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TGFβ1 suppresses EGF-induced increase in nuclear type 1 protein phosphatase activity at the G_1/S transition of hepatocyte proliferation

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Abstract Nuclear type 1 protein phosphatase (PP1) activity in primary culture of EGF-stimulated hepatocytes was significantly and transiently increased at the G_1/S transition, being about 2.5-fold, while that in non-stimulated hepatocytes showed almost no change. On the other hand, non-nuclear PP1 activity was gradually increased until the G_1/S transition, but the activity showed no difference between EGF-stimulated and non-stimulated hepatocytes. Under growth-inhibited conditions in the presence of TGFβ1, the increase in nuclear PP1 activity was completely suppressed, whereas non-nuclear PP1 activity was little affected. Such close correlation between nuclear PP1 activity and growth factor-induced positive or negative growth signaling strongly suggests an involvement of PP1 in progression from G_1 to S phase of hepatocytes. On Western immunoblotting using antisera for PP1α, PP1γ1, and PP1δ, no isoform showed any change in amount under these conditions. Mechanism(s) of growth-associated alterations in nuclear PP1 activity is discussed.

Key words: Type 1 protein phosphatase; Epidermal growth factor; Transforming growth factor β1; DNA synthesis; Hepatocyte

1. Introduction

There are several lines of evidence that epidermal growth factor (EGF) stimulates replicative DNA synthesis and that transforming growth factor β1 (TGFβ1) strongly inhibits DNA synthesis in many mammalian cells including hepatocytes [1,2]. Recently it has become clear that phosphorylation of the retinoblastoma gene product (RB) during G_1 is essential for progression from G_1 into S phase [3]. In addition, it has been shown that TGFβ1 causes the accumulation of unphosphorylated RB and the inhibition of Cdk2 and Cdk4 in late G_1, resulting in G_1 arrest [4]. However, the regulatory mechanisms at the molecular level by which the effects of EGF and TGFβ1 are exerted still remain to be elucidated.

Protein phosphatases, which functionally counteract protein kinases, play important roles in regulating the cell cycle. Type 1 and type 2A protein phosphatases (PP1 and PP2A) have been implicated in the control of the mitotic events in yeasts and mammalian cells [5]. However, it remains unclear yet whether PP1 and/or PP2A is involved in the progression from G_1 into S in mammalian cells. We have reported that mRNA and protein levels of PP1α are increased in poorly differentiated ascites hepatomas [6,7,8,9], suggesting that PP1α is closely linked to malignant potency and/or hepatocyte proliferation [10]. We have also demonstrated that nuclear PP1 activity is elevated at 12 h after partial hepatectomy, the time corresponding to the G_1/S transition of hepatocyte cell cycle [11].

Therefore, we examine the effects of EGF and TGFβ1 on PP1, in particular, those at the G_1/S transition in primary culture of hepatocytes. Here we show that TGFβ1 inhibits an increase in nuclear PP1 activity caused by EGF at the G_1/S transition, while expression levels of three PP1 isoforms, PP1α, PP1γ1, and PP1δ [12], remain constant.

2. Materials and methods

2.1. Materials

Leibovitz L-15 tissue culture medium (L-15), Dulbecco's modified Eagle medium (DMEM) with high glucose and Ham's F-12 (F-12) were obtained from Kyokuto Pharmaceutical Industries (Tokyo, Japan). ITX-premix (insulin, transferrin, and selenious acid) and EGF were purchased from Collaborative Reseach, Inc (Lexington, MA) and TGFβ1 from human platelets was from R&D systems Inc. (Minneapolis, MN). Collagenase was from Yakult Honsha Co. Ltd. (Tokyo, Japan). Collagen was from Collagen Corp. (Palo Alto, CA). Percoll was obtained from Pharmacia (Uppsala, Sweden).

