Studies on the role of prolyl oligopeptidase in mouse trophoblast stem cell differentiation

(マウス胎盤幹細胞分化における
プロリルオリゴペプチダーゼ機能に関する研究)

A DISSERTATION

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CONTENTS

ABBREVIATIONS

α-MSH, α-melanocyte stimulating hormone

ANOVA, analysis of variance

bHLH, basic helix-loop-helix

CaMKI, calcium/calmodulin-dependent protein kinase I

cAMP, cyclic adenosine monophosphate

CBB, coomassie brilliant blue

Cd276, cluster of differentiation 276

Cdx2, caudal type homeobox

Cebpα, CCAAT/enhancer binding protein alpha

DMSO, dimethyl sulfoxide

E, embryonic day

EDTA, ethylenediamine tetraacetic acid

EPC, ectoplacental cone

ExE, extraembryonic ectoderm

FGF4, fibroblast growth factor 4

GAP43, growth associated protein 43

Gapdh, glyceraldehyde-3-phosphate dehydrogenase

Gcm1, glial cells missing homolog 1

Glis3, glis family zinc 3

Hand1, heart and neural crest derivatives-expressed protein 1

ICM, inner cell mass

IP_3, inositol trisphosphate

Kiss1, kiss-1 metastasis-suppressor

KYP-2047, 4-phenylbutanoyl-L-prolyl-2(S)-cyanopyrrolidine

LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride

Mash2, mammalian achaete-scute family bHLH transcription factor 2

MCA, methyl-coumaryl-7-amide

MMC-MEF, mitomycin C-treated mouse embryonic fibroblast

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PAGE, poly acrylamide gel electrophoresis

PBS, phosphate buffered saline

PI3K, phosphoinositide 3-kinase

Pl1, placental lactogen 1 (prolactin family 3, subfamily d, member 1)

POP, prolyl oligopeptidase

PPARγ, peroxisome proliferator-activated receptor γ

qRT-PCR, quantitative reverse transcription-polymerase chain reaction

S.D., standard deviation

SDS, sodium dodecyl sulfate

SpT, spongiotrophoblast

SUAM-14746, 3-({4-[2-(E)-styrylphenoxy] butanoyl}-L-4-hydroxyprolyl)-thiazolidine

SynT, syncytiotrophoblast

TGC, trophoblast giant cell

TSC, trophoblast stem cell

GENERAL INTRODUCTION

Prolyl oligopeptidase (POP, E.C.3.4.21.26, prolyl endopeptidase, post proline cleaving enzyme; gene symbol *Prep*) is a serine endopeptidase which has unique enzymatic activity and various physiological roles. After its discovery as an oxytocin-cleaving enzyme in human uterus (Walter et al. 1971), the POP protein was purified and characterized in many mammalian tissues such as lamb kidney (Koida & Walter 1976), rabbit brain (Orlowski et al. 1979), rat brain (Kato et al. 1980), bovine brain (Tate 1981) and porcine muscle (Moriyama et al. 1988). POP was also found and purified in non-mammalian species, bacteria (Yoshimoto et al. 1980), archaea (Harwood et al. 1997), fungi (Sattar et al. 1990) and plants (Yoshimoto et al. 1987a), so it is now recognized that most tissues in most species express POP although some exceptions were reported (Myöhänen et al. 2008b, Schulz et al. 2005). As results of these initial characterization of the POP protein, POP's biochemical aspects were revealed. For example, the molecular mass and isoelectric point are around 70,000 and 5.0 respectively, and as an enzyme, POP preferentially digests a peptide bond at the carboxyl side of a proline residue in a peptide smaller than 30 amino acids (Moriyama et al. 1988). POP is mainly localized in cytoplasm (Dresdner et al. 1982) but present also in nucleus (Moreno-Baylach et al. 2008, Ohtsuki et al. 1994), bound to membrane, and even secreted from some types of cells or tissues (Kimura et al. 1998, Soeda et al. 1984, Tenorio-Laranga et al. 2008). In 1998, the three dimensional structure of the porcine muscle POP protein was reported for the first time by X-ray crystal structure analysis. POP is composed of two domains: a peptidase domain with α/β-hydrolase fold and a β-propeller domain in which seven β-sheet blades formed a cylindrical structure (Fülöp et al. 1998). The specificity of POP to small peptides is due to the β-propeller domain ensuring an access to the active site for short peptides but not for proteins.

In 1991, cDNA cloning was successful for the first time in porcine brain (Rennex et al.

