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Significance of oestrogen-related receptor gamma on biliary epithelial cells in the pathogenesis of primary biliary cirrhosis

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ABSTRACT

Aims: Oestrogen has been speculated to play an important role in the pathogenesis of primary biliary cirrhosis (PBC), which mainly affects middle- and old-aged females, because biliary epithelial cells (BECs) are known to express oestrogen receptors (ERs). Oestrogen-related receptors (ERRs) are constitutively active without oestrogen and competitively inhibit the ER-dependent effects of oestrogen. We clarified the effects of oestrogen and the significance of ERRs along with their association with the pathogenesis of cholangiopathy in PBC. **Methods:** We investigated the expression of ERs and ERRs and the apoptosis-related cell kinetics in BECs using cultured human BECs and human liver specimens. **Results:** Although cultured human BECs and the interlobular bile ducts in the liver expressed ER β , in cultured BECs, oestrogen treatment did not induce significant cell proliferation but increased the expression of a negative cell proliferation regulator (14-3-3 σ protein). The cultured BECs constantly expressed ERR α and ERR γ , and oestrogen down-regulated the ERR γ expression. Furthermore, the ERR γ expression was determined in the intrahepatic bile ducts and was stronger in the middle- and old-aged females, particularly those with PBC, than in the younger females. The ERR γ ligand activated a transcription factor, SP1, and enhanced the expression of the pro-apoptotic Bcl-2 family molecules and Bcl-2 inhibitor-induced apoptosis in cultured BECs. **Conclusion:** Although oestrogen down-regulates the ERR γ expression, the increased ERR γ expression under oestrogen-deficient conditions increases the susceptibility to Bcl-2 family-mediated apoptosis in cultured human BECs of females, particularly those with PBC. Understanding the oestrogen-mediated cell kinetics is important for elucidating the pathogenesis of cholangiopathy in PBC.

INTRODUCTION

Primary biliary cirrhosis (PBC) mainly affects middle-aged females, where histologically, the interlobular bile ducts are damaged and reveals characteristics such as chronic non-suppurative destructive cholangitis (CNSDC), followed by progressive bile duct loss.^{1,2} There is considerable evidence that the damage of bile ducts is mediated by auto-reactive T cells, monocytes and natural killer cells.³⁻⁵ Biliary apoptosis and apoptoses are regarded as the major effectors in the immune-mediated loss of bile ducts in PBC, and aberrant Fas (CD95) expression and dysregulation of the Bcl-2 family are observed in the damaged bile ducts, including CNSDC in PBC.⁶⁻⁸ In contrast, gender-based differences in the incidence of PBC have been speculated to be associated with hormonal factors, including oestrogen.⁹⁻¹² Oestrogen, a sex steroid hormone, exhibits important biological functions, including proliferative effects, in target tissues. Oestrogen-stimulated growth in normal cells requires the oestrogen receptor (ER). Although bile ducts have not been considered a target of hormones, it was demonstrated that biliary epithelial cells (BECs), particularly rodent BECs, expressed ERs and oestrogen and have been speculated to play important roles in the pathogenesis of PBC.¹⁰⁻¹³

ER forms homo- or hetero-dimers consisting of ER α and ER β , which bind to an oestrogen-response element (ERE) and affect cell proliferation as well as the promotion of apoptosis and cell differentiation, respectively.¹⁴ Both ER α and ER β are observed in bile ducts in the early stages of PBC, but the disappearance of ER α in bile ducts during the cirrhotic stage and oestrogen deficiency are speculated to accompany the evolution of PBC toward ductopenia.¹¹ As the mechanism of cell proliferation via ER α (or ERs), an oestrogen response molecule, oestrogen-responsive finger protein (Efp), targets the 14-3-3 σ protein for proteolysis as an ubiquitin ligase and stimulates cell growth.¹⁵ 14-3-3 σ is a negative regulator of cell cycle progression and is important for maintaining G2 arrest by sequencing phosphorylated Cdc2-cyclin B1 from the nucleus

into the cytosol.¹⁶ Moreover, Efp is predominantly expressed in the tissues and cells targeted by oestrogen, including the mammary gland and uterine epithelial cells.¹⁷ In contrast to ER, oestrogen-related receptors (ERRs) are constitutively active members of the nuclear hormone receptor superfamily without oestrogen or without binding to ERs. Although the functional mechanism of ERRs is complicated, ERRs bind to ERE as well as ERR-response element (ERRE), modulating oestrogen-induced and ER-dependent oestrogen effects via competition with ERs.^{18,19}

In this study, we investigated ER and ERR expression in human BECs along with the apoptosis-associated cell kinetics to clarify the effects of oestrogen and its association with the pathogenesis of cholangiopathy in PBC.

MATERIALS AND METHODS

Cell culture

Two human intra-hepatic BEC lines were established from the explanted livers of females with PBC (BEC1 and BEC2).²⁰ The cell lines were confirmed to be BECs by the expression of the biliary-type cytokeratins, CK7 and CK19, and BECs were used between passages 6 and 10 to maintain their characteristics. Moreover, cDNA samples in BEC1 at passage 3 was also used to confirm the stable expression of receptors and molecules examined in this study. As the positive control for ERs and ERRs, the breast cancer cell line MCF-7 was purchased from the Health Science Research Resources Bank (Osaka, Japan).²¹

Cell viability on treatment with oestrogen and an ERR γ agonist

Cultured cells were treated with oestrogen (17 β -Oestradiol, E2, 10–200 pg/ml, Calbiochem, Darmstadt, Germany), GSK4716 (selective agonist at ERR β and ERR γ , 10 μ M, Sigma-Aldrich, Japan, Tokyo, Japan), Bcl-2 inhibitor (167 μ M, Calbiochem) and/or a pan-caspase inhibitor (40 μ M,

Calbiochem). Cell proliferation was measured 24 h later using the DNA–IdU labelling and detection kit (Takara, Ohtsu, Japan) or the tetrazolium salt WST-1 (Roche, Penzberg, Germany).

Treatment with E2 and the ERR γ agonist

To evaluate the regulation of ERs and ERRs after stimulation with oestrogen and an ERR agonist, in serum- and phenol red-free conditions, cultured cells were treated with E2 (200 pg/ml) for 3 h or 24 h, and total RNA samples were used for the following molecular analysis. Reverse transcription (RT)-polymerase chain reaction (PCR) and real-time PCR were performed for the detection and measurement of mRNAs of ERs (α and β), Efp, 14-3-3 σ , ERRs (α , β and γ) and the Bcl-2 family members. Total RNA was isolated using the RNeasy Total RNA System (Qiagen, Hilden, Germany) following the manufacturer's instructions. For real-time PCR, quantitative analyses according to standard protocols using the SYBR Green PCR Master Mix and MX3000P (Stratagene, Tokyo, Japan) were performed. The specific primers for ERs, ERRs and GAPDH (positive control) were designed as presented in Table 1.

Evaluation of transcription factors

To evaluate the influence of ERR γ , the activation of ER-related transcription factors, ER, activator protein-1 (AP1)²² and stimulating protein-1 (SP1)²³ were measured on the basis of the DNA-binding capacity of these transcription factors using a sensitive multi-well colorimetric assay according to the instruction manual (TransAM ER, AP1 and SP1 kits, respectively, Active Motif Japan, Tokyo, Japan).

Patients and liver tissue preparation

A total of 55 needle liver specimens were obtained from 25 patients with PBC (histological stage 1/2/3

= 3/14/8 and cholangitis activities CA0/1/2/3 = 2/6/4/13 by Nakanuma's grading and staging system,²⁴ mean age, 52 years, range, 42–80 years, all females) and from 30 females with no PBC (controls), including 3 patients with non-specific reactive changes, 13 with HCV-related chronic hepatitis, 1 with HBV-carrier, 8 with drug-induced liver injury and 5 with fatty liver (mean age, 49 years, range, 33–64 years). Moreover, as control biliary diseases, surgically resected or explanted livers were also obtained from 3 patients with primary sclerosing cholangitis (PSC) and 4 with extrahepatic biliary obstruction.

Immunohistochemistry

The expression of ER β , Efp, 14-3-3 σ and ERR γ was immunohistochemically assessed. The deparaffinised sections were microwaved in 10-mM citrate buffer for 20 min in a microwave oven. Following the blocking of endogenous peroxidase, the deparaffinised sections were incubated overnight at 4°C with the primary antibody against ER β (rabbit IgG, 2.5 μ g/ml, Abcam, Tokyo, Japan), Efp (mouse IgG1, 1 μ g/ml, BD, Tokyo, Japan), 14-3-3 σ (mouse IgG1, 5 μ g/ml, Abcam) or ERR γ (mouse IgG2a, 1 μ g/ml, PPRX, Tokyo, Japan) and then at room temperature for 1 h with goat anti-mouse or anti-rabbit immunoglobulin conjugated to a peroxidase-labelled dextran polymer (Envision, Dako Japan, Tokyo, Japan). After a benzidine reaction, sections were counterstained with haematoxylin or methyl green. As negative controls, normal mouse IgG1, mouse IgG2a or rabbit IgG was used as the primary antibody.

For semi-quantitative evaluation of the immunohistochemistry of ERR γ , 2 or 3 portal tracts containing interlobular bile ducts, including damaged bile ducts, were selected in each section for assessment, and the nuclear immunoreactivity in each bile duct was semi-quantitatively graded as follows: 0, absence of expression; 1, \leq 1/3 positive cells; 2, 1/3–2/3 positive cells and 3, \geq 2/3 positive cells. The final score indices for each patient were defined as the mean of individual cases.

Statistical analysis

Data were analysed using Welch's t-test or the paired t-test; $p < 0.05$ was considered statistically significant.

RESULTS

Effects of oestrogen on the proliferation of cultured BECs

RT-PCR revealed that ER β mRNA was amplified in human BECs cultured in standard medium, although the levels were lower than those in the positive control (MCF-7 cells; Fig. 1). In contrast, no clear amplification of ER α was evident. These expressions in BECs were stable until at least passage 9 (Supplemental figure 1). The stimulation with E2 did not induce significantly increased proliferation of human BECs, irrespective of the E2 levels (1–200 pg/ml; Fig. 2). In addition, the human BECs constantly expressed mRNAs of Efp and 14-3-3 σ , as detected by RT-PCR (Fig. 1). These expressions in BECs were stable until at least passage 9 (Supplemental figure 1). Moreover, quantitative analysis using real-time PCR revealed that E2 (200 pg/ml) did not affect the Efp expression but up-regulated the 14-3-3 σ expression (Fig. 3). Furthermore, immunohistochemistry revealed that BECs in the interlobular bile ducts constantly expressed ER β , Efp and 14-3-3 σ (Fig. 4). In addition, nuclear expression of ER β was determined in BECs. In contrast, the expression of Efp and 14-3-3 σ revealed a cytoplasmic pattern and was strong and weak, respectively, but did not change significantly with disease and age.