2.2. Isolation of rat hepatocytes and cell culture

Adult male Wistar rats (Japan SLC Inc.) weighing about 200 g were used to isolate hepatocytes by a collagenase perfusion technique [13] and viable hepatocytes were purified with Percoll [14]. The viability of purified hepatocytes was greater than 85% (assessed by Trypan blue dye exclusion). The cells were suspended in L-15 supplemented with 0.5 mg/ml insulin, 1 mg/ml albumin, and 100 μg/ml streptomycin, and then plated on collagen-coated dishes at a cell density of 2×10^5 cells/35-mm dish or 2×10^6 cells/100-mm dish. Two hours after start of culture (plating), the medium was changed to a mixture of F-12 and DMEM (1:1) supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenious acid, 10^{-7} M dexamethasone, the antibiotics, and 20 ng/ml EGF. The medium was renewed at 24 h after plating.

2.3. Measurement of DNA synthesis

DNA synthesis was determined by measurement of [^{3}H]thymidine

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Abbreviations: PP1, type 1 protein phosphatase; EGF, epidermal growth factor; TGFβ1, transforming growth factor β1; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; 2ME, 2-mercaptoethanol.

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incorporation into DNA. Cells (5×10^4) were cultured in the absence or presence of EGF (20 ng/ml) and various concentrations of TGFβ1 in a 12-well plate (22-mm diameter). The cultures were treated with 1 μCi of [^{3}H]thymidine for 4 h prior to harvest. Then the incorporation into DNA was measured as described previously [15].

2.4. Subcellular fractionation

Nuclei were isolated essentially by the method of Blobel and Potter [16] with a slight modification as described by Kuret et al. [17]. Cells (1×10^7) growing on five 100-mm dishes were harvested at the indicated times by trypsinizing and scraping. Cells were then pelleted at 2,000 × *g*, suspended in 300 μl of buffer A (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF) containing 0.25 M sucrose and 0.1% NP-40, and homogenized by 5 × 5 strokes on ice in a glass–Teflon homogenizer. The resulting homogenate was mixed with 600 μl of buffer A containing 2.3 M sucrose. Subsequently the mixture was underlaid by 300 μl of buffer A containing 2.3 M sucrose and centrifuged at 124,000 × *g* for 30 min. The resulting supernatant was termed non-nuclear fraction. The nuclear pellets were resuspended in 300 ml of buffer A containing 0.25 M sucrose and 0.1% NP-40, syringed through a 25-gauge needle five times to remove the outer nuclear membrane, and pelleted by centrifugation for 5 min at 800 × *g*. The white nuclear pellets were resuspended in 75 μl of buffer B (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 0.1% 2ME, 0.1 mM PMSF, 1 mM benzamidine, and 10 mM EDTA), and then the nuclei were lysed by adding 25 μl of buffer C (50 mM Tris-HCl, pH 7.5, 0.1% 2ME, and 2 M NaCl). The resulting suspension was termed nuclear fraction. To examine the purity of isolated nuclear fraction, the activity of glucose-6-phosphatase, microsome marker enzyme, was determined. The activity per 2×10^6 cells and specific activity in microsome were 0.182 unit and 0.21 unit/mg protein, respectively. In contrast, those in nuclear fraction were 0.0012 unit and 0.013 unit/mg protein, respectively. One unit of activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of phosphate per min. Thus, the contamination of microsome into the nuclear fraction was negligible.

2.5. Phosphatase assay

PP1 activity was measured by the method of Cohen, P. et al. [18] with a slight modification as described in detail previously [19]. ^{32}P-Labeled rabbit skeletal muscle phosphorylase *a* (10^6 cpm/nmol) was used as a substrate. PP1 was defined as the phosphorylase phosphatase activity that is sensitive to inhibitor-2. Nuclear PP1 activity was defined as the activity of the lysed nuclear fraction minus that of the resuspended nuclear pellets just before lysis. One unit of activity (U) was defined as the amount of enzyme that catalyzes the release of 1 μmol of phosphate per min.

2.6. Western immunoblotting

Subcellular fractions of hepatocytes were electrophoresed on a 12% polyacrylamide gel by Laemmli's procedure [20] and proteins were electrophoretically transferred to a nitrocellulose filter. The filter was incubated with blocking solution and then with rabbit antibodies against synthetic peptides identical to the carboxyl-termini of PP1α, PP1γ1, and PP1δ for 1 h at room temperature as described previously [8]. After washes in PBS containing 0.1% Tween 20, the filter was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG for 1 h. Detection of the immune signal was done by using ECL Western blotting detection system (Amersham).