1991) and *Flavobacterium* (Yoshimoto et al. 1991), and since then, the primary structure of POP cDNA had been determined in various tissues from many species, including bacteria (Chevallier et al. 1992), human T cells (Shirasawa et al. 1994), bovine brain (Yoshimoto et al. 1997) and mouse Swiss 3T3 cells (Ishino et al. 1998). From these study, it has been revealed that mammalian POP mRNA is about 3 kb long and encodes a protein composed of 710 amino acids containing the catalytic triad of a typical serine protease (Ser554, Asp641 and His680). In addition, the amount of POP mRNA is different among the species. For example, high POP expression is reported in the porcine heart and muscle (Rennex et al. 1991), the human skeletal muscle (Goossens et al. 1997, Shirasawa et al. 1994), and the rat thymus (Kimura & Takahashi 2000). In 1999, the genome structure of the mouse POP gene was reported: The gene spans a 92-kb genomic region on chromosome 10B2-B3, including 15 exons and a TATA-less, GC-rich promoter (Kimura et al. 1999). By these data, genetic analyses were made possible to investigate the function of POP.

Physiological roles of POP have been proposed or determined by its expression and localization patterns, experiments with POP-specific inhibitors, and generation and analyses of POP-deficient mice. Initially, the expression pattern was important information to predict the function of POP. During follicular development in rodent ovaries, high levels of POP expression was observed at earlier stages, suggesting that POP is involved in the control of folliculogenesis (Kimura & Takahashi 2000, Kimura et al. 1998). The enzymatic activity is increased during postnatal liver development from day 0 to day 8 in rat, suggesting its role in liver development (Matsubara et al. 1998). The activity is decreased in human cerebellar granule cells as they proliferated, implying a role in cell differentiation in the brain (Moreno-Baylach et al. 2008). The changes in the POP mRNA distribution in the mouse testis during sexual maturation indicate the possibility that POP is implicated in the regulation of

male meiosis (Kimura et al. 2002). By these reports, the importance of POP has been recognized in reproduction, development and differentiation.

Many chemical compounds that specifically inhibit POP enzymatic activity have been synthesized by academia and industrial groups and used for functional analyses of this protease. The most famous experiments were performed to investigate its role in the brain; Z-Pro-Prolinal and JTP-4819 recovered learning and memory in scopolamine-induced amnesia model rats (Toide et al. 1995, Yoshimoto et al. 1987b) and rats with forebrain lesion (Shishido et al. 1998). In cultured cells, POP is localized in nuclei and the treatment with an inhibitor, ZTTA, inhibits DNA synthesis and imaginal disc differentiation in mouse and fly (Ishino et al. 1998, Ohtsuki et al. 1994, Ohtuski et al. 1997). Moreover, Z-Pro-Prolinal attenuates sperm motility in mouse and herring testes (Kimura et al. 2002, Yoshida et al. 1999), and prevents translocation of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and formation of reactive oxygen species (Puttonen et al. 2006). These findings indicate the involvement of POP in the regulation of sperm motility and in the stress response. Another famous POP-specific inhibitor, 4-phenylbutanoyl-L-prolyl-2(S)-cyanopyrrolidine (KYP-2047), increases concentrations of dopamine and decreases dopamine transporter immunoreactivity in the striatal tissue, suggesting a role in regulating the nigrostriatal dopaminergic system (Jalkanen et al. 2014). The same inhibitor also decreases the extracellular acetylcholine level in rat striatum (Jalkanen et al. 2014). 3-({4-[2-(E)-styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) suppresses the proliferation of NB-1 human neuroblastoma cells and increases the cell population at the G0/G1 phase (Sakaguchi et al. 2011). It is therefore likely that POP is a multifunctional molecule which plays important physiological roles in various cells and tissues.

Several POP-deficient mice were established, and exhibited various phenotypes especially in two strains. The most famous POP knockdown mouse was generated by the gene trap method which trapped the 2nd intron of the mouse POP gene. It showed parent of origin dependent obesity caused by the defect in the processing of α-melanocyte stimulating hormone (α-MSH) (Perroud et al. 2009, Warden et al. 2009), a decrease of synaptic spine density in hippocampus CA1 region (D'Agostino et al. 2013), the disturbance in secretion levels of insulin and glucagon (Kim et al. 2014), and a decrease of testis size and sperm motility (Dotolo et al. 2016). The other POP knockout mouse was generated by homologous recombination to delete the 3rd exon, and showed the defect in growth cone dynamics of neurons (Di Daniel et al. 2009) and extraordinary synaptic plasticity (Höfling et al. 2016).

Genetic mutation in the POP gene was also reported in *Dictyostelium* and caused the abnormal accumulation of inositol trisphosphate (IP_3), suggesting its involvement in cell signaling (Williams et al. 1999). Therefore, POP is now considered to participate in many physiological events and may have unknown functions in different tissues from different species.

