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Thyroid Hormone Is Necessary for Expression of Constitutive Androstane Receptor in Rat Hepatocytes

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Running title: CAR expression needs thyroid hormone

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Abbreviations used; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; ChIP, chromatin immunoprecipitation; cryo-SH, cryopreserved small hepatocyte; CYP, cytochrome P450; Dio1, 5'-deiodinase type 1; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ME1, malic enzyme 1; MH, mature hepatocyte; PB, phenoberbital; PTU, 6-propyl-2-thiouracil; PXR, pregnane X receptor; RXR, retinoid X receptor; SH, small hepatocyte; sub-SH, subcultured small hepatocyte; T_3, 3,5,3'-triiodothyronine; T_4, thyroxine; TH, thyroid hormone; Thrsp, thyroid hormone responsive gene; TSH, thyroid stimulating hormone; TR, thyroid hormone receptor.

Abstract

Small hepatocytes are hepatocyte progenitor cells that possess the capability of maturation and cryopreservation. When cryopreserved rat small hepatocytes were cultured in serum-free medium, the protein expression and the inducibility of CYP 1A1/2, CYP2E1, and CYP3A were maintained but those of CYP2B1 were lost. In this study we investigated the cause of the loss of CYP2B1 expression in cryopreserved small hepatocytes by RT-PCR, immunoblotting, and chromatin-immunoprecipitation assay. Expression of mRNA and protein of the nuclear receptor, constitutive androstane receptor (CAR), which regulates the expression of CYP2B1, was inhibited in the serum-free culture of cryopreserved small hepatocytes, whereas they were expressed in that of subcultured small hepatocytes. Serum application dramatically induced CAR expression in the culture of cryopreserved small hepatocytes. The addition of very low concentrations of thyroid hormones (THs; T_3, $5x10^{-12}$ M; T_4, $5x10^{-12}$~$5x10^{-10}$ M) to the medium also induced the expression of CAR and CYP2B1. Moreover, CYP2B1 expression was induced by administration of phenobarbital. In rats with hypothyroidism induced by thyroidectomy and 6-propyl-2-thiouracil treatment, the expression of CAR and CYP2B1 was strongly repressed. Although THs do not directly regulate the expression of CAR, they may be important for rat hepatocytes to regulate CYP2B1 through CAR expression in the physiological condition.

Introduction

Cytochrome P450s (CYPs) constitute a superfamily of mono-oxygenases that play a key role in either the detoxification or the metabolic activation of xenobiotics (Guengerich and McDonald 1990; Wrighton and Stevens 1992; Gonzalez and Gelboin 1994). The CYPs involved in xenobiotic metabolism are rich in hepatocytes. In vivo, many of the constitutive CYPs are actually inducible by xenobiotics such as aromatic hydrocarbons, drugs, alcohol, etc. However, in traditional culture systems, it is very difficult to maintain the CYP expression of hepatocytes and the activity of most CYPs is rapidly lost after plating (Bissell and Guzelian 1980). In cryopreserved hepatocytes isolated from both rodents and humans, hepatic differentiated functions, including CYP activities, also decrease immediately after plating (Jackson et al. 1985; Loretz et al. 1989; Sun et al. 1990; Chesne et al. 1993; de Sousa et al. 1996; Swales et al. 1996; Garcia et al. 2003).

Small hepatocytes (SHs) have been identified as proliferating cells with hepatic characteristics (Mitaka et al. 1992, 1995; Tateno and Yoshizato 1996). We showed that a single SH could clonally proliferate to form colonies (Mitaka et al. 1999). It was also reported that maturation of the proliferating SHs was induced by the application of Engelbreth-Holm-Swarm sarcoma-derived matrix (EHS gel; Matrigel®) (Sugimoto et al. 2002). Moreover, several CYP proteins were expressed and induced in SHs even after more than one month of culture (Miyamoto et al. 2005). On the other hand, we reported that SHs could be cryopreserved for more than 6 months and that, even after thawing, the cells could maintain the capability of the growth and the maturation (Ikeda et al. 2002). We also examined the expression of CYP1A1/2, CYP2E1, and CYP3A in thawed SHs even after the cells were cultured in a serum-free medium for more than a month (Ooe et al. 2006). However, CYP2B1 was not expressed in the culture even if hepatic maturation of SHs was induced by Matrigel and induction by phenobarbital was carried out (Ooe et al. 2006).

Nuclear receptors such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), retinoid X receptor (RXR), and pregnane X receptor (PXR) are related to the transcription of CYP and other enzyme genes (Ma 2001; Honkakoski et al. 1998; Kliewer 2003). CAR and RXR play a central role in constitutive expression and the induction of CYP2B. The translocation of CAR from the cytoplasm to the nucleus was observed after phenobarbital treatment (Kawamoto et al. 1999). In the nucleus CAR forms heterodimer with RXR and binds to DNA sequence called phenoberbital (PB)-responsive enhancer module (Honkakoski et al. 1998).

The aim of this study is to clarify the reason why no expression of CYP2B1 occurs in cryopreserved SHs (cryo-SHs). The results revealed that CAR expression was inhibited in the cryo-SHs when the cells were cultured in serum-free medium. The expression of CAR and CYP2B1 was recovered by the addition of serum. Furthermore, the physiological concentration of thyroid hormones (THs) could be substituted for the serum. Here, we show the importance of THs for the expression of CAR and CYP2B1 in a physiological condition.

Methods

Animals

Male F344 rats (Sankyo Lab Service, Tokyo, Japan), weighing 170-240 g, were used. Thyroidectomized F344 rats (male, 7 weeks old) were purchased from Sankyo Lab Service. All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to Sapporo Medical University guidelines. They received a diet and drinking water *ad libitum*, and were maintained in an SPF animal facility at 23±1°C on a standard 12-hr light-dark cycle. Intraperitoneal administration of phenobarbital (PB; Wako Pure Chem. Co., Tokyo, Japan, 25 mg/kg body weight) was repeated every day for 4 times before preparation of mature hepatocytes (MHs). The surgically operated rats were used within 2 weeks after the operation. 6-Propyl-2-thiouracil (PTU; SIGMA Chem. Co., St Louis, MO) treatment was performed by the addition of 0.1% (w/v) PTU to drinking water for 2 weeks before preparation of MHs. PTU treatment of thyroidectomized rats was done only for one week. Serum were obtained before the liver perfusion and stored at -20˚C until use. The concentration of 3,5,3'-triiodothyronine (T_3), thyroxine (T_4), and thyroid stimulating hormone (TSH) in serum was determined on the ADVIA Centaur immunoassay system (Bayer, Leverkusen, Germany).

Isolation and Culture of SHs

Hepatic cells were isolated by the two-step collagenase perfusion method. Cell suspension was centrifuged at 50 x *g* for 1 min. The supernatant was used for preparing SHs and the pellet for MHs. Details of the isolation and culture procedure for SHs and MHs were previously described (Mitaka et al. 1999). Finally, 6 x 10^4 viable cells/cm^2 were seeded on a 100-mm dish and cultured in Dulbecco's modified Eagle's medium (DMEM; SIGMA Chem. Co.) supplemented with 20 mM HEPES, 25 mM $NaHCO_3$, 30 mg/L L-proline, 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT), 10 mM nicotinamide (Katayama Chemical Industries Co., Osaka, Japan), 1 mM ascorbic acid 2-phosphate (Wako Pure Chem.

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Co.), 10 ng/ml epidermal growth factor (EGF; Collaborative Research Inc., Lexington, MA), 0.5 mg/L insulin, 10^{-7} M dexamethasone, and antibiotics. After 4 days of culture, 1% (v/v) dimethyl sulfoxide (DMSO; Aldrich Chem. Co., Milwaukee, WI) was added to the medium.

Subculture and Cryopreservation of SH Colonies

As previously reported (Ooe et al. 2006), SH colonies consisting of 30-50 cells were observed at day 14 after plating. To collect SHs, the colonies were detached from dishes and immediately replated (subculture) or cryopreserved at –80°C until use. The cells that were cryopreserved for more than 1 month were used. SH colonies (3-5 x 10^3 colonies/60-mm dish) were replated on new dishes coated with rat tail collagen. One day after replating, the medium was replaced with serum-free modified DMEM supplemented with or without TH. PB was used as inducer for CYP2B. Ten days after replating, fresh medium containing 2 mM PB was added. To enhance the CYP2B expression, the medium containing PB was renewed every day for 3 consecutive days before harvest. Some dishes were treated with Matrigel (1 mg/60-mm dish; Becton Dickinson, Bedford, MA) from 7 days after replating.

RNA Preparation and RT-PCR

For total RNA extraction, the cells were washed with PBS twice and treated with 1 ml of Isogen (Nippon Gene Co., Tokyo, Japan). RNA concentration was determined by measuring absorption at 260 nm. Reverse transcription was performed by OmniScript RT kit (Qiagen, Hilden, Germany) according to the manufacture's instruction. cDNA was amplified with Taq polymerase (Fermentas Life Sciences, Burlington, Canada). Sequences of forward and reverse primers, annealing temperature and number of reaction cycle are listed in **Table 1**. Amplicon was separated by 1% (w/v) agarose gel and detected with ethidium bromide. Glycerol 3 phosphate dehydrogenase (G3PDH) was used as quantitative control for the estimation of relative expression.