2.7. Protein determination

Protein concentrations were measured by the procedure of Bradford [21], using bovine serum albumin as the standard.

3. Results

3.1. DNA synthesis of cultured hepatocytes

As shown in Fig. 1A, hepatocytes treated with EGF started DNA synthesis 36–40 h after plating, reached a peak at 44 h, when about 80% of the cells incorporated [^{3}H]thymidine into DNA (data not shown). These data indicate that the time 36–40 h corresponds to the G_1/S transition in EGF-stimulated hepatocytes. Next, TGFβ1 was added at different times (2, 24, 36, and 40 h) and various concentrations (0.5, 1.5, and 3 ng/ml), then DNA synthesis at 44 h was measured. As shown in Fig. 1B, TGFβ1 added at 2 h and 24 h caused about 95% reduction of DNA synthesis. However, TGFβ1 added at 36 h inhibited only 30–40% and that added at 40 h showed no effect.

3.2. PP1 activity in subcellular fractions of EGF-stimulated hepatocytes

In non-nuclear fractions (Fig. 2A), PP1 activity either with or without EGF treatment was gradually increased until 40 h and then decreased. There was no difference between the activities with and without EGF. In nuclear fractions (Fig. 2B), PP1 activity with or without EGF treatment showed almost no change until 36 h. Thereafter the activity with EGF treatment was significantly and transiently increased at 40 h, being about 2.5-fold over the activities at 12 h and 24 h and the corresponding control (no EGF) activity, while the activity without EGF

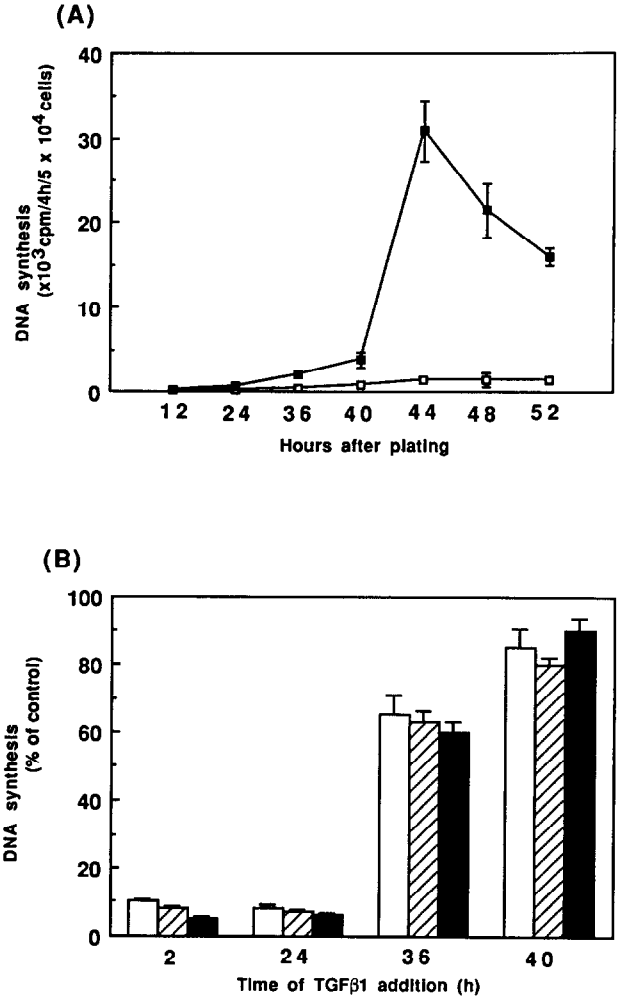

Fig. 1. DNA synthesis of cultured hepatocytes treated with EGF and EGF plus TGFβ1. (A) [^{3}H]Thymidine incorporation of hepatocytes in the presence (■) or absence (□) of EGF was measured at the indicated times after plating. (B) 0.5 (*open bars*), 1.5 (*hatched bars*), and 3.0 ng/ml (*solid bars*) of TGFβ1 were added to the culture containing EGF at the indicated times. Cells (5×10^4) were harvested at 44 h and then [^{3}H]thymidine incorporated was measured. Control denotes [^{3}H]thymidine incorporation at 44 h in EGF-stimulated hepatocytes. Error bars indicate one standard deviation in four separate experiments.

treatment showed only slight increase. Then both activities maintained almost similar levels to those at 36 h.

