Mouse prolyl oligopeptidase plays a role in trophoblast stem cell differentiation into trophoblast giant cell and spongiotrophoblast

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ABSTRACT

Introduction: Prolyl oligopeptidase (prolyl endopeptidase, Prep), a multifunctional protease hydrolyzing -Pro-X- peptide bonds, is highly expressed in the mouse placenta, but the function during development is not known. We explored the possibility of Prep's involvement in placental differentiation.

Methods: We cultured trophoblast stem cells (TSCs) derived from the E6.5 mouse embryo and investigated the detailed expression pattern of Prep during their differentiation. Prep-specific inhibitors were added to the TSC culture, and the effect on the differentiation was assessed by microscopic observation and the expression of marker gene for each placental cell.

Results: During TSC differentiation for 6 days, Prep was constantly detected at mRNA, protein, and activity levels, and the protein was found mainly in the cytoplasm. The addition of 30 μM and 10 μM SUAM-14746, a Prep-specific inhibitor, effectively inhibited the differentiation into spongiotrophoblasts (SpTs) and trophoblast giant cells (TGCs), while the TSC viability was not affected. 5 μM SUAM-14746 impaired the differentiation into SpTs, and 1 μM SUAM-14746 exhibited no effects. Another Prep-specific inhibitor, KYP-2047, did not affect the differentiation. We confirmed efficient inhibition of Prep enzymatic activity in TSCs by both inhibitors.

Conclusion: The dose-dependent effect of SUAM-14746 on TSCs suggests that Prep plays an important role in the differentiation into SpTs and TGCs in the mouse placenta.

Keywords: prolyl endopeptidase, SUAM-14746, placenta, protease

Abbreviations: DMSO, dimethyl sulfoxide; FGF4, fibroblast growth factor 4; KYP-2047, 4-Phenylbutanoyl-L-Prolyl-2(S)-Cyanopyrrolidine ; MCA, 4-methyl-coumaryl-7-amide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, Phosphoinositide 3-kinase; Prep, prolyl endopeptidase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; S.D., standard deviation; SpT, spongiotrophoblast; SUAM-14746, 3-({4-[2-(E)-Styrylphenoxy] butanoyl}-L-4-hydroxyprolyl)-thiazolidine; SynT, syncytiotrophoblast; TGC, trophoblast giant cell; TSC, trophoblast stem cell

1. Introduction

Prolyl oligopeptidase (also known as prolyl endopeptidase, E.C.3.4.21.26; gene symbol *Prep*) is a serine endopeptidase, which cleaves peptides shorter than 30-mer at the carboxyl side of a proline residue [1]. Prep was discovered as an oxytocin-cleaving uterine enzyme in human [2], and is now known to be expressed in various tissues of many species from bacteria to mammals [3]. Prep is related to various physiological events such as learning and memory [4, 5], cell signaling [6, 7], sperm motility [8–10], and cell proliferation and differentiation [11–13], so it is recognized as a multifunctional molecule. We previously reported that the mouse ovary and placenta express *Prep* mRNA at higher levels than other tissues [14, 15], but there are no reports of the function of Prep in the placenta.

The mouse placenta is composed of three layers, maternal decidua, junctional zone, and labyrinth, and three types of cells are known to be functionally important: trophoblast giant cell (TGC), spongiotrophoblast (SpT), and syncytiotrohoblast (SynT). TGC show polyploidy, characteristic of the large nucleus and cytoplasm resulting from endoreduplication [16, 17], and contributes to maintaining pregnancy by synthesizing hormones and growth factors. SpT plays a pivotal role in fetus viability [18], and SynT, a multinuclear cell formed by cell to cell fusion, functions to exchange nutrients between the embryo and the mother [19]. While the normal formation of these cells is important for the mouse placental function, the molecular mechanism(s) responsible for their differentiation is not fully understood [20, 21].

We previously reported that a high level of Prep was localized in SpTs and TGCs of the mouse placenta [14, 15]. Combined with earlier and recent works showing the involvement of Prep in cell differentiation [11, 12], we hypothesized that Prep had an important function in placental differentiation. Since Prep-knockout mice were not lethal and no phenotype was so far reported in the placenta [10, 22–28], the first step to investigate Prep's role in placental differentiation should be done

with the trophoblast stem cell (TSC) culture system. Mouse TSCs can be maintained as undifferentiated cells in the presence of fibroblast growth factor 4 (FGF4), and by removing it, we can easily induce the differentiation into three trophoblast subtypes, TGC, SpT, and SynT [29]. Therefore, the TSC culture system is an ideal tool for analyzing the function and differentiation of the mouse placenta.

In this study, as an initial step for understanding the function of Prep in the mouse placenta, we induced the TSC differentiation in the presence of a Prep-specific inhibitor, 3-({4-[2-(E)-Styrylphenoxy] butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746), and investigated whether TSCs successfully differentiated into each type of placental cell. The addition of SUAM-14746 significantly inhibited the differentiation into SpTs and TGCs, which suggests that Prep plays an important role in the mouse placental differentiation into SpTs and TGCs.

2. Materials and methods

2.1. TSC culture, induction of differentiation, and treatment with Prep-specific inhibitors

TSCs derived from the E6.5 mouse embryo were kindly provided by Dr. Satoshi Tanaka [29] and maintained as reported [30]. To induce the differentiation, 2×10^5 TSCs were seeded on a 35-mm dish, and FGF4 was removed on the next day. A Prep-specific inhibitor, SUAM-14746 [31] (Peptide Institute, Osaka, Japan) or 4-phenylbutanoyl-l-prolyl-2(S)-cyanopyrrolidine (KYP-2047) [32] (Sigma-Aldrich, St. Louis, MO), was added on the removal of FGF4. An equal volume of dimethyl sulfoxide (DMSO) was used as a control. Both inhibitors inhibit Prep activity by binding to the active site and blocking the access of substrates.

2.2. Quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNAs were prepared and qRT-PCR was performed as previously described [14, 33]. All the data were normalized to *Aip*. The value at day 0 was set to 1.0 in Fig. 2A, and in other figures, the highest value was set to 1.0 in each experiment. Primer sequences are shown in Table 1.