The regulatory mechanism of POP gene expression is important because aberrant expression of POP was reported to be the potential cause of some cancer types and neurodegenerative diseases such as Alzheimer's disease and bipolar disorder (García-Horsman et al. 2007, Goossens et al. 1996, Mantle et al. 1996, Williams 2004). Gene regulation of POP has been mostly investigated by our laboratory, and we found that a CpG island in exon 15 is an epigenetically regulated potential enhancer and that a long noncoding RNA transcribed from downstream of the gene upregulated the POP expression in the mouse ovary (Matsubara et al. 2010, 2014). Given that the tissue distribution of POP is different between species as described above, the gene regulatory mechanism is significant to consider

species-specific physiological functions of POP.

Because POP is a serine protease, the identification of its physiological substrates is essential to understand the molecular mechanism of POP's functions, and researchers have made tremendous efforts to solve this issue for a long time (García-Horsman et al. 2007). However, while POP can cleave many biologically active peptides *in vitro*, the only substrate that is determined to be processed *in vivo* is α-MSH (Perroud et al. 2009, Warden et al. 2009). On the other hand, recent studies revealed that POP functions as an interacting molecule and protease activity is not required for the protein-protein interaction. The first protein that was reported to interact with POP is PEP-19 in porcine brain (Brandt et al. 2005). Then, growth associated protein 43 (GAP43) was found to interact with POP in HEK-MSRII cells and mouse brain, and the interaction was involved in the regulation of neural growth cone plasticity (Di Daniel et al. 2009). The interaction between Gapdh and POP was also reported in human neuroblastoma cell line NB-1, and was involved in nuclear translocation and cell death (Matsuda et al. 2013). α-Synuclein, a molecule that is involved in the Parkinson's disease and other synucleinopathies, was also found to interact with POP in mouse neuroblastoma cell line N2A, suggesting the possibility that POP is a therapeutic target (Savolainen et al. 2015). Thus, POP may function as an interacting protein more frequently than as a protease, but obviously, more analyses are necessary for completely understanding the functions of POP in various tissues and cells.

In the mouse, the ovary and placenta express higher levels of POP mRNA than other tissues (Matsubara et al. 2010, 2011, 2013, 2014). More specifically, ovarian granulosa cells and placental trophoblast giant cells (TGCs) and spongiotrophoblasts (SpTs) (Matsubara et al. 2010, 2011) exhibit high levels of POP signals. While the ovarian POP was suggested to control the folliculogenesis (Kimura & Takahashi 2000, Kimura et al. 1998), there have been

no reports about the function of placental POP and no phenotype was described in the knockout mouse database (KOMP, https://www.komp.org/). Nonetheless, the placenta is the most important organ to maintain pregnancy in eutherian mammals, and such high expression of POP in the mouse placenta shows the possibility that the mouse is suitable for investigating the significance of POP in development. This is why I focused on the function of POP in the mouse placenta.

The placenta plays essential roles in fetal development by supporting fetus, transferring nutrients and gases between the mother and fetus, producing and secreting some hormones, and protecting from pathogens (Robbins & Bakardjiev 2012). Eutherian mammals, but not monotremata and marsupialia, generate the placenta during gestation, and there are several variations in its structure between species. In general, the placenta is classified into four categories by the morphological difference: zonary, diffuse, cotyledonary and discoid placenta (Eidem et al. 2017, Furukawa et al. 2014). Zonary placenta is observed in dog and cat, and has the cylindrical structure surrounding the fetus (Miglino et al. 2006). Diffuse placenta is made up of villi diffusely scattered over almost the whole surface of the chorion, in horse and pig (Roberts & Bazer 1988). Cotyledonary placenta has many separated villous patches called cotyledons localized in the surface of chorion, observed in cow and sheep (Assheton 1905, Björkman 1969). Discoid placenta has chorionic villi that are arranged in a round shape plate in human and mouse (King 1992).

In mice, the inner cell mass (ICM) and trophectoderm are differentiated in the early blastocyst stage at embryonic day (E) 3.5, and the placenta is formed from the latter. By around E5.0, the polar trophectoderm becomes the extraembryonic ectoderm (ExE), which is further differentiated into two layers, chorion and ectoplacental cone (EPC) before E6.0. Chorion will become labyrinthine cells and syncytiotrophoblasts (SynTs), while EPC will

become the SpT and TGC.