3.3. Effect of TGFβ1 on PP1 activity

TGFβ1 showed almost no effect on non-nuclear PP1 activity at 40 h (Fig. 3A). In contrast, TGFβ1 suppressed the increase in nuclear PP1 activity caused by EGF at 40 h in proportion to its growth-inhibitory effect (Figs. 1B and 3B). Namely, when TGFβ1 was added at 2 h or 24 h, it completely inhibited DNA synthesis and at the same time suppressed the nuclear PP1 activity to the level of non-stimulated hepatocytes; when TGFβ1 was added at 36 h, it inhibited DNA synthesis by only 30–40% and also suppressed 30–40% of the increase in nuclear PP1 activity caused by EGF.

3.4. Western immunoblotting

To investigate whether the changes in nuclear PP1 activity in EGF-stimulated hepatocytes is through an accumulation of PP1 protein, we performed Western immunoblotting of nuclear fractions at 36 h, 40 h, and 44 h using rabbit antisera for PP1α, PP1γ1, and PP1δ. As shown in Fig. 4A, the specificity of the antisera used was confirmed by peptide-competition test. The amounts of three isoforms together remained almost constant from 36 h to 44 h in EGF-stimulated hepatocytes (Fig. 4B and C). Moreover, TGFβ1 showed no effect on the amount of any nuclear PP1 isoform at 40 h after plating (Fig. 5). Although we examined mRNA and protein levels of PP1 isoforms in more expanded time course (6 h to 48 h after plating) in EGF-stimulated hepatocytes, neither mRNA nor protein level of any PP1 isoform showed obvious change (data not shown).

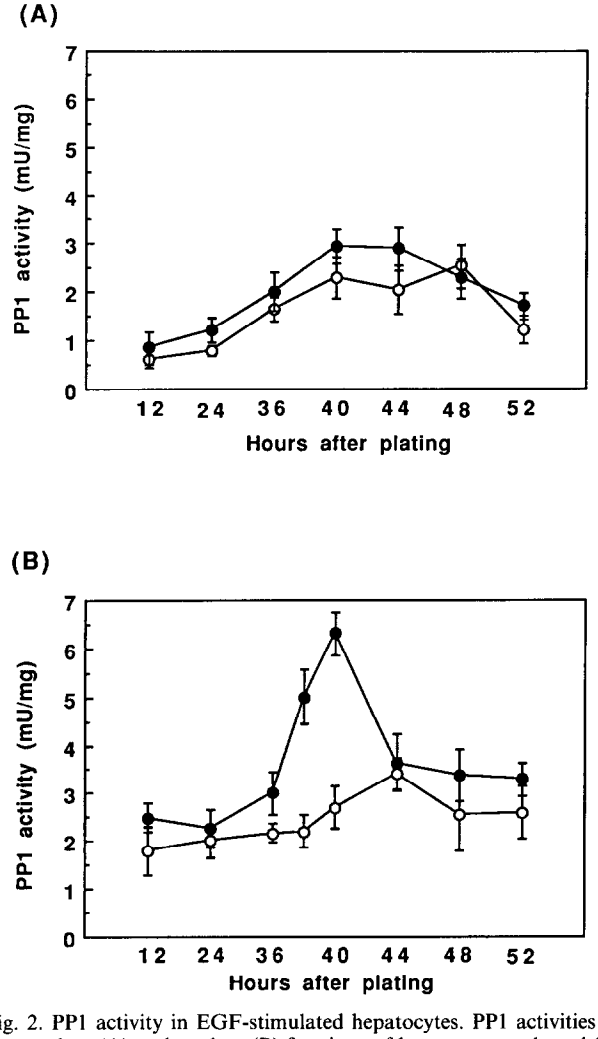

Fig. 2. PP1 activity in EGF-stimulated hepatocytes. PP1 activities in non-nuclear (A) and nuclear (B) fractions of hepatocytes cultured for the indicated times in the presence (●) or absence (○) of EGF were measured. Data were compiled from three or four separate experiments.

