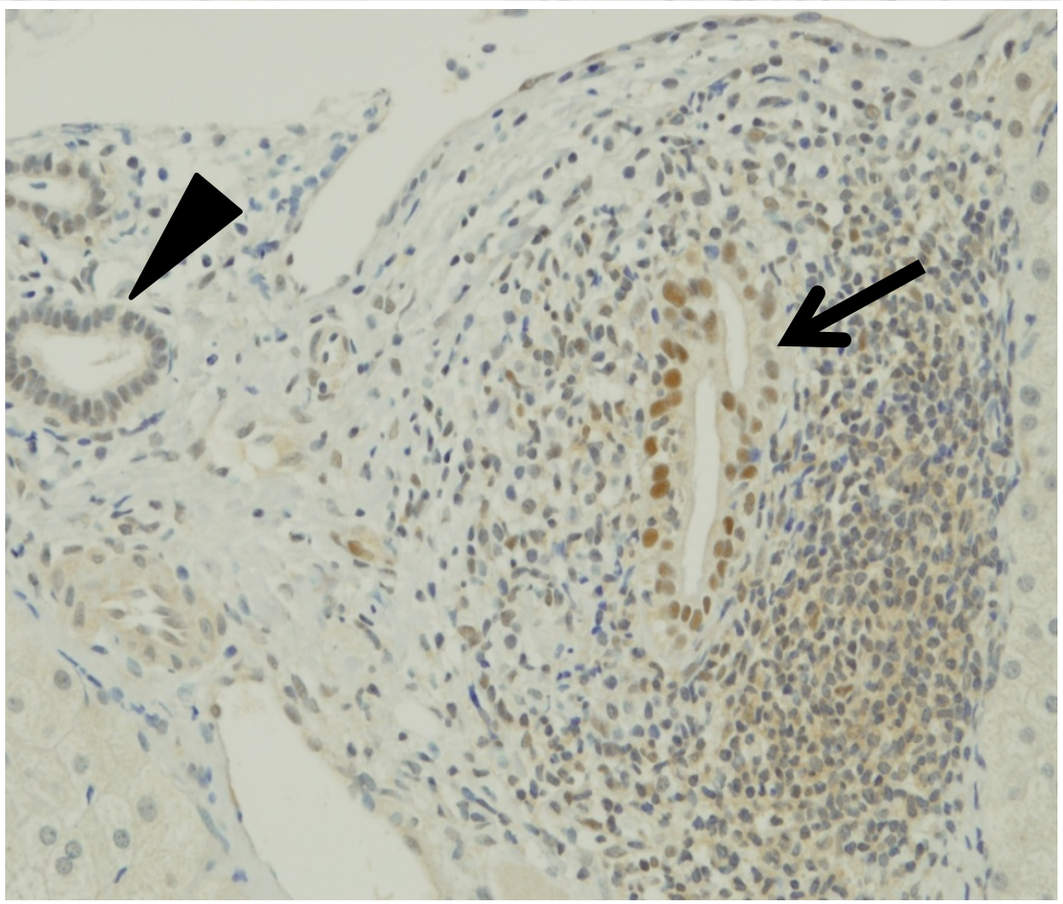
CH-C

PGC-1





Bcl-3



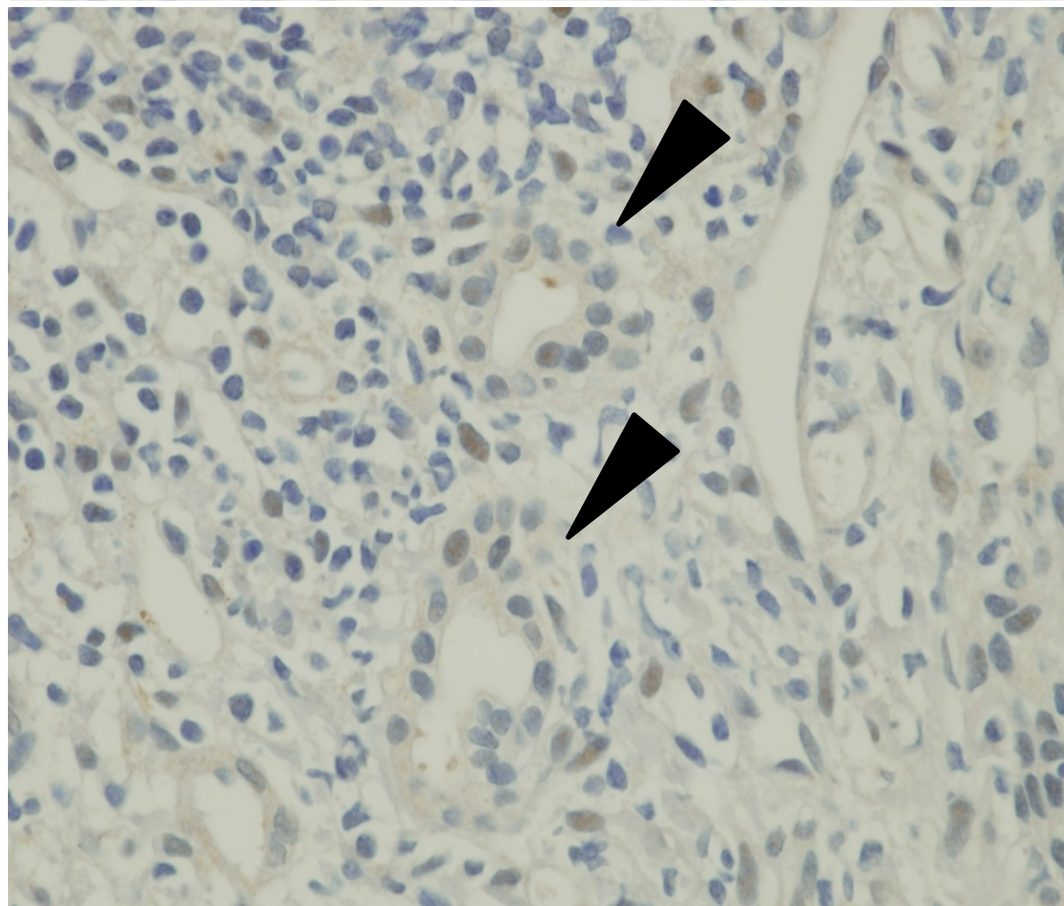


Fig.1A