2.3. Preparation of soluble extract, whole cell extract, and nuclear, cytoplasmic, and membrane fractions

The cells were suspended in phosphate buffered saline, and the soluble extract was collected by the freeze-thaw method [34]. To prepare the whole cell extract, the cells were mixed with an equal volume of 2× lysis buffer (0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 0.5 % sodium deoxycholate, 2% NP-40, and 2 mM ethylenediamine tetraacetic acid) containing 1× proteinase inhibitor (Roche, Basel, Switzerland), incubated on ice for 5 min, and sonicated to shear nucleic acids. After centrifugation, the supernatant was used as the whole cell extract. Nuclear, cytoplasmic, and membrane fractions were prepared as previously described [15]. Protein concentration was measured with a Pierce bicinchoninic acid protein assay reagent kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instruction.

2.4. Western blot analysis

Western blot analysis was performed as previously described [15], using the purified anti-Prep antibody (52 ng/ml at a final concentration [15]) and the anti-Actb polyclonal antibody (1:1000 dilution, GTX109639, Genetex, Irvine, CA) as primary antibodies. As secondary antibodies, goat anti-chicken IgY antibody (1:10,000 dilution, 103-035-155, Jackson ImmunoResearch, West Grove, PA) and anti-mouse IgG antibody (1:5,000 dilution, NA934V, GE healthcare, Piscataway, NJ) were used for Prep and Actb, respectively. The signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck, Darmstadt, Germany) and quantified by the Image J software [35] to normalize the Prep band intensity to Actb. To confirm the specificity, we also

performed this experiment by using the anti-Prep antibody preincubated with an excess amount of recombinant Prep protein.

2.5. Measurement of Prep enzyme activity

The specific Prep activity toward Suc-Gly-Pro-Leu-Gly-Pro-4-methyl-coumaryl-7-amide (MCA) (Peptide Institute) was measured as previously described [36]. In brief, soluble TSC extracts (3 μg for Fig 2; 30 μg for Figs 4, 6) with or without a Prep-specific inhibitor were reacted in 0.1 M Tris-HCl (pH 8.0) containing 10 mM β-mercaptoethanol with 0.1 mM substrate in a volume of 500 μl at 37°C for 20 min. The reaction was stopped by the addition of 2.5 ml of 30 mM sodium acetate buffer (pH 4.3) containing 100 mM monochloroacetic acid. Prep activity was assessed by measuring the released fluorophore 7-amino-4-methylcoumarin using an excitation wavelength of 370 nm and an emission wavelength of 460 nm.

2.6. Cell viability assay

TSC viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using MTT Cell Count kit (Nakalai tesque, Kyoto, Japan) according to the manufacturer's recommendations with slight modifications. 0.2×10^5 cells were seeded onto each well of a 24-well plate, and the differentiation was induced with 10 μM or 30 μM SUAM-14746 or DMSO (control). After cultured for 4 or 6 days, the cells were treated with MTT reagent for 3 hr at 37°C. Then, the solubilization solution was added and the cells were incubated at 37°C for overnight. The amount of formazan product was measured using a spectrophotometer (Eppendorf, Hamburg, Germany) at 550 nm absorbance.

2.7. Statistical analysis

Results were represented as the average ± standard deviation (S.D.), and their statistical

significance was analyzed by Student's *t* test, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or Dunnett's test. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Expression of marker genes during TSC differentiation

We first checked the expression of marker genes by qRT-PCR. The genes were *Cdx2* for undifferentiated cells, *Gcm1* for SynT, *Tpbpα* for SpT, and *Pl1* for TGC. *Cdx2* expression was decreased immediately after the induction (Fig. 1A), and *Gcm1* expression was increased to reach a peak at day 2, followed by a reduction (Fig. 1B). *Tpbpα* and *Pl1* expression were gradually increased during the differentiation and became the highest at day 6 (Fig. 1C and D). These expression patterns verified that TSC differentiation was successful as in other reports [29, 37, 38].

3.2. Prep expression and enzymatic activity during TSC differentiation

We investigated Prep expression and enzymatic activity during TSC differentiation. By qRT-PCR, *Prep* mRNA was significantly increased after the induction and reached a peak at day 4 (Fig. 2A). By Western blot analysis with both soluble and whole cell extracts, Prep protein was detected at a constant level during TSC differentiation (Fig. 2B and C). Soluble and whole cell extracts were used because Prep is mainly localized in the cytosol but membrane-bound and nuclear forms also exist in some cell types [12, 15]. In Western blot analysis with TSC extracts, we sometimes detected extra bands at upper and lower positions of a main 73 kDa band, but they were not specific Prep signals as revealed by blotting with pre-absorbed antibody (Fig. 2D). By Western blot analysis with subcellular fractions of TSCs at day 0 and day 6, the Prep signal was observed mainly in the cytoplasm and at lower levels in the membrane fraction, but not in the nucleus (Fig. 2E). The Prep enzymatic activity was detected

during the entire period of TSC differentiation, and the TSC sample at day 4 showed low but significantly higher activity than that at day 0 (Fig. 2F). These results indicated that Prep mRNA, protein, and enzymatic activity were all detected at high levels and the Prep protein was mostly localized in the cytoplasm during TSC differentiation.

3.3. TSC differentiation in the presence of SUAM-14746

To examine a function of Prep in TSC differentiation, we added a Prep-specific inhibitor, SUAM-14746, at various concentrations, and the cell morphology and marker gene expression were examined. When we added 30 μM SUAM-14746 and cultured for 4 days, there were many TGC-like cells that had large cytoplasms and nuclei in the control cells (Fig. 3A center), compared with undifferentiated cells (Fig. 3A left). However, the number and percentage of the TGC-like cells dramatically decreased in the presence of 30 μM SUAM-14746 (Fig. 3A right).

We then examined the marker gene expression at day 2 and day 6 by qRT-PCR. With the addition of SUAM-14746 at 10 μM and 30 μM, *Cdx2* expression was not different from the control (Fig. 3B). *Gcm1* expression was increased at day 2, similarly to the control cells, but at day 6, it was decreased in a dose-dependent manner (Fig. 3C). *Tpbpa* and *Pl1* expression were considerably decreased in TSCs cultured with SUAM-14746 at day 6 compared to the control (Fig. 3D and E). With the addition of 5 μM and 1 μM SUAM-14746, we did not observe any difference in *Cdx2* expression between the cells with the inhibitor and the control cells (Fig. 3F), and *Gcm1* expression was decreased in a dose-dependent manner at day 6 (Fig. 3G). *Tpbpα* expression was significantly decreased by 5 μM SUAM-14746 at day 6 (Fig. 3H), and *Pl1* expression was not changed by this concentration (Fig. 3I). 1 μM SUAM did not affect the differentiation of any types of cells compared to control.