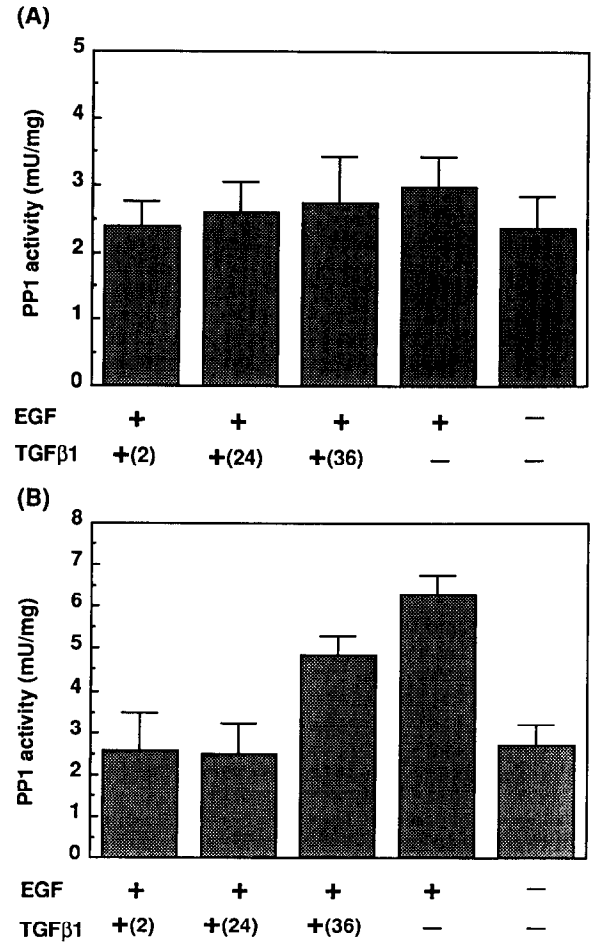

Fig. 3. Effect of TGFβ1 on PP1 activity. TGFβ1 was added at a concentration of 1.5 ng/ml and at the times (h) indicated in parentheses. Cells (1×10^7) were harvested at 40 h and their non-nuclear (A) and nuclear (B) fractions were assayed for PP1 activity. The activities at 40 h in EGF-stimulated and non-stimulated hepatocytes are also presented. Results were compiled from four separate experiments.

4. Discussion

In the present study, first we have demonstrated that nuclear PP1 activity is significantly and transiently increased at the G_1/S transition in EGF-stimulated hepatocytes, but not in non-stimulated hepatocytes. This finding is in accord with our previous results of in vivo regenerating livers [11]. Second, to further explore a relationship between nuclear PP1 and cell growth at the G_1-S phase, we examined the nuclear PP1 activity in the presence of TGFβ1, a negative growth factor whose final target molecule(s) is believed to be in late G_1 [4]. We have shown that