PBC

CH,C

ERRα

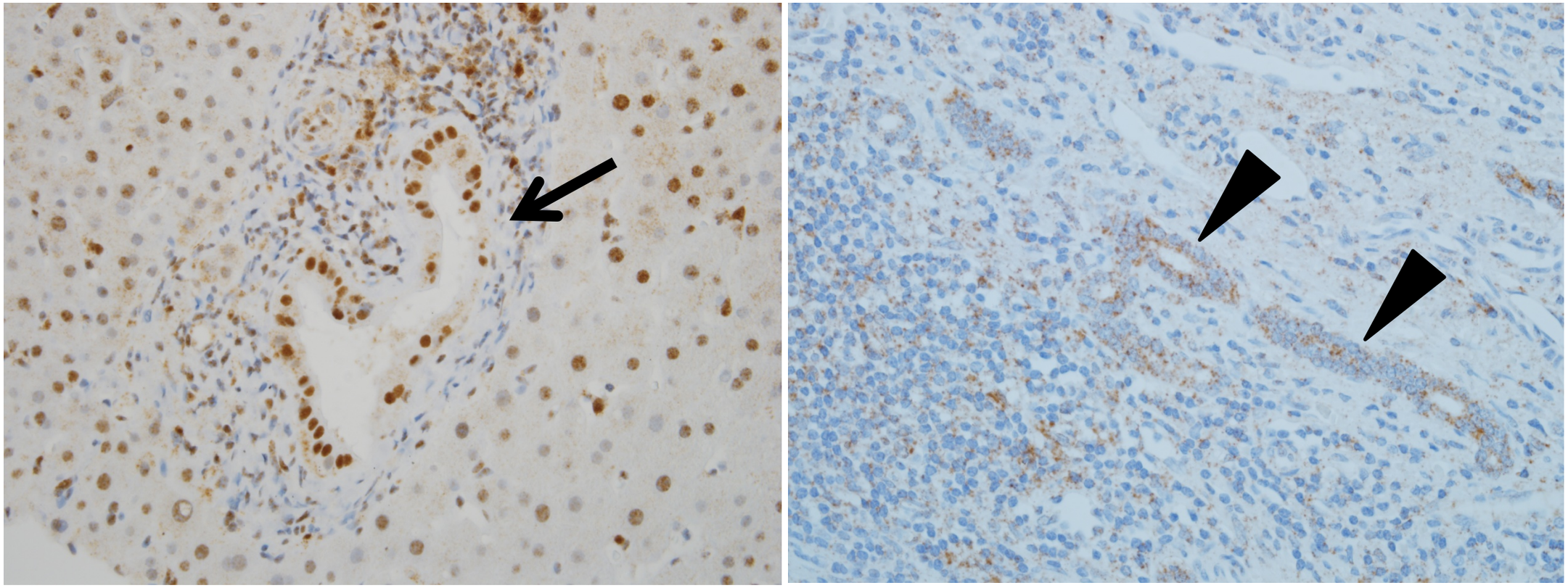
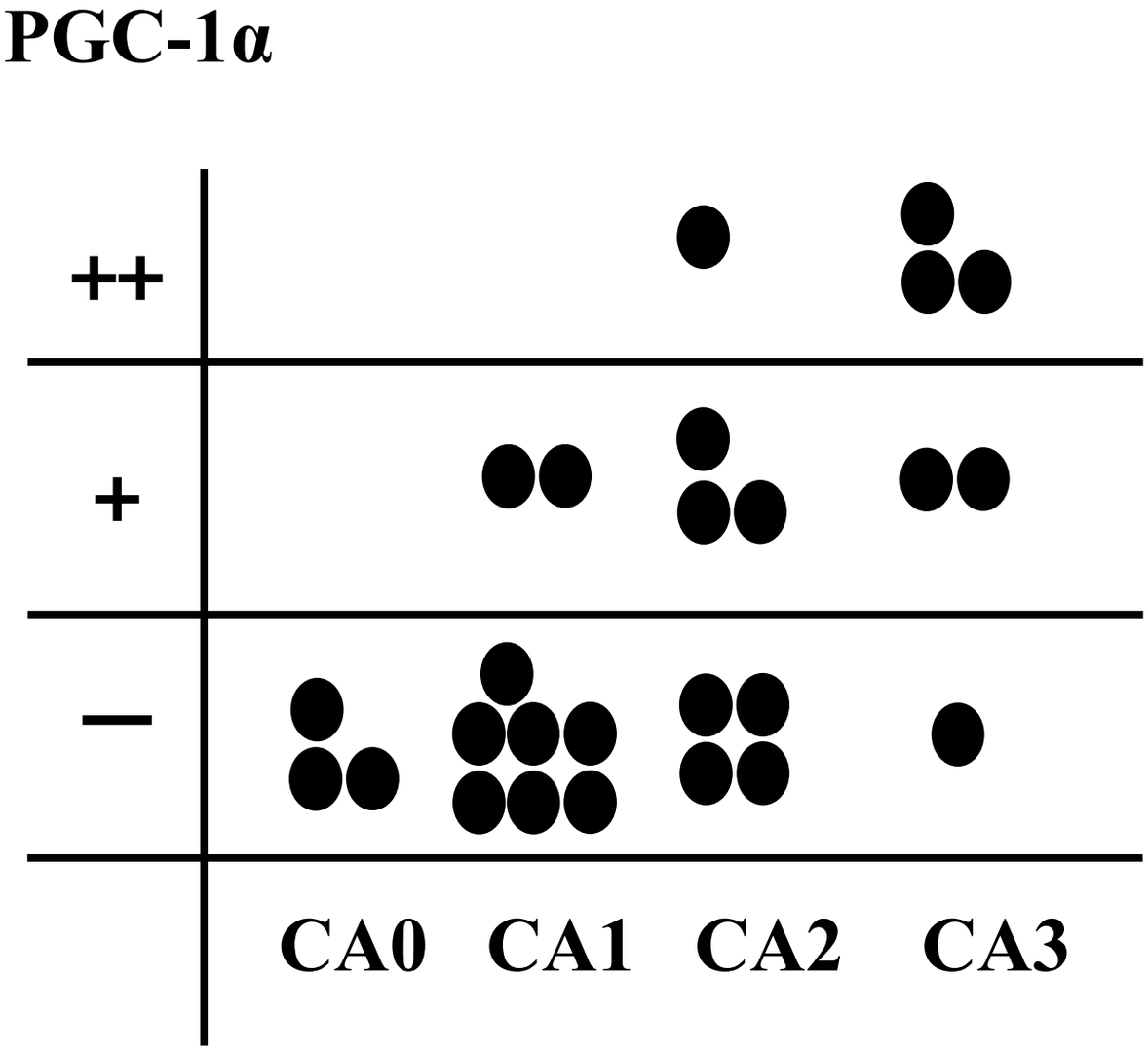


Fig.1A (continued)



