A molecular mechanism of mouse placental spongiotrophoblast differentiation regulated by prolyl oligopeptidase

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Running title: POP regulates *Ascl2* in mouse TSC

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Summary

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

Keywords: prolyl oligopeptidase, SUAM-14746, trophoblast stem cell, spongiotrophoblast, *Ascl2*

Introduction

The placenta is an organ which mediates the communication between embryo and mother in eutherian mammals, by producing and secreting hormones and supplying nutrients from mother (Cross, 2006). For normal embryo development, the placental formation is critical, as many reports showed that a placental defect led to embryonic lethality (Rielland et al., 2008; Rossant and Cross, 2001). However, the mechanism of placental differentiation is not fully understood.

In mouse, the placenta is composed of three layers: maternal decidua, junctional zone, and labyrinthine layer (Watson and Cross, 2005). While these layers include various cell types, three cell types have been well studied: trophoblast giant cell (TGC) and spongiotrophoblast (SpT) in the junctional zone and syncytiotrophoblast (SynT) in the labyrinth. TGC and SpT are derived from ectoplacental cone and formed in the placenta at around E7.5 and E9.5, respectively (Hu and Cross, 2010). The failure of their differentiation was repeatedly proved to be essential for embryo development (Cross, 2005; Hu and Cross, 2010; Watson and Cross, 2005).

We recently reported that mouse prolyl oligopeptidase (POP, E.C.3.4.21.26, also known as prolyl endopeptidase; gene symbol *Prep*), a multifunctional serine protease, contributes to differentiation of TGC and SpT, using the trophoblast stem cell (TSC) system (Maruyama et al., 2017). TSCs are the cells that can be easily differentiated into TGC, SpT, and SynT *in vitro*, and widely used in the study of placental differentiation (Latos and Hemberger, 2014; Tanaka et al., 1998). By these data, we suggested the importance of POP in placental differentiation, but the molecular mechanism was not clear.

Here we attempted to elucidate the mechanism of TSC differentiation into SpT, by focusing on three genes that showed abnormal SpT phenotypes in their knockout mice (Cross, 2005; Rossant and Cross, 2001). A basic helix-loop-helix (bHLH) transcription factor, *Achaete-scute family bHLH transcription factor 2* (*Ascl2*, also known as *Mash2*), is a famous regulator of SpT formation, and the

knockout mice completely lack the SpT layer and the embryos die at around E10.5 (Guillemot et al., 1994; Guillemot et al., 1995; Tanaka et al., 1997). *Aryl hydrocarbon receptor nuclear translocator* (*Arnt*)-deficient mice show the reduction in SpT and labyrinthine layers, and the embryos die between E9.5 and E10.5 (Adelman et al., 2000; Kozak et al., 1997). *Epidermal growth factor receptor* (*Egfr*)-deficient mice also resulted in the reduced SpT layer and the embryonic lethality until E12.5, although milder phenotypes were reported depending on the strain or genetic background (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). These indicate that these three genes may participate in SpT differentiation, which in turn suggests that POP regulates the TSC differentiation via controlling any of these genes.

In this study, we tested the possibility that POP regulated the TSC differentiation via controlling any of *Ascl2*, *Arnt*, and *Egfr* genes by using a POP-specific inhibitor, 3-({4-[2-(E)-Styrylphenoxy] butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) (Saito et al., 1991) in the TSC culture system.

Materials and methods

TSC culture, induction of differentiation, and treatment with inhibitors

TSCs were kindly provided by Dr. Satoshi Tanaka (Tanaka et al., 1998) and maintained as previously reported (Himeno et al., 2008). The TSC differentiation was performed as previously described (Maruyama et al., 2017). To induce the differentiation, 2×10^5 TSCs were seeded on a 35-mm dish, and on the next day, fibroblast growth factor 4 (FGF4) was removed to start the differentiation. A POP-specific inhibitor, SUAM-14746 (Peptide Institute, Osaka, Japan), or a PI3K-specific inhibitor, LY294002 (Wako Pure Chemical, Osaka, Japan), was added on the removal of FGF4. An equal volume of dimethyl sulfoxide (DMSO) was used as a control.

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

Total RNAs were prepared from the cultured cells at day 2 and 6, and from undifferentiated TSCs as the cells at day 0. After treatment with TurboDNase (Thermo Fisher Scientific, Walthum, USA), cDNA was synthesized by reverse transcription with the oligo(dT) primer and 500 ng of each RNA, and quantitative PCR was performed using the ABI Prism 7300 real-time PCR system (Matsubara et al., 2010). All the data were normalized to *Aryl hydrocarbon receptor interacting protein* (*Aip*) and the highest value was set to 1.0 in each experiment. Primer sequences are shown in Table 1.