Quantitative real-time PCR

RNA was reverse transcribed using an OmniScript RT kit (Qiagen) and random hexamers. Real-time PCR analyses were carried out on TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for rat CAR (Rn00576085_m1), rat CYP2B1 (Rn01457875_m1), rat HNF4α (Rn00573309_m1) and rat G3PDH (Rn99999916_s1). Reaction solutions (50 μL) containing cDNA samples (corresponding to 100 ng of total RNA), 1 x TaqMan Universal Master Mix (Applied Biosystems), 900 nM primers and 200 nM probes were analyzed on an ABI Prism 7500 (Applied Biosystems). After denaturation at 94˚C for 10 min, the reaction was repeated 40 times with denaturation at 94˚C for 15 sec,

annealing at 60˚C and extension for 1 min. Relative expression was determined during the log-linear phase of amplification. G3PDH was used for the normalization of relative expression.

Western Blot Analysis

For immunoblots, the dishes were washed with PBS twice and then treated with 1 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% (v/v) Triton X-100, 5 μg/ml leupeptin, 5 μg/ml pepstatin A) for 1 hr at 4˚C. The cells were scraped and used for protein extraction. The concentration of proteins was measured using a BCA assay kit (Pierce, Rockford, IL). Samples (5 μg/lane) were separated by SDS-PAGE. A goat anti-CYP2B1 (Daiichi Pure Chem. Co, Tokyo, Japan) antibody was used for immunoblots. The details of the method were previously described (Ooe et al. 2006).

Chromatin-immunoprecipitation (ChIP) assay

ChIP assay was performed using a QuikChIP kit (IMGENEX, San Diego, CA) according to the manufacturer's instructions. Briefly, 4-day-cultured MHs or 14-day-cultured cryo-SHs were fixed with 1% formaldehyde at 37˚C for 15 min. Cells were washed twice with ice-cold PBS and resuspended in 1 ml of lysis buffer. Sonication was performed to reduce the DNA length to between 200 and 1,000 bp. A rabbit anti-TRα antibody (10 μg; Santa Cruz Biotechnology Inc, Santa Cruz, CA) and chromatin solution (500 μl) were used for ChIP assay and PCR. The sequences of the forward and reverse primers, annealing temperature and number of reaction cycles are listed in **Table 1**. The amplicon was separated using 1% (w/v) agarose gel and detected with ethidium bromide.

Statistical Analysis

Statistical analysis was performed using Student's *t*-test. Data acquired from three independent experiments were analyzed. A *P*-value of <0.05 was considered significant.

Results

CYP2B1 expression in rat cryo-SHs

Our previous report showed that the expression of CYP2B1 was repressed in cultured cryo-SHs, though it was maintained in subcultured SHs (sub-SHs). Therefore, we first confirmed whether mRNA and protein of CYP2B1 could be expressed in the various conditions of SHs. Western blot analysis showed that only small amount of CYP2B1 protein was detected in the frozen and cultured cryo-SHs (**Fig. 1A**). No induction was observed in the cryo-SHs even after PB treatment. The low expression was observed in spite of the

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periods of cryopreservation (data not shown). On the other hand, sub-SHs could maintain the protein expression. As shown in **Figure 1B**, the loss of CYP2B1 mRNA expression was also observed in cryo-SHs, whereas the sub-SHs maintained the expression. No induction of the gene was observed in the cryo-SHs, even after PB treatment.

Expression of nuclear receptors

The gene expression of CYP2B1 is regulated by nuclear receptors such as CAR and RXR (Honkakoski et al. 1998). To clarify the reason why the cryo-SHs lost the ability of CYP2B1 expression, we examined the expression of the nuclear receptors. As shown in **Figure 2**, CAR mRNA was scarcely expressed in cryo-SHs even after the induction by PB and maturation with Matrigel, whereas as much RXRα mRNA was expressed in cryo-SHs as in MHs. Although, unlike in MHs, PB could not induce RXRα expression in the cryo-SHs, these results showed that low expression of CAR might be involved in the poor expression of CYP2B1. Comparing the previous (Miyamoto et al. 2005; Ooe et al. 2006) and present experiments, the difference of the experimental procedure was whether cells were cryopreserved or not. Thus, we compared the expression of major nuclear receptors involved in the regulation of CYP expression between sub-SHs and cryo-SHs. As shown in **Figure 3**, in serum-free culture, the expression of CAR was much higher in sub-SHs than in cryo-SHs. AhR, PXR and RXRα were expressed in both cell types as much as in MHs. Next, we also examined the effect of FBS on the expression of nuclear receptors. The expression of CAR was restored when cryo-SHs were cultured in the medium supplemented with 10% (v/v) FBS (**Fig. 3**). The addition of FBS to the medium could prevent the loss of CYP2B1 expression in cryo-SHs and, when the addition was performed at any time of the culture, the gene was always induced soon after it (data not shown). However, the addition of FBS affected neither the expression of CAR in sub-SHs nor that of AhR, PXR, and RXRα in cryo-SHs.

Effects of TH on the expression of CAR and CYP2B1

Next, we examined which factors in serum were critical for the regulation of CAR gene. The data of Genebank shows that a TH responsive element (TRE)-like sequence exists in about 1.3 kb up-stream of the gene. Therefore, we investigated whether TH could induce the CAR expression in cultured cryo-SHs. Both T_3 and T_4 possess the TH activity *in vivo*. Physiological activity of T_3 is stronger than that of T_4, and T_3 concentration in serum is lower than that of T_4 (Brent 1994). As shown in **Figure 4A**, the most effective doses of T_3 and T_4 to the expression of CAR mRNA were $5x10^{-12}$ M and $5x10^{-12}$ M to $5x10^{-10}$ M, respectively.

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The dose-dependent induction by THs was not observed in the experiment and the range for the effectiveness of THs was very narrow. Similarly, the expression of CYP2B1 was also correlated to that of CAR. A high concentration of THs has induced neither CAR nor CYP2B1 expression.

The time courses of CAR and CYP2B1 mRNA expression were examined in the medium supplemented with or without $5x10^{-12}$ M T_3. As shown in **Figure 4B**, in the cells cultured without T_3 the mRNA expression of CAR was very low and that did not change during culture. The addition of T_3 to the medium significantly induced the expression of CAR from 6 days after the treatment and it increased until day 10. The maximum expression of CAR mRNA was about 70% as much as that in MHs. The protein expression of CYP2B1 was examined in SHs cultured in the medium containing T_3. As shown in **Figures 5A and 5B**, CYP2B1 protein was induced when cryo-SHs were treated with 5×10^{-12} M T_3, whereas the protein was scarcely expressed in cryo-SHs when the cells were treated both without T_3 and with 1×10^{-8} M T_3. Moreover, an administration of PB in the cells treated with 5×10^{-12} M T_3 resulted in the induction of CYP2B1. However, neither induction was observed in the cells treated without T_3 or a high concentration of T_3.

The expression of thyroid hormone receptors and deiodinase in cryo-SHs

To confirm the reactivity of cryo-SHs to TH, we investigated the expression of thyroid hormone-related genes. RT-PCR analysis indicated that TH receptor (TR) α was expressed as strongly in cryo-SHs as in MHs (**Fig. 6**). TR β was also expressed in cryo-SHs but the amount was very low. It is known that T_4 is converted into T_3 by the action of the enzyme 5'-deiodinase type 1 (Dio1) in the liver (Köhrle 2000). Dio1 was also expressed in cryo-SHs, corresponding to the reactivity of cryo-SHs to T_3. TH-responsive genes such as malic enzyme (ME) 1, Dio1, and thyroid hormone responsive gene spot 14 (Thrsp) (Song et al. 1988; Liaw and Towle 1984) were clearly up-regulated in cryo-SHs treated with 1×10^{-8} M T_3. However, the addition of FBS to the medium did not induce the up-regulation of those genes in cryo-SHs. Cryo-SHs were shown to be able to respond to TH.

CAR and CYP2B1 expression in hypothyroid rats

As it is important to examine whether hypothyroid rats lose the expression of CAR and CYP2B1, we investigated the expression of both genes in the liver and measured the concentration of THs in serum of rats with combined thyroidectomy and PTU-treatment. To dramatically decrease the T_3 concentration in the serum, MHs were prepared from rat livers treated with both thyroidectomy and PTU. All rats treated with both treatments were

dead within 2 weeks after the treatment. Therefore, we examined the expression of CAR and CYP2B1 in the liver of the rats treated with the combination at day 7, whereas either thyroidectomized or PTU-treated rats were examined at day 14. As shown in **Table 2**, total T_3 concentrations in the serum of thyroidectomized and PTU-treated rats were about 0.35 nM and under 0.15 nM, respectively. In the rats treated with both treatments T_3 concentration and T_4 in the serum were both under measurable level. As shown in **Figure 7**, either thyroidectomy or PTU-treatment did not significantly decrease both CAR and CYP2B1 expression. However, both expressions were dramatically reduced in hepatocytes of the rats treated with the combination of thyroidectomy and PTU although HNF4 expression of the cells was maintained and the low concentration of TH in the serum might result in the induction of TSH.