We checked whether this inhibitor actually inhibited Prep enzymatic activity in TSCs. By the enzyme assay, Prep activity in TSC extracts collected at day 0 and 6 was greatly decreased by 1-30 μM SUAM-14746 (Fig. 4). Thus, our results demonstrated that SUAM-14746 specifically impaired

151 the TSC differentiation into SpTs and TGCs.

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153 *3.4. Cell Viability in the Presence of SUAM-14746*

154 To test the cytotoxicity of SUAM-14746, we checked the TSC viability by MTT assay. After the
155 differentiation induction for 4 days and 6 days with 10 μM or 30 μM SUAM-14746, we treated the
156 cells with MTT and performed the assay. The viability was not significantly different between the cells
157 with SUAM-14746 and the control cells (Fig. 5). Thus, this inhibitor did not affect the TSC viability.

158

159 *3.5. TSC differentiation with another Prep-specific inhibitor, KYP-2047*

160 Finally, we examined the effect of another Prep-specific inhibitor, KYP-2047, on TSC
161 differentiation. We induced the TSC differentiation with 60 μM KYP-2047 and collected total RNAs
162 at day 6. Unexpectedly, *Tpbpα* and *Pl1* expression were not different between the control cells and
163 TSCs treated with KYP-2047 (Fig. 6A and B), while this inhibitor efficiently decreased Prep activity
164 in TSC extracts (Fig. 6C and D). These data indicated that KYP-2047 did not inhibit the TSC
165 differentiation into SpTs and TGCs.

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167

168 **4. Discussion**

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170 During TSC differentiation, *Prep* mRNA was significantly increased at day 4, whereas the Prep
171 protein amount and enzymatic activity were fairly constant. In addition, we observed a slight but
172 significant increase in Prep activity at day 4, but the Prep protein amount was constant during TSC
173 differentiation. This kind of discrepancy was reported in several studies [3], and is considered to be
174 due to the stability of *Prep* mRNA, the translational efficiency, rapid turnover of Prep protein, or the
175 existence of endogenous Prep inhibitors [39–41]. Thus, such post-transcriptional regulation of the

176 *Prep* gene may be present during TSC differentiation.

177 We detected the Prep protein in both cytoplasm and membrane fractions but not in the nuclei in
178 TSCs before and after differentiation. While this is consistent with our previous result showing the
179 Prep protein in the cytoplasm and membrane of trophoblast cell lineages within the mouse placenta
180 [15], it is the nuclear Prep that was suggested to play roles in cell differentiation [11, 12]. Considering
181 that the relation of membrane-bound Prep to cell differentiation is not reported and that most Prep was
182 present in the cytoplasm of TSC, the cytoplasmic Prep is likely to function in TSC differentiation.

183 Of particular interest is that SUAM-14746 had no effects on the initiation of TSC differentiation
184 whereas TSCs did not complete the differentiation into TGCs and SpTs. Because multinuclear SynTs
185 generally accounted for only a small percentage of cells in TSC differentiation, the results indicated
186 that most cells were neither undifferentiated cells nor any of TGCs, SpTs, and SynTs in the presence of
187 SUAM-14746. We do not know what specific differentiation state the cells possessed, but they could
188 have been in a transitory state or had become cell types unrelated to the placenta.

189 The expression of a SynT marker gene, *Gcm1*, was significantly decreased by the addition of
190 SUAM-14746 at day 6. However, given that *Gcm1* expression was not affected by SUAM-14746 at
191 day 2, this inhibitor probably had no effect on SynT differentiation, and we think that Prep is unlikely
192 to be involved in TSC differentiation into SynTs. The reason why it was decreased at day 6 is unclear
193 but might be due to change in the relative percentage of each cell type during the culture. The detailed
194 mechanism of SynT differentiation will require further investigation.

195 In contrast, the TSC differentiation into SpTs and TGCs was specifically inhibited by the addition
196 of SUAM-14746 at day 6. The dose-dependent decrease of *Tpbpα* and *Pl1* expression supports the
197 specific effect of SUAM-14746. Our previous data demonstrating the Prep localization at higher levels
198 in SpTs and TGCs in the mouse placenta are also consistent with its role in the differentiation into
199 these cells [15]. In addition, several studies reporting Prep's involvement in cell differentiation [11,
200 12] support the idea that Prep has a fundamental function via the regulation of various types of cell

differentiation. Collectively, our current data strongly suggest that Prep plays an important role in the regulation of placental differentiation into TGCs and SpTs.

Then, by what kind of mechanism does Prep control the TSC differentiation? It is notable that the phosphoinositide 3-kinase (PI3K)/Akt pathway is involved in activation of the *Mash2* gene, a key factor for SpT differentiation [18, 42, 43]. Because Prep regulates the PI3K/Akt pathway [7], it may be involved in some aspect of controlling the pathway promoting SpT differentiation. In the case of TGC differentiation, Prep may be related to the regulation of pathways including inositol trisphosphate [6, 44, 45]. Prep is known to function as an interacting molecule [22, 46–48], and therefore it is plausible it controls TSC differentiation through interaction with other proteins.

While SUAM-14746 efficiently inhibited the TSC differentiation into SpTs and TGCs, another Prep-specific inhibitor, KYP-2047, had no effect. Since both inhibitors greatly decreased Prep enzymatic activity, Prep is likely involved in TSC differentiation as an interacting protein but not as a protease. The binding of the two inhibitors may cause different conformational changes in the Prep protein with different implications for differentiation. For example, SUAM-14746 may lead to dissociation between Prep and its interacting proteins, but KYP-2047 may not result in such changes. Therefore, the two inhibitors, SUAM-14746 and KYP-2047, probably have different properties, and it is SUAM-14746 that inhibits TSC differentiation into SpTs and TGCs.

In conclusion, a Prep-specific inhibitor, SUAM-14746, specifically impaired the TSC differentiation into SpTs and TGCs, which strongly suggests that Prep plays an important role in the differentiation of SpTs and TGCs in the mouse placenta. This is the first report to indicate the involvement of Prep in placental differentiation.

Conflicts of Interest

The authors certify that they have nothing to disclose.