Statistical analysis

Results were represented as the average ± standard deviation (SD), and their statistical significance was analyzed by Student's *t* test or 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.

Results

We first examined the expression patterns of *Ascl2*, *Arnt*, and *Egfr* during normal TSC differentiation. By qRT-PCR, *Ascl2* mRNA was significantly increased at day2 and decreased at day 6 (Fig. 1*A*), while mRNA levels of the other two genes gradually increased to reach a peak at day 6 (Fig. 1*D* and *F*). The pattern of *Ascl2* gene was in agreement with previous reports (Hughes et al., 2004; Tanaka et al., 1998). Then, we treated TSCs with a POP-specific inhibitor, SUAM-14746, at 10 μM and 30 μM because these concentrations were effective on TSC differentiation in our previous study (Maruyama et al., 2017). Compared to the control experiment with DMSO, *Ascl2* expression was significantly reduced by both concentrations of SUAM-14746 at day 2 (Fig. 1*B* and *C*). In contrast, the

inhibitor did not change the expression of *Arnt* and *Egfr* (Fig. 1*E* and *G*).

To further confirm the result, we checked an effect of SUAM-14746 on the expression of *Flt1*, a downstream gene of *Ascl2* (El-Hashash et al., 2010; Guillemot et al., 1994). *Flt1* expression was increased during the normal differentiation to become the highest at day 6, and by the addition of 10 μM and 30 μM SUAM-14746, it significantly reduced at day 6 compared to the control (Fig. 2*A* and *B*). These results indicate that POP is involved in regulation of the *Ascl2* gene during mouse TSC differentiation into SpT.

We next attempted to reveal the signaling pathway POP regulated. Because POP is involved in the regulation of PI3K-Akt signaling pathway which controls the *Ascl2* gene expression (Duan et al., 2014; Takao et al., 2012), we investigated whether this pathway was related to SpT differentiation regulated by POP. We added a PI3K-specific inhibitor, LY294002, to TSC culture system at 10 μM, and checked the expression of *Ascl2* and *Tpbpα*. However, qRT-PCR showed no significant difference in their expression between TSCs treated with DMSO and LY294002 (Fig. 3*A* and *B*). These suggested that the regulation of *Ascl2* expression by POP was not through the PI3K-Akt pathway.

Discussion

Our present data clearly showed the downregulation of *Ascl2* by adding a POP-specific inhibitor. The *Ascl2* gene encodes a transcription factor which is critical for SpT formation (Guillemot et al., 1994; Guillemot et al., 1995; Johnson et al., 1992). *Flt1* is one of its downstream genes and was downregulated by the inhibitor, although this gene itself is dispensable for placental formation (Hirashima et al., 2003). Moreover, another downstream gene, *Tpbpα*, which is unrelated to *Flt1* and essential for vascularization of the placenta (Hu and Cross, 2011), was also significantly decreased by 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 *Tpbpα* 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.

POP was reported to be localized in nuclei of some cell types (Ishino et al., 1998; Ohtsuki et al., 1994; Venditti and Minucci, 2018), but cytoplasmic POP is more likely to be involved in regulation of the *Ascl2* gene because it is predominantly present in cytoplasm of TSCs (Maruyama et al., 2017). Cytoplasmic POP is not only a protease that digests -Pro-X- peptide bonds in bioactive peptides but also functions as an interacting protein regardless of its enzyme activity (García-Horsman et al., 2007; Myöhänen et al., 2009), and there are some possible pathways from POP to *Ascl2*. In cancerous cells, the *Ascl2* gene is controlled by Wnt/β-catenin and PI3K-Akt pathways (Jubb et al., 2006; Takao et al., 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 other pathways. In any case, POP probably controls *Ascl2* through changing the phosphorylation status of proteins in these pathways since it was reported to regulate the protein phosphorylation (Duan et al., 2014; Williams et al., 1999). Moreover, it is interesting that *Ascl2* is activated by a placenta-specific transcription factor, AP-2γ (Sharma et al., 2016), and POP is also suggested to be controlled by AP-2γ (Matsubara et al., 2013). The SpT differentiation may be regulated by an AP-2γ-POP-Ascl2 pathway.

In conclusion, the addition of SUAM-14746 significantly decreased the expression of *Ascl2* and its related genes in mouse TSCs, which suggests that POP plays a role in SpT differentiation via regulating the *Ascl2* gene. Some strains of POP-deficient mice showed embryonic lethality and growth 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

and embryo.

