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1	A molecular mechanism of mouse placental spongiotrophoblast differentiation regulated by
2	prolyl oligopeptidase
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10	Running title: POP regulates Ascl2 in mouse TSC
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17 Summary

19	In eutherian mammals, the placenta plays critical roles in embryo development by supplying nutrients
20	and hormones and mediating the interaction with mother. To establish the fine connection between
21	mother and embryo, the placenta needs to be formed normally, but the mechanism of placental
22	differentiation is not fully understood. We previously revealed that mouse prolyl oligopeptidase (POP)
23	plays a role in trophoblast stem cell (TSC) differentiation into two placental cell types,
24	spongiotrophoblast (SpT) and trophoblast giant cell. Here, we focused on the SpT differentiation and
25	attempted to elucidate a molecular mechanism. Among Ascl2, Arnt, and Egfr genes that are
26	indispensable for SpT formation, we found that a POP-specific inhibitor, SUAM-14746, significantly
27	decreased the Ascl2 expression, which was consistent with a significant decrease in expression of Flt1,
28	a downstream gene of Ascl2. Although this downregulation was unlikely to be mediated by the PI3K-
29	Akt pathway, our results indicate that POP controls TSC differentiation into SpT via regulating the
30	Ascl2 gene.
31	
32	Keywords: prolyl oligopeptidase, SUAM-14746, trophoblast stem cell, spongiotrophoblast, Ascl2

35 Introduction

37	The placenta is an organ which mediates the communication between embryo and mother in
38	eutherian mammals, by producing and secreting hormones and supplying nutrients from mother
39	(Cross, 2006). For normal embryo development, the placental formation is critical, as many reports
40	showed that a placental defect led to embryonic lethality (Rielland et al., 2008; Rossant and Cross,
41	2001). However, the mechanism of placental differentiation is not fully understood.
42	In mouse, the placenta is composed of three layers: maternal decidua, junctional zone, and
43	labyrinthine layer (Watson and Cross, 2005). While these layers include various cell types, three cell
44	types have been well studied: trophoblast giant cell (TGC) and spongiotrophoblast (SpT) in the
45	junctional zone and syncytiotrophoblast (SynT) in the labyrinth. TGC and SpT are derived from
46	ectoplacental cone and formed in the placenta at around E7.5 and E9.5, respectively (Hu and Cross,
47	2010). The failure of their differentiation was repeatedly proved to be essential for embryo
48	development (Cross, 2005; Hu and Cross, 2010; Watson and Cross, 2005).
49	We recently reported that mouse prolyl oligopeptidase (POP, E.C.3.4.21.26, also known as prolyl
50	endopeptidase; gene symbol Prep), a multifunctional serine protease, contributes to differentiation of
51	TGC and SpT, using the trophoblast stem cell (TSC) system (Maruyama et al., 2017). TSCs are the
52	cells that can be easily differentiated into TGC, SpT, and SynT in vitro, and widely used in the study of
53	placental differentiation (Latos and Hemberger, 2014; Tanaka et al., 1998). By these data, we
54	suggested the importance of POP in placental differentiation, but the molecular mechanism was not
55	clear.
56	Here we attempted to elucidate the mechanism of TSC differentiation into SpT, by focusing on
57	three genes that showed abnormal SpT phenotypes in their knockout mice (Cross, 2005; Rossant and
58	Cross, 2001). A basic helix-loop-helix (bHLH) transcription factor, Achaete-scute family bHLH
59	transcription factor 2 (Ascl2, also known as Mash2), is a famous regulator of SpT formation, and the

60	knockout mice completely lack the SpT layer and the embryos die at around E10.5 (Guillemot et al.,
61	1994; Guillemot et al., 1995; Tanaka et al., 1997). Aryl hydrocarbon receptor nuclear translocator
62	(Arnt)-deficient mice show the reduction in SpT and labyrinthine layers, and the embryos die between
63	E9.5 and E10.5 (Adelman et al., 2000; Kozak et al., 1997). Epidermal growth factor receptor (Egfr)-
64	deficient mice also resulted in the reduced SpT layer and the embryonic lethality until E12.5, although
65	milder phenotypes were reported depending on the strain or genetic background (Miettinen et al.,
66	1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). These indicate that these three genes may
67	participate in SpT differentiation, which in turn suggests that POP regulates the TSC differentiation via
68	controlling any of these genes.
69	In this study, we tested the possibility that POP regulated the TSC differentiation via controlling
70	any of Ascl2, Arnt, and Egfr genes by using a POP-specific inhibitor, 3-({4-[2-(E)-Styrylphenoxy]
71	butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) (Saito et al., 1991) in the TSC culture
72	system.
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74	
75	Materials and methods
76	
77	TSC culture, induction of differentiation, and treatment with inhibitors
78	TSCs were kindly provided by Dr. Satoshi Tanaka (Tanaka et al., 1998) and maintained as
79	previously reported (Himeno et al., 2008). The TSC differentiation was performed as previously
80	described (Maruyama et al., 2017). To induce the differentiation, 2×10^5 TSCs were seeded on a 35-mm
81	dish, and on the next day, fibroblast growth factor 4 (FGF4) was removed to start the differentiation. A
82	POP-specific inhibitor, SUAM-14746 (Peptide Institute, Osaka, Japan), or a PI3K-specific inhibitor,
83	LY294002 (Wako Pure Chemical, Osaka, Japan), was added on the removal of FGF4. An equal
84	volume of dimethyl sulfoxide (DMSO) was used as a control.