A mature mouse placenta is composed of three layers, maternal decidua, junctional zone and labyrinth. Maternal decidua is derived from maternal endometrium and connects the placenta with mother uterus. Labyrinth is the layer at the fetus side directly connected to umbilical cord, and required for exchanging nutrient, gas and fetus body waste. Junctional zone is located between maternal decidua and labyrinth, containing maternal vascular channels and the spiral artery. There are three types of functionally important cells in the mouse placenta: TGC, SpT and SynT (Cross 2005, Watson & Cross 2005). TGC shows the large cytoplasm and nucleus which is polypoid because of endoreduplication (Barlow and Sherman 1972, Kuhn et al. 1991, MacAuley et al. 1998), and synthesizes and secretes hormones (ex. placental lactogen (Soares et al. 1996)) and angiogenic factors (ex. vansccular endothelial growth factor (Vuorela et al. 1997)). SpT is positioned in junctional zone and generates some proteases (ex. matrix metalloprotease and urokinase-type plasminogen activator (Teesalu et al. 1998, 1999)). SynT has the characteristic of multinucleation by cell fusion and is involved in molecular transport between fetus and mother (Cross 2000, Cross et al. 2003).

Because the placenta plays an important role in the fetus development, the failure in placental development leads to embryonic lethality. There are so many genes that affect the placental formation, as revealed by knockout mice were dead (Hu & Cross 2010, Rossant & Cross 2001, Watson & Cross 2005). Among such genes, what we call ‘master genes’ showed especially severe phenotypes when they were deleted. For example, *heart and neural crest derivatives-expressed protein 1* (*Hand1*)-deficient embryos die by E7.5 owing to reducing the number of TGC (Riley et al. 1998, Scott et al. 2000). *Mammalian achaete-scute family bHLH transcription factor 2* (*Mash2*)-deficient embryos die by E10, because SpT is not

differentiated in the mutant placenta (Guillemot et al. 1994). Similarly, because of the lack of functional labyrinth layer, *glial cells missing homolog 1* (*Gcm1*)-deficient embryos die by around E10 (Cross et al. 2000, Schreiber et al. 2000). However, studying these essential genes only revealed the initial step of the placental formation, and the whole mechanism of placental differentiation should come from the studies of many other genes.

In contrast to the genes showing clear phenotypes by knockout, some genes showed moderate phenotypes. For example, HOP/NECC1 (-/-) placentas exhibited propagation of TGC layers and reduction of SpTs, but the embryo was not lethal (Asanoma et al. 2007). In addition, there are so many genes that showed no placental phenotypes by genetic mutation even though they were expressed in placenta. For example, krüppel-like zinc finger protein, *Glis family zinc 3* (*Glis3*), is expressed in placenta (Kim et al. 2003), but phenotypes in *Glis3*-deficient mice show the abnormality of insulin level and the polycystic kidney (Kang et al. 2009, Watanabe et al. 2009), and no placental phenotypes have been reported. Similarly, *Cluster of Differentiation 276* (*Cd276*), an immune checkpoint molecule, is expressed in the placenta (Sun et al. 2002), but *Cd276*-deficient mice showed the abnormal physiology of T-helper cells (Suh et al. 2003). *Kiss-1 metastasis-suppressor* (*Kiss1*) is famous for reproduction-associated neuropeptide and highly expressed in placenta, but placental phenotype is not found in *Kiss1*-deficient mice (Herreboudt et al. 2015). Instead, the knockout mice showed a decrease of the gonadotropin concentration and the growth failure in ovary and testis (D'Anglemont de Tassigny et al. 2007, Lapatto et al. 2007). These indicate the difficulty in the investigation of the molecular mechanism underlying the placental function and development, and another useful system is required.

In 1998, trophoblast stem cell (TSC) was established from both blastocysts at E3.5 and ExE at E6.5, to recapitulate the placental differentiation in mice. TSCs can be maintained as

undifferentiated state in the presence of fibroblast growth factor 4 (FGF4), and by removing it, they recapitulate the placental development *in vitro*, by differentiating into three trophoblast subtypes, TGC, SpT and SynT (Tanaka et al. 1998). It was reported that, in the TS cell differentiation, approximately 50% of the differentiated cells are TGCs whereas the percentage of SynT cells are assumed to be less than 5% (Hughes et al. 2004). Some studies demonstrated that the addition of activin facilitates the TSC differentiation into SynTs in the absence of FGF4 (Natale et al. 2009) and that retinoic acid promotes TGC differentiation (Yan et al. 2001). Thus, TSCs are used as a powerful tool for analyzing the function and differentiation of the placenta because we can easily induce the differentiation.