Author contributions

Y.M. and A.P.K. designed the study. Y.M. and S.M. performed the experiments. Y.M. analyzed the data. Y.M., S.M., and A.P.K. wrote the paper.

Acknowledgements

We thank Dr. Satoshi Tanaka at Tokyo University for kindly gifting TSCs. This work was supported by Grant-in-aid for JSPS Fellows 24-6128 from Japan Society for the Promotion of Science. We are very grateful to Dr. Kevin Wakeman for his suggestions on grammatical issues and to Dr. Masayo Soma for her valuable advice on statistical analyses.

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Figure legends

Fig. 1. The expression pattern of marker genes for each type of placental cell by qRT-PCR.
qRT-PCR was performed with total RNAs prepared from TSCs at indicated days. The expression level was normalized with that of the *Aip* gene, and the highest value in each data set was set to 1.0. The graph shows the relative expression of each marker gene after induction of differentiation. All the data are presented as mean ± S.D. from six independent experiments, and the statistical significance was

analyzed by one-way ANOVA followed by Tukey's post hoc test. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$, ****$P < 0.0000001$ compared with day 0. A: *Cdx2* for the undifferentiated cell, B: *Gcm1* for SynT, C: *Tpbp*α for SpT, and D: *Pl1* for TGC.

Fig. 2. Prep expression and enzymatic activity during TSC differentiation. A: *Prep* mRNA expression by qRT-PCR. The *Prep* expression level was normalized with a reference gene, *Aip*, and the value at day 0 was set to 1.0. All the data are presented as mean ± S.D. from six independent experiments, and were analyzed by one-way ANOVA followed by Tukey's post hoc test. †$P < 0.01$ compared with day 0. *Prep* mRNA expression significantly increased after the induction, reached a peak at day 4, and decreased thereafter. B, C: Western blot analysis for analyzing the Prep protein amount. 10 μg of soluble extract (B) or whole cell extract (C) from TSCs at indicated days were used. Specific Prep bands are indicated by arrows. The signal intensity was measured using Image J software, and the Prep signal was normalized to Actb. The relative Prep protein amount is shown in the upper graph, and the image below the graph is a representative blot result from three independent experiments. The data were analyzed by one-way ANOVA followed by Tukey's post hoc test, but Prep levels did not significantly change during TSC differentiation. D: Western blot analysis for specificity of the anti-Prep antibody. The anti-Prep antibody was pre-absorbed with recombinant Prep protein and used for Western blot analysis with soluble and whole cell extracts from TSCs at day 6. Two or three bands were detected, but only a main band disappeared in each sample with pre-absorbed antibody. The main Prep band is indicated by black arrowheads, and the other bands by white arrowheads. E: Western blot analysis with cytoplasmic, membrane, and nuclear fractions of TSCs. In both TSCs at day 0 and day 6, the Prep signal was mainly detected in the cytoplasmic fraction and at a lower level in the membrane but not in the nucleus. F: Prep enzymatic activity against an MCA substrate, Suc-Gly-Pro-Leu-Gly-Pro-MCA. The data are presented as mean ± S.D. from three independent experiments, and were analyzed by one-way ANOVA followed by Tukey's post hoc test. †$P < 0.01$

compared with day 0. The activity was significantly increased at day 4.

Fig. 3. The effects of SUAM-14746 on TSC differentiation. A: Morphology of TSCs by microscopic observation. Undifferentiated cells (left), control cells at day 4 (middle), and cells with 30 μM SUAM-14746 at day 4 (right) are shown. Many TGC-like cells are observed in the control, but not in the cells treated with the inhibitor. The white bar represents 500 μm. B-E: The marker gene expression in TSCs treated with SUAM-14746 by qRT-PCR. Total RNAs were purified from TSCs treated with 30 μM and 10 μM SUAM-14746 or DMSO (control) for indicated days. There was no difference in *Cdx2* expression (B), and *Gcm1* expression was significantly decreased at day 6 (C). *Tpbpα* and *Pl1* expression were considerably decreased at day 6 (D, E). F-I: The marker gene expression in TSCs with 10 μM, 5 μM, and 1 μM SUAM-14746 for indicated days. 5 μM SUAM-14746 significantly affected the expression of *Gcm1* and *Tpbpα* at day 6. The data are presented as mean ± S.D. from three independent experiments, and the statistical significance was analyzed by one-way ANOVA followed by Dunnett's test to compare the samples with SUAM-14746 to the control. ‡$P < 0.05$, ‡‡$P < 0.01$, ‡‡‡$P < 0.001$, ‡‡‡‡$P < 0.0001$, ‡‡‡‡‡$P < 0.00000001$ compared to the cells with DMSO.

Fig. 4. The inhibitory effects of SUAM-14746 on Prep in TSCs. Soluble extracts of TSCs at day 0 (A) and day 6 (B) were incubated with 30 μM, 10 μM, 5 μM, 1μM SUAM-14746, or DMSO (control), and Prep enzymatic activity was measured. The value of control was set to 100 in both data sets, and relative Prep enzymatic activity is shown. All concentrations of SUAM-14746 efficiently inhibited Prep activity. The data are presented as mean ± S.D. from three independent experiments, and the statistical significance was analyzed by one-way ANOVA followed by Dunnett's test to compare the samples with SUAM-14746 to the control. ¶$P < 0.000000000001$, ¶¶$P < 0.0000000000000001$ compared to the control.

Fig. 5. The effects of SUAM-14746 on TSC viability. 30 μM and 10 μM SUAM-14746 or an equal volume of DMSO (control) was added to the medium at the time of induction. The cells were cultured for 4 or 6 days and subject to the MTT assay. The cell viability was represented by the amount of formazan product which was measured by a spectrophotometer at 550 nm absorbance. The data are averages from three independent experiments, and were analyzed by Student's *t* test to compare the sample with SUAM-14746 at the indicated concentration to the control. The addition of SUAM-14746 at both concentrations made no significant difference in the absorbance value from the control cells at day 4 and day 6.