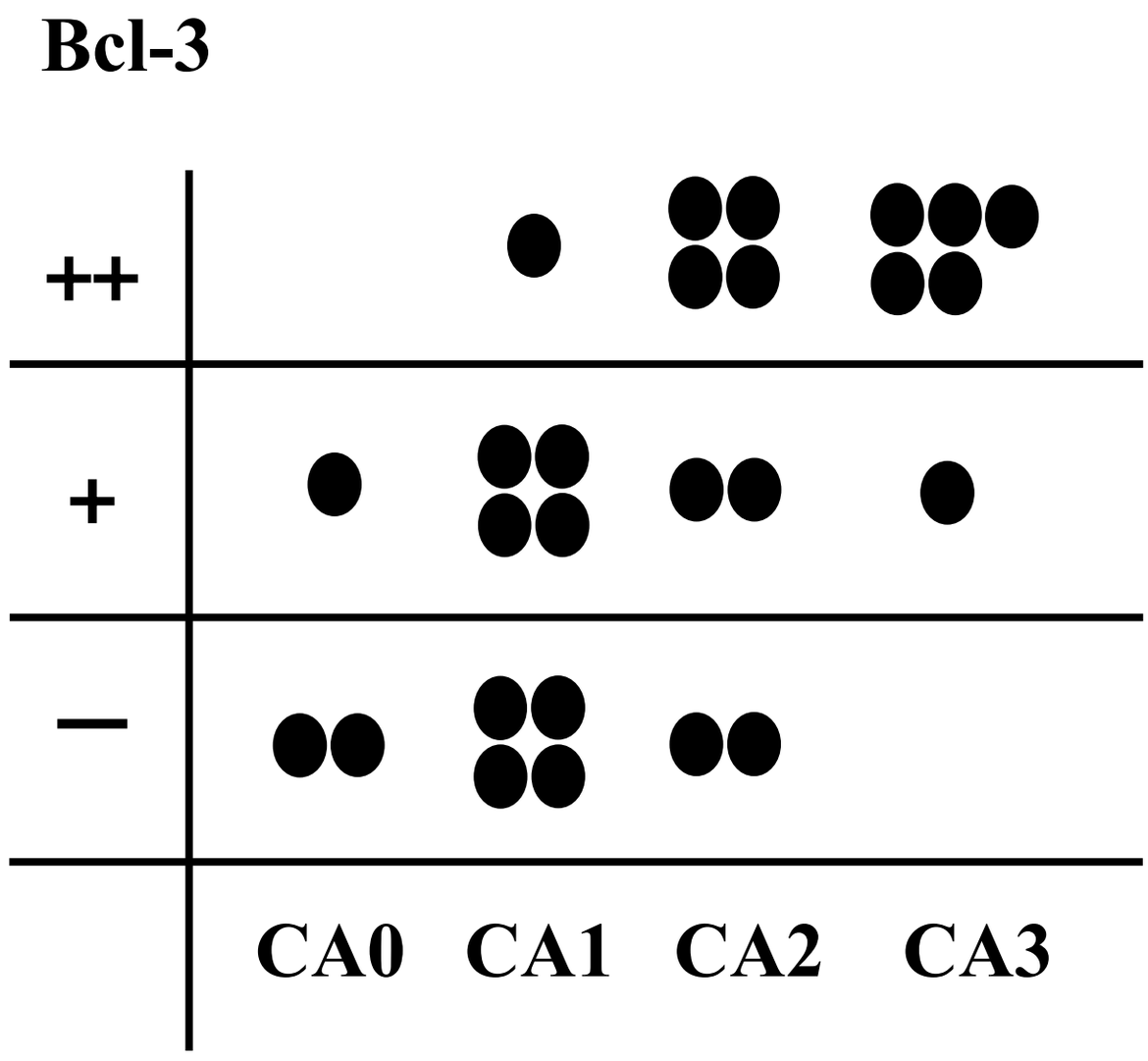
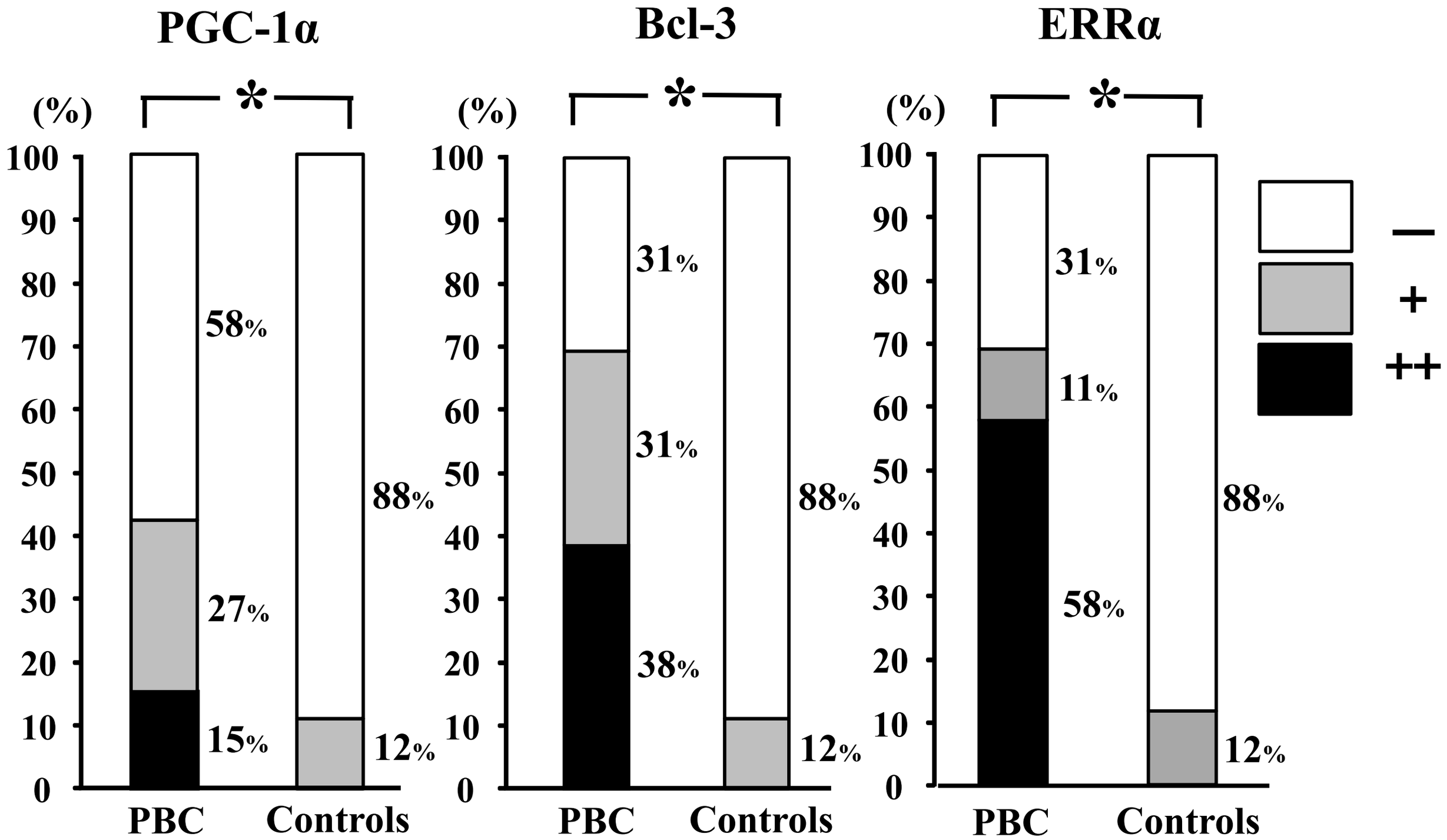