ERR expression in BECs and its regulation by oestrogen

RT-PCR demonstrated the mRNA expression of ERR α and ERR γ but not ERR β in the human BECs

that were cultured in a standard medium (Fig. 1). These expressions in BECs were stable until at least passage 9 (Supplemental figure 1). Moreover, real-time PCR demonstrated that E2 (200 pg/ml) did not affect the expression of ERs (α and β) and $ERR\alpha$ but significantly decreased the $ERR\gamma$ expression (Fig. 5).

$ERR\gamma$ expression in liver tissue

Immunohistochemistry demonstrated the nuclear expression of $ERR\gamma$ in several BECs of the interlobular bile ducts and bile ductules, hepatocytes and infiltrating inflammatory cells. BECs in the damaged bile ducts, including CNSDC in PBC, were particularly positive for $ERR\gamma$, but negative bile ducts were also scattered in young non-PBC patients (Figs. 6A and 6B). However, because the immunohistochemistry for $ERR\gamma$ did not work in surgical and explanted liver specimens, the data of PSC and extrahepatic biliary obstruction were excluded in the following analysis. Furthermore, semi-quantitative analysis revealed the $ERR\gamma$ expression in the interlobular bile ducts to be stronger in the middle- or old-aged females (>50 years) than in the younger females (≤ 50 years) and that in the middle- and old-aged females, PBC expression was up-regulated (Fig. 6C). Moreover, among the middle- or old-aged females with PBC, those with severe cholangitis activity (CA3) revealed high $ERR\gamma$ expression in the bile ducts compared with those with no, mild, or moderate activities (CA0–CA2; Fig. 6C).

Effects of the $ERR\gamma$ agonist in cultured BECs

Among ER-related transcription factors, as indicated by the results of DNA-binding capacity assays in Fig. 7, the $ERR\gamma$ agonist GSK4716 activated the nuclear factor SP1 but not ER or AP1 in cultured human BECs. Because the promoters of many pro- and anti-apoptotic genes have been found to contain SP1-binding elements,²⁵ we evaluated the regulation of the Bcl-2 family by GSK4716.

GSK4716 up-regulated the expression of the 2 pro-apoptotic proteins Bax and Bad but not that of the pro-apoptotic protein Bid or any other anti-apoptotic factors, including Mcl-1, Bcl-XL and Bcl-2 (Fig. 8). To investigate the effects of the ERR γ agonist on the cell kinetics of cultured human BECs, we analysed cell viability using the WST-1 assay. As displayed in Fig. 9, treatment with GSK4716 or with the Bcl-2 inhibitor alone did not reduce the cell viability after 24 h, but together, the ERR γ agonist and Bcl-2 inhibitor significantly reduced the cell viability; moreover, this reduction was inhibited by pre-treatment with the pan-caspase inhibitor.

DISCUSSION

One possible reason why PBC mainly affects females is that oestrogen worsens or attenuates the factors involved in the pathogenesis of this disease. Alvaro et al. used cultured rat BECs and reported that BECs (cholangiocytes) possessed both ER α and ER β and that oestrogen treatment induced a significant increase in cell proliferation, suggesting that oestrogen deficiency was closely associated with the pathogenesis of PBC and that replacement treatment with oestrogen may attenuate the disease activity. However, our study revealed that human BECs had ER β , but the functional expression of ER α was faint, and cell proliferation was not significantly increased by oestrogen treatment. The difference in the response to oestrogen between humans and rats is possibly because of the differences in ER expression between the 2 species,²⁶ particularly ER α expression.

14-3-3 σ is a negative regulator of cell proliferation, and Efp proteolyzes 14-3-3 σ and promotes cell proliferation.¹⁵ The present study used cultured human BECs and human liver tissues and revealed that BECs constantly expressed both Efp and 14-3-3 σ , suggesting that the proliferating activity of human BECs is negatively regulated by 14-3-3 σ and that oestrogen could induce the proliferation of BECs. However, the presence of oestrogen did not result in any significant increase

in the proliferative activity. As a possible explanation for this discrepancy, we speculated the presence of other negative regulatory systems as well as a lack of functional expression of ER α . Therefore, we evaluated the regulation of Efp and 14-3-3 σ expression and found that oestrogen up-regulated the expression of 14-3-3 σ but not Efp. These findings suggest that oestrogen-induced Efp activation proteolyzes 14-3-3 σ and simultaneously induces the expression of 14-3-3 σ , thereby affecting the negative regulation of oestrogen-induced cell proliferation. Although oestrogen-induced Efp activation generally targets 14-3-3 σ for proteolysis, inducing an increase in the proliferative activity of the affected cells, it is unclear why E2 could not affect the proliferative activity of BECs by the up-regulation of 14-3-3 σ expression. It is possible that the difference in these E2 functions is associated with the functions of the 2 oestrogen receptors ER α and ER β . Moreover, although Efp expression was constantly found in the interlobular bile ducts of all patients, including pre-menopausal females, the distinct down-regulation of 14-3-3 σ was not determined in any age or disease, which supports the presence of the up-regulation system of 14-3-3 σ in BECs.

ERRs are constitutively active members of the nuclear hormone receptor superfamily without oestrogen or without binding to ERs and inhibit oestrogen-induced and the ER-dependent effects of oestrogen by competing with ERs.^{18,19} The present study used cultured human BECs and revealed that BECs expressed ERR α and ERR γ , thereby suggesting that these ERRs could regulate biliary oestrogen signalling via ERs. Moreover, the presence of oestrogen down-regulated the ERR γ expression; in addition, the ERR γ levels in the interlobular bile ducts was lower in the younger females than in the middle- and old-aged females, suggesting that the bile ducts of younger females are easily affected by oestrogen considering a relative insusceptibility to the inhibitory effects of ERR γ . In contrast, the bile ducts among the middle- and old-aged females, particularly those with marked activity of chronic cholangitis (CA3)²⁴ in PBC, revealed significantly increased the ERR γ expression compared with those in the non-PBC females. Thus, under the conditions of low serum

oestrogen levels in the middle- and old-aged females, ERR γ function is speculated not to compete with oestrogen.

Next, to clarify the significance of highly expressed ERR γ in the damaged bile ducts of PBC, we first examined the ER-related transcription factors activated by ERR γ in human BECs. The ERR γ agonist GSK4716 activated SP1 but not ER or AP1 in BECs. Although ER, AP1 and SP1 are all known ER-related transcription factors,^{22,23} ERR γ was associated with SP1 in human BECs, suggesting an interaction between ER and ERR γ in the intra-cellular signalling via SP1 and its related transcription. Next, because the SP1-binding site is located within the promoter regions of several apoptosis-related molecules,²⁵ we evaluated the expression of the Bcl-2 family members associated with apoptosis. GSK4716 induced the up-regulation of the 2 pro-apoptotic molecules Bad and Bax but not of any anti-apoptotic molecules in human BECs. As a mechanism of the bile duct loss in PBC, several previous studies have demonstrated that enhanced biliary apoptosis was associated with the Bcl-2 family and Fas (CD95).^{6,7} Our previous study has revealed that the expression of a pro-apoptotic factor, Bax, was predominant in the damaged bile ducts in PBC compared with that of the anti-apoptotic Mcl-1 and Bcl-XL.²⁷ Moreover, combined treatment with the ERR γ agonist and Bcl-2 inhibitor significantly reduced the viability of the cultured human BECs, and this reduction was completely rectified by pre-treatment with a caspase inhibitor, suggesting that the reduced cell viability is caused by enhanced biliary apoptosis. Therefore, it is speculated that the increased susceptibility to biliary apoptosis caused by ERR γ - and SP1-associated transcriptional signalling is closely associated with the pathogenesis of bile duct loss in PBC.

In conclusion, the functional expression of ERRs in human BECs is suggested to play a role in regulating the effects of oestrogen by competing with ERs. In the younger females, the ERR γ expression in the bile ducts is inhibited by increased serum levels of oestrogen, and the ER-dependent effects of oestrogen could be predominant. In contrast, increased the ERR γ expression

followed by increased susceptibility to biliary apoptosis in the bile ducts, particularly the damaged bile ducts, of middle- or old-aged females, including post-menopausal females, is an important finding to understand the pathogenesis of cholangiopathy in PBC.

Take home messages

- Human biliary epithelial cells express ER β , and oestrogen treatment does not induce significant cell proliferation.
- Although oestrogen down-regulates the ERR γ expression, the increased ERR γ expression under oestrogen-deficient conditions increases the susceptibility to Bcl-2 family-mediated apoptosis in human BECs of females, particularly those with PBC.
- Understanding this oestrogen-mediated cell kinetics is important for elucidating the pathogenesis of cholangiopathy in PBC.

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Table 1 Primers used for RT-PCR and quantitative PCR

Transcript	Primers	Product size
ER α	Forward 5'-TTTGCATCCAATTGTGCCTGA-3' Reverse 5'-TGTTGTTTCTAGGGTGCACAGCTC-3'	103 bp
ER β	Forward 5'-GATGCTCCTGTCCCACGTCA-3' Reverse 5'-CACTGCTCCATCGTTGCTTCA-3'	111 bp
Efp	Forward 5'-GCATCTGCCACATCTGCCTG-3' Reverse 5'-GCTGTTGACCCTCTTCTCCTCTTC-3'	296 bp
14-3-3 σ	Forward 5'-AGAGACACAGAGTCCGGCATTGG-3' Reverse 5'-TCCACCTTCTCCCGGTA CTACGC-3'	290 bp
ERR α	Forward 5'-CTTGGCCCTTGCCAATTC-3' Reverse 5'-CCGGCTTCATACTCCAGCAG-3'	102 bp
ERR β	Forward 5'-CAGGCAGATCTCACCCAGCA-3' Reverse 5'-TGAGGTCACTCGCAGCAACAC-3'	148 bp
ERR γ	Forward 5'-TGAAACATCAACCCAGTGCT-3' Reverse 5'-AAGGGATGTGCCAATTTCTG-3'	196 bp
Bad	Forward 5'-CCGAGTGAGCAGGAAGACTC-3' Reverse 5'-GGTAGGAGCTGTGGCGACT-3'	205 bp
Bid	Forward 5'-GCAGGCCTACCCTAGAGACA-3' Reverse 5'-GTCCATCCCATTTCTGGCTA-3'	181 bp
Bax	Forward 5'-TTTGCTTCAGGGTTTCATCC-3' Reverse 5'-ATCCTCTGCAGCTCCATGTT-3'	162 bp
Mcl-1	Forward 5'-TGCTGGAGTAGGAGCTGGTT-3' Reverse 5'-CCTCTTGCCACTTGCTTTTC-3'	176 bp
Bcl-XL	Forward 5'-GTAAACTGGGGTCGCATTGT-3' Reverse 5'-TGGATCCAAGGCTCTAGGTG-3'	146 bp
Bcl-2	Forward 5'-TGTGGATGACTGAGTACC-3' Reverse 5'-AGGAGAAATCAAACAGAGG-3'	117 bp
GAPDH	Forward 5'-GCACCGTCAAGGCTGAGAAC-3' Reverse 5'-ATGGTGGTGAAGACGCCAGT-3'	142 bp

FIGURE LEGENDS

Fig. 1 The expression of oestrogen receptor (ER) α , ER β , oestrogen-responsive finger protein (Efp), 14-3-3 σ , oestrogen-related receptor (ERR) α , ERR β and ERR γ . RT-PCR analysis using cultured biliary epithelial cells (BECs) and MCF-7 (used as positive controls) was performed for 40 cycles except for GAPDH (35 cycles) to survey the presence or absence of amplifications and confirm the absence of non-specific amplifications. BECs expressed mRNAs of ER β , Efp, 14-3-3 σ , ERR α , ERR γ and the positive control (GAPDH). However, ER α expression is faint, and no amplification of ERR β is evident. Each PCR product provides bands of the appropriate molecular weight. N/C, distilled water instead of reverse transcriptase (negative control) for reverse transcription.