In chapter 1, to clarify the function of POP in placenta, especially during placental differentiation, I used TSC and POP-specific inhibitors and analyzed their impact on POP. SUAM-14746, a POP-specific inhibitor, dramatically arrested the TSC differentiation into SpT and TGC, so I suppose that POP plays a role in the differentiation into SpT and TGC in the mouse placenta. In chapter 2, I tried to clarify the molecular mechanism of POP in SpT and TGC differentiation. SUAM-14746 decreased the expression of *Mash2*, a SpT master controlling gene, although it was not controlled by phosphoinositide 3-kinase (PI3K)-Akt signaling. Thus, the regulation of SpT differentiation by POP was suggested to be through regulating *Mash2* expression. By these analyses, I clarified a novel function of POP in the mouse placenta, and established a good experimental system for controlling the TSC differentiation by a POP-specific inhibitor. These findings provide the important information about POP's role not only in the TSC differentiation but also in mouse development.

CHAPTER 1

MOUSE PROLYL OLIGOPEPTIDASE PLAYS A ROLE IN TROPHOBLAST STEM CELL DIFFERENTIATION INTO TROPHOBLAST GIANT CELL AND SPONGIOTROPHOBLAST

ABSTRACT

POP, a multifunctional protease hydrolyzing -Pro-X- peptide bonds, is highly expressed in the mouse placenta. To explore the possibility of POP's involvement in placental differentiation, I used TSCs and POP-specific inhibitors. During TSC differentiation for 6 days, POP was constantly detected at mRNA, protein, and activity levels, and the protein was found mainly in the cytoplasm. I added a POP-specific inhibitor, SUAM-14746, to the TSC culture system at various concentrations, and assessed its effect on the differentiation by microscopic observation and by checking the expression of marker gene for each placental cell. The addition of 30 μM and 10 μM SUAM-14746 effectively inhibited the differentiation into SpTs and 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 POP-specific inhibitor, KYP-2047, surprisingly did not affect the differentiation, although it effectively inhibited POP enzymatic activity and caused the conformational change in the POP-inhibitor complex, just like SUAM-14746. The dose-dependent effect of SUAM-14746 on TSCs suggests that POP plays an important role in the differentiation into SpTs and TGCs in the mouse placenta.

INTRODUCTION

POP is a serine endopeptidase, which cleaves peptides shorter than 30-mer at the carboxyl side of a proline residue (Moriyama et al. 1988, Wilk 1983). POP was discovered as an oxytocin-cleaving uterine enzyme in human (Walter et al. 1971), and is now known to be expressed in various tissues of many species from bacteria to mammals (Yoshimoto et al. 1987a, Rennex et al. 1991, Chevallier et al. 1992, Robinson et al. 1995, Ishino et al. 1998, Kimura and Takahashi 2000). POP is related to various physiological events such as learning and memory (Yoshimoto et al. 1987b, Toide et al. 1995, Shishido et al. 1998), cell signaling (Williams et al. 1999), sperm motility (Yoshida et al. 1999, Kimura et al. 2002, Dotolo et al. 2016), and cell proliferation and differentiation (Matsubara et al. 1998, Moreno-Baylach et al. 2008, Ohtsuki et al. 1994, Sakaguchi et al. 2011), so it is recognized as a multifunctional molecule. Our group previously reported that the mouse ovary and placenta express POP at higher levels than other tissues (Matsubara et al. 2010, 2011, 2013, 2014), but there are no reports of the function of POP in the placenta.

The placenta is a feto-maternal organ which has a role in the appropriate embryonal growth through exchanging nutrients and gases and by synthesizing and secreting growth factors and hormones (Cross 2006). The placenta is composed of three layers, maternal decidua, junctional zone, and labyrinth, and the three types of cells are known to be functionally important: TGC, SpT, and SynT. TGC shows polyploidy, characteristic of the large nucleus and cytoplasm resulting from endoreduplication (Barlow and Sherman 1972, Kuhn et al. 1991, MacAuley et al. 1998), and contributes to maintaining pregnancy by synthesizing hormones and growth factors. SpT plays a pivotal role in fetus viability (Guillemot et al. 1995, Cross 2005), and SynT, a multinucleated cell formed by cell to cell

fusion (Cross 2000, Cross et al. 2003), functions to exchange nutrients between the embryo and the mother. There are many unanswered questions regarding events in the placenta, including genomic imprinting (Constância et al. 2002), cell fusion in trophoblast syncytialization (McCoy et al. 2000), infiltration into endometrium (Vicovac et al. 1993), and the differentiation of TGCs and SpTs.