ChIP assays

To confirm whether TR could directly regulate CAR expression, we performed ChIP assays using MHs and cryo-SHs cultured with and without T_3. The promoter region of the rat ME1 gene, in which the existence of TRE is well known, was selected to show that the ChIP assay was accurately performed. As shown in **Figure 8A**, the binding of TRα to TRE (Petty et al. 1989) in the promoter region of the gene was detected in cryo-SHs as well as MHs. Moreover, the binding of TRα was enhanced by T_3 treatment. These data indicated that TR could bind to TRE in the gene in cryo-SHs as in MHs. However, as shown in **Figure 8B**, the binding of TRα to the TRE-like sequence that exists in the promoter region of the rat CAR gene was not detected in cryo-SHs even after $1x10^{-8}$ M T_3 treatment.

Discussion

We previously reported that primary cultured SHs maintained the protein expression of CYPs1A1, 2B1, 2E1, 3A2, and 4A1 for more than one month and that the expression increased with the maturation of SHs (Miyamoto et al. 2005). The enzymatic activity of CYPs1A, 2B, and 3A could be also measured and increased with their maturation. In addition, both expression and activity of the CYPs were maintained after long-term cryopreservation (Ooe et al. 2006). However, CYP2B1 was hardly detected in the culture of cryo-SHs and administration of PB could not induce the expression. The present study showed that nuclear receptor CAR expression was repressed when cryo-SHs were cultured in serum-free conditions, and that the expression was restored by the addition of serum. On the other hand, AhR, PXR and RXRα were expressed in the cells cultured with or without serum

as much as in MHs. AhR and PXR act as transcription factors of CYP1A1 and CYP3A in a ligand-specific manner, respectively (Ma 2001; Kliewer 2003). RXR forms a heterodimer with CAR, PXR or other nuclear receptors in nucleus and can activate the transcription of their target genes (Waxman 1999). Considering that nuclear receptors other than CAR were expressed in cryo-SHs, it is reasonable that CYPs regulated by nuclear receptors other than CAR were maintained in the cells (Ooe et al. 2006).

When a relatively large number of SHs exists in the culture dish, as SHs produce adequate amount of serum proteins, SHs can proliferate in a serum-free medium (Sugimoto et al. 2002; Miyamoto et al. 2005; Ooe et al. 2006). Therefore, whenever SH colonies are replated on new dishes, the cells are routinely cultured in a serum-free medium. In these culture conditions, although many nuclear receptors could be expressed in the cryo-SHs, only CAR disappeared. This loss was reversed by the addition of FBS. The FBS used in the present experiment contained about 1 nM of total T_3 and 100 nM of total T_4 (**Table 2**). The present study revealed that the effective dosage of T_3 ($5x10^{-12}$ M) or T_4 ($5x10^{-12}$~$5x10^{-10}$ M) for the induction of CAR expression was close to the concentrations of free-T_3 and free-T_4 in the serum of rats (Nauman et al. 1967; Refetoff et al. 1970). We think that the recovery of CAR expression in the experiment was due to free and protein-conjugated THs contained in FBS. TR α may have a role in CAR expression because this gene was expressed even after cryopreservation and serum did not alter the expression. On the other hand, the expression of TR β in cryo-SHs was lower than that in MHs and the addition of serum increased its expression only a little. As CAR expression was restored by serum, TRβ might not be directly related to the regulation of the CAR gene. Furthermore, T_3 and T_4 possess TH activity *in vivo* and T_4 is converted into T_3 by the action of Dio1 in the liver (Köhrle 2000). Considering that T_4 also had the ability to induce CAR, Dio1 might play a role in this pathway. Induction of CAR expression by T_4 might be due to the conversion of T_3 by Dio1 expressed in cryo-SHs.

Induction of CYP2B1 mRNA by T_3 was also observed in the cells and the expression increased with time in culture. As some SHs in colonies can mature and their hepatic differentiated functions also increase (Sugimoto et al. 2002), it is feasible that CAR and CYP2B1 expression increased with time in culture. A discrepancy was observed in the timing of the expression. The expression of CYP2B1 was delayed compared to that of CAR. The maximum expression of CYP2B1 was observed at day 14 and was about 60% of that in MHs, whereas that of CAR was at day 10 and about 70% of that in MHs. Our preliminary

experiments suggested that the delay of CYP2B1 expression might be due to the nuclear translocation of RXR. By immunostaining the accumulation of RXR protein was only observed in the nucleus of large-sized SHs, which were matured SHs, not small-sized SHs (data not shown). In our culture conditions, especially without Matrigel, maturation of SHs gradually progresses and the degree of the maturation in cultured SHs depends on the number of large cells and piled-up cells (Sugimoto et al. 2002). Although T_3 supply induces CAR expression and both CAR and RXR proteins accumulate in the cytoplasm of SHs, the lesser translocation of RXR into nuclei may limit the expression of CYP2B1. Thus, CAR expression might be dependent on the existence of THs, whereas CYP2B1 might require both CAR expression and a differentiated condition of the cells.

Although it is known that CAR is involved in the metabolism of THs (Maglich et al. 2003; Qatanani et al. 2005), there is no report that THs can affect CAR expression. It is only known that a TRE-like sequence exists in the upstream of the CAR promoter region. In the present experiment, although binding of TR to the TRE sequence in the promoter region of the ME1 gene and amplification of the expression by T_3 were confirmed in cryo-SHs as well as MHs, we could not obtain proof of the direct binding of TRα to the TRE-like sequence in the promoter region of the CAR gene. However, it is true that a limited concentration of THs can induce CAR expression in cryo-SHs and the regulation of CAR expression is not well understood. At present, although we do not know the exact mechanism of CAR expression by THs, unknown indirect mechanisms of regulation may exist in SHs. There is a possibility that the mechanism of CAR gene regulation is different between MHs and SHs. To clarify this issue and the characteristics of SHs, we need to perform further experiments.

Although sub-SHs could maintain CAR expression in serum-free culture, cryo-SHs could not. However, cryo-SHs could recover the expression of CAR in the presence of THs and the expression of other nuclear receptors was maintained in the cryo-SHs. In addition, the duration of cryopreservation (1 week to 1 year) did not affect this phenomenon and MHs that were cryopreserved by the same procedure as SHs possessed CYP2B1 protein (data not shown). Moreover, cryopreserved human hepatocytes were reported to maintain the activity and the expression of many metabolic enzymes (Shitara et al. 2003; Richert et al. 2006). These results indicate that the loss of the CAR expression may result from the procedure of cryopreservation of SHs. Although it is well known that cryopreservation subjects cells to intensive stress, most damaged functions may be recovered with time if suitable culture conditions are employed (Richert et al. 2006). The reason why only CAR was repressed in

cryo-SH is not clear. However, this phenomenon may involve the immaturity of SHs. On the other hand, THs seem to function to both maintain CAR expression in MHs and reverse CAR repression in cryo-SHs. When sub-SHs and cryo-SHs were compared, the addition of THs was necessary for reexpression of CAR in cryo-SHs, though it was not necessary for expression of CAR in sub-SHs. Otherwise, an extremely low level of THs in serum caused CAR repression *in vivo*. This discrepancy may also be due to the difference between MHs and SHs. Another hypothesis is that enough THs for the expression of CAR are preserved in sub-SHs as protein conjugates and that they are lost as a result of the procedure of cryopreservation. In any case, further investigations concerning the pathway of CAR expression are necessary to clarify the mechanism of TH functions in CAR expression.

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Footnotes

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Figure Legends

Figure 1. Expression of CYP2B1 in MHs and SHs. (A) Immunoblots for CYP2B1 protein in MHs and SHs. Proteins (5 μg/lane) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. MHs were prepared from a normal rat or a PB-treated rat liver. SH colonies were isolated at day 14, replated on a new dish, and cultured for 14 days (Sub). Some colonies were cryopreserved for more than 1 month at -80°C. Frozen colonies (F) were thawed and cultured for 14 days (Cryo) with (+PB) or without 2 mM PB. (B) Detection of CYP2B1 transcripts in MHs and SHs by RT-PCR analysis. Total RNA was extracted from the cells prepared for the experiment used in (A). Sequences of the forward and the reverse primers, annealing temperatures and the number of reaction cycles are listed in **Table 1**.

Figure 2. Expression of CAR and RXRα in MHs and cryo-SHs. Total RNA was extracted from cryo-SHs with or without 2 mM PB, and SHs treated with both 2 mM PB and Matrigel. Total RNA of MHs was also prepared from normal rat and PB treated rat liver. CAR and RXRα mRNAs were detected by RT-PCR analysis. Sequences of the forward and the reverse primers, annealing temperatures and the number of reaction cycles are listed in **Table 1**.

Figure 3. Expression of nuclear receptors in SHs. Total RNA was extracted from MHs, Sub-SH, and cryo-SHs cultured in the medium with or without 10% (v/v) FBS. CAR, AhR, PXR and RXRα mRNAs were detected by RT-PCR analysis. Sequences of the forward and the reverse primers, annealing temperatures and the number of reaction cycles are listed in **Table 1**.