86	Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis
87	Total RNAs were prepared from the cultured cells at day 2 and 6, and from undifferentiated TSCs
88	as the cells at day 0. After treatment with TurboDNase (Thermo Fisher Scientific, Walthum, USA),
89	cDNA was synthesized by reverse transcription with the oligo(dT) primer and 500 ng of each RNA,
90	and quantitative PCR was performed using the ABI Prism 7300 real-time PCR system (Matsubara et
91	al., 2010). All the data were normalized to Aryl hydrocarbon receptor interacting protein (Aip) and the
92	highest value was set to 1.0 in each experiment. Primer sequences are shown in Table 1.
93	
94	Statistical analysis
95	Results were represented as the average \pm standard deviation (SD), and their statistical
96	significance was analyzed by Student's t test or one-way analysis of variance (ANOVA) followed by
97	Tukey's post hoc test or Dunnett's test. P value less than 0.05 was considered statistically significant.
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99	
100	Results
101	
102	We first examined the expression patterns of Ascl2, Arnt, and Egfr during normal TSC
103	differentiation. By qRT-PCR, Ascl2 mRNA was significantly increased at day2 and decreased at day 6
104	(Fig. 1A), while mRNA levels of the other two genes gradually increased to reach a peak at day 6 (Fig.
105	1D and F). The pattern of Ascl2 gene was in agreement with previous reports (Hughes et al., 2004;
106	Tanaka et al., 1998). Then, we treated TSCs with a POP-specific inhibitor, SUAM-14746, at 10 μ M
107	and 30 μ M because these concentrations were effective on TSC differentiation in our previous study
108	(Maruyama et al., 2017). Compared to the control experiment with DMSO, Ascl2 expression was
109	significantly reduced by both concentrations of SUAM-14746 at day 2 (Fig. 1B and C). In contrast, the

110	inhibitor did not change the expression of Arnt and Egfr (Fig. 1E and G).
111	To further confirm the result, we checked an effect of SUAM-14746 on the expression of <i>Flt1</i> , a
112	downstream gene of Ascl2 (El-Hashash et al., 2010; Guillemot et al., 1994). Flt1 expression was
113	increased during the normal differentiation to become the highest at day 6, and by the addition of 10
114	μ M and 30 μ M SUAM-14746, it significantly reduced at day 6 compared to the control (Fig. 2A and
115	B). These results indicate that POP is involved in regulation of the Ascl2 gene during mouse TSC
116	differentiation into SpT.
117	We next attempted to reveal the signaling pathway POP regulated. Because POP is involved in the
118	regulation of PI3K-Akt signaling pathway which controls the Ascl2 gene expression (Duan et al.,
119	2014; Takao et al., 2012), we investigated whether this pathway was related to SpT differentiation
120	regulated by POP. We added a PI3K-specific inhibitor, LY294002, to TSC culture system at 10 μ M,
121	and checked the expression of Ascl2 and Tpbpa. However, qRT-PCR showed no significant difference
122	in their expression between TSCs treated with DMSO and LY294002 (Fig. 3A and B). These suggested
123	that the regulation of Ascl2 expression by POP was not through the PI3K-Akt pathway.
124	
125	
126	Discussion
127	
128	Our present data clearly showed the downregulation of Ascl2 by adding a POP-specific inhibitor.
129	The Ascl2 gene encodes a transcription factor which is critical for SpT formation (Guillemot et al.,
130	1994; Guillemot et al., 1995; Johnson et al., 1992). Flt1 is one of its downstream genes and was
131	downregulated by the inhibitor, although this gene itself is dispensable for placental formation
132	(Hirashima et al., 2003). Moreover, another downstream gene, $Tpbpa$, which is unrelated to $Flt1$ and
133	essential for vascularization of the placenta (Hu and Cross, 2011), was also significantly decreased by
134	the same inhibitor (Maruyama et al., 2017). These indicate that POP is directly or indirectly involved

in regulation of the pathway originated from *Ascl2* leading to SpT differentiation in the placenta.
However, it is also possible that POP controls glycogen trophoblast differentiation because all of *Ascl2*, *Flt1*, and *Tpbpa* genes are expressed in both SpT and glycogen trophoblast. We can not
completely rule out the possibility, but a low level of POP expression in glycogen trophoblast is not
necessarily consistent with the hypothesis (Matsubara et al., 2011). Thus, we strongly suggest that
POP regulates the *Ascl2* gene pathway and thereby the SpT differentiation.