Fig. 6. The effect of 60 μM KYP-2047 on TSC differentiation. A, B: *Tpbpα* and *Pl1* expression in TSCs cultured for 6 days and in undifferentiated cells with 60 μM KYP-2047 or DMSO (control) by qRT-PCR. The data are averages from three independent experiments, and were analyzed by Student's *t* test to compare the sample with 60 μM KYP-2046 to the control. Unlike SUAM-14746, 60 μM KYP-2047 did not affect the expression of the two marker genes. C, D: The inhibitory effects of 60 μM KYP-2047 on Prep enzymatic activity in TSCs at day 0 (C) and day 6 (D). The data are averages from three independent experiments, and were analyzed by Student's *t* test to compare the sample with KYP-2047 to the control. §$P < 0.000000000000001$ compared to the control.

Table 1 Primers used in this study.

Gene	Species	Forward primer	Reverse primer	Refseq ID
Prep	mouse	GGAATCGATGCTGCTGATTA	CCATCCAGCTTTATGCCTTT	NM_011156
Cdx2	mouse	TGGAGCTGGAGAAGGAGTTT	CTGCGGTTCTGAAACCAAAT	NM_007673
Gcm 1	mouse	AACACCAACAACCACAACTCC	CAGCTTTTCCTCTGCTGCTT	NM_008103
Tpbpa	mouse	TGGATGCTGAACTGCAAGAG	TCCGTCTCCTGGTCATTTTC	NM_009411
Pl1	mouse	TTGGCCGCAGATGTGTATAG	TCGTGGACTTCCTCTCGATT	NM_008864
Aip	mouse	GAGGACGGGATCCAAAAGC	CTGTGCAGCGTCCGAAAGT	NM_016666

Fig. 1.

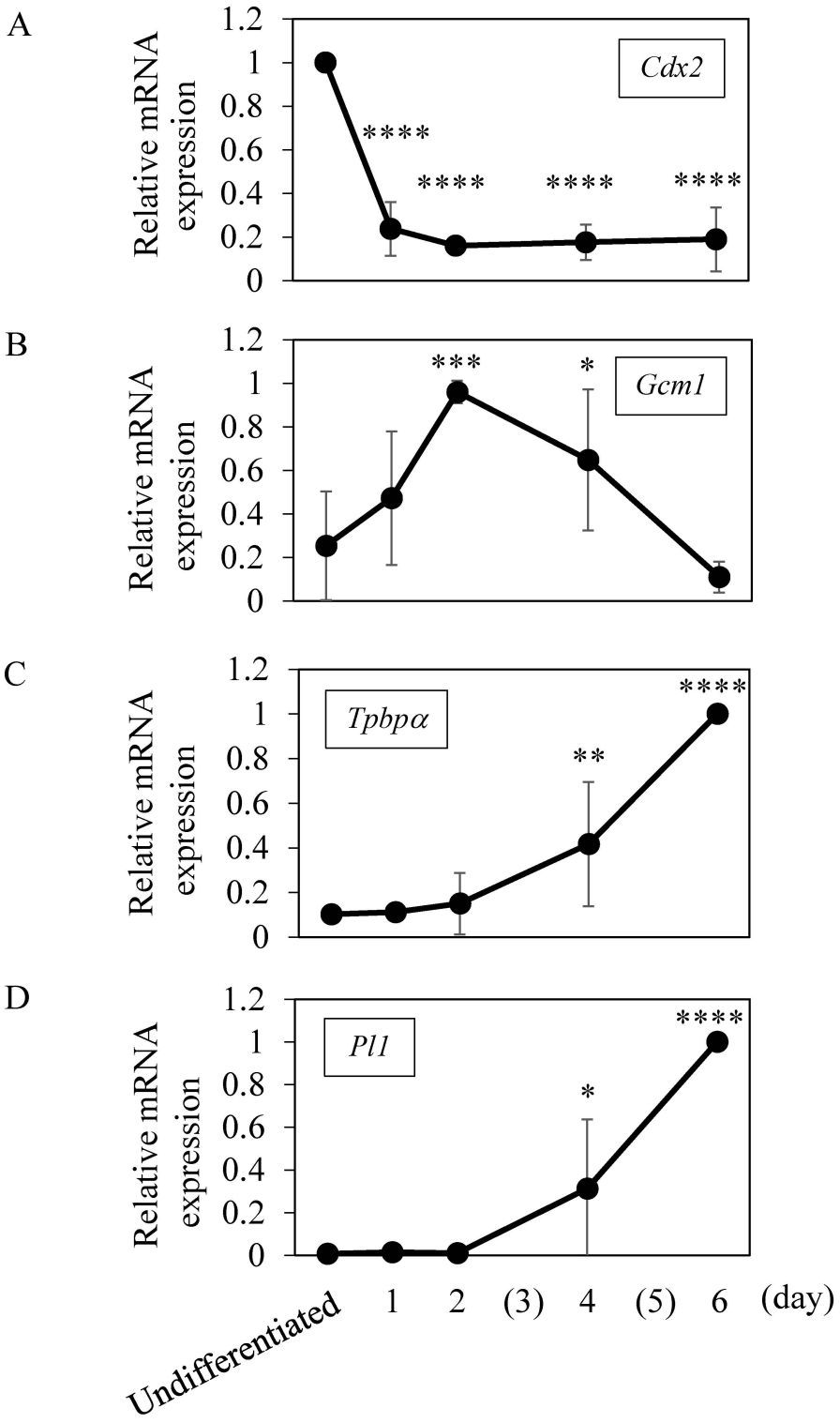

A
B
C
D
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
Cdx2
****
****
****
****
Gcm1
***
*
Tpbpα
**
****
Pl1
*
****
Undifferentiated
1
2
(3)
4
(5)
6
(day)

Fig. 2.