Figure 4. Induction of CAR and CYP2B1 mRNAs by THs in cryo-SHs. (A) Expression of CAR and CYP2B1 in cryo-SHs under various concentrations of T_3 or T_4. Total RNA was extracted from the cells cultured for 14 days and used for quantitative real-time PCR analyses. Three independent experiments were carried out. The relative expression compared to MH was calculated and standardized with G3PDH expression. Bars show standard deviations and asterisks indicate significant differences between control and each concentration (* $p<0.05$; ** $p<0.01$). (B) Time courses of CAR and CYP2B1

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expression in cryo-SHs treated with or without TH. Total RNA was extracted from the cells cultured SHs with or without $5x10^{-12}$ M T_3 at days 0, 3, 6, 10, and 14. Quantitative real-time PCR analyses were conducted. Three independent experiments were carried out. The relative expression compared to MH was calculated and standardized with G3PDH expression. Bars show standard deviations and asterisks indicate significant differences between 0 and each day (*p<0.05; **p<0.01).

Figure 5. Expression and induction of CYP2B1 in cryo-SHs. (A) Immunoblots for CYP2B1 protein in MH and cryo-SHs treated with T_3. Proteins (5 μg/lane) were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. Cryo-SHs were cultured in the medium supplemented with 0, $5x10^{-12}$, or $1x10^{-8}$ M of T_3 for 14 days. Three independent experiments were performed. (B) Relative expressions of CYP2B1 compared to MH were calculated. Bars show a standard deviation and asterisks indicate significant differences (*p<0.05).

Figure 6. Expression of thyroid hormone-related genes in cryo-SHs. Expression of thyroid hormone-related genes in MHs and cryo-SHs. Thyroid hormone receptors, deiodinase (Dio) 1, malic enzyme (ME) 1, and thyroid hormone-responsive gene spot 14 (Thrsp) mRNAs were detected by RT-PCR analysis. Cryo-SHs were cultured in the medium supplemented with 0, $5x10^{-12}$ M T3 (+), $1x10^{-8}$ M T_3 (++) or 10% (v/v) FBS for 14 days. Sequences of the forward and the reverse primers, annealing temperatures and the number of reaction cycles are listed in **Table 1**.

Figure 7. Expression of CAR and CYP2B1 in thyroidectomized and/or PTU-treated rat livers. Total RNA was prepared from MHs isolated from rats whose thyroids were surgically resected (T) and/or treated with PTU, and quantitative real-time PCR was carried out. The relative expression compared to the normal rat was calculated and standardized with G3PDH expression. Three independent experiments were performed. Bars show standard deviations and asterisks indicate significant differences between normal rats and each concentration (*p<0.05).

Figure 8. Chromatin-immunoprecipitation (ChIP) assays. The genomic DNA-protein complex was isolated from 4–day cultured MHs or 2-week cultured cryo-SH. An anti-TRα

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antibody was used for immunoprecipitation (IP). (A) The promoter region of the rat ME1 gene containing the TRE sequence was amplified. Cells were cultured with $1x10^{-8}$ Mor without T_3. (B) The promoter region of the rat CAR gene containing the putative TRE sequence was amplified. This figure shows representative data of 4 experiments. Cells were cultured with 0 M, 5x10-^{12} M, or $1x10^{-8}$ M T_3. To detect the sequence, 4 sets of primers were prepared. The sequences of the forward and reverse primers, annealing temperatures and the number of reaction cycles are listed in **Table 1**. Genomic DNA samples used for ChIP assays were also amplified (In).

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Table 1 Primer list and PCR conditions

Gene		Sequence (5'-3')	Cycle	Annealing Temp. (°C)	Amplicon size (bp)
CAR	Sense	ATGACAGCTACTCTAACACTAGAG	27	56	1076
	Anti-sense	CAGCTGCAAATCTCCCCAAGCAGC			
RXR	Sense	ACCCCGTGAGCAGCAGTGAGG	32	60	992
	Anti-sense	CCCAATGGATCGCAGTGCAGG			
AhR	Sense	CCGTCCATCCTGGAAATTCGAACC	30	60	350
	Anti-sense	CCTTCTTCATCCGTTAGCGGTCTC			
PXR	Sense	ATGAGACCTGAGGAGAGGTGGAAC	30	60	1007
	Anti-sense	TCCTCCTCACGCAGCTGTAGCTTC			
CYP2B1	Sense	GGGACACCCAAAGTCCCGTGG	27	56	867
	Anti-sense	GGAAACCATAGCGGAGTGTGG			
TRα	Sense	ACGTTGCTACAGAGGCCCATC	32	56	700
	Anti-sense	AGGAAGAGTGGGGGGAAGAGTT			
TRβ	Sense	GAAAATCGCCTTCCAGCCTGG	32	56	619
	Anti-sense	GCTCTGGCTTATGCCCAATGG			
Dio1	Sense	ATGGGGCTGTCCCAGCTATGG	32	56	567
	Anti-sense	AGCAGATGTGCTGCCCGCAGG			
ME1	Sense	CCTGAACCCCAAAACAAAGA	32	56	263
	Anti-sense	CCCATGAGTCTGTGGGCTAA			
Thrsp	Sense	CCTCATCAGATCCAACAGGATG	32	56	212
	Anti-sense	GCAGGGACGCTGTATTTACCTC			
G3PDH	Sense	ACCACAGTCCATGCCATCAC	22	56	451
	Anti-sense	TCCACCACCCTGTTGCTGTA			

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Table 1 Primer list and PCR conditions (continued)

Gene		Sequence (5'-3')	Cycle	Annealing Temp. (°C)	Amplicon size (bp)
ME1-promoter	Sense	TGGACCTGTGCCCTCTAACAC	30	56	379
	Anti-sense	TCCCGCGTCAGCAGGTAGC			
CAR-promoter 1	Sense	GATAGTGACAGTGTACTCATAC	35	56	424
	Anti-sense	CCAGGGTGTACAAGCCTAGCC			
CAR-promoter 2	Sense	GGAAGCTCCAGAATAAAGAGAG	35	56	502
	Anti-sense	GAGATCCCACTTTCTTACAAGG			

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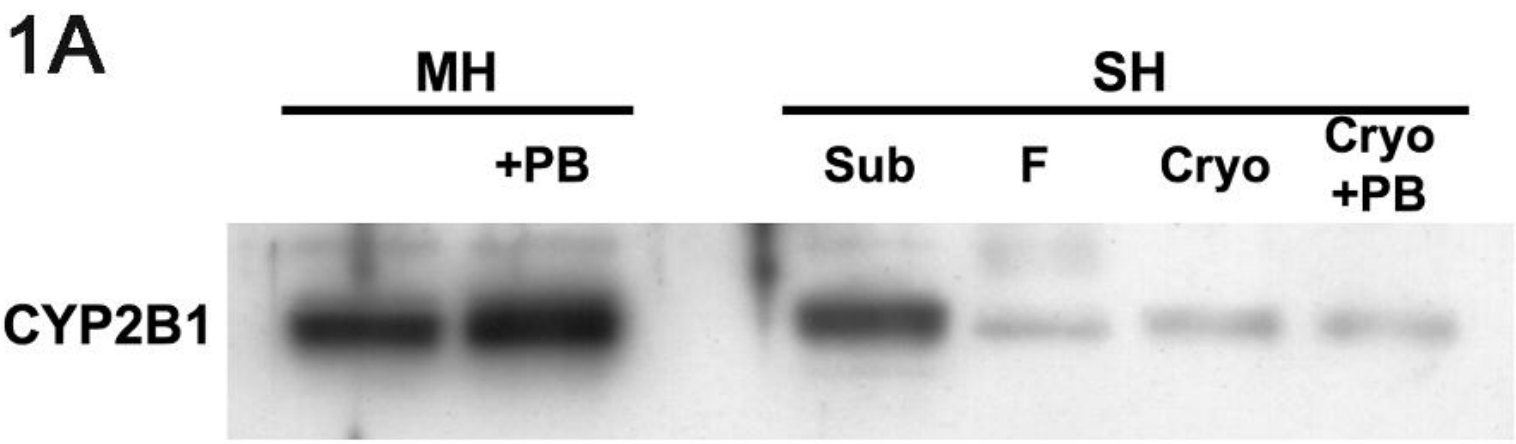
Table 2 Thyroid hormones and TSH level in serum

Sample	Total T_3 conc. (nM)	Total T_4 conc. (nM)	TSH (μU/ml)
Normal rat	1.34±0.10	53.7±3.9	0.44±0.22
Thyroidectomized rat	0.35±0.05	<3.9	33.07±8.20
PTU treated rat	<0.15	<3.9	40.77±10.19
Thyroidectmized and PTU treated rat	<0.15	<0.39	48.16±21.78
Fetal Bovine serum	1.06±0.05	101.8±3.6	n.d.