Fig. 2 Assessment of cell viability using the DNA-IdU labelling. Human biliary epithelial cells cultured in serum- and phenol red-free media were treated with oestrogen (17 β -Oestradiol, E2, 10–200 pg/ml), and the degree of cell proliferation was measured 24 h later. Results were obtained from 2 independent experiments and were shown relative to the levels without oestrogen treatment. No significant differences were observed, irrespective of the presence of oestrogen. The bars indicate the mean \pm S.E.M values.

Fig. 3 The effects of oestrogen on the expression of oestrogen-responsive finger protein (Efp) and 14-3-3 σ . Human biliary epithelial cells cultured in serum- and phenol red-free media were treated with oestrogen (17 β -Oestradiol, E2, 10–200 pg/ml) for 3 h. Quantitative analysis using real-time PCR revealed that oestrogen significantly up-regulated the 14-3-3 σ mRNA

expression by 2.7 ± 0.6 -fold (mean \pm S.E.M) but not the Efp expression. Results obtained from 2 independent experiments are presented relative to the levels without oestrogen treatment (vehicle control). The bars indicate the mean \pm S.E.M values. * <0.05 .

Fig. 4 Immunohistochemical staining of oestrogen receptor β (ER β , A), oestrogen-responsive finger protein (Efp, B) and 14-3-3 σ (C). A: Primary biliary cirrhosis (PBC). Biliary epithelial cells in the normal interlobular bile ducts (arrow) and bile ductules express ER β in the nucleus. B: HCV-related chronic hepatitis. Strong cytoplasmic expression of Efp is evident in the bile ducts (arrow). C: Primary sclerosing cholangitis. Marked expression of Efp is found in the bile ducts (arrow). D: PBC. Damaged bile ducts expressed 14-3-3 σ in the cytoplasm (arrow). The staining patterns are constant, and significant differences are evident among the diseases and age groups.

Fig. 5 The effects of oestrogen on the oestrogen receptors (ERs) and oestrogen-related receptors (ERRs). Human biliary epithelial cells cultured in serum- and phenol red-free media were treated with oestrogen (17 β -Oestradiol, E2, 200 pg/ml) for 3 h. Quantitative analysis using real-time PCR revealed a relative decrease in the ERR γ mRNA expression by 0.39 ± 0.17 -fold (mean \pm S.E.M), compared with no treatment (non, vehicle control). In contrast, ER α , ER β and ERR α were not affected by oestrogen. The results were obtained from 2 independent experiments and were shown relative to the levels without oestrogen treatment. The bars indicate the mean \pm S.E.M values. * <0.05 .

Fig. 6 Immunohistochemistry of oestrogen-related receptor γ (ERR γ). A: Primary biliary cirrhosis (PBC). The immunoreaction to ERR γ was restricted to the nucleus, and most biliary epithelial cells were positive in the damaged bile duct (CNSDC, arrow). B: HCV-related

chronic hepatitis. The positive and negative cells are inter-mingled in the mildly damaged bile ducts (hepatic bile duct injury, arrow). C: Semi-quantitative analysis demonstrated that the ERR γ expression was higher in the inter-lobular bile ducts of the middle- or old-aged females (>50 years) than in those of the younger females (\leq 50 years). In the middle- or old-aged females, the ERR γ expression was predominantly evident in PBC when compared with the non-PBC diseased livers (controls). Moreover, among the middle- or old-aged females with PBC, the patients with severe cholangitis activity (CA3 by Nakanuma's grading system for PBC) revealed high ERR γ expression in the bile ducts when compared with those with no, mild or moderate activities (CA0–CA2). The bars indicate the mean \pm S.E.M values. * <0.05.

Fig. 7 The 3 transcription factors oestrogen receptor (ER), activator protein-1 (AP1) and stimulating protein-1 (SP1) and the DNA binding assay of the ERR γ agonist GSK4716 (10 μ M)-induced activation in the cultured human biliary epithelial cells. The results are presented as optical density (OD). The activation of ER and AP1 by GSK4716 was similar to that without treatment (vehicle control). In contrast, although the baseline levels of SP1 (vehicle) were slightly increased, GSK4716 increased the SP1 activation by 2.3-fold. Data using cultured human cells (BEC1) are presented. P/C, positive controls were used in the enclosed samples of each kit. Each DNA-transcription factor binding activity was effectively competed for by the wild-type consensus oligonucleotide but not by the mutated oligonucleotide (data not shown).

Fig. 8 The effects of ERR γ on the mRNA expression of the Bcl-2 family. The human biliary epithelial cells were treated with the ERR γ agonist GSK4716 (10 μ M) for 3 h. Quantitative analysis using real-time PCR revealed that among the pro-apoptotic molecules, the

expression of Bad and Bax (A) significantly increased by 1.75 ± 0.21 - and 1.50 ± 0.11 -fold (mean \pm S.E.M), respectively, compared with no treatment (vehicle control). However, the pro-apoptotic protein Bid (A) and the anti-apoptotic factors Mcl-1, Bcl-XL and Bcl-2 (B) were not affected by the ERR γ agonist. The results were obtained from 2 independent experiments and were shown relative to the levels without GSK4716. The bars indicate the mean \pm S.E.M values. * <0.05.

Fig. 9 The effects of the ERR γ agonist GSK4716 on Bcl-2 inhibitor-induced apoptosis. Combined treatment with GSK4716 (10 μ M) and the Bcl-2 inhibitor (167 μ M) significantly reduced the viability of the cultured human biliary epithelial cells for 24 h, and this reduction was rectified by pre-treatment with pan-caspase inhibitor (40 μ M), although GSK4716 or the Bcl-2 inhibitor alone did not reduce cell viability. The results were obtained from 2 independent experiments using the WST-1 assay. The bars indicate the mean \pm S.E.M. * <0.05.

Supplemental figure 1. The expression of oestrogen receptor (ER) α , ER β , oestrogen-responsive finger protein (Efp), 14-3-3 σ , oestrogen-related receptor (ERR) α , ERR β and ERR γ mRNAs in cultured biliary epithelial cell 1 (BEC1) at the passage indicated. Quantitative analysis using real-time PCR revealed that the expressions of all receptors/molecules were maintained, though the expression of ERR γ was halved between passages 3 and 6. Results are presented as relative ratio (%) to those in MCF-7 (used as positive controls).