A high level of POP is found in SpTs and TGCs of the mouse placenta (Matsubara et al. 2010, 2011). Combined with earlier and recent works showing the involvement of POP in cell differentiation (Moreno-Baylach et al. 2008, Ohtsuki et al. 1994, Yamakawa et al. 1994), I hypothesized that POP has an important function in placental differentiation. Since POP-knockout mice are not lethal and no phenotype is so far reported in the placenta (D'Agostino et al. 2013, Dotolo et al. 2016, Höfling et al. 2016, Kim et al. 2014, Perroud et al. 2009, Warden et al. 2009), the first step to investigate POP's role in placental differentiation should be done with the TSC culture system. TSCs can be maintained as undifferentiated cells in the presence of FGF4, and by removing it, we can easily induce the differentiation into three trophoblast subtypes, TGC, SpT, and SynT (Tanaka et al. 1998). Therefore, the TSC culture system is an ideal tool for analyzing the function and differentiation of the placenta.

In this chapter, as an initial step for understanding the function of POP in the placenta, I induced the TSC differentiation in the presence of a POP-specific inhibitor, 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 POP plays an important role in the placental differentiation into SpTs and TGCs.

MATERIALS AND METHODS

TSC Culture, Induction of Differentiation, and Treatment with POP-Specific Inhibitors

TSCs were kindly provided by Dr. Satoshi Tanaka at Tokyo University (Tanaka et al. 1998) and kept at 37°C with 5% CO_2 as reported (Himeno et al. 2008). A frozen stock of TSCs was thawed and cultured on mitomycin C-treated (Wako Pure Chemical, Osaka, Japan) mouse embryonic fibroblast (MMC-MEF) in a 10-cm dish with TS medium (RPMI 1640 (Wako Pure Chemical) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 100 μM 2-mercaptoethanol (Wako Pure Chemical), 50 U/ml penicillin, 50 μg/ml streptomycin, 25 ng/ml FGF4 (Sigma-Aldrich), and 1.5 μg/ml heparin (Sigma-Aldrich)). Heparin was added to stabilize FGF4. I also prepared MMC-MEF-conditioned medium; TS medium was kept on MMC-MEFs for 3 days and was filtered after that.

Before the induction of differentiation, MMC-MEFs were removed as follows. TSCs on MMC-MEFs were washed with phosphate buffered saline (PBS), treated with 0.25% trypsin/1 mM ethylenediamine tetraacetic acid (EDTA) for 5 min at 37°C, and re-suspended in 70CM + F4H medium (30% of TS medium, 70% of MMC-MEF-conditioned medium, 25 ng/ml FGF4, and 1.5 μg/ml heparin). The cells were spread onto a new 10-cm dish and incubated for 45 min at 37°C, and the floating TSCs were cultured on a new dish. To completely remove MMC-MEFs, I repeated this process once again, when the cells became nearly confluent, before using them for experiments. On the day before starting the differentiation, I spread 2×10^5 TSCs on a 35-mm dish and cultured overnight. Then, I changed the medium to the one without FGF4 and heparin after the wash with PBS. The cells were cultured for 6 days and collected at indicated days. The medium was changed every other day.

For treatment of TSCs with a POP-specific inhibitor, SUAM-14746 (Saito et al. 1991) (Peptide Institute, Osaka, Japan) or KYP-2047 (Jarho et al. 2004) (Sigma-Aldrich), I added each inhibitor at the indicated concentration or an equal volume of dimethyl sulfoxide (DMSO), a control, when I removed FGF4 and heparin. The cells were cultured for 6 days, and the medium was changed every other day.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNAs were purified from cultured cells at indicated time points using ISOGEN II (Nippon gene, Tokyo, Japan) and treated with TURBO DNase (Ambion, Austin, TX), and cDNAs were synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), according to manufacturers' instructions. Quantitative PCR was performed as previously described (Matsubara et al. 2010). All the data were normalized to *Aip*. The value at day 0 was set to 1.0 in Fig. 1-2A, and in other figures, the highest value was set to 1.0 in each experiment. Primer sequences are listed in Table 1.

Preparation of Soluble Extract, Whole Cell Extract, and Nuclear, Cytoplasmic, and Membrane Fractions

The cells were collected in PBS, frozen and thawed three times, and centrifuged at 15,000 rpm for 5 min at 4°C. The supernatants were used as the soluble extract. To prepare the whole cell extract, cells were suspended in ice-cold PBS containing 1× proteinase inhibitor (Roche, Basel, Switzerland) and mixed with the same 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 EDTA) containing 1× proteinase inhibitor. The sample was incubated on ice for 5 min, sonicated to shear nucleic acids, and centrifuged at 15,000 rpm for 10 min at 4°C. The resulting supernatant was used as

the whole cell extract. Nuclear, cytoplasmic, and membrane fractions were prepared as previously described (Matsubara et al. 2011). Protein concentration was measured with a Pierce bicinchoninic acid protein assay reagent kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instruction.