T_3; 3,5,3'-triiodothyronine, T_4; thyroxine, TSH; thyroid stimulating hormone, PTU; 6-propyl-2-thiouracil

1A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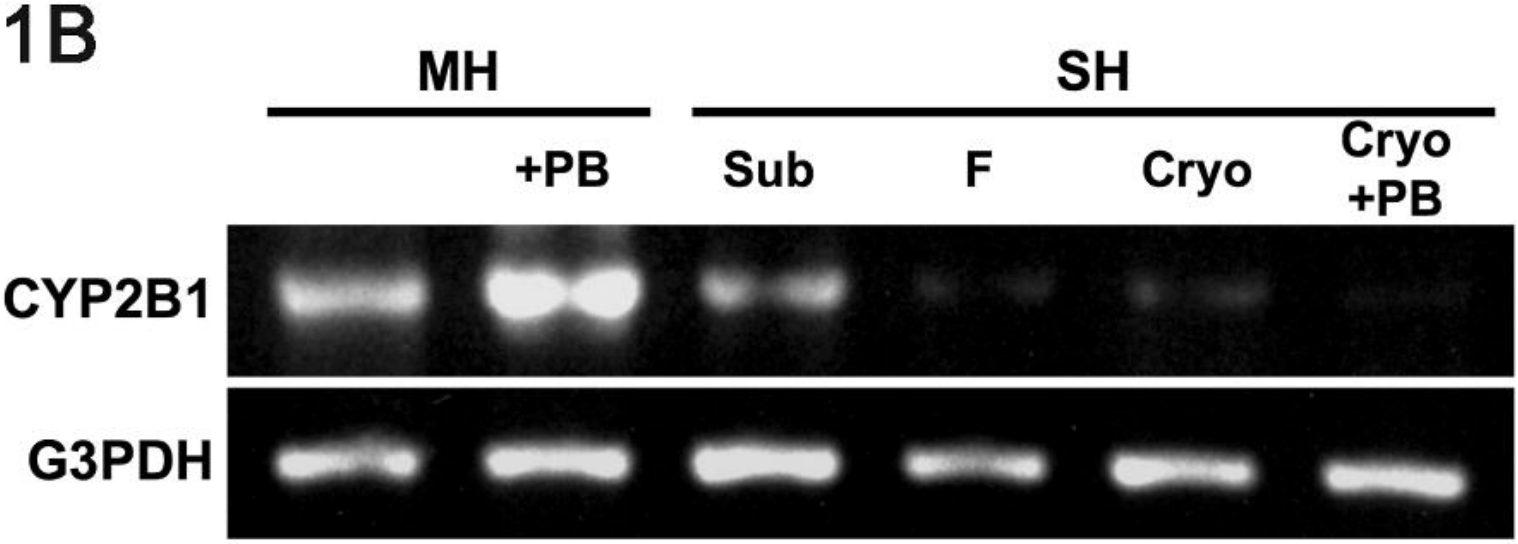
SH
+PB
Sub
F
Cryo
Cryo +PB
CYP2B1

1B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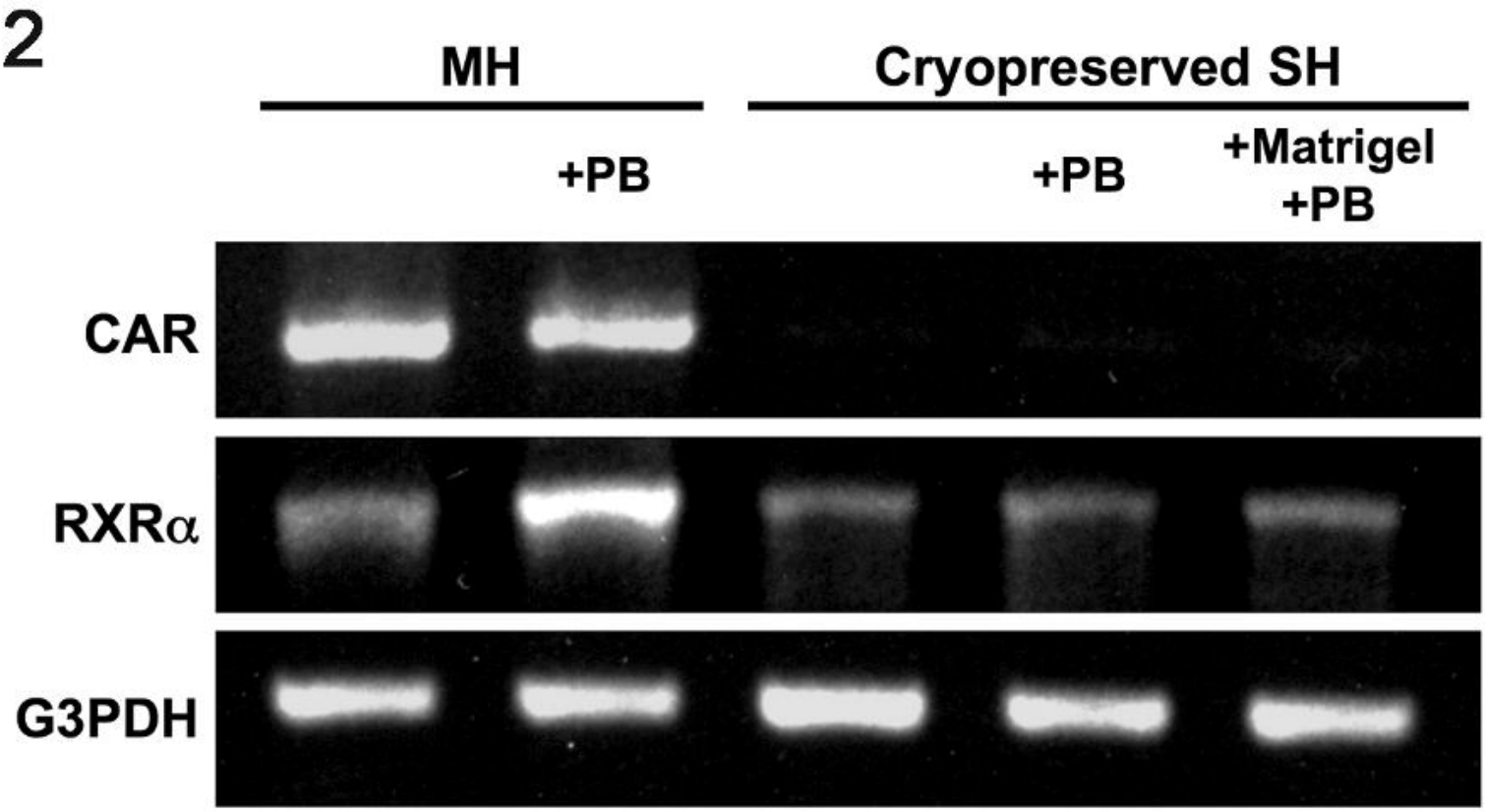
+PB
Sub
F
Cryo
Cryo +PB
CYP2B1
G3PDH

2



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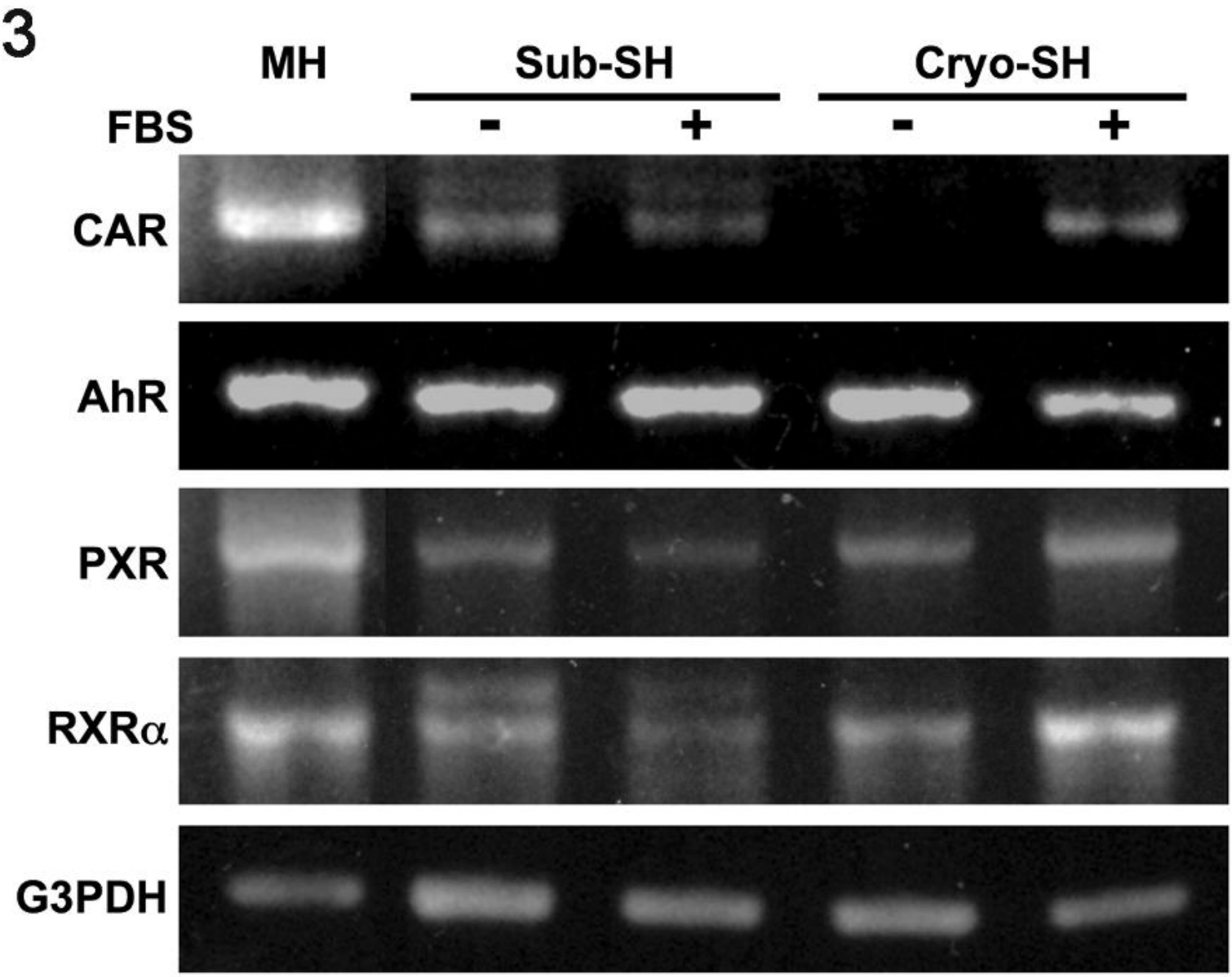