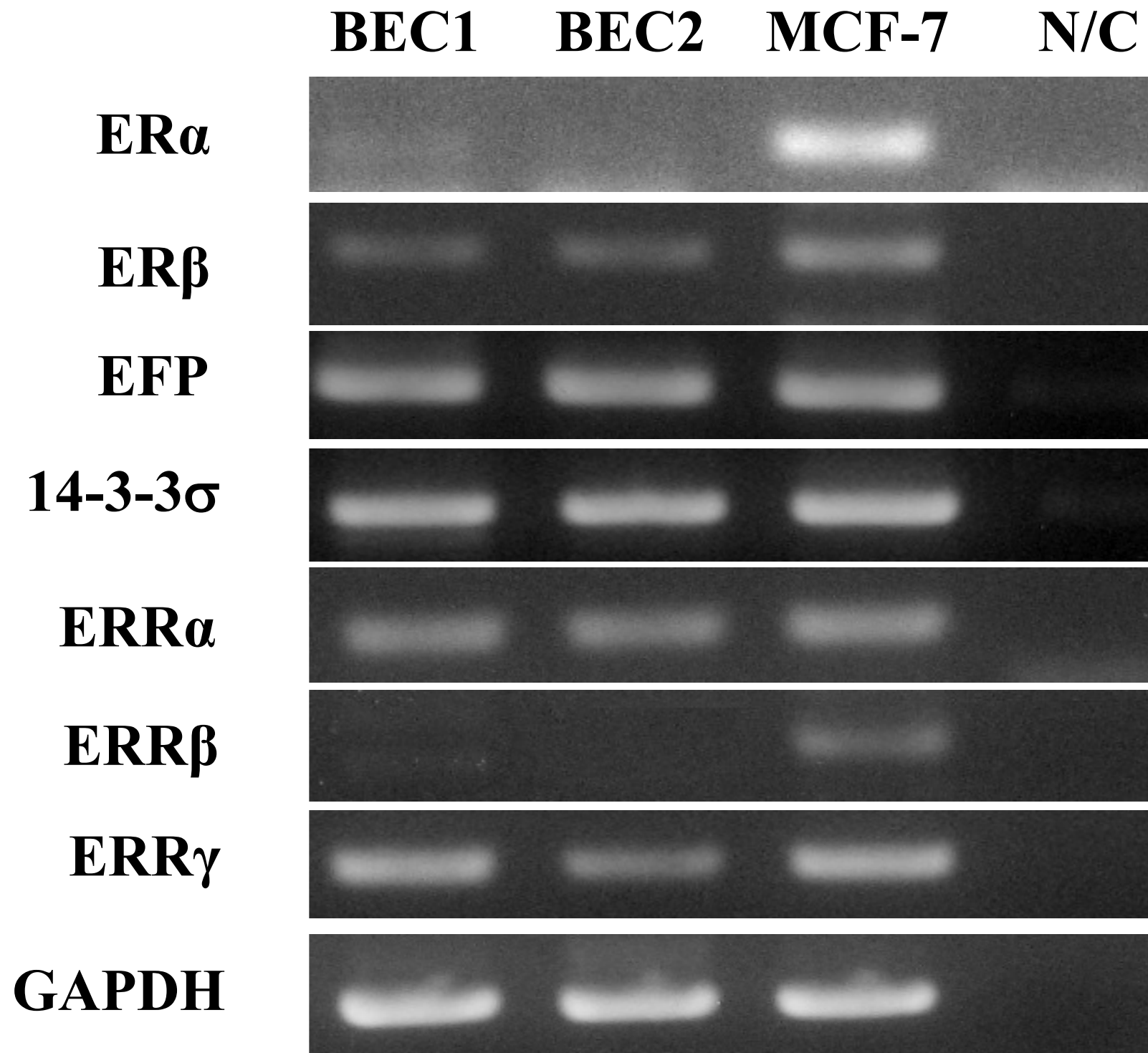


Fig.1

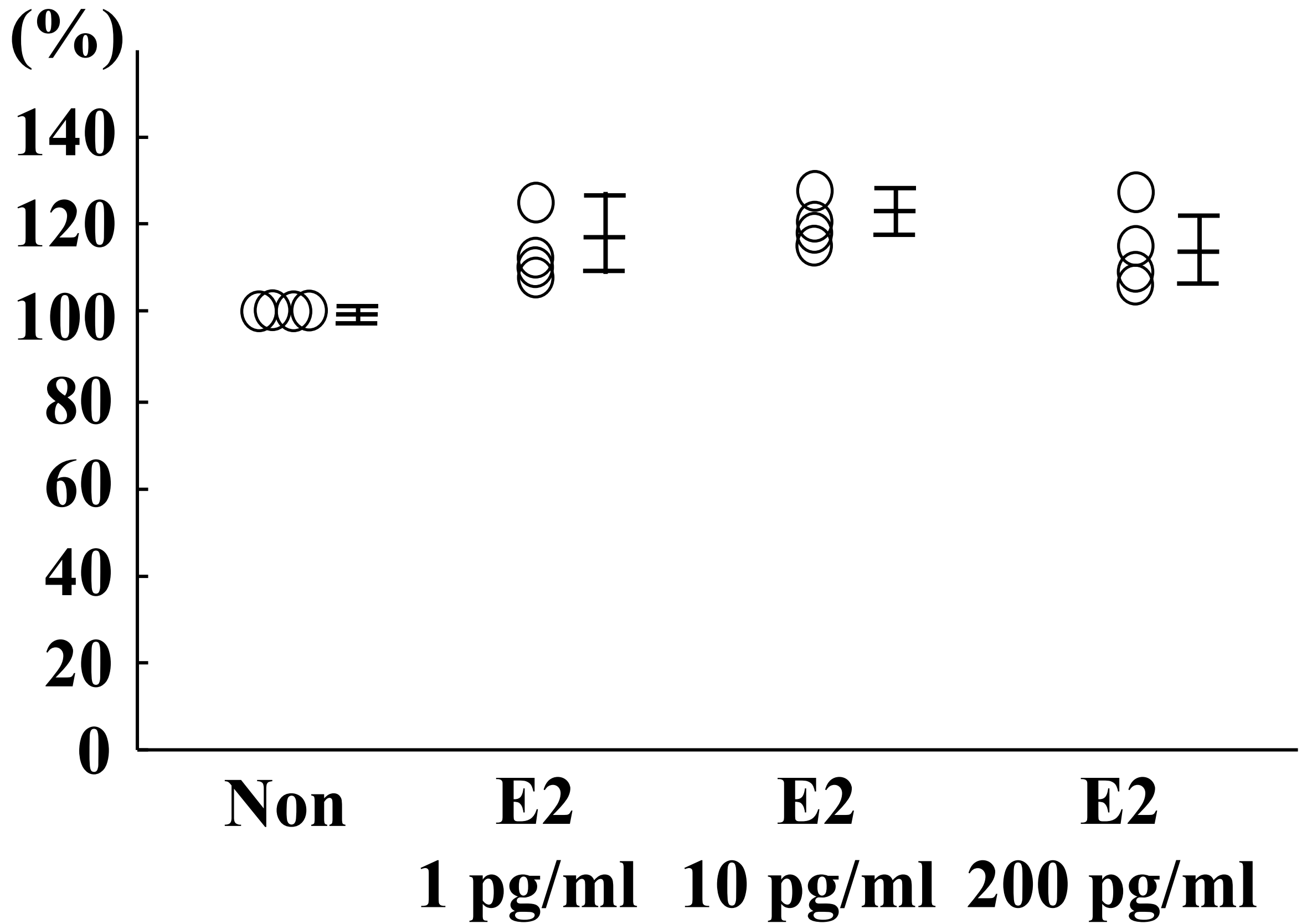


Fig.2

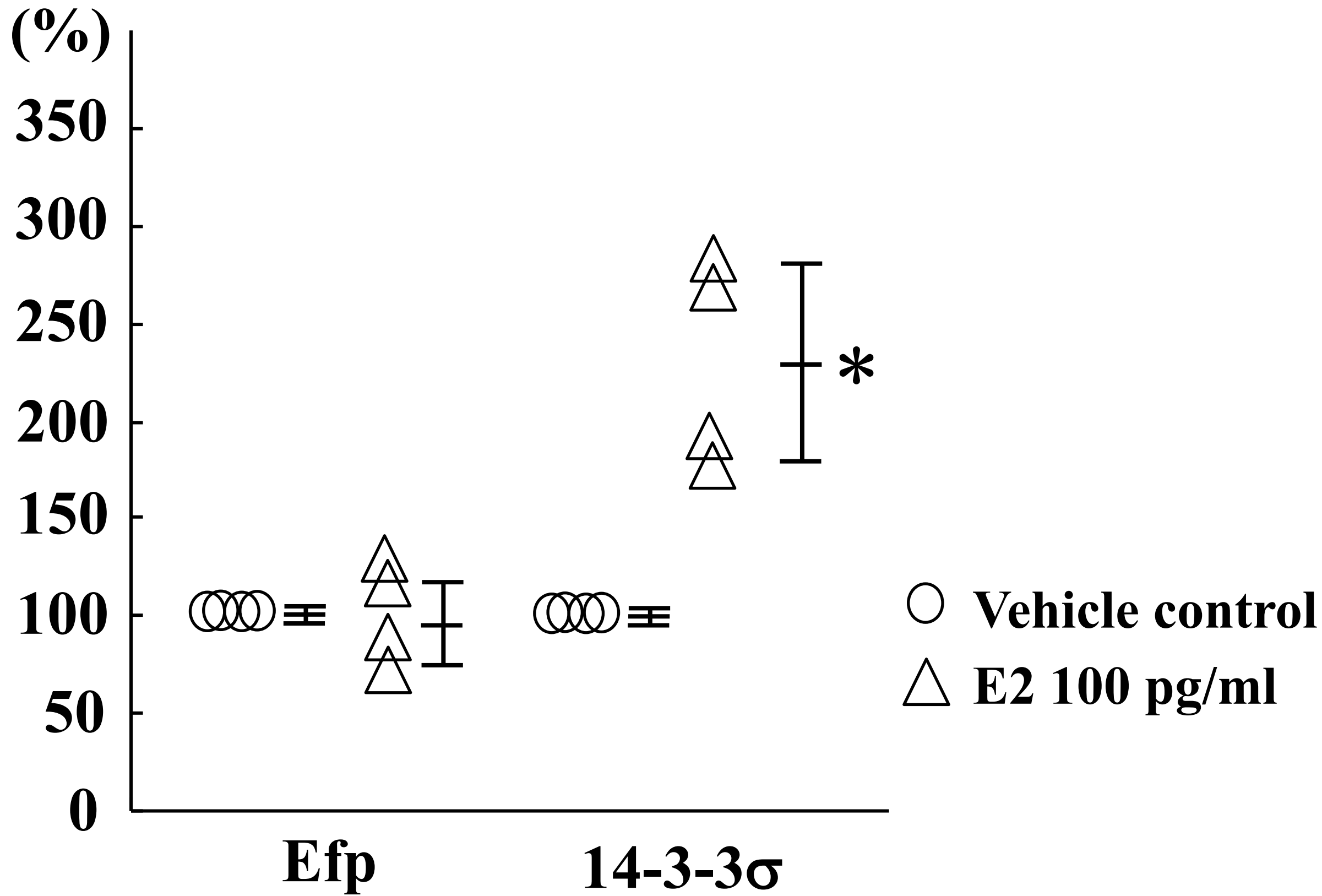
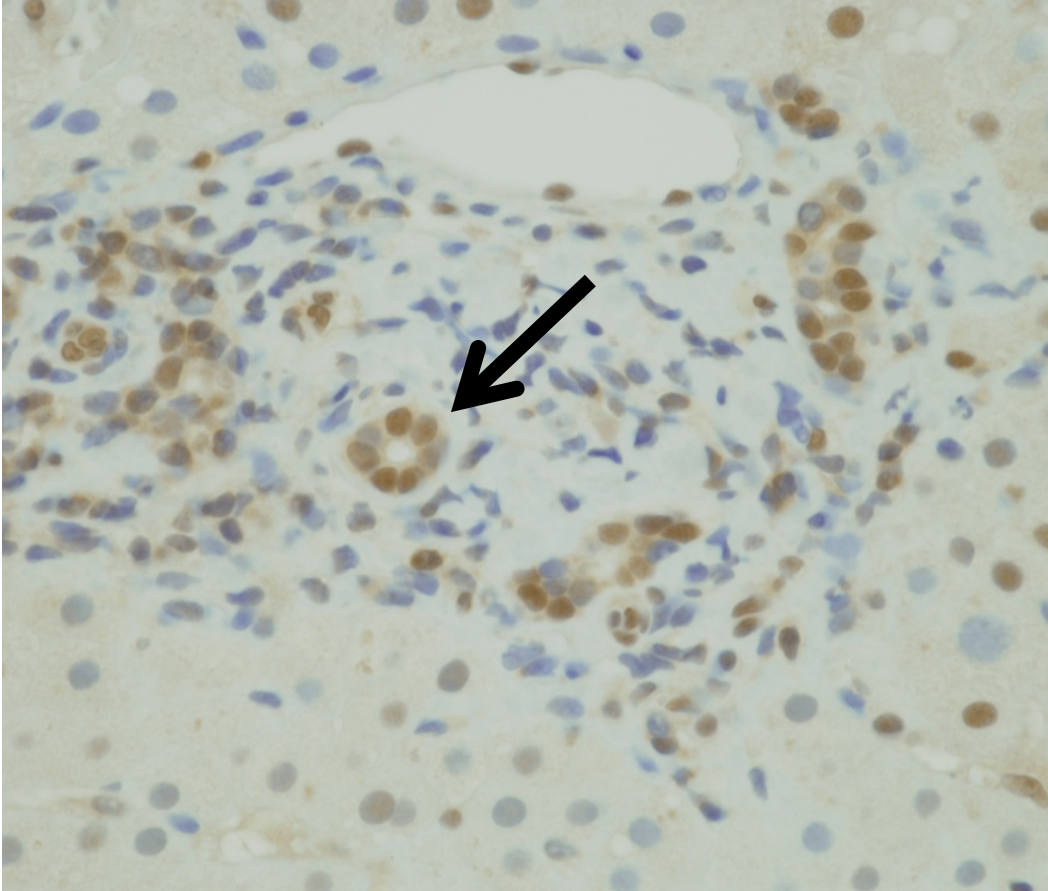
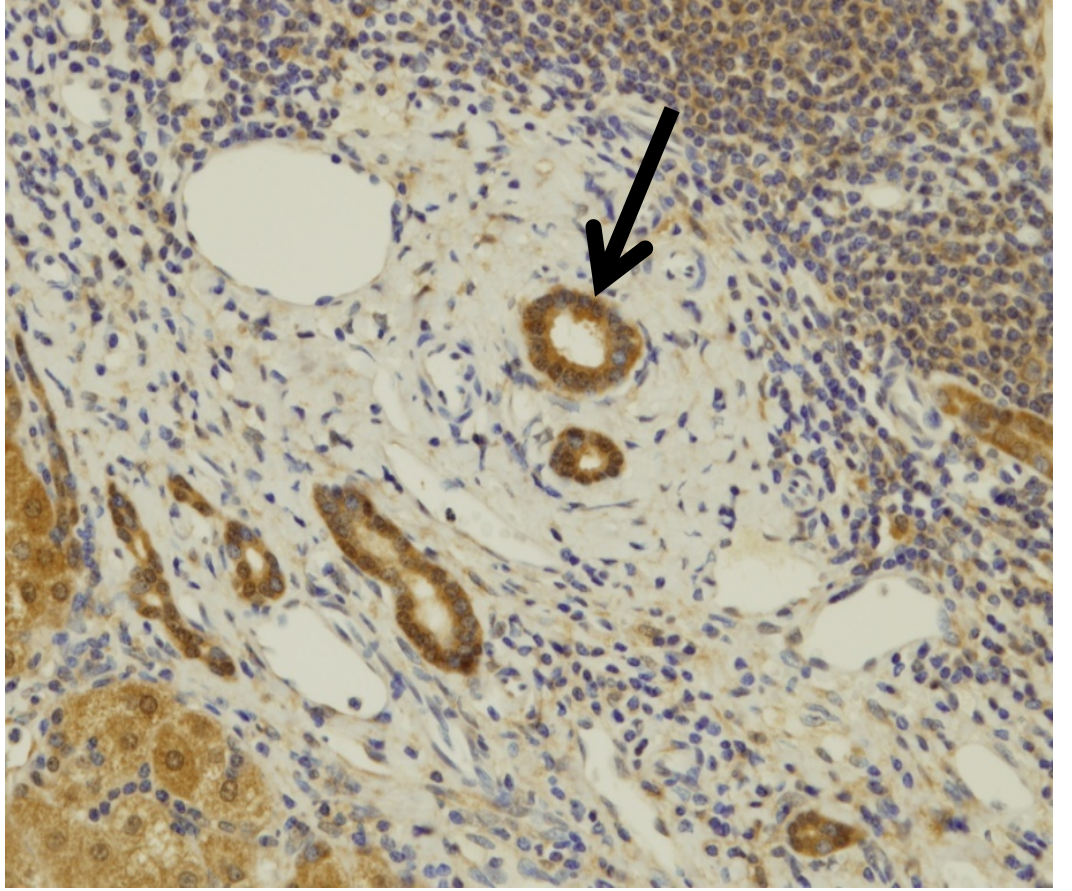