Western Blot Analysis

Western blot analysis was performed as previously described (Matsubara et al. 2011). In this study, I used the purified anti-POP antibody (52 ng/ml at a final concentration (Matsubara et al. 2011)) and anti-β-actin polyclonal antibody (1:1000 dilution, Genetex, Irvine, CA) as primary antibodies, and goat anti-chicken IgY antibody (1:5000 dilution, Jackson ImmunoResearch, West Grove, PA) and anti-mouse IgG antibody (1:5000 dilution, GE healthcare, Chalfont St Giles, England) as secondary antibodies for POP and β-actin, respectively. The signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck, Darmstadt, Germany) and quantified by the Image J software to normalize the POP band intensity to β-actin. To confirm the specificity, I also performed this experiment by using the anti-POP antibody pre-incubated with an excess amount of recombinant POP protein.

Measurement of POP Enzyme Activity

Soluble extracts were prepared as above, and the specific POP activity toward Suc-Gly-Pro-Leu-Gly-Pro-4-methyl-coumaryl-7-amide (MCA) (Peptide Instisute) was measured as previously described (Kimura et al. 1998).

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 on the next day, the differentiation was started by removing FGF4 and heparin from the medium, and at the same time, 10 μM or 30 μM SUAM-14746 or an equal volume of DMSO (control) was added. The cells were cultured for 4 or 6 days and treated with MTT reagent for 3 hr at 37°C. After the addition of the solubilization solution and the incubation at 37°C for overnight, the amount of formazan product was measured using a spectrophotometer (Eppendorf, Hamburg, Germany) at 550 nm absorbance.

Preparation of Recombinant POP Protein

An open reading frame of the mouse POP gene was subcloned into pET30a expression vector (Novagen, Madison, WI) (Kimura et al. 2002). The recombinant POP was produced as a fusion protein with a vector-derived N-terminal sequence containing His-tag and an S-protein. The *Escherichia coli* strain, Rosseta, was transformed with the expression vector, and were grown at 37°C in Luria-Bertani medium with 10 μg/ml kanamycin and 34 μg/ml chloramphenicol. When the absorbance at 600 nm reached 0.6, isopropyl β-D(–)-thiogalactopyranoside was added at a final concentration of 1 mM, and the bacteria were cultured for another 20 hr at room temperature. Harvested cells were suspended in 50 mM Tris-HCl (pH 8.0) containing 10 μg/ml DNase I, lysed by freezing and thawing them three times, and centrifuged at 9,000 rpm for 15 min at 4°C. The supernatant was filtered with Minisart filter unit (0.45 μm pore size, Sartorius Biolab Products, Goettingen, Germany) and subjected to Ni^{2+} affinity chromatography (GE Healthcare Biosciences, Piscataway, NJ). The

recombinant POP protein was eluted with the buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 50 mM histidine.

Native-Poly Acrylamide Gel Electrophoresis (PAGE)

I mixed 4 μg of the recombinant POP protein with an excess amount of an inhibitor (SUAM-14746 or KYP-2047), or an equal volume of DMSO (control), and incubated for 1 hr at room temperature. The samples were electrophoresed in a 10% polyacrylamide gel without using sodium dodecyl sulfate (SDS) and 2-mercaptoethanol. The gel was stained with coomassie brilliant blue (CBB) buffer (45% methanol, 10% acetic acid, 0.25% CBB-R) for 1 hr, and destained by the treatment with destaining buffer (45% methanol, 10% acetic acid) for 30 min twice.

Statistical Analysis

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

Expression of Marker Genes during TSC Differentiation

To confirm whether the TSC differentiation was successfully induced, I performed the qRT-PCR analysis to detect a marker gene for each type of placental cell. The genes I checked were *caudal type homeobox 2* (*Cdx2*) for undifferentiated cells, *Gcm1* for SynT, *trophoblast specific protein alpha* (*Tpbpα*) for SpT, and *prolactin family 3, subfamily d, member 1* (*Pl1*) for TGC. The mRNA level was normalized to that of *Aip*, a housekeeping gene, and the highest value in each data set was set to 1.0. I collected total RNAs from TSCs at 1, 2, 4, and 6 days after the induction was started by removing FGF4, and as a sample at day 0, I used undifferentiated TSCs. *Cdx2* expression was decreased immediately after the induction (Fig. 1-1A), and *Gcm1* expression was increased to reach a peak at day 2, followed by a reduction (Fig. 1-1B). *Tpbpα* and *Pl1* expression was gradually increased during the differentiation and became the highest at day 6 (Fig. 1-1C and D). These expression patterns were consistent with the data reported in previous studies (Hughes et al. 2004, Natale et al. 2009, Parast et al. 2009, Takao et al. 2012, Tanaka et al. 1998), which indicated that the TSC differentiation was successful.