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Conflicts of Interest

The authors certify that they have nothing to disclose.

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Figure legends

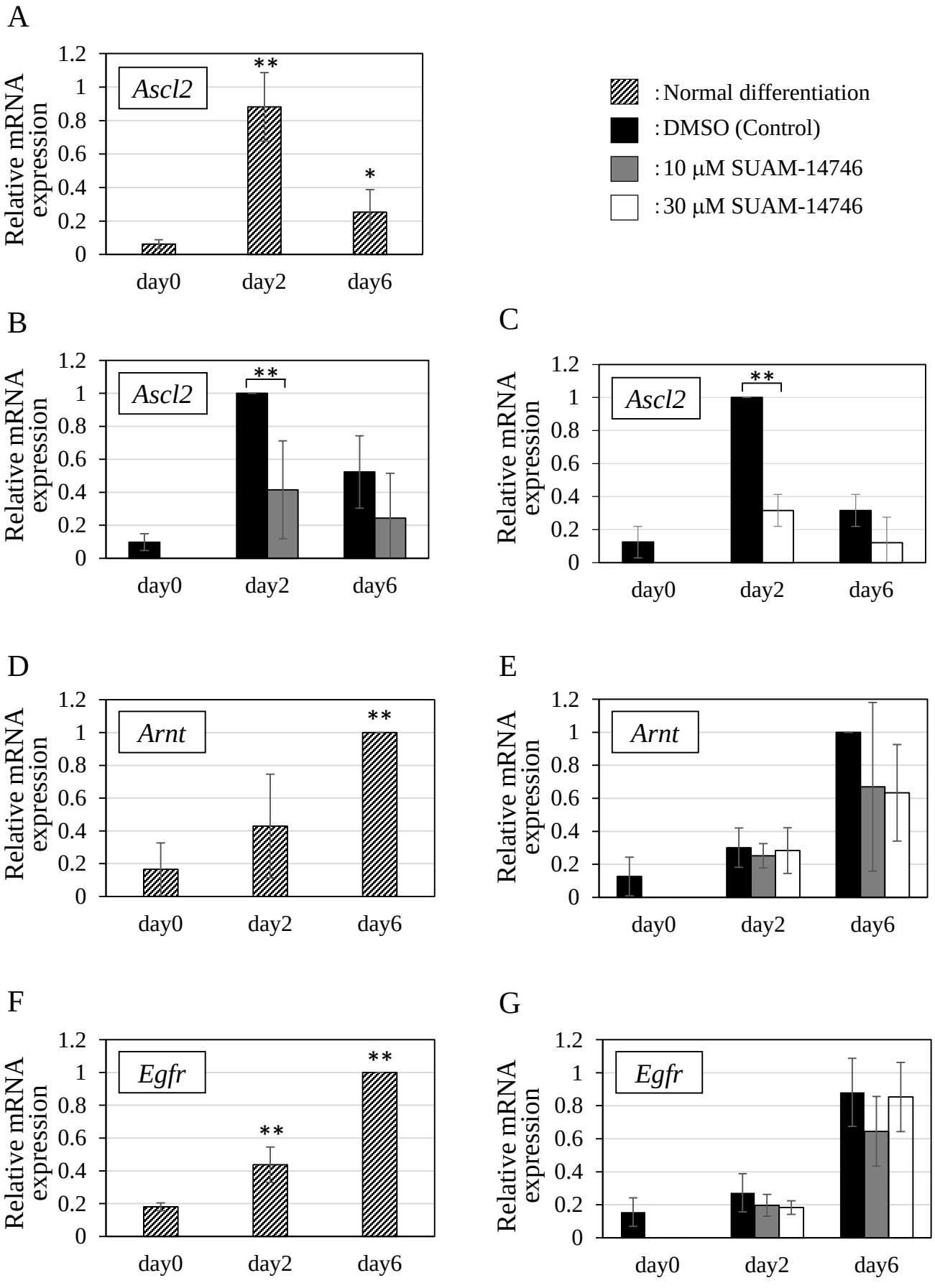
Figure 1 The effects of SUAM-14746 on expression patterns of *Ascl2*, *Arnt*, and *Egfr* genes. The relative expression of (*A*) *Ascl2*, (*D*) *Arnt*, and (*F*) *Egfr* during normal TSC differentiation is shown as mean ± SD, and the data were analyzed by one-way ANOVA followed by Tukey's post hoc test. **P* < 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 μM 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

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

Figure 2 The effect of SUAM-14746 on *Flt1* expression. The relative expression of *Flt1* during normal TSC differentiation (*A*) and in TSCs cultured with 10 or 30 μM SUAM-14746 or DMSO (control) (*B*) was analyzed by qRT-PCR. Both concentrations of SUAM-14746 significantly reduced the expression of *Flt1* at day 6. The data are presented as mean ± SD, and were analyzed by one-way ANOVA followed by (*A*) Tukey's post hoc test or (*B*) Dunnett's test. $^{**}P < 0.01$ compared to (*A*) day 0 or (*B*) the cells with DMSO. n=3.