Fig.3

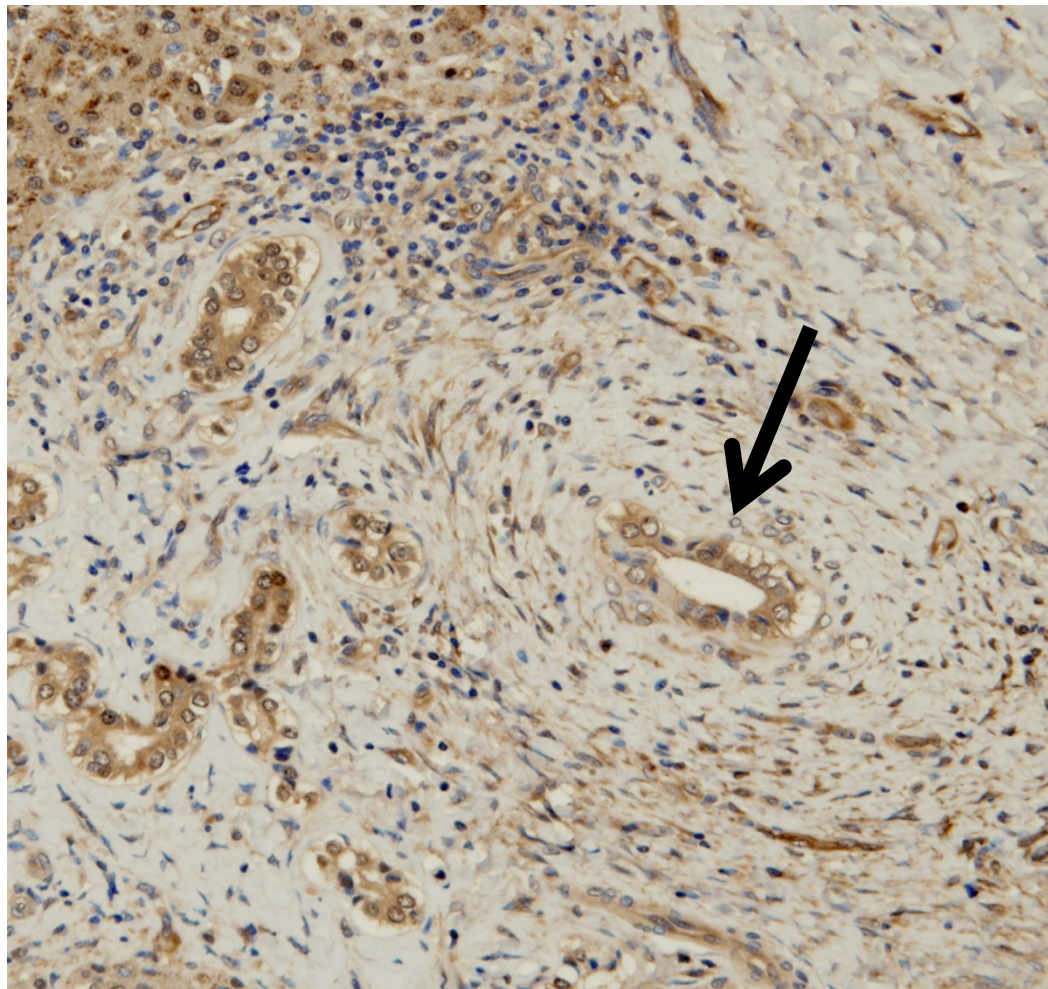
A



B



C



D

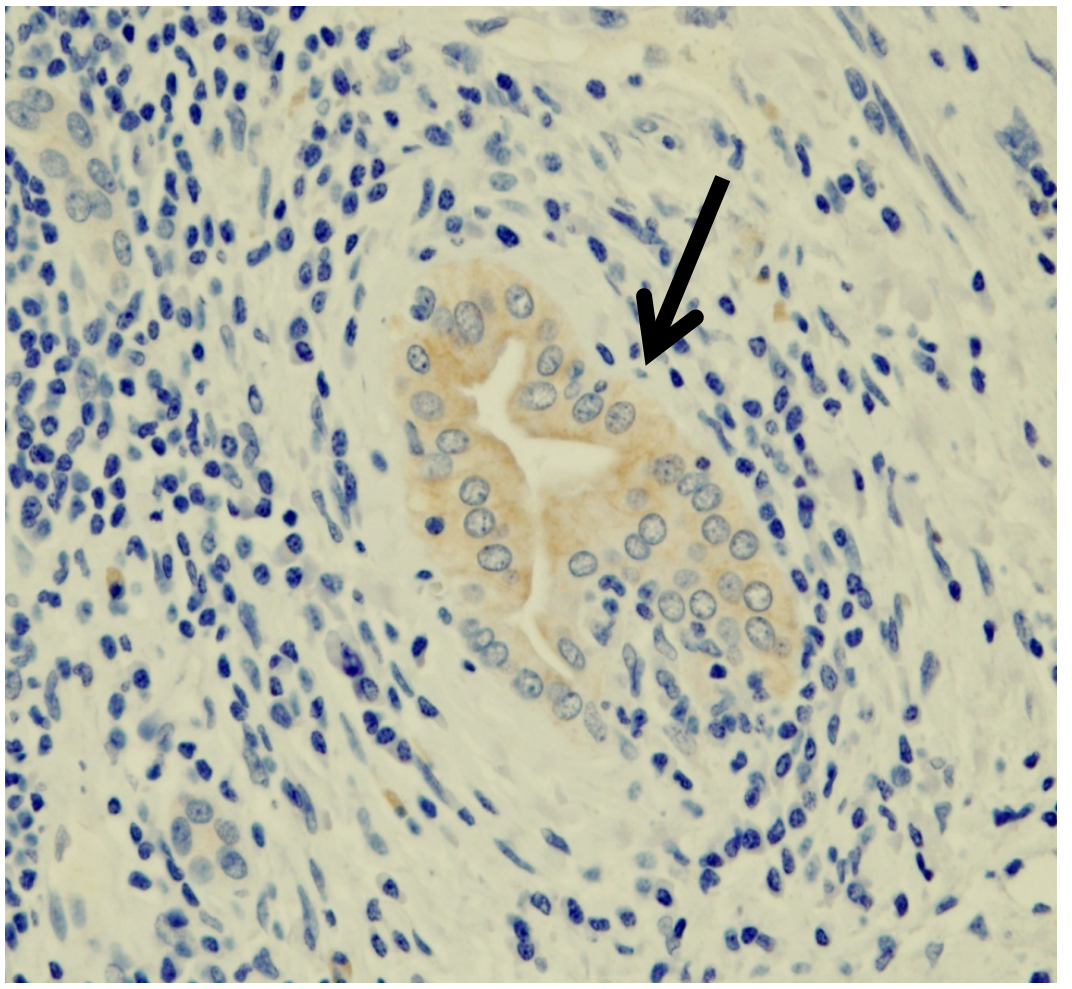


Fig.4

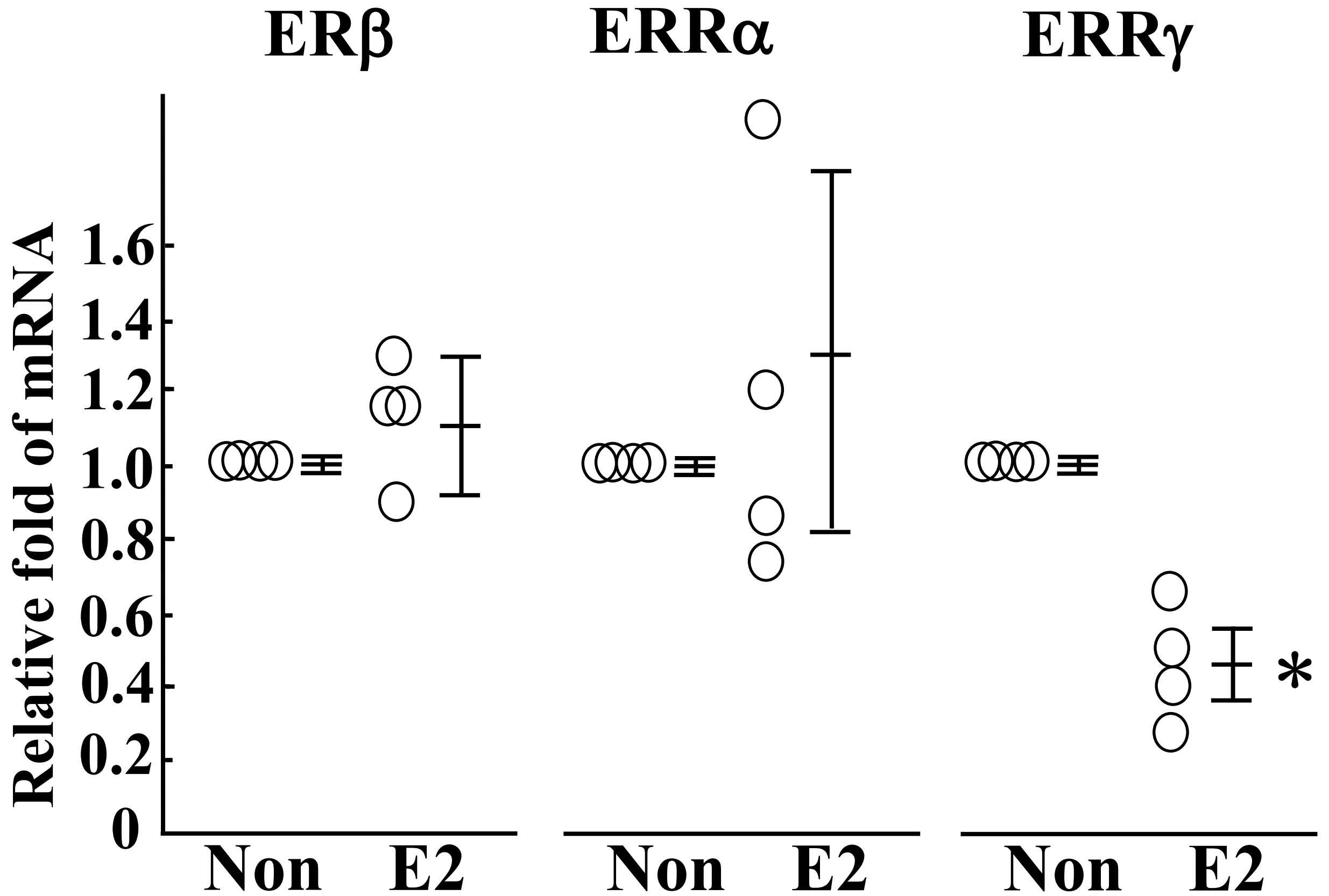
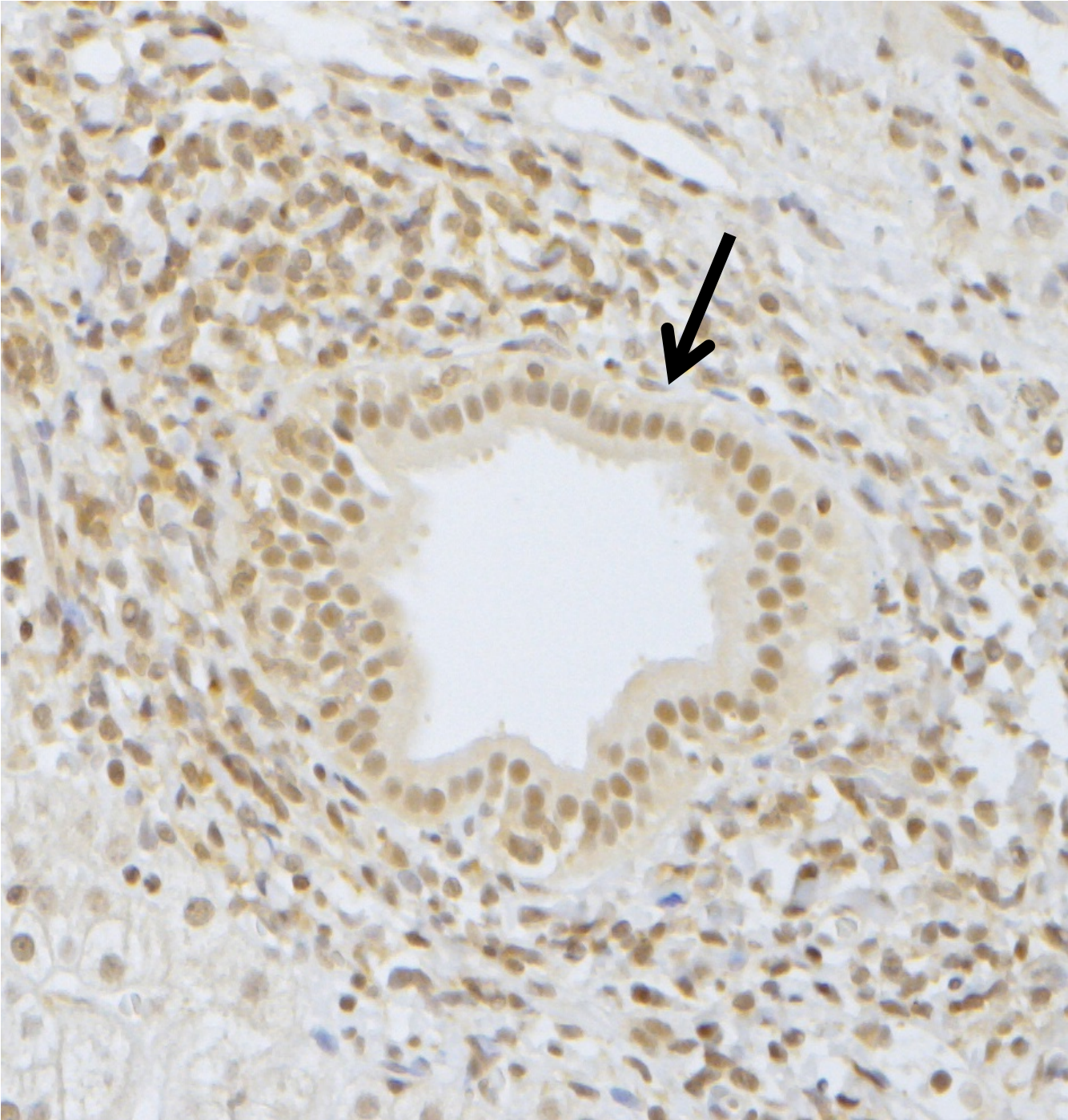
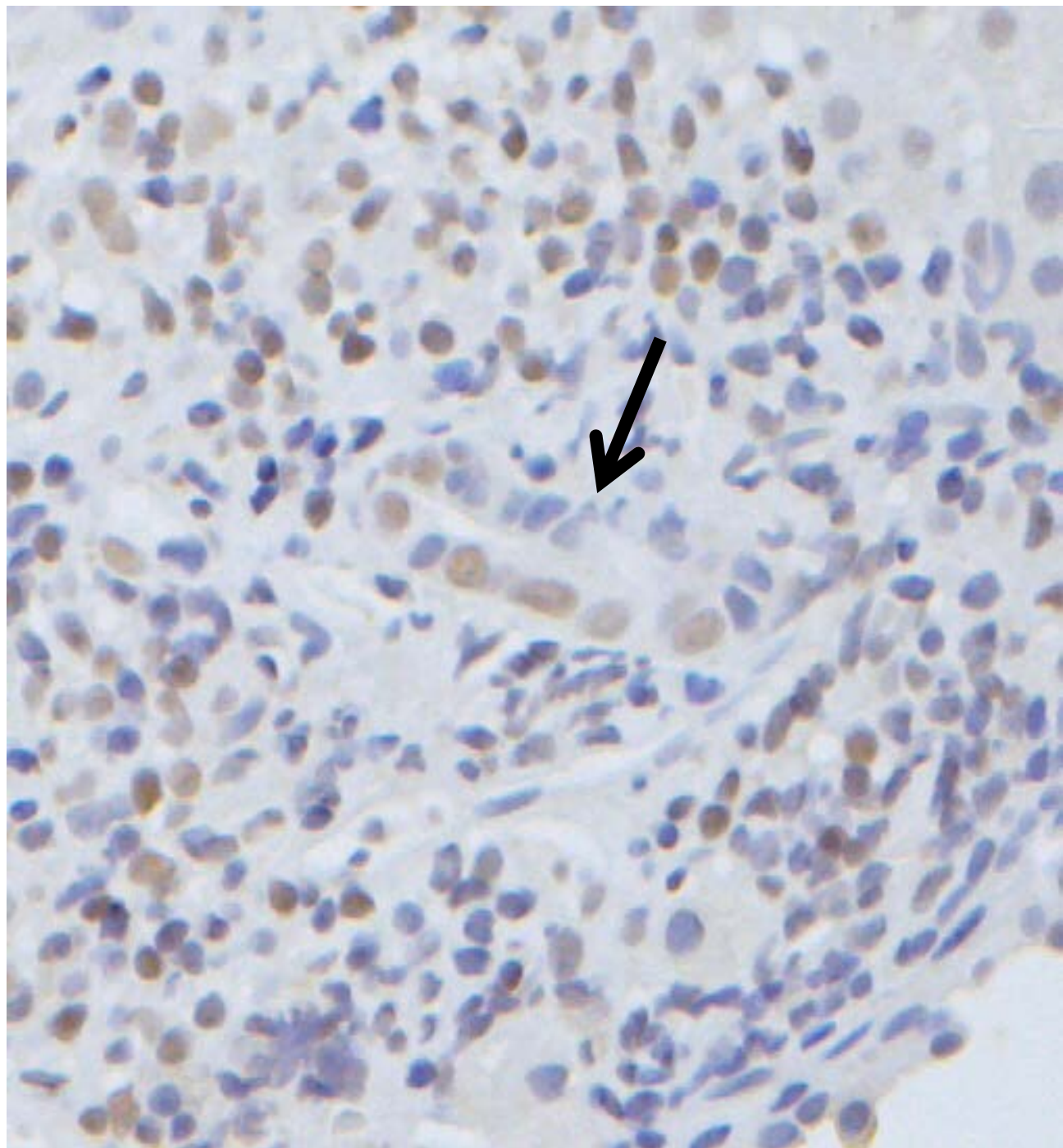