POP Expression and Enzymatic Activity during TSC Differentiation

I investigated POP expression and enzymatic activity during TSC differentiation. I induced the TSC differentiation and collected total RNAs or cell extracts at 1, 2, 4, and 6 days after that. Total RNAs and cell extracts from undifferentiated cells were collected as the samples at day 0. By qRT-PCR using *Aip* as an internal control, POP mRNA was significantly increased after the induction, reached a peak at day 4, and decreased thereafter (Fig. 1-2A).

The relative POP protein amount was measured by western blot analysis using the anti-POP antibody (Matsubara et al. 2011) and the anti-Actb antibody as a reference. The specificity of anti-POP antibody was checked by western blotting with pre-absorbed antibody (Fig. 1-2B). Although this antibody sometimes detected extra bands at upper and lower positions of a main band with TSC extracts, they were not specific POP signals. I prepared soluble cell extracts and whole cell extracts, and in both extracts, POP protein was detected at a constant level during TSC differentiation (Fig. 1-2C and D). Because POP protein was detected in the membrane fraction as well as in the cytoplasm in the mouse placenta (Matsubara et al. 2011), I investigated the POP localization in TSCs. I fractionated TSCs at day 0 and day 6 into cytoplasmic, membrane, and nuclear fractions and performed western blot analysis. The POP signal was observed mainly in the cytoplasm and at lower levels in the membrane fraction, but not in the nucleus (Fig. 1-2E). Then, I measured POP enzymatic activity with a POP-specific synthetic substrate. The activity was detected at high levels during the entire period of TSC differentiation, and the TSC sample at day 4 showed barely but significantly higher activity than that at day 0 (Fig. 1-2F).

These results indicated that POP mRNA, protein, and enzymatic activity were all detected throughout the entire period of TSC differentiation, and the POP protein was mainly localized in the cytoplasm of TSCs. These suggested that POP played some role in TSC differentiation.

TSC Differentiation with SUAM-14746

To examine a function of POP in TSC differentiation, I used a POP-specific inhibitor, SUAM-14746. I added the inhibitor or its solvent, DMSO as a control, to the culture media from which FGF4 was removed to induce the differentiation. First, I observed cell

morphology under the microscope at day 4. In the control experiment, I observed many TGC-like cells which had large cytoplasms and nuclei (Fig. 1-3A center), compared with undifferentiated cells (Fig. 1-3A left). In contrast, the number and percentage of the TGC-like cells dramatically decreased by adding 30 μM SUAM-14746 (Fig. 1-3A right). These suggested that the TSC differentiation was inhibited by SUAM-14746.

Then, I examined the marker gene expression by qRT-PCR. Here I checked TSCs at day 2 and day 6 only, because *Gcm1* expression was high at day 2 and *Tpbpα* and *Pl1* expression was at a peak at day 6 (Fig. 1-1). By the addition of SUAM-14746 at 10 μM and 30 μM, *Cdx2* expression was the highest in undifferentiated cells and decreased at day 2 and day 6, and this pattern was not different from the control (Fig. 1-3B). Interestingly, *Gcm1* expression was increased at day 2 even in the presence of SUAM-14746 at 10 μM and 30 μM, similarly to the control cells, although at day 6, it was decreased in a dose-dependent manner (Fig. 1-3C). *Tpbpa* and *Pl1* expression was considerably decreased in TSCs cultured with SUAM-14746 at day 6 compared to the control (Fig. 1-3D and E). These indicated that TSC differentiation into SpTs and TGCs was inhibited by the addition of 10 μM and 30 μM SUAM-14746 but SynTs normally differentiated with these concentrations of SUAM-14746.

I next investigated the effect of SUAM-14746 on TSC differentiation at lower concentrations. The addition of 5 μM and 1 μM SUAM-14746 to the TSC system did not induce any difference in *Cdx2* expression between the cells with the inhibitor and the control cells (Fig. 1-3F). *Gcm1* expression was not changed by 5 μM and 1 μM SUAM-14746 at day 2, although at day 6, it was decreased in a dose-dependent manner (Fig. 1-3G). *Tpbpα* expression was significantly decreased by 5 μM SUAM-14746 at day 6 (Fig. 1-3H), and *Pl1* expression was not changed by these concentrations of the inhibitor (Fig. 1-3I). 1 μM SUAM-14746 did not affect any marker genes.

I checked whether this inhibitor actually inhibited POP activity in TSCs. By the enzyme assay, POP activity in TSC extracts collected at day 0 and 6 was efficiently decreased by 1-30 μM SUAM-14746 (Fig. 1-4A and B). Thus, these results demonstrated that SUAM-14746 specifically impaired the TSC differentiation into SpTs and TGCs