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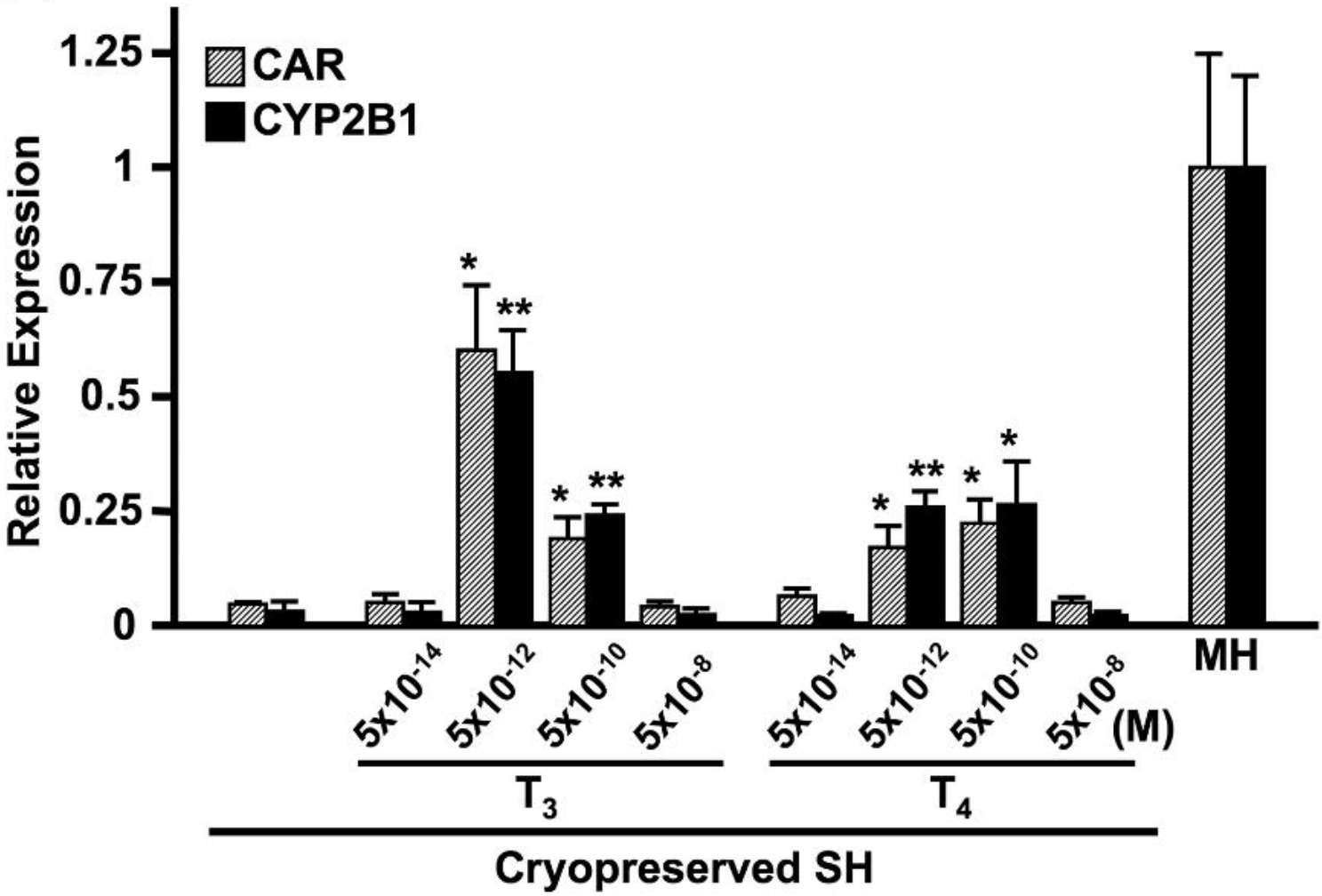
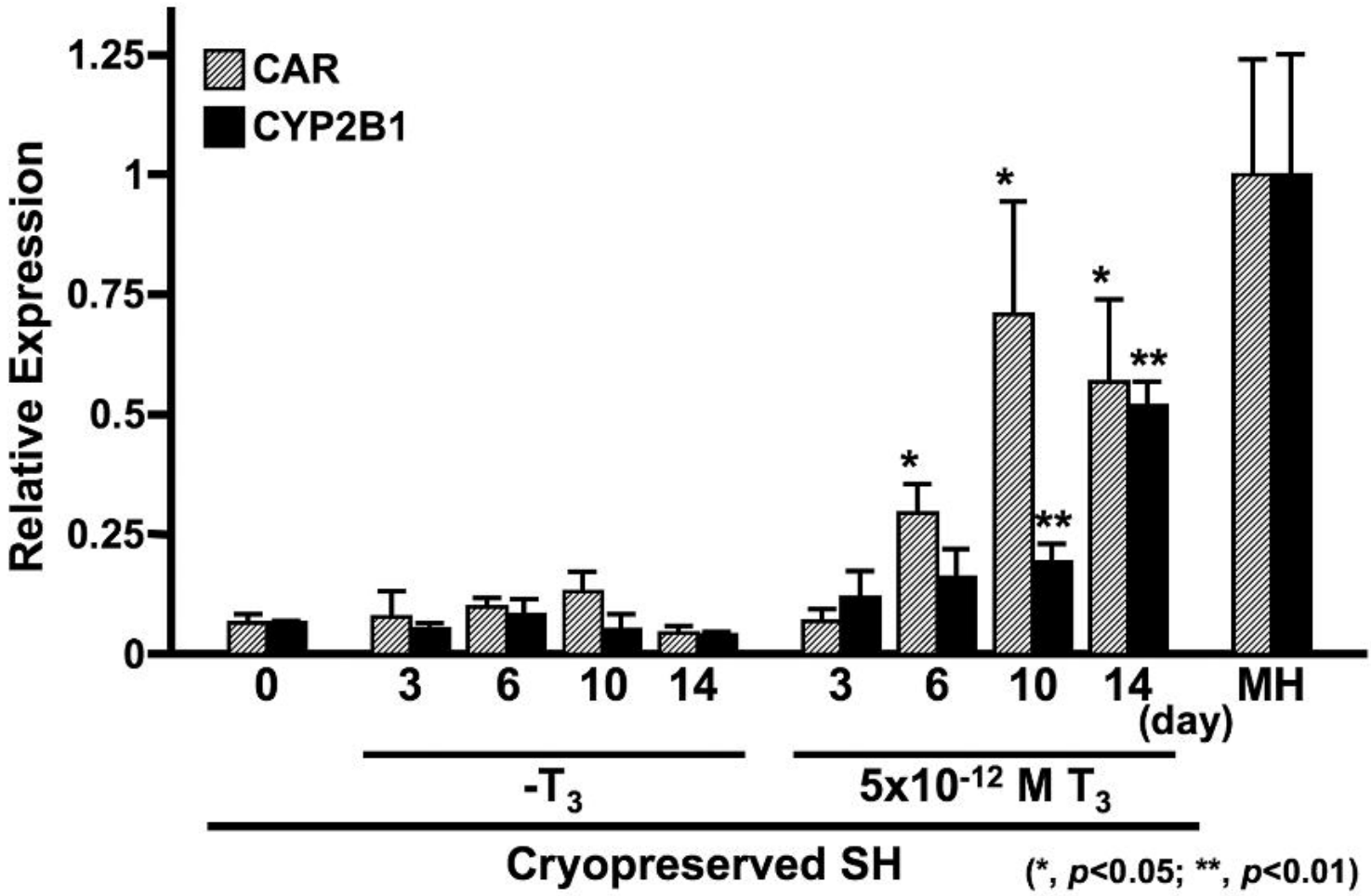

CAR
CYP2B1
Relative Expression
1.25
1
0.75
0.5
0.25
0
*
**
*
**
*
**
*
*
5x10-14
5x10-12
5x10-10
5x10-8
5x10-14
5x10-12
5x10-10
5x10-8
(M)
MH
T3
T4
Cryopreserved SH