Fig.5

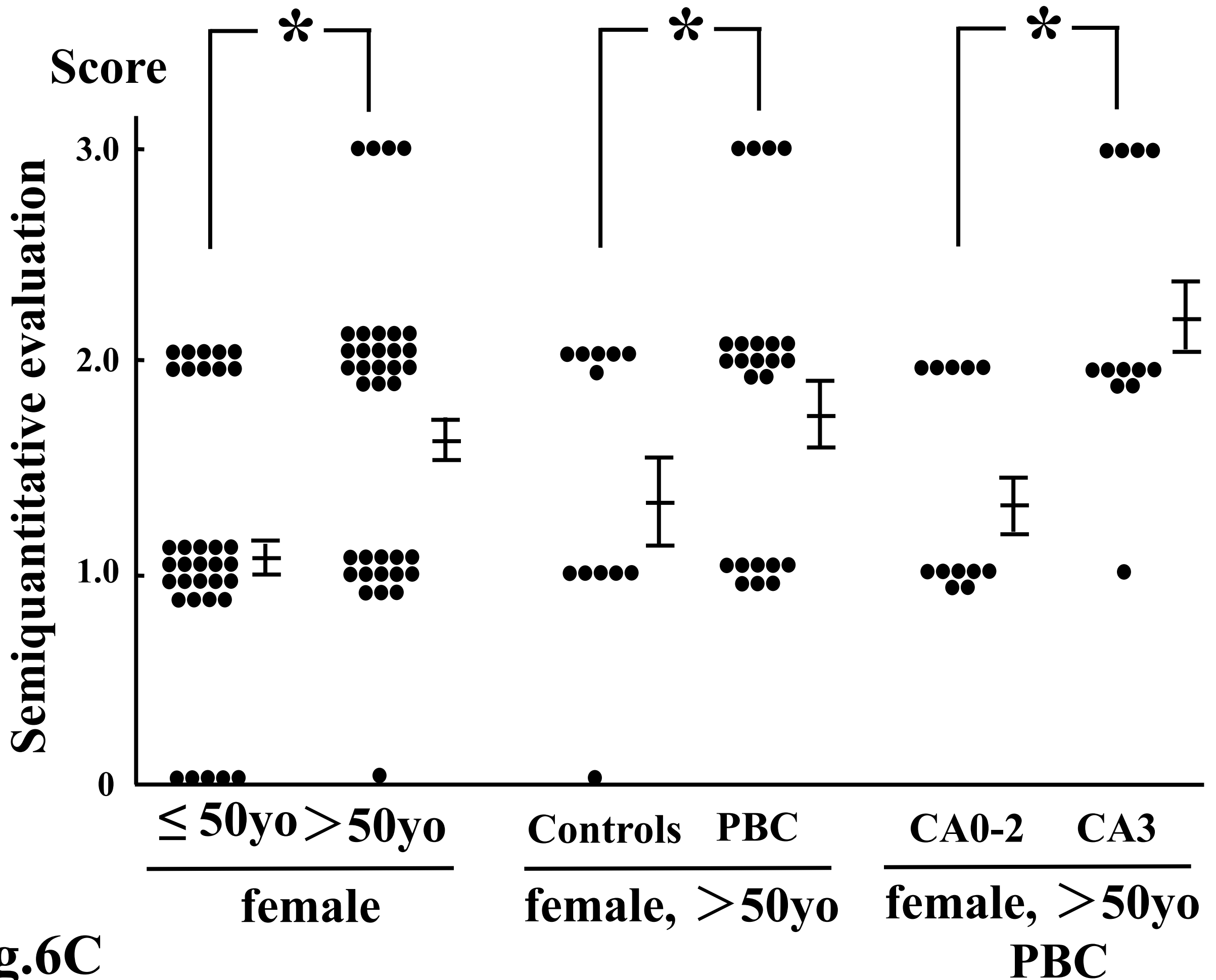


A



B

Fig.6



(OD)

