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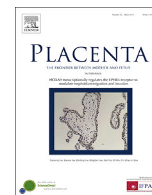

Mouse prolyl oligopeptidase plays a role in trophoblast stem cell differentiation into trophoblast giant cell and spongiotrophoblast

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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.
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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

Abbreviations: ANOVA, analysis of variance; 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.

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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
Gcm1	mouse	AACACCAACAACCACAACTCC	CAGCTTTTCCTCTGCTGCTT	NM_008103
Tpbpα	mouse	TGGATGCTGAACTGCAAGAG	TCCGTCTCCTGGTCATTTTC	NM_009411
Pl1	mouse	TTGGCCGCAGATGTGTATAG	TCGTGGACTTCCTCTCGATT	NM_008864
Aip	mouse	GAGGACGGGATCCAAAAGC	CTGTGCAGCGTCCGAAAGT	NM_016666

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 [10,22–27], 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 [28]. 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 [28] and maintained as reported [29]. 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 [30] (Peptide Institute, Osaka, Japan) or 4-phenylbutanoyl-l-prolyl-2(S)-cyanopyrrolidine (KYP-2047) [31] (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 [14,32]. 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.

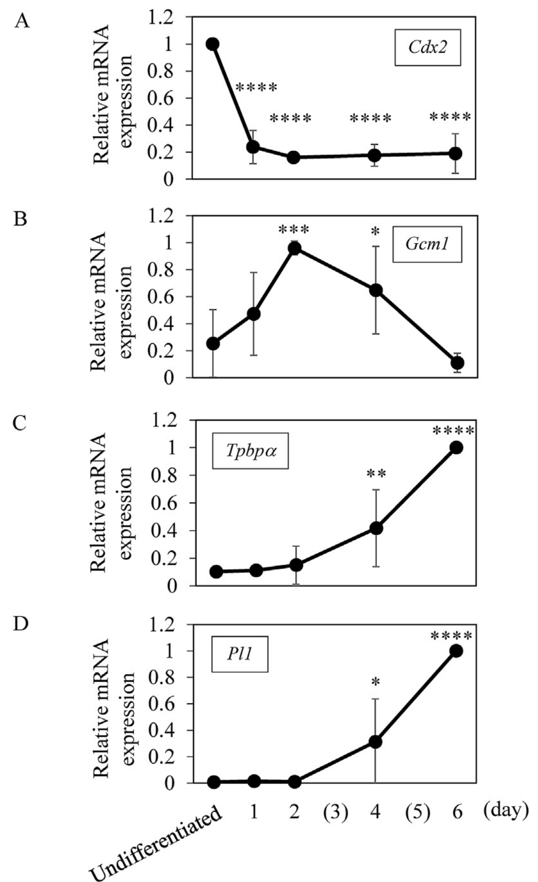

Fig. 1. The expression pattern of marker genes for each type of placental cell by qRT-PCR. qRT-PCR was performed with total RNAs prepared from TSCs at indicated days. The expression level was normalized with that of the *Aip* gene, and the highest value in each data set was set to 1.0. The graph shows 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: *Tpbpα* for SpT, and D: *Pl1* for TGC.

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 [33]. 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,

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