**Fig.1B**

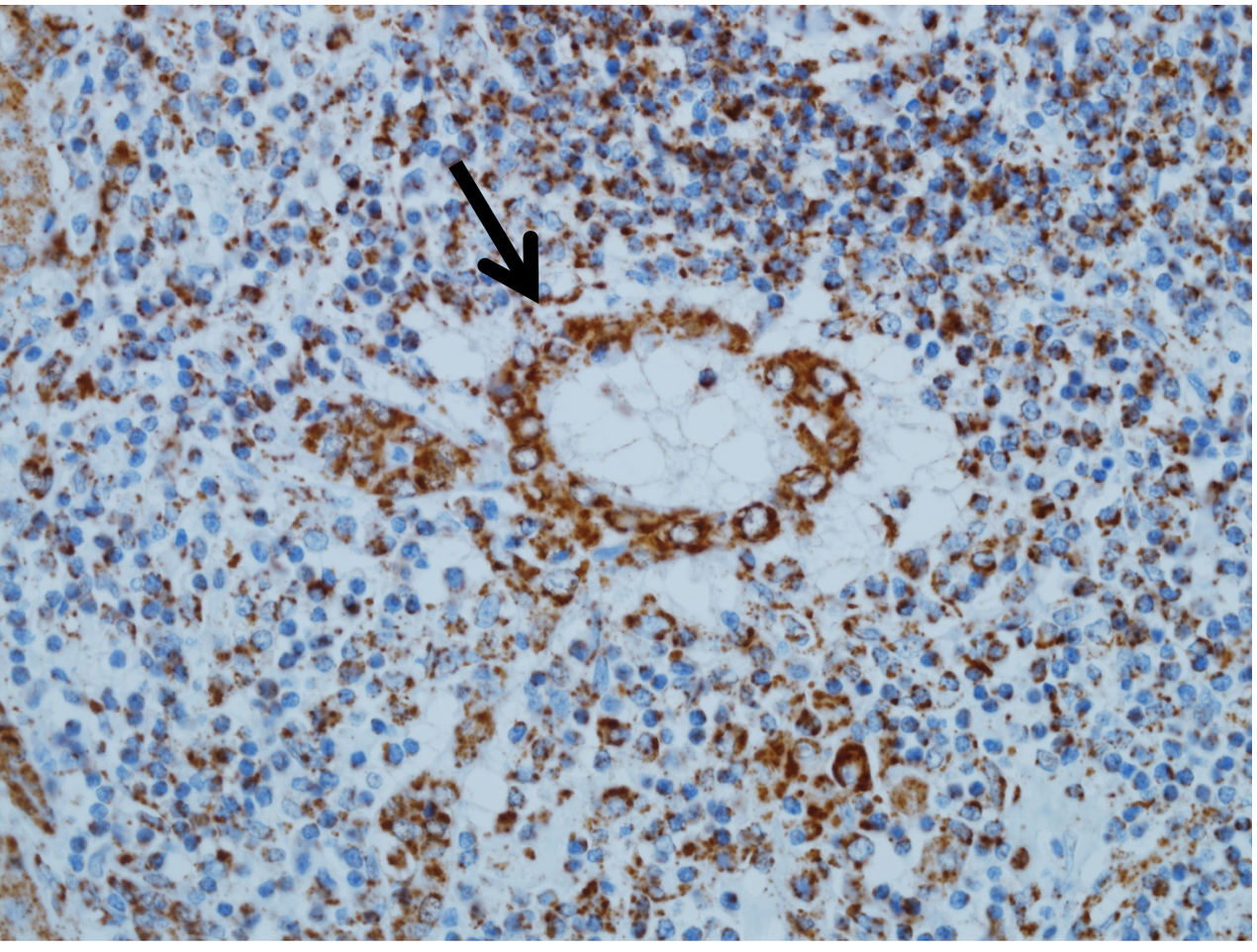
**< 0.05 (Spearman's correlation coefficient by rank test)**




*; < 0.05 (Mann-Whitney U test)