ER

AP1

SP1

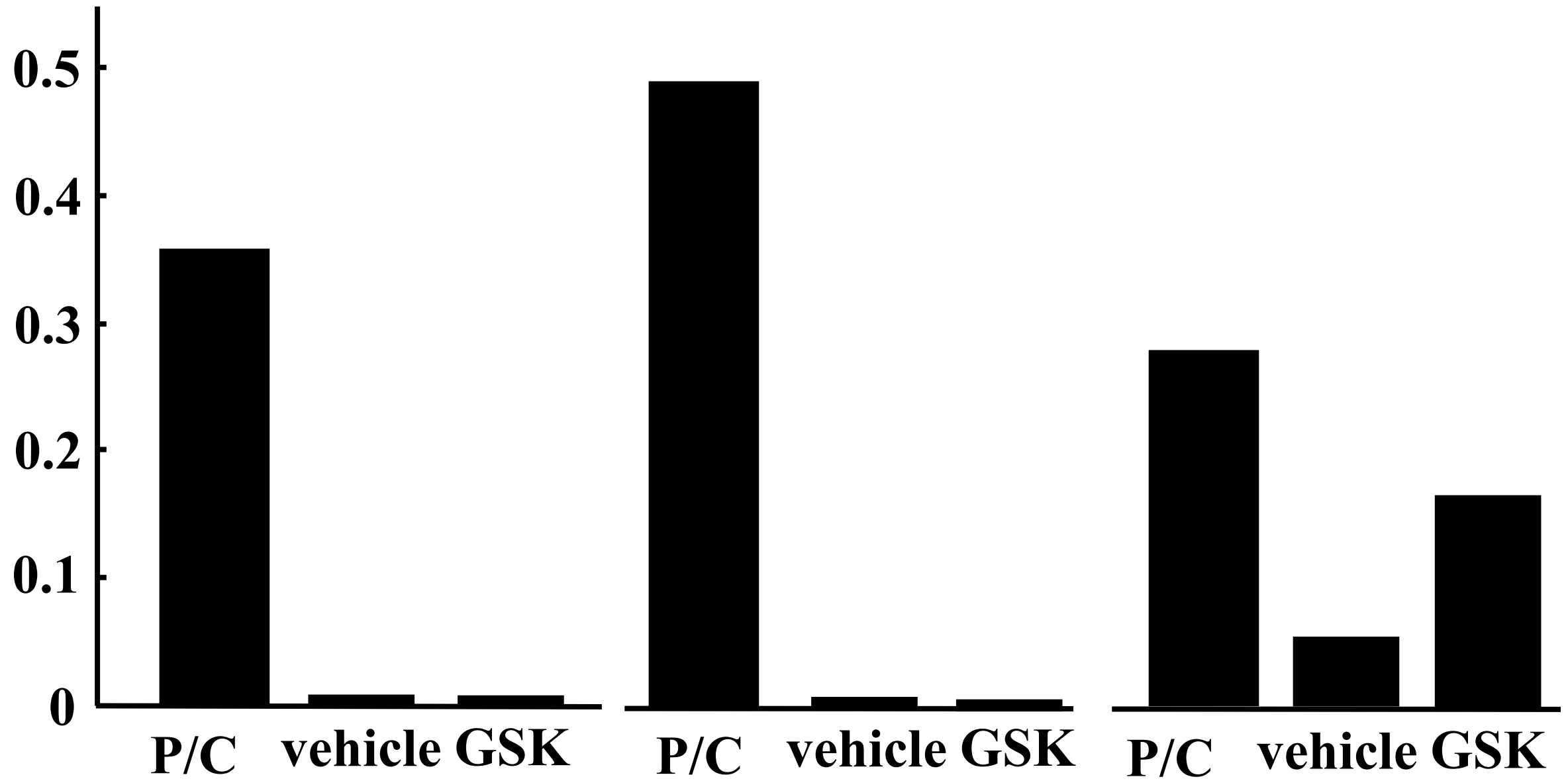


Fig.7

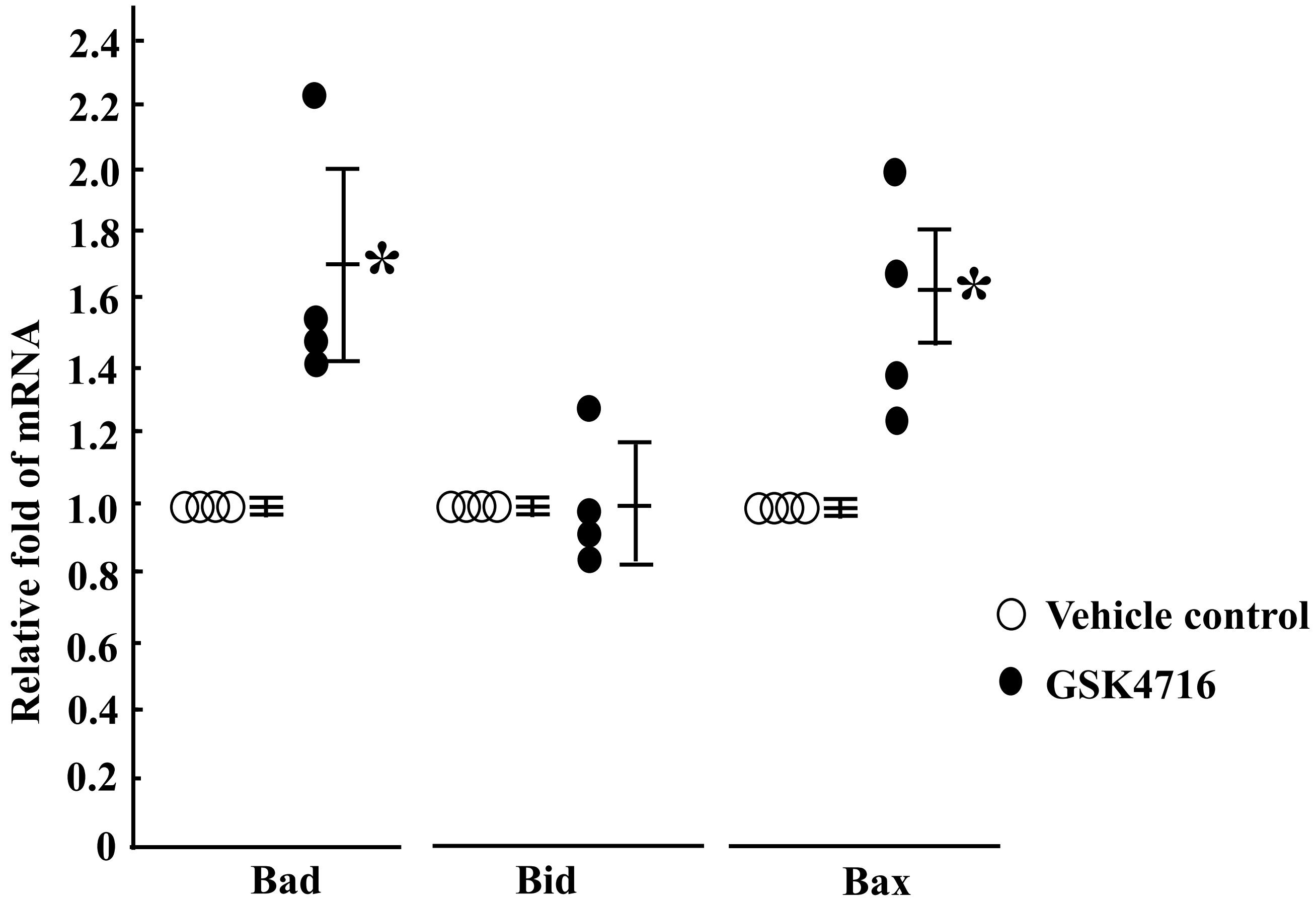


Fig.8A

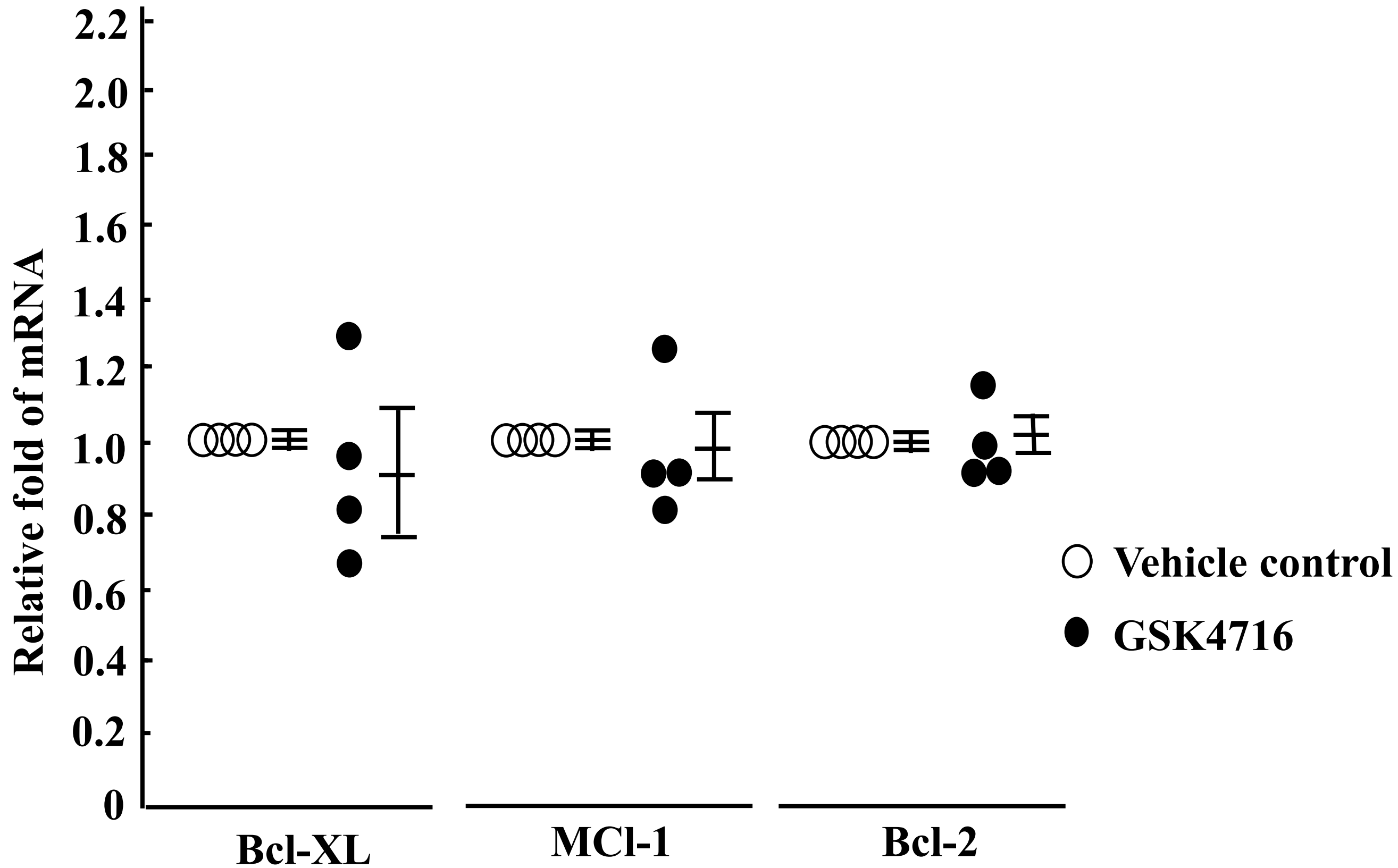