Figure 3 The effect of LY294002 on expression of SpT marker genes. (*A*) *Ascl2* and (*B*) *Tpbpα* expression in TSCs supplemented with 10 μM LY294002 or DMSO (control) at day 2 and 6 and in undifferentiated cells (day 0) was analyzed by qRT-PCR. 10 μM LY294002 did not significantly affect the expression of SpT marker genes. n=5.

Figure 1


A
B
C
D
E
F
G
Ascl2
Arnt
Egfr
Relative mRNA expression
1.2
1
0.8
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day0
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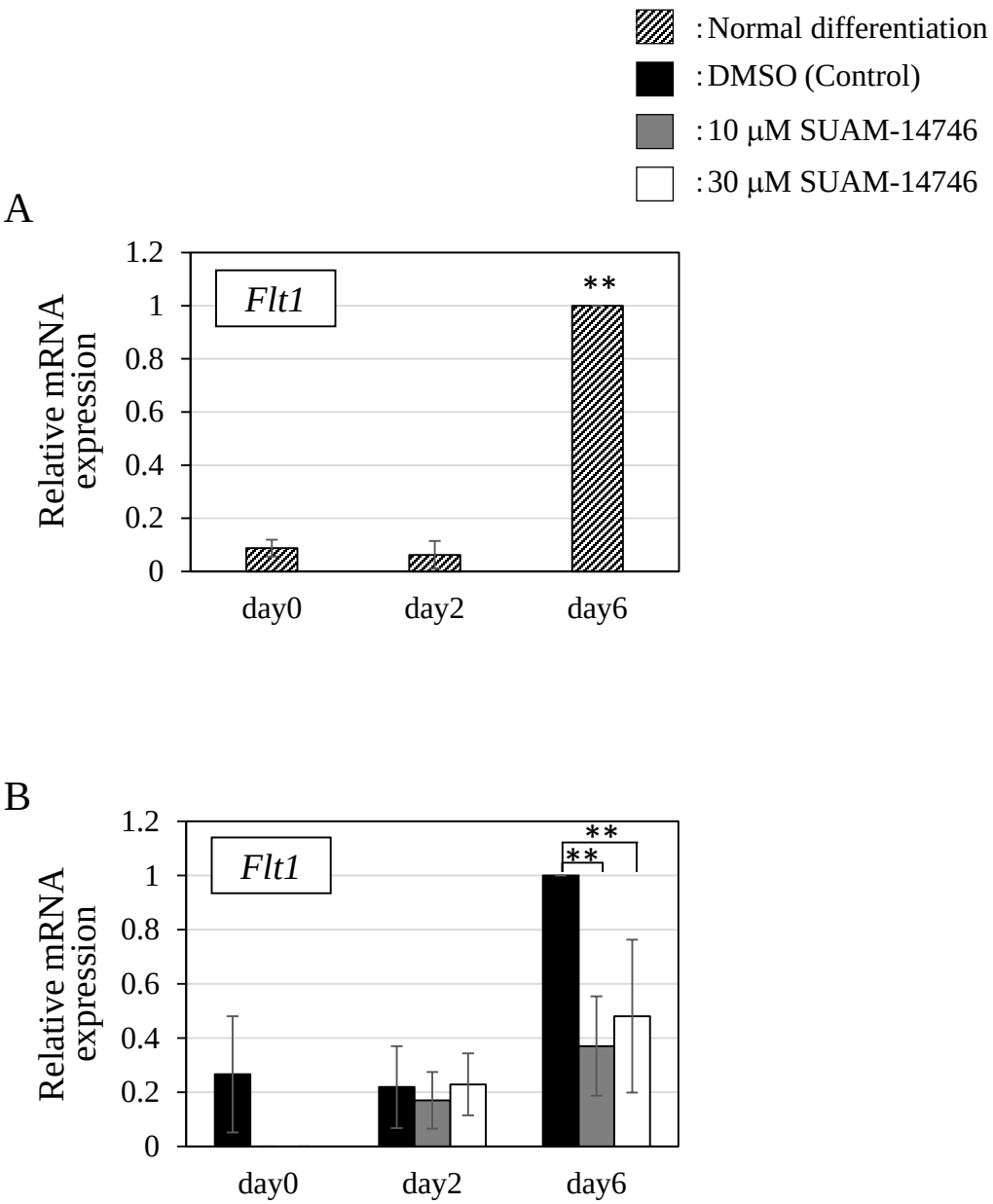
*
: Normal differentiation
: DMSO (Control)
: 10 μM SUAM-14746
: 30 μM SUAM-14746