Fig.1C

PDC-E1α



PDK4

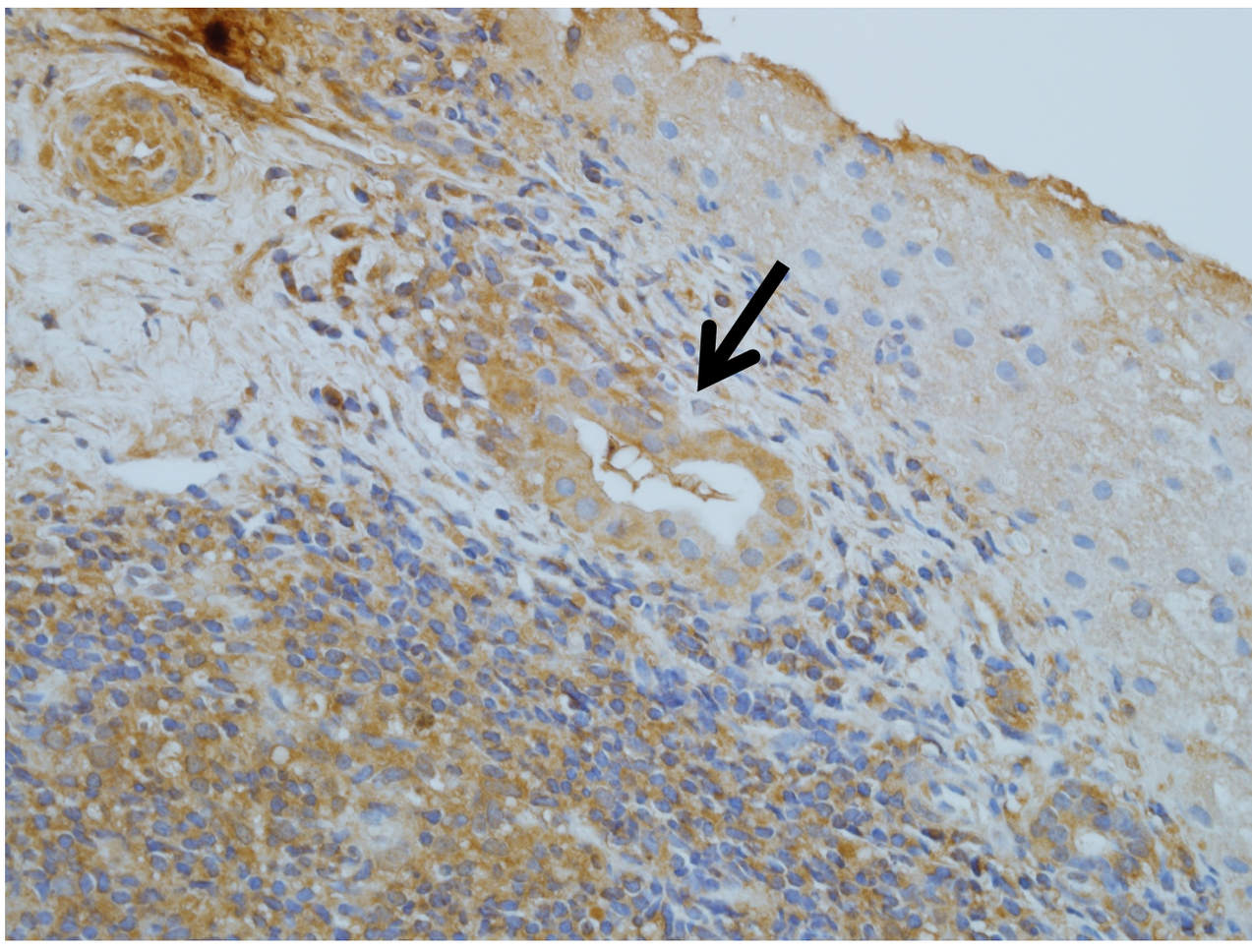
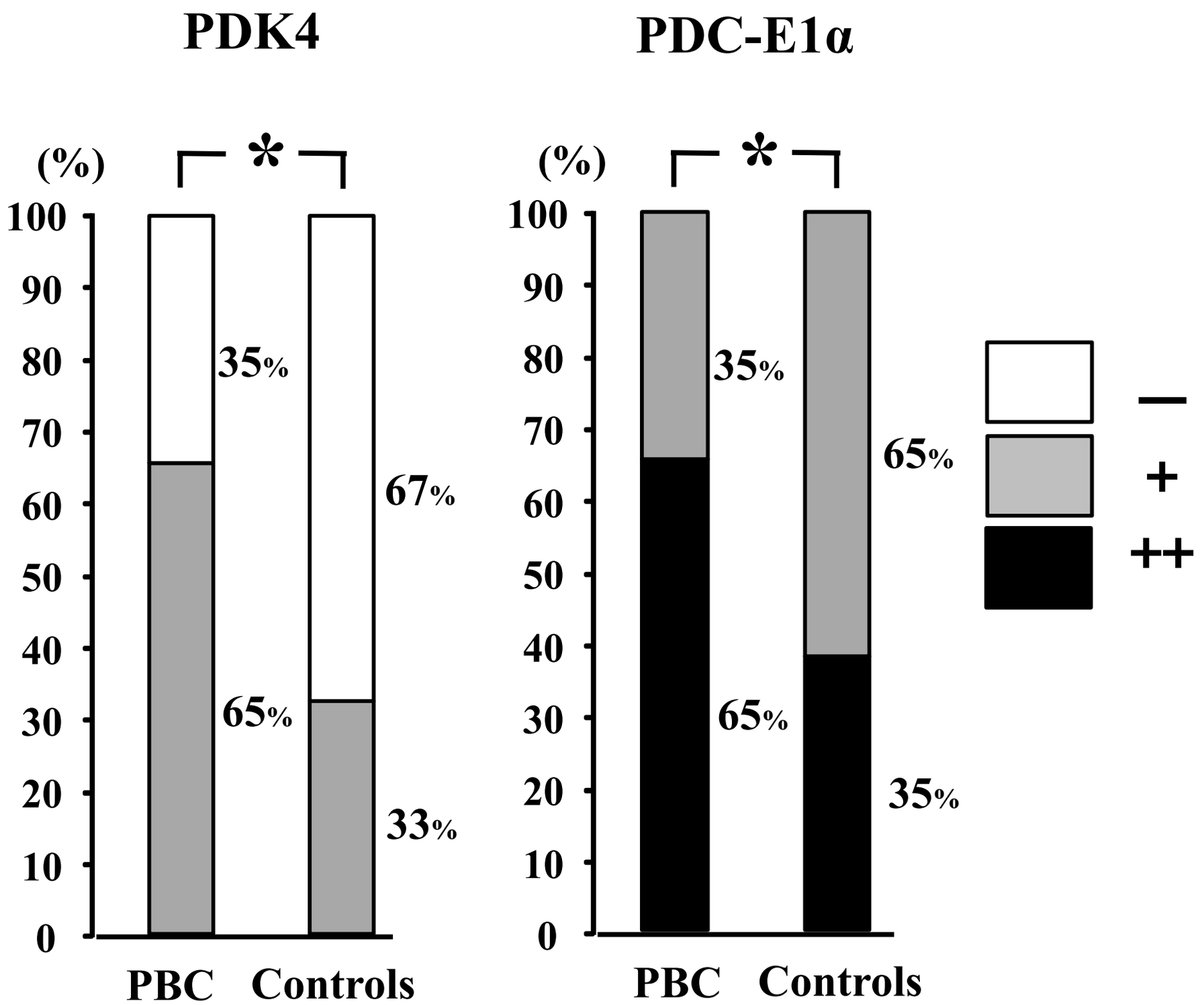
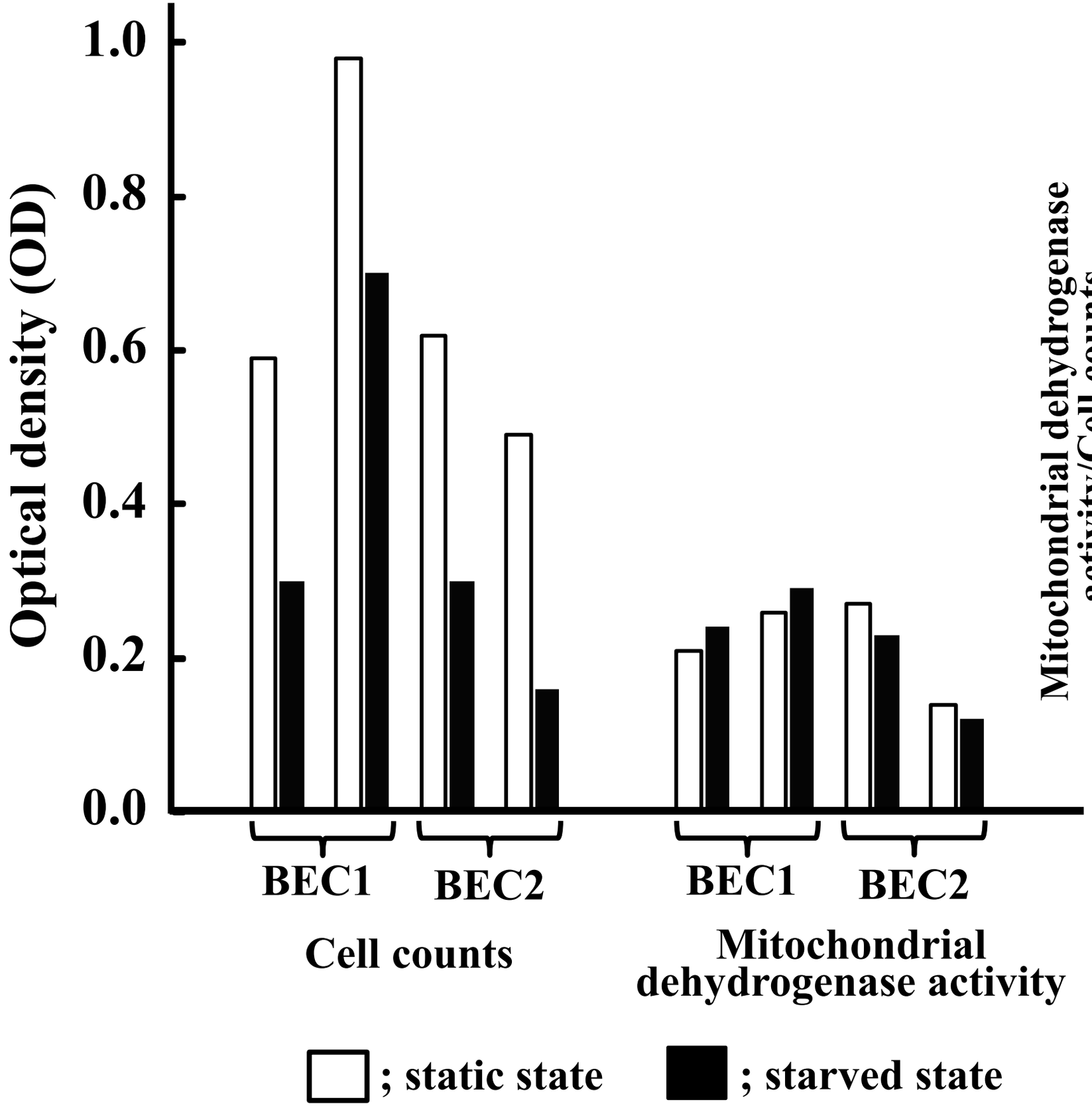