4B


CAR
CYP2B1
Relative Expression
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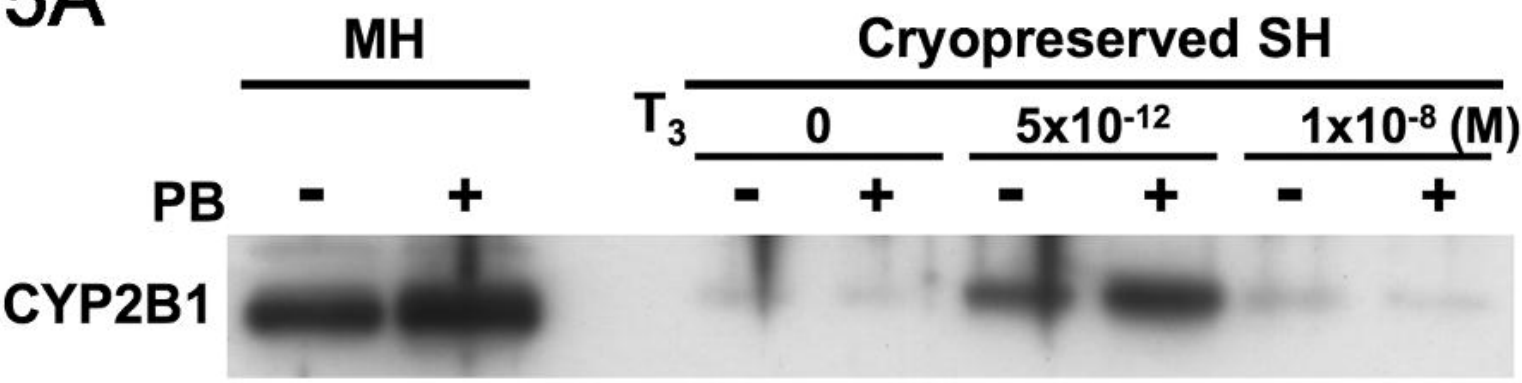
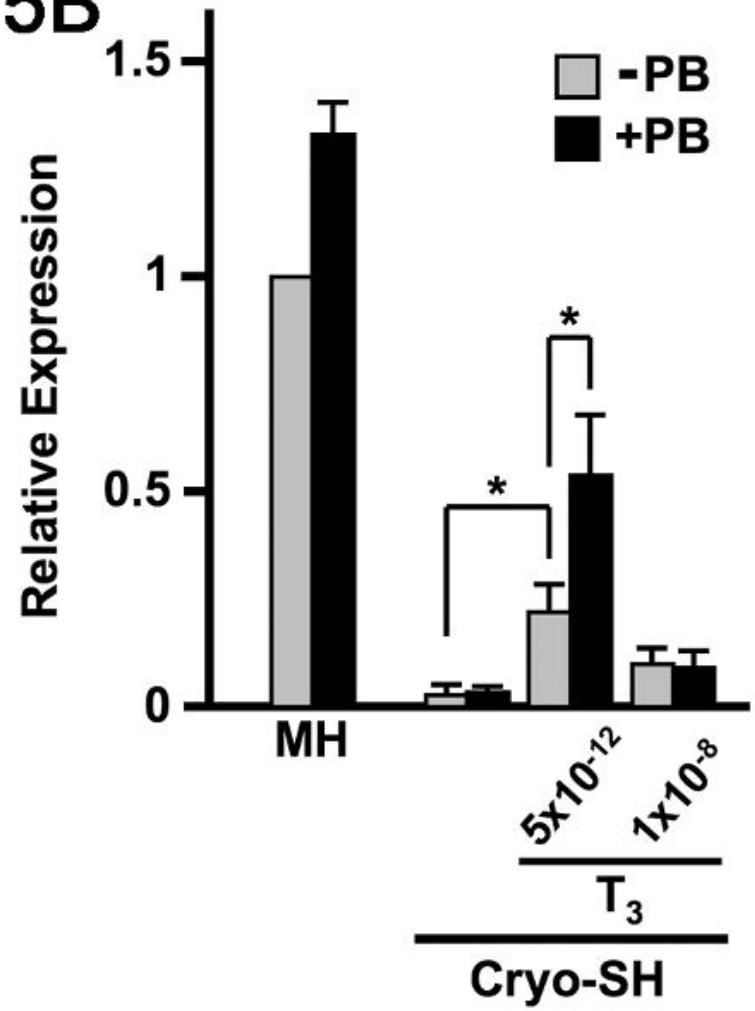
1
0.75
0.5
0.25
0
0
3
6
10
14
3
6
10
14
MH
(day)
-T3
5x10-12 M T3
Cryopreserved SH
(*, p<0.05; **, p<0.01)

5A


MH
Cryopreserved SH
T3
0
5x10-12
1x10-8 (M)
PB
-
+
-
+
-
+
-
+
CYP2B1

5B


Relative Expression
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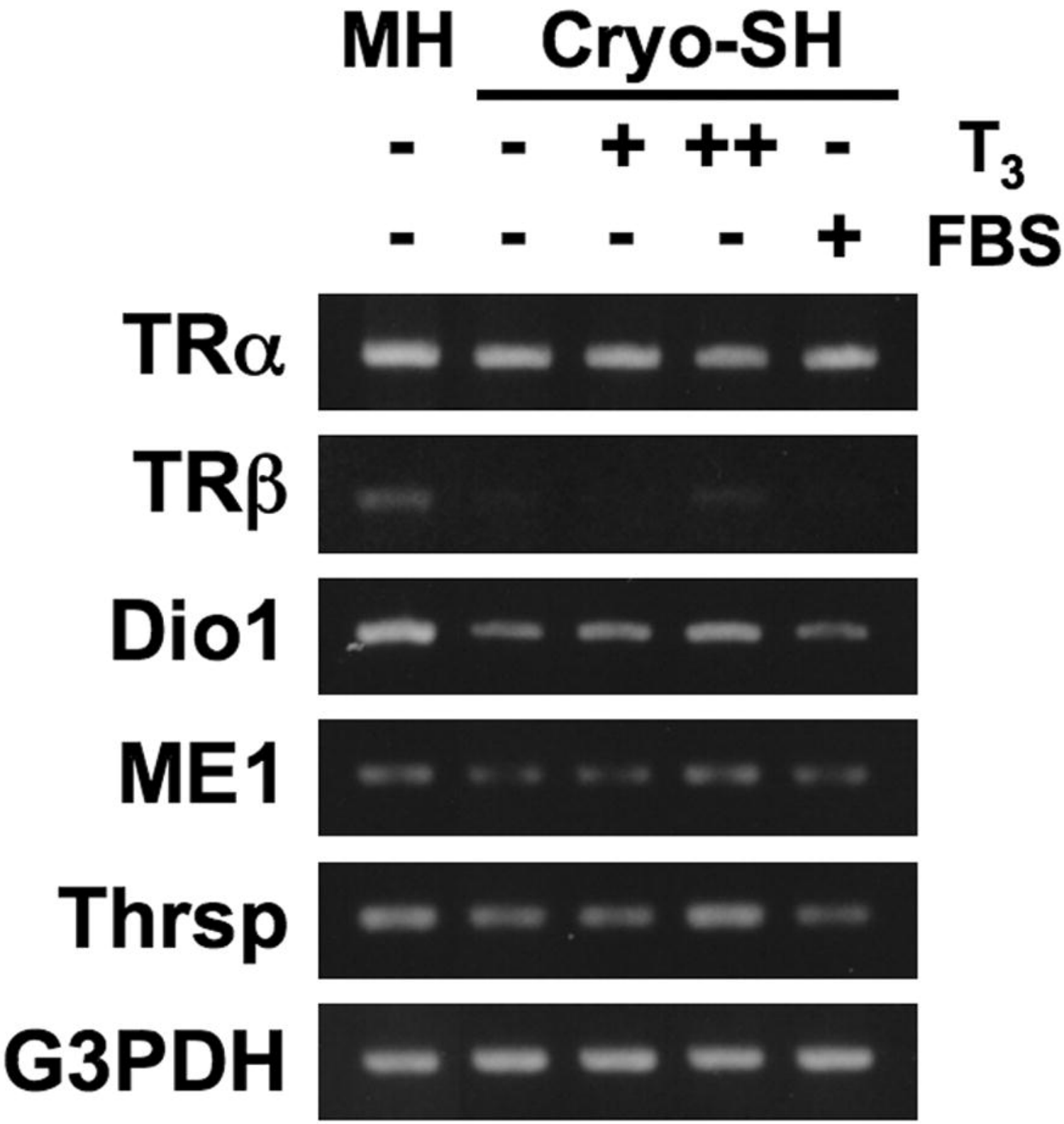
1
0.5
0
-PB
+PB
*
*
MH
5x10-12
1x10-8
T3
Cryo-SH