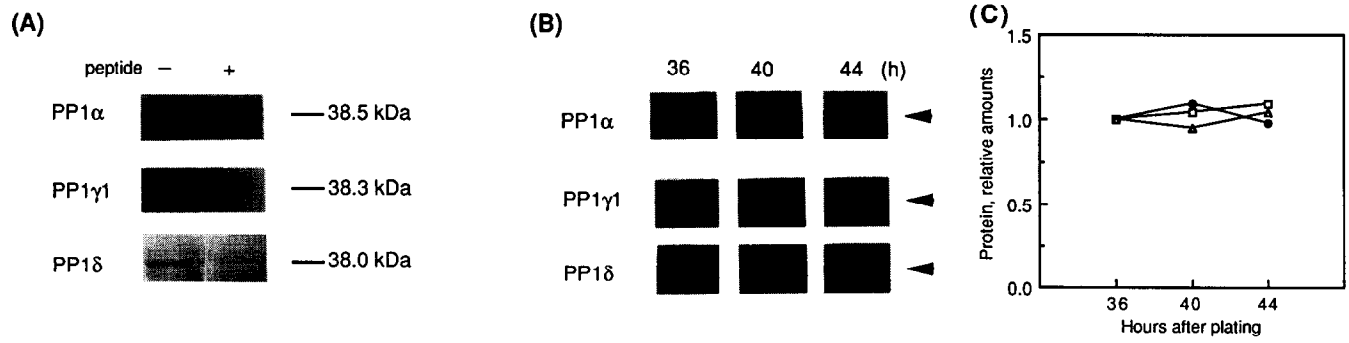

Fig. 4. Specificity of the antisera and protein expressions of nuclear PP1 isoforms. (A), peptide-competition test was performed. Each antiserum was preincubated with the corresponding peptide (final 10 μM) and then the blots of nuclear fraction of hepatocytes were incubated with the neutralized antisera. (B), Western immunoblotting analysis using the peptide antisera for PP1α, PP1γ1, and PP1δ was performed for nuclear fractions of 36-h, 40-h, and 44-h cultured cells. The fractions containing 3 μg of protein were subjected to SDS-PAGE on a 12% polyacrylamide gel and transferred to a nitrocellulose filter. Representative result was shown among three separate experiments. (C), the relative amounts of PP1α (●), PP1γ1 (□), and PP1δ (△) were quantified by densitometric scanning. Results are expressed as a ratio for the amount at 36 h.

TGFβ1 suppresses the increase in nuclear PP1 activity at the G_1/S transition of EGF-stimulated hepatocytes, in a growth inhibition-dependent manner. Therefore it seems that nuclear PP1 activity is subjected to stimulatory or inhibitory regulation by EGF or TGFβ1, respectively, and thus nuclear PP1 is involved in progression from G_1 into S phase of hepatocytes.

Recently Gruppuso et al. have reported that growth arrest in human keratinocytes by TGFβ1 involves activation of PP1 [22], which is incompatible with our results. The intracellular events in response to EGF plus TGFβ1 might be different between hepatocytes and keratinocytes. In fact, they have shown in keratinocytes that protein content per cell is increased after exposure to TGFβ1. However, in our experiments with hepatocytes, protein content showed no change after plating (data not shown), which is consistent with the report previously published [23].

On Western immunoblotting performed in order to investigate the mechanism of the changes in nuclear PP1 activity, the amounts of three PP1 isoforms, PP1α, PP1γ1, or PP1δ, did not change in either EGF-treated or EGF plus TGFβ1-treated hepatocytes. Therefore, the mechanisms by which EGF enhances nuclear PP1 activity and TGFβ1 suppresses the increase may be attributed to post-translational processes such as: (1) modification of catalytic subunit of PP1 itself, for example, phosphorylation-dephosphorylation; (2) binding or release of regulatory subunit(s).

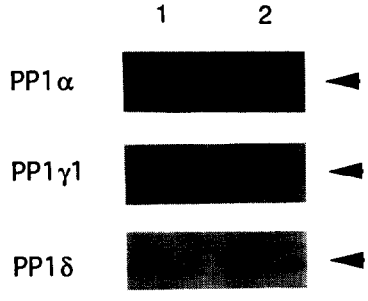

Fig. 5. Effect of TGFβ1 on protein expression of PP1 isoforms in the nucleus. TGFβ1 (1.5 ng/ml) was added to the medium containing EGF at 24 h after plating. Cells were harvested at 40 h and then their nuclear fractions (3 μg protein/lane) were subjected to Western immunoblotting using the peptide antisera. Lane 1 = EGF alone; lane 2 = EGF plus TGFβ1.

So far, there are several reports that catalytic subunit of PP1 is phosphorylated in vitro by v-abl tyrosine kinase [24], cdc2 kinase [25], and $pp60^{v\text{-}src}$ [26], resulting in modulation of PP1 activity. Regarding regulatory subunit of nuclear PP1, the existence of nuclear inhibitor protein of PP1, termed NIPP-1, has been reported [27] in addition to inhibitor-2 [28].

Recently, we have demonstrated that mRNA and protein levels of PP1α were specifically increased in poorly differenciated ascites hepatomas compared with normal liver [7,8,9]. In contrast, in growth-stimulated hepatocytes induced by EGF, both mRNA and protein levels of PP1α remained constant during the culture. The significance of this difference remains unknown. Further study to elucidate the significance of the different expression of PP1 isoforms between hepatocytes and hepatomas will provide a clue to understanding of malignant growth as well as normal proliferation of hepatocytes.

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