Fig.2A




* < 0.05 (Mann-Whitney's U test)

Fig.2B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1.0
0.8
0.6
0.4
0.2
0.0
BEC1
BEC2
BEC1
BEC2
Cell counts
Mitochondrial dehydrogenase activity
; static state
; starved state

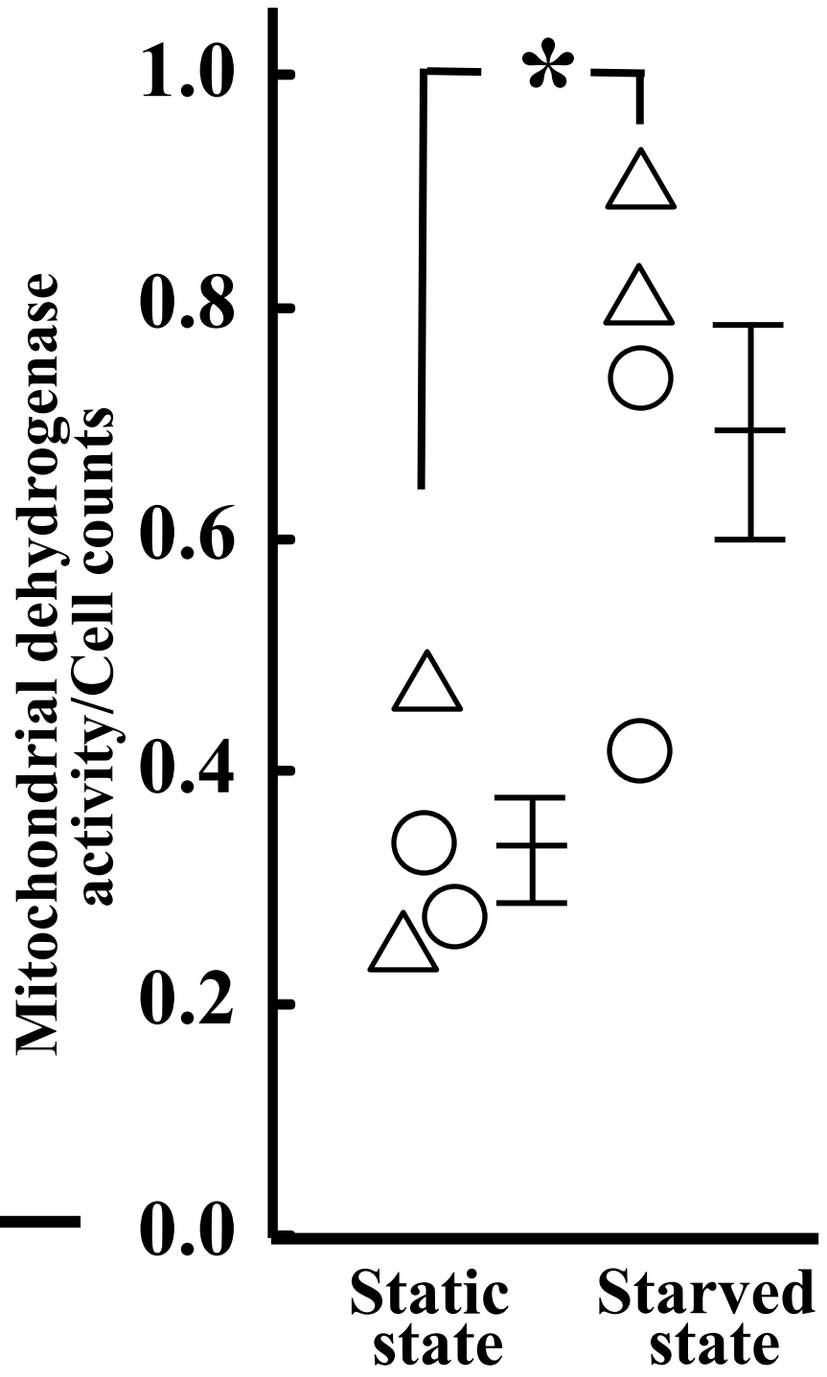

Mitochondrial dehydrogenase activity/Cell counts
1.0
0.8
0.6
0.4
0.2
0.0
*
Static state
Starved state
Mitochondrial dehydrogenase activity per cell
○, BEC1; △, BEC2
*; <0.05 (Paired t test)

**Fig.3**

(A)