6



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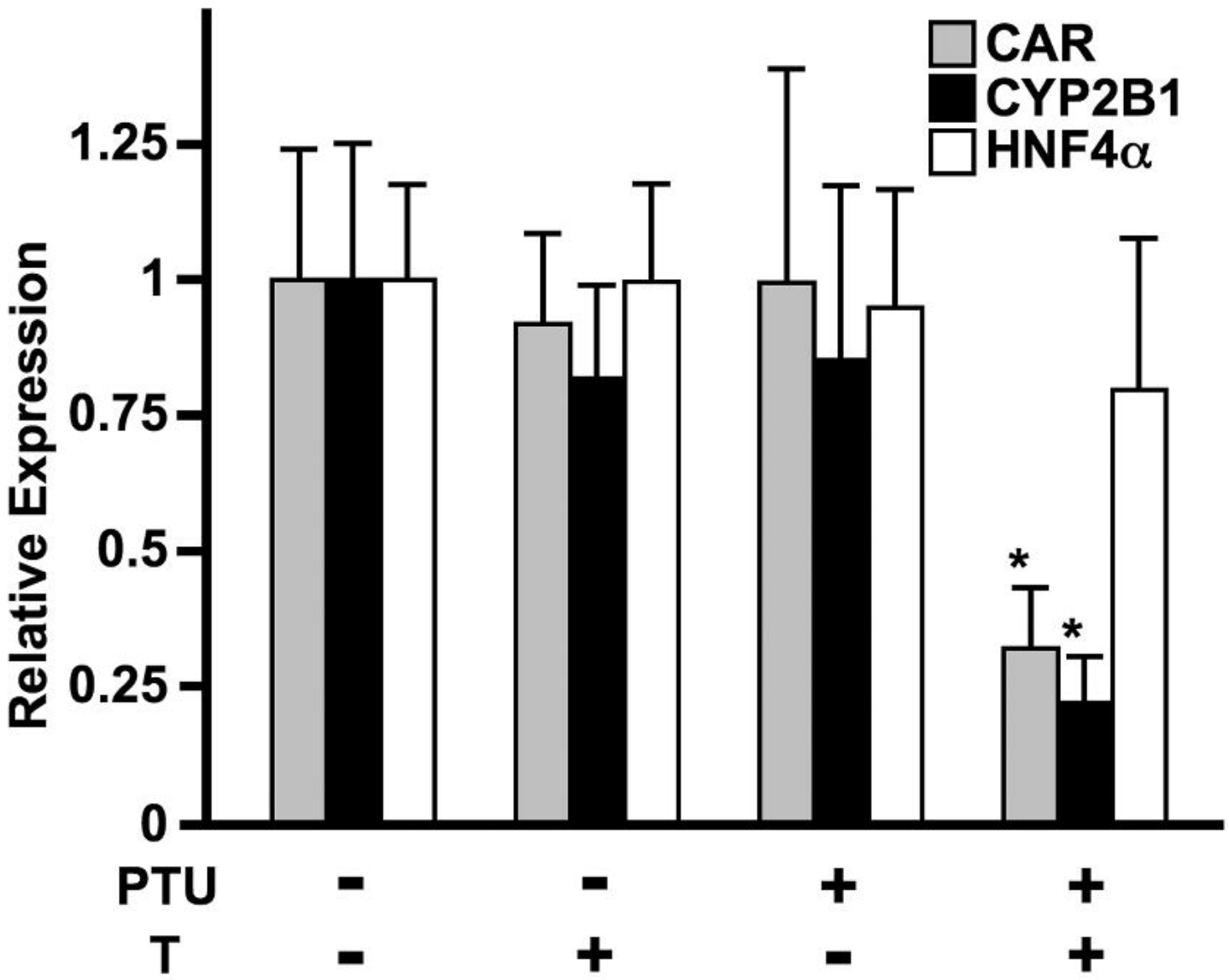
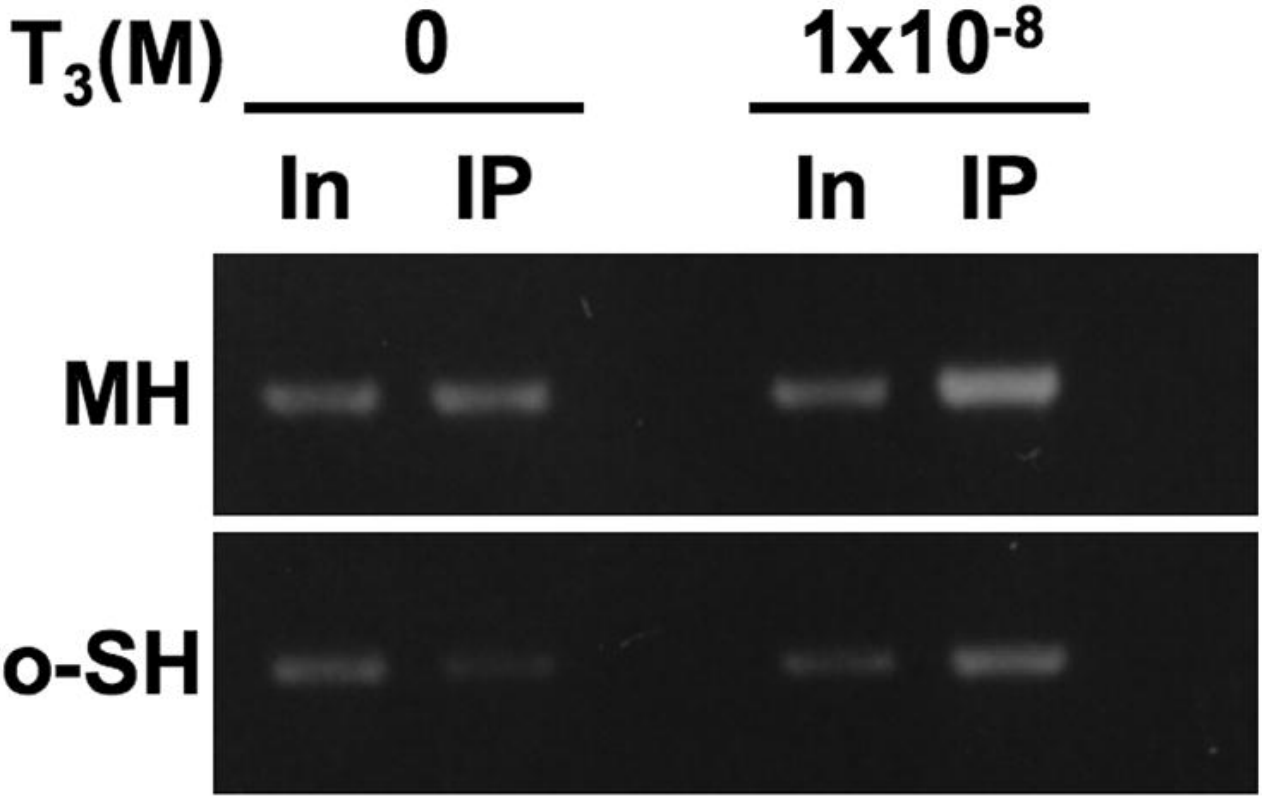
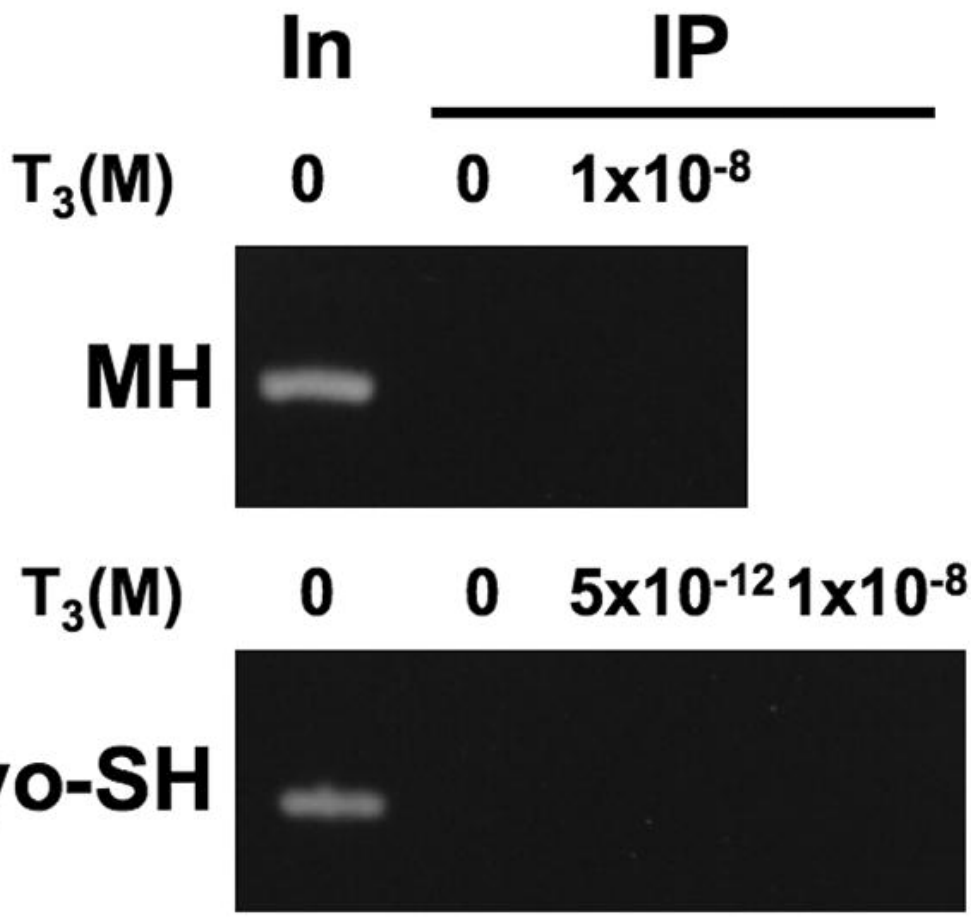


8A


T3(M)
0
1x10-8
In
IP
In
IP
MH
Cryo-SH

8B


In
IP
T3(M)
0
0
1x10-8
MH
T3(M)
0
0
5x10-12
1x10-8
Cryo-SH