Fig.8B

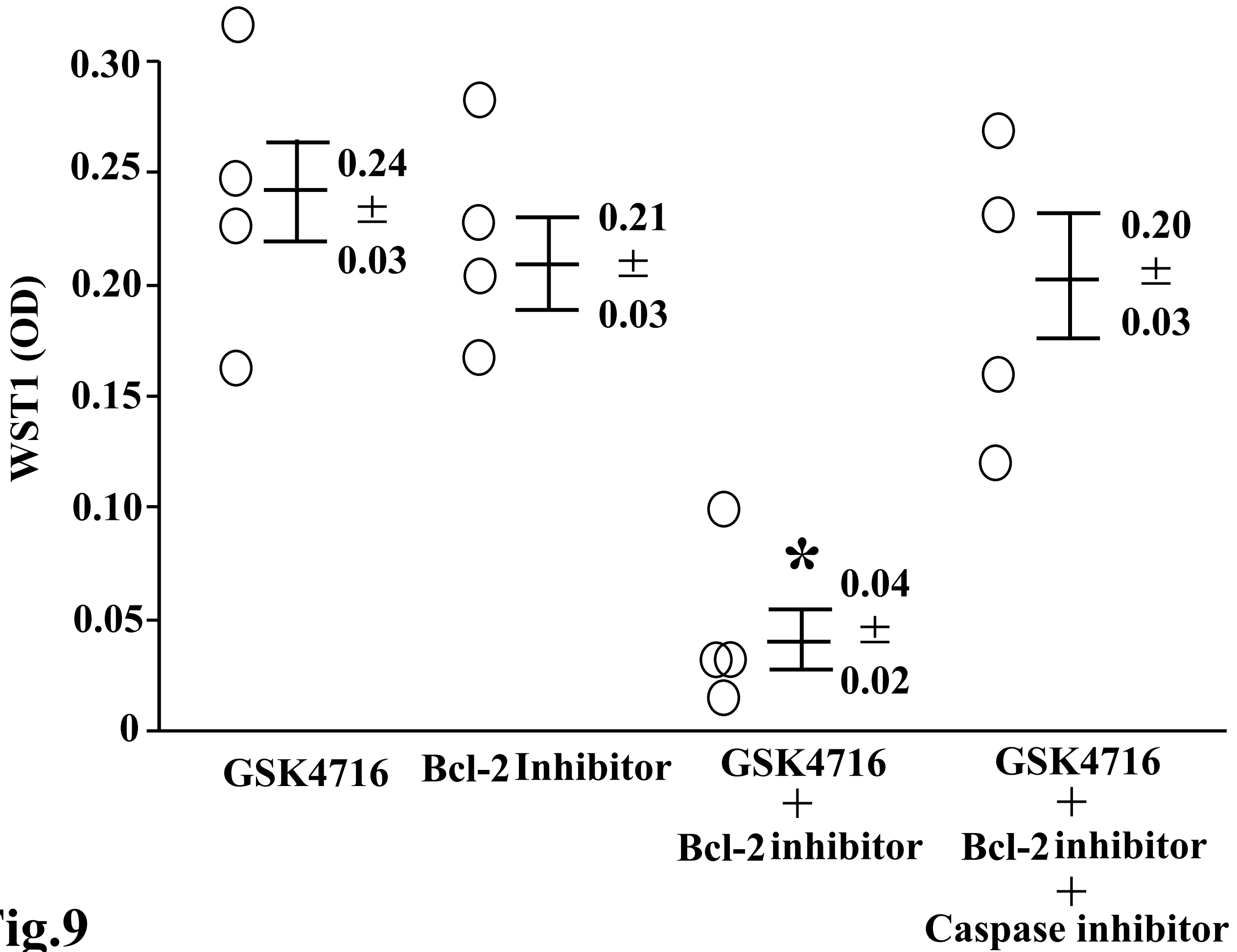
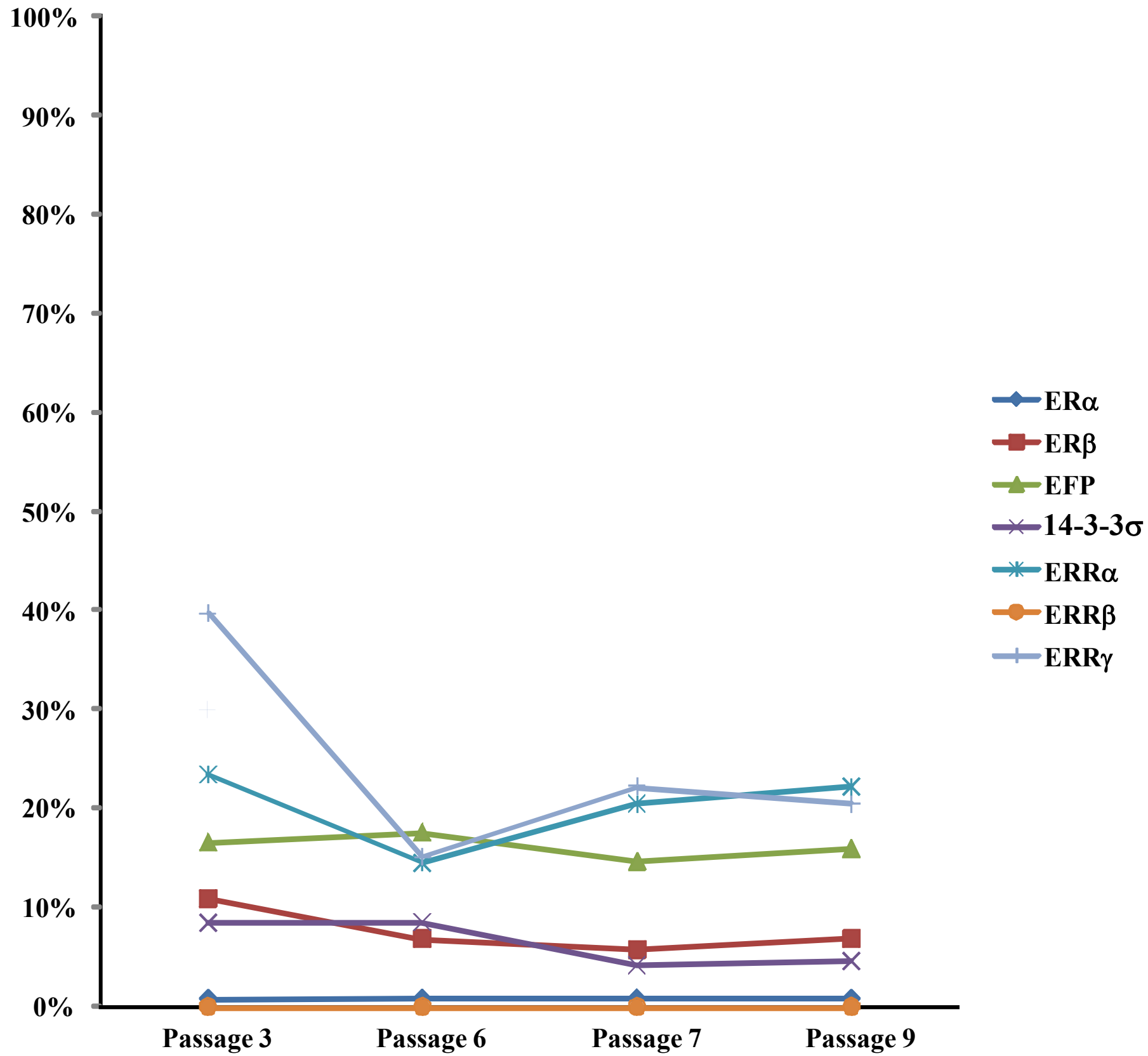


Fig.9



Supplemental figure 1