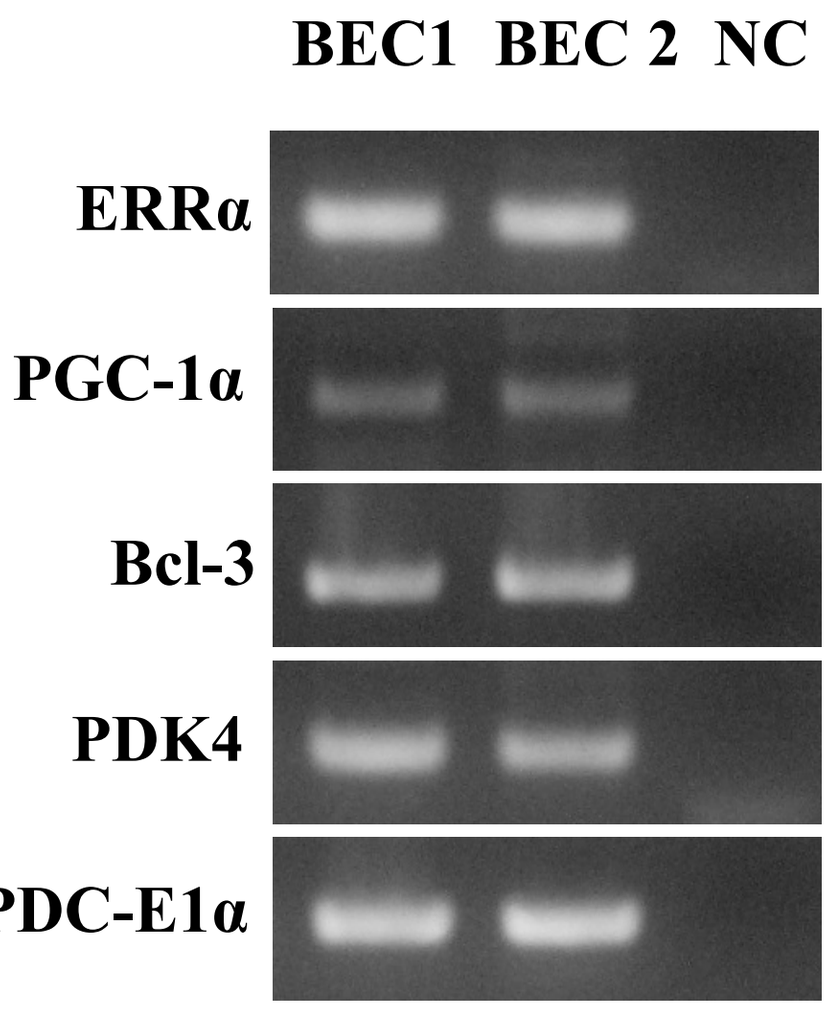
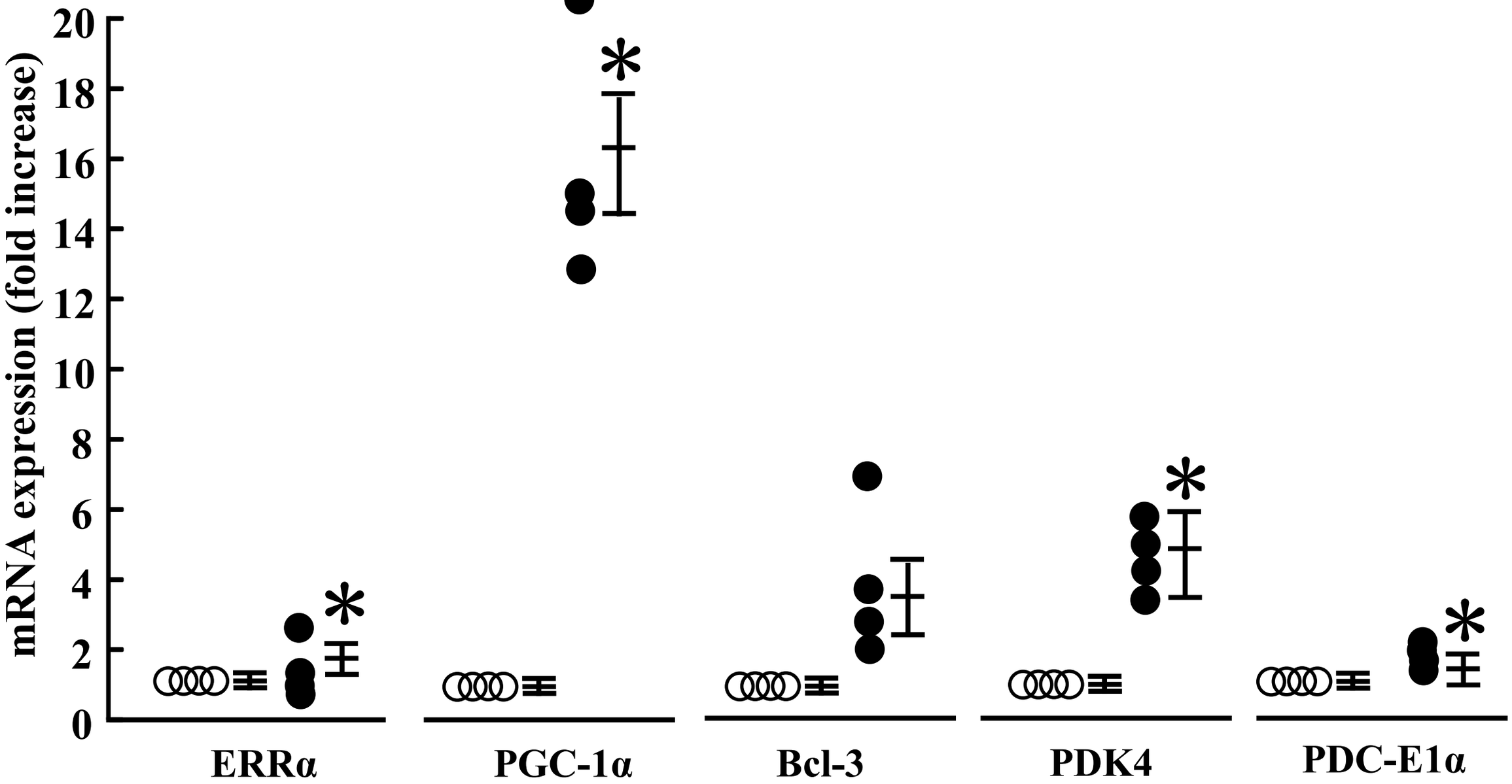