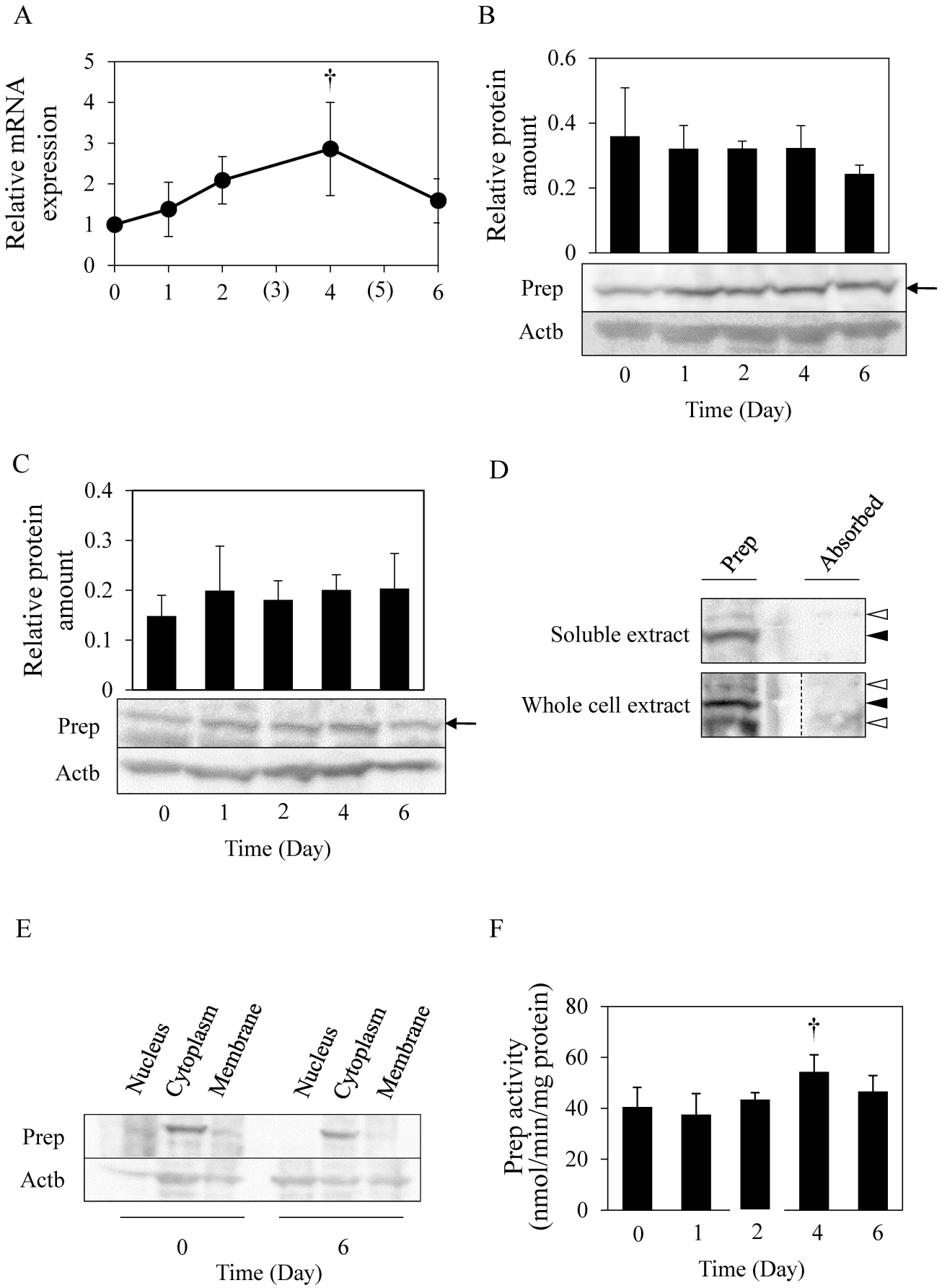

A
Relative mRNA expression
5
4
3
2
1
0
†
0
1
2
(3)
4
(5)
6
B
Relative protein amount
0.6
0.4
0.2
0
Prep
Actb
0
1
2
4
6
Time (Day)
C
Relative protein amount
0.4
0.3
0.2
0.1
0
Prep
Actb
0
1
2
4
6
Time (Day)
D
Prep
Absorbed
Soluble extract
Whole cell extract
E
Nucleus
Cytoplasm
Membrane
Nucleus
Cytoplasm
Membrane
Prep
Actb
0
6
Time (Day)
F
Prep activity (nmol/min/mg protein)
80
60
40
20
0
†
0
1
2
4
6
Time (Day)

Fig. 3.