Figure 2


: Normal differentiation
: DMSO (Control)
: 10 μM SUAM-14746
: 30 μM SUAM-14746
A
Flt1
Relative mRNA expression
1.2
1
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0.4
0.2
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day2
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B
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Relative mRNA expression
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day2
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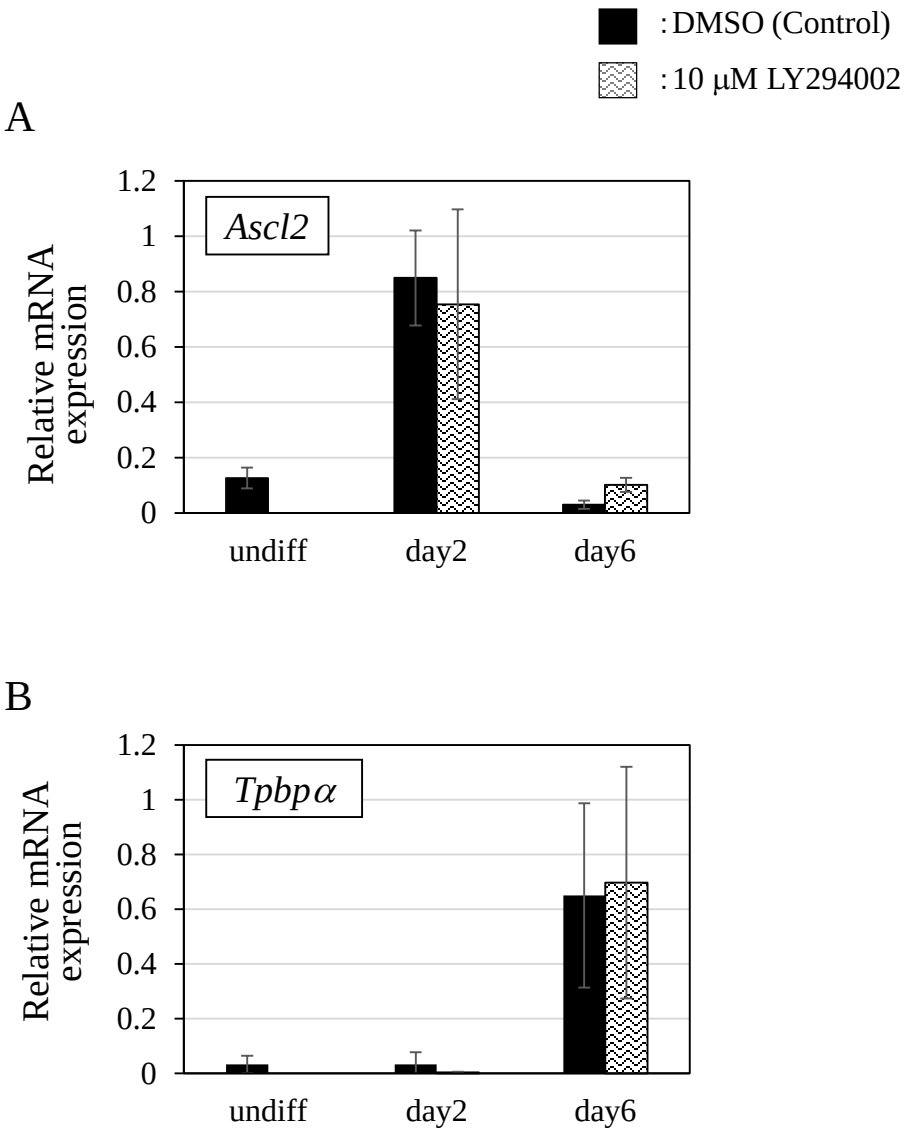
Figure 3


: DMSO (Control)
: 10 μM LY294002
A
Ascl2
Relative mRNA expression
1.2
1
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B
Tpbpα
Relative mRNA expression
1.2
1
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Table 1 Primers used in this study

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