Fig.4A

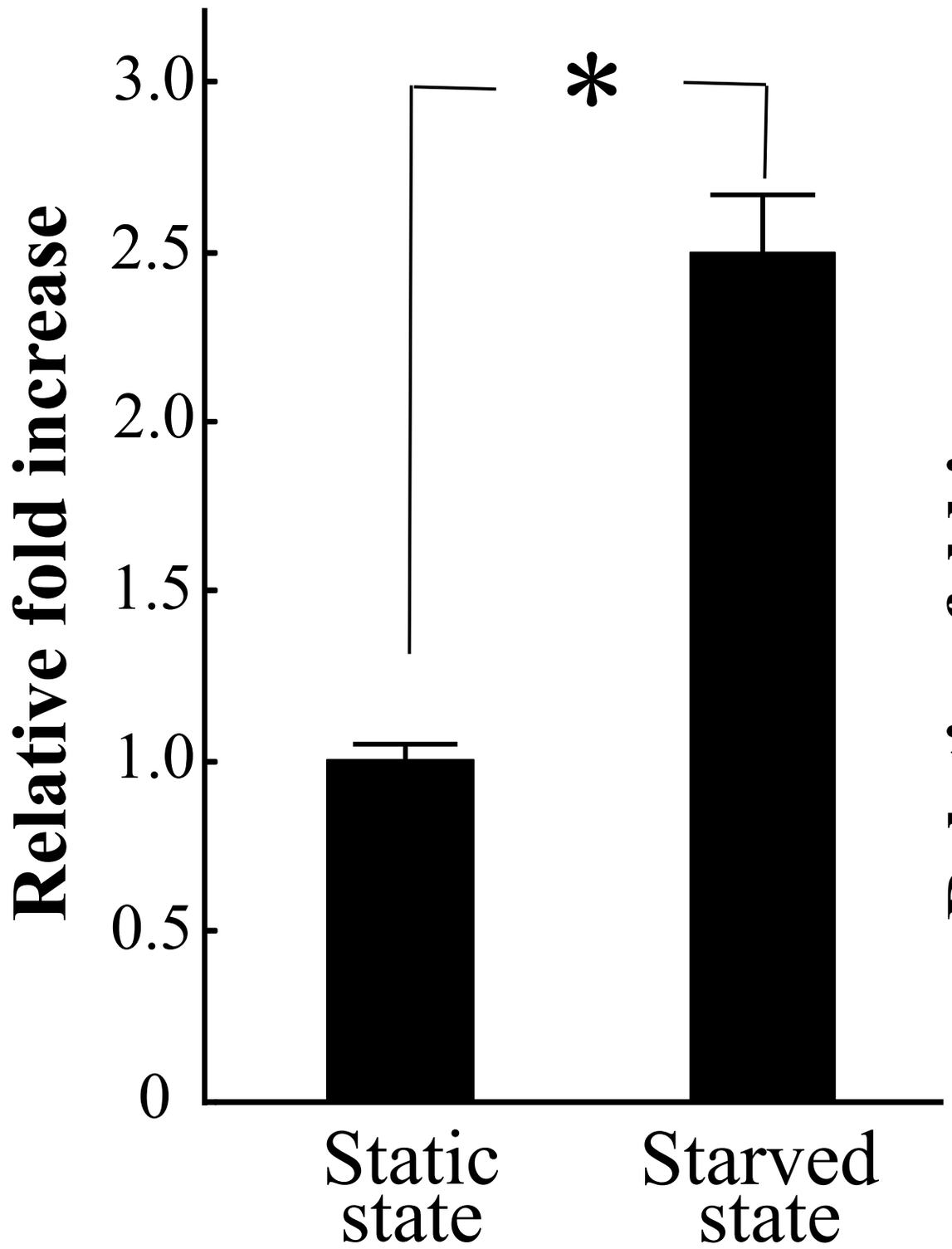
(B)




○; static state ● ; starved state * ; <0.05

Fig.4B

(C)



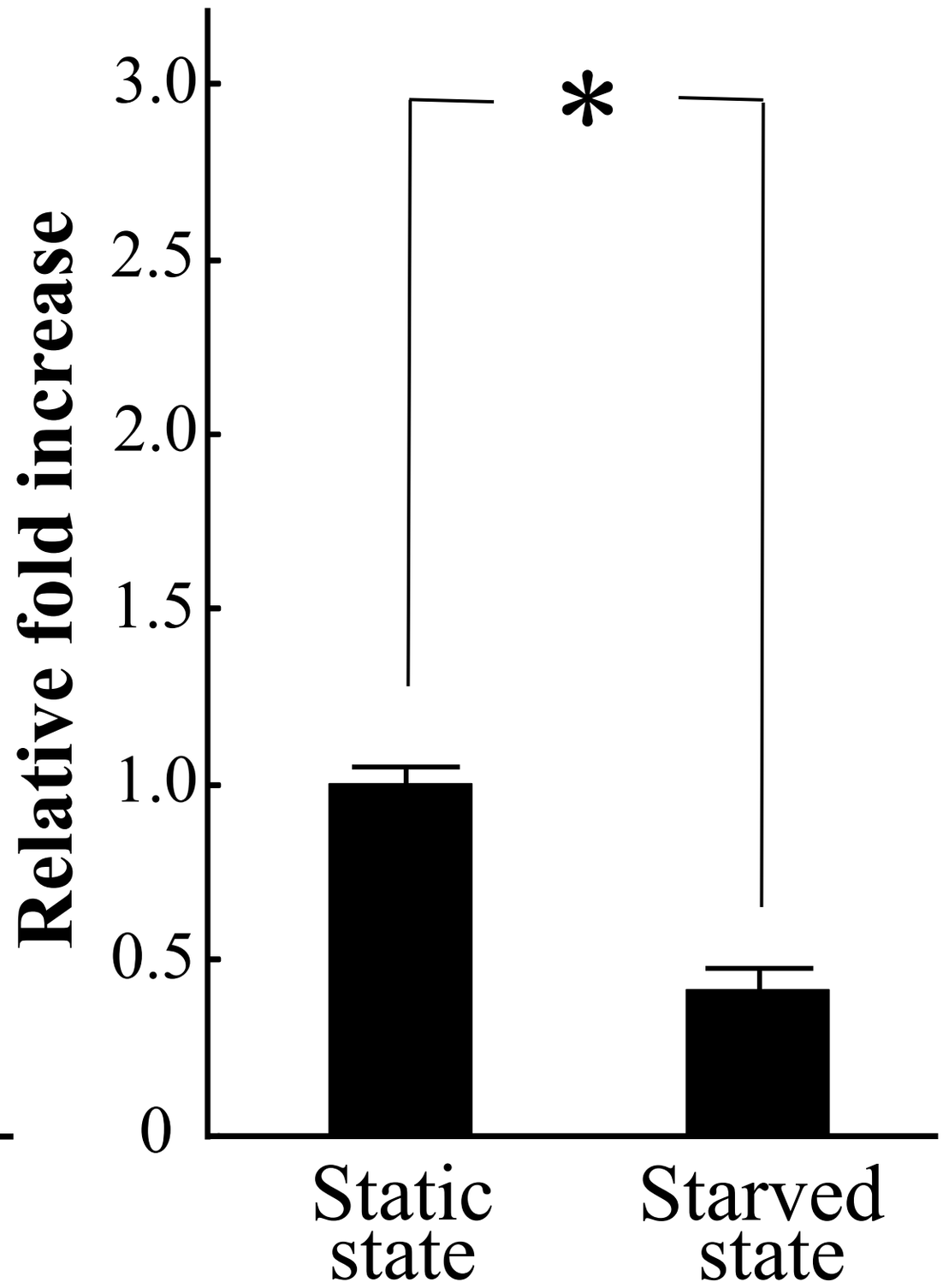




**Fig.4C**

**＊ ; <0.05 (Paired *t* test)**