141 POP was reported to be localized in nuclei of some cell types (Ishino et al., 1998; Ohtsuki et al., 142 1994; Venditti and Minucci, 2018), but cytoplasmic POP is more likely to be involved in regulation of 143 the Ascl2 gene because it is predominantly present in cytoplasm of TSCs (Maruyama et al., 2017). 144 Cytoplasmic POP is not only a protease that digests -Pro-X- peptide bonds in bioactive peptides but 145 also functions as an interacting protein regardless of its enzyme activity (García-Horsman et al., 2007; 146 Myöhänen et al., 2009), and there are some possible pathways from POP to Ascl2. In cancerous cells, 147 the Ascl2 gene is controlled by Wnt/β-catenin and PI3K-Akt pathways (Jubb et al., 2006; Takao et al., 148 2012; Zhou et al., 2015), and the relation of ERK pathway was also pointed out (Sharma et al., 2016). Here, we found that the PI3k-Akt pathway was unlikely to be related, so POP may be involved in 149 150 other pathways. In any case, POP probably controls Ascl2 through changing the phosphorylation status 151 of proteins in these pathways since it was reported to regulate the protein phosphorylation (Duan et al., 152 2014; Williams et al., 1999). Moreover, it is interesting that Ascl2 is activated by a placenta-specific transcription factor, AP-2y (Sharma et al., 2016), and POP is also suggested to be controlled by AP-2y 153 154 (Matsubara et al., 2013). The SpT differentiation may be regulated by an AP- 2γ -POP-Ascl2 pathway. 155 In conclusion, the addition of SUAM-14746 significantly decreased the expression of Ascl2 and 156 its related genes in mouse TSCs, which suggests that POP plays a role in SpT differentiation via 157 regulating the Ascl2 gene. Some strains of POP-deficient mice showed embryonic lethality and growth 158 retardation on IMPC (http://www.mousephenotype.org/) and KOMP (https://www.komp.org/) databases, possibly due to the failure of SpT formation and abnormal communication between mother 159

160	and embryo.
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169	Conflicts of Interest
170	
171	The authors certify that they have nothing to disclose.
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- 275
- 276
- 277 Figure legends
- 278

- 280 relative expression of (A) Ascl2, (D) Arnt, and (F) Egfr during normal TSC differentiation is shown as
- 281 mean \pm SD, and the data were analyzed by one-way ANOVA followed by Tukey's post hoc test. **P* <
- 282 0.05, **P < 0.01 compared to day 0. n=3. To investigate the effects of a POP-specific inhibitor, we
- added 10 or 30 μ M SUAM-14746 or DMSO (control) to TSCs. Total RNAs were isolated from the
- cells at day 0, 2, and 6, and the expression of (B, C) Ascl2, (E) Arnt and (G) Egfr was analyzed by

Figure 1 The effects of SUAM-14746 on expression patterns of Ascl2, Arnt, and Egfr genes. The

285 qRT-PCR. These data are presented as mean \pm SD, and were analyzed by one-way ANOVA followed 286 by Dunnett's test. **P < 0.01 compared to the cells with DMSO. n=3.

287

288	Figure 2 The effect of SUAM-14746 on <i>Flt1</i> expression. The relative expression of <i>Flt1</i> during
289	normal TSC differentiation (A) and in TSCs cultured with 10 or 30 μ M SUAM-14746 or DMSO
290	(control) (B) was analyzed by qRT-PCR. Both concentrations of SUAM-14746 significantly reduced
291	the expression of <i>Flt1</i> at day 6. The data are presented as mean \pm SD, and were analyzed by one-way
292	ANOVA followed by (A) Tukey's post hoc test or (B) Dunnett's test. $**P < 0.01$ compared to (A) day 0
293	or (B) the cells with DMSO. $n=3$.
294	
295	Figure 3 The effect of LY294002 on expression of SpT marker genes. (A) Ascl2 and (B)
296	<i>Tpbpa</i> expression in TSCs supplemented with 10 μ M LY294002 or DMSO (control) at day 2 and 6
297	and in undifferentiated cells (day 0) was analyzed by qRT-PCR. 10 μ M LY294002 did not
298	significantly affect the expression of SpT marker genes. n=5.
299	

Figure 1



day6





Figure 3



0.6

0.4

0.2

0

undiff

day2

day6

Table 1 Primers us	ed in this study
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Gene	Species	Forward primer	Reverse primer	Refseq ID
Ascl2	mouse	TCTCTCGGACCCTCTCTCAG	GGACCCCGTACCAGTCAAG	NM_008554
Arnt	mouse	GGCGGCGACTACAGCTAAC	CAGCTCCTCCACCTTGAATC	NM_009709
Egfr	mouse	TGTGGGCCTGACTACTACGA	CACCAATGCCTATGCCATTAC	NM_207655
Flt1	mouse	GGATGCAGGGGACTATACGA	AAGCGAGGACACGGACTTT	NM_010228
Tpbpa	mouse	TGGATGCTGAACTGCAAGAG	TCCGTCTCCTGGTCATTTTC	NM_009411
Aip	mouse	GAGGACGGGATCCAAAAGC	CTGTGCAGCGTCCGAAAGT	NM_016666