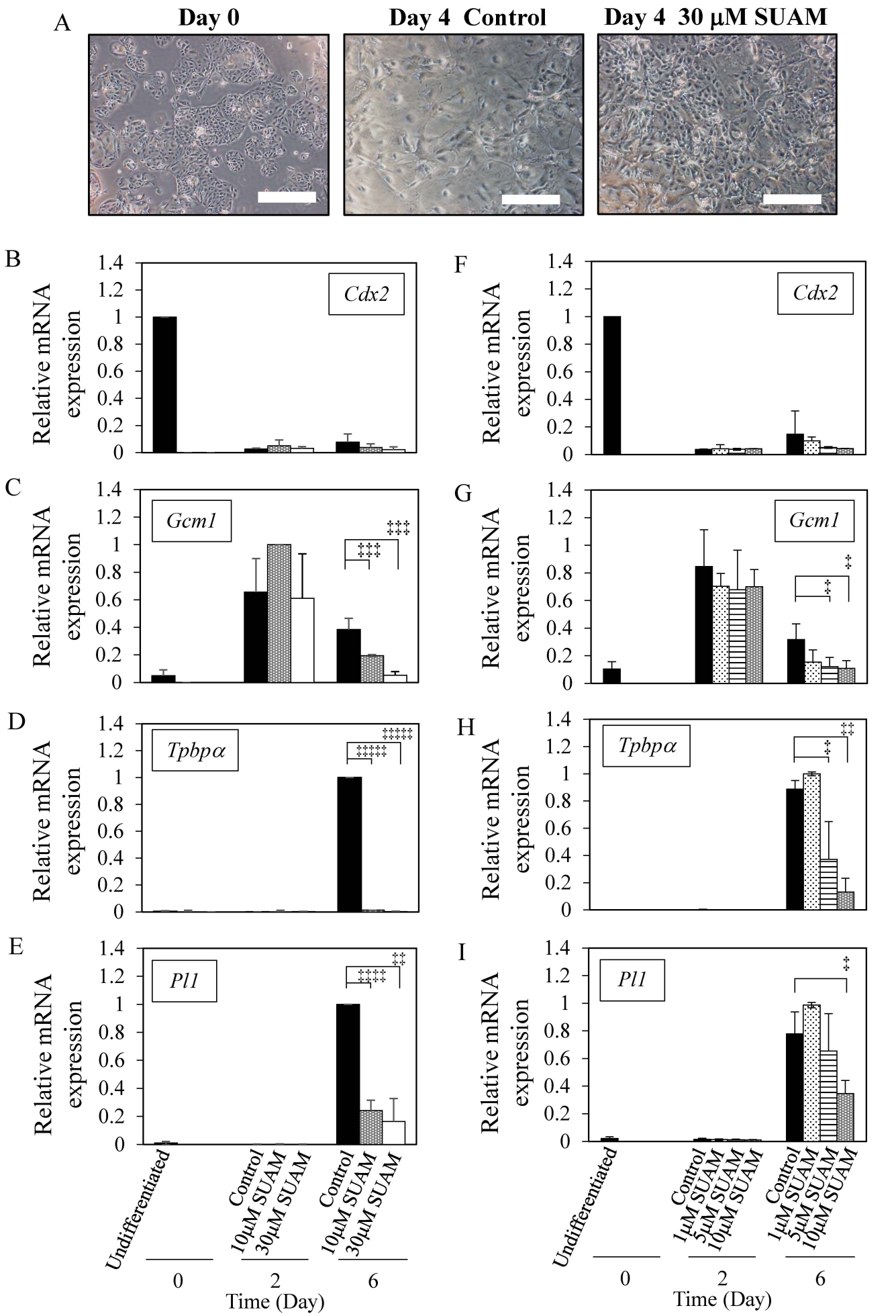

A
Day 0
Day 4 Control
Day 4 30 μM SUAM
B
C
D
E
F
G
H
I
Relative mRNA expression
Cdx2
Gcm1
Tpbpα
Pl1
Undifferentiated
Control
10μM SUAM
30μM SUAM
1μM SUAM
5μM SUAM
10μM SUAM
0
2
6
Time (Day)

Fig. 4.

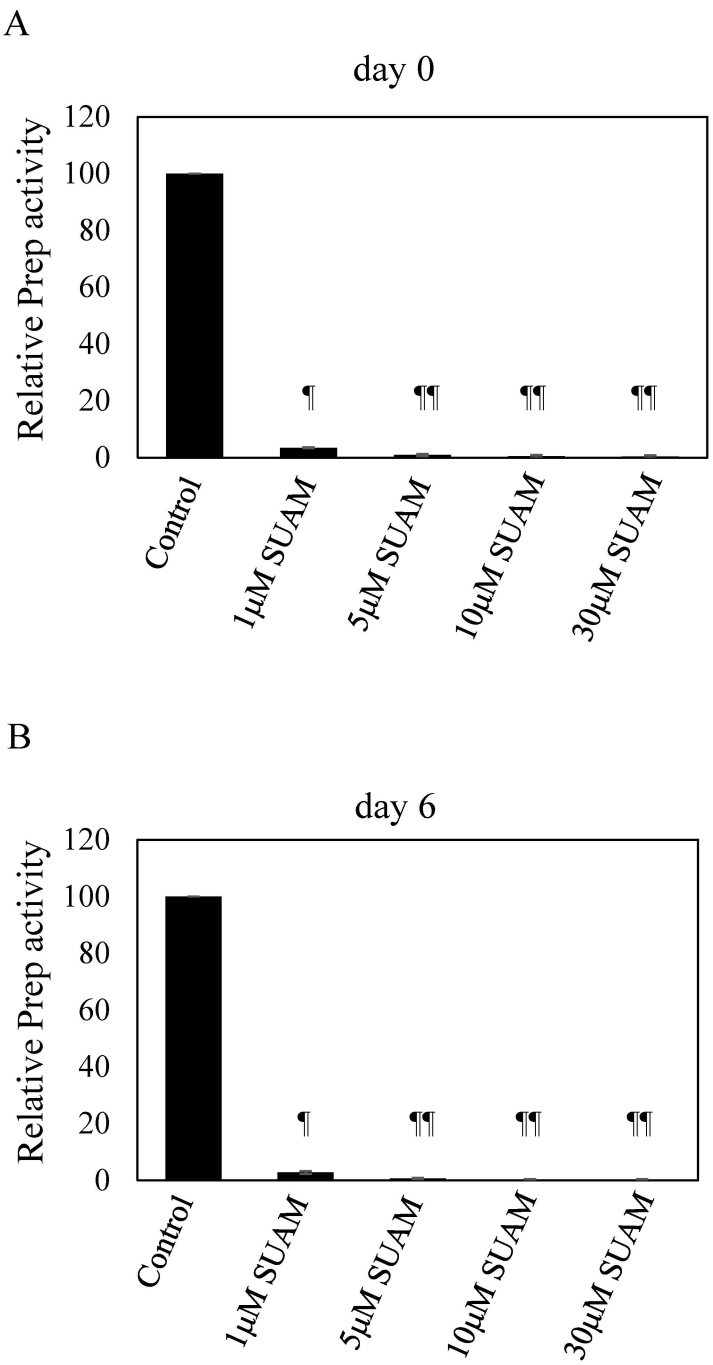

A
day 0
Relative Prep activity
120
100
80
60
40
20
0
¶
¶¶
¶¶
¶¶
Control
1μM SUAM
5μM SUAM
10μM SUAM
30μM SUAM
B
day 6
Relative Prep activity
120
100
80
60
40
20
0
¶
¶¶
¶¶
¶¶
Control
1μM SUAM
5μM SUAM
10μM SUAM
30μM SUAM

Fig. 5.

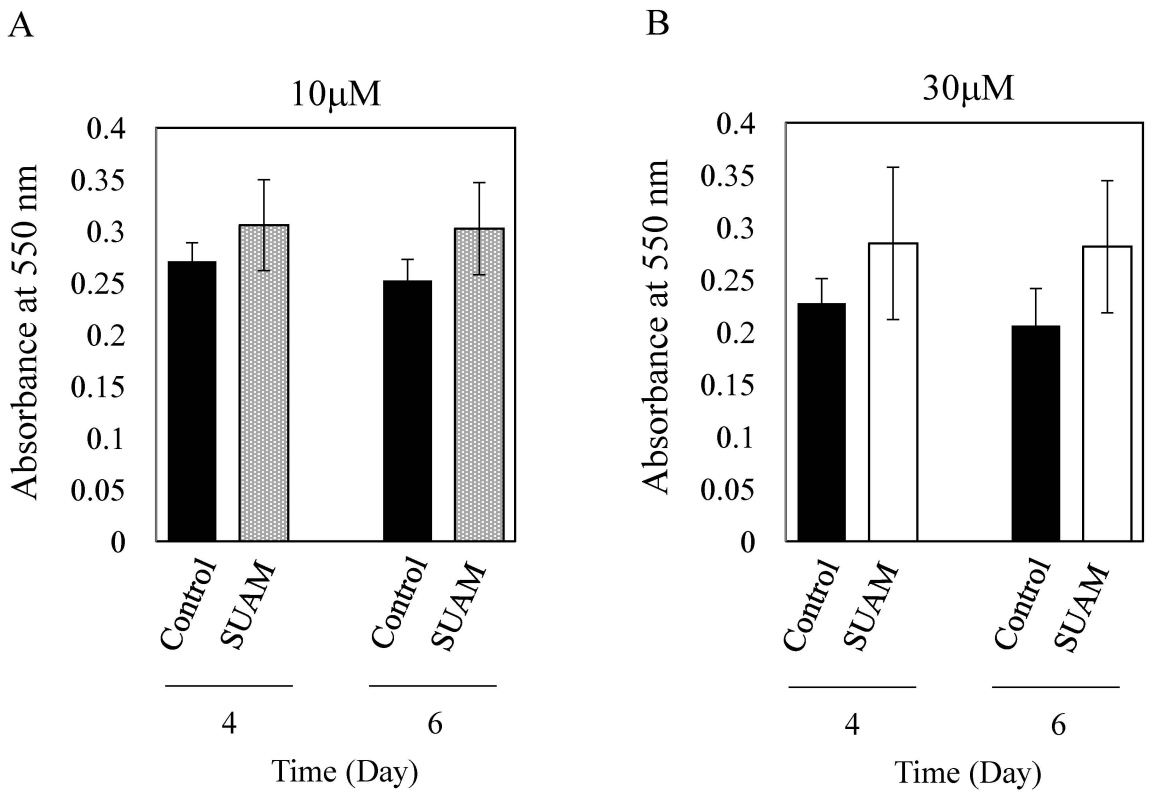
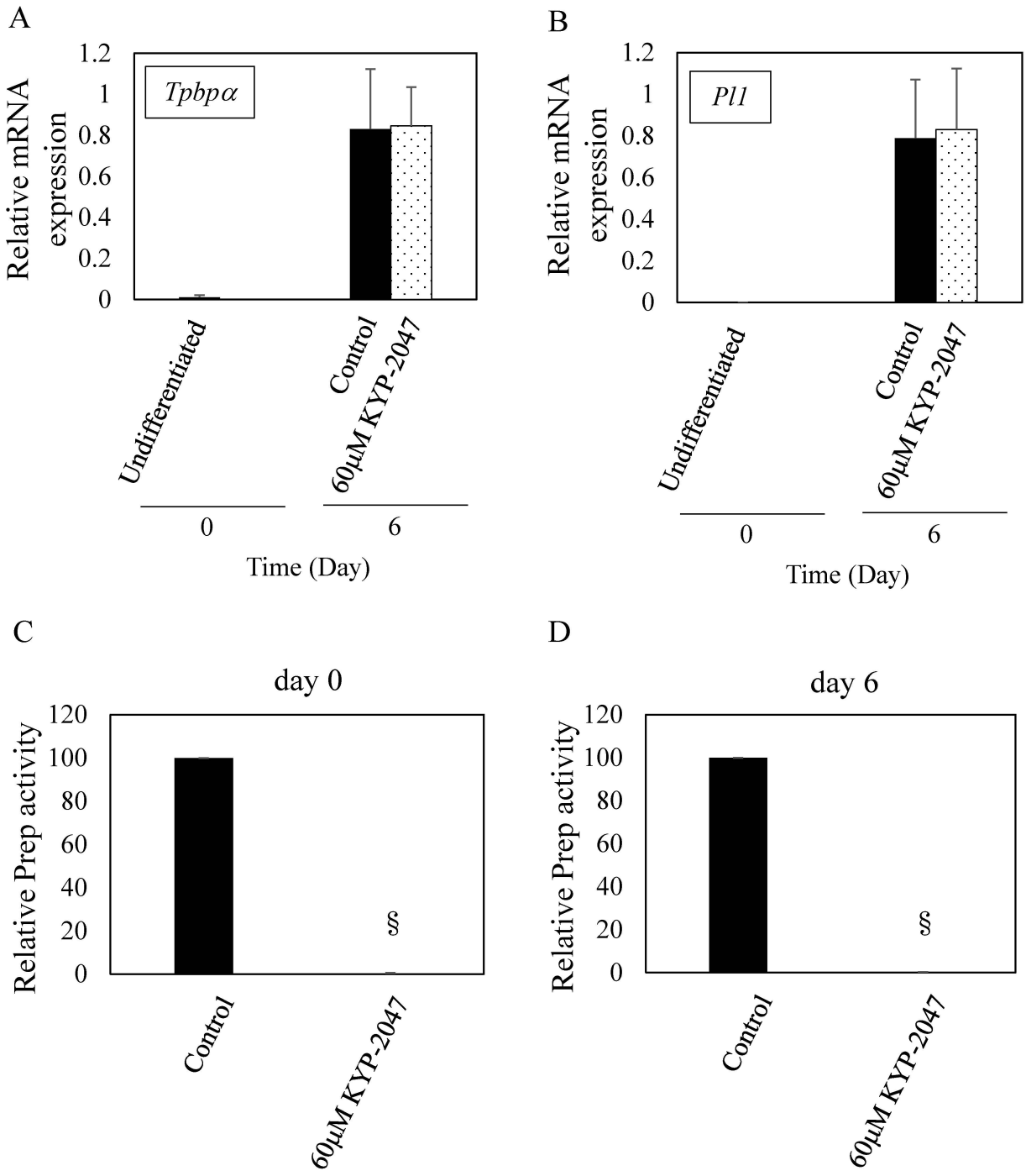

A
10μM
Absorbance at 550 nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
SUAM
Control
SUAM
4
6
Time (Day)
B
30μM
Absorbance at 550 nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
SUAM
Control
SUAM
4
6
Time (Day)

Fig. 6.


A
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
Tpbpα
Undifferentiated
Control
60μM KYP-2047
0
6
Time (Day)
B
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
Pl1
Undifferentiated
Control
60μM KYP-2047
0
6
Time (Day)
C
day 0
Relative Prep activity
120
100
80
60
40
20
0
§
Control
60μM KYP-2047
D
day 6
Relative Prep activity
120
100
80
60
40
20
0
§
Control
60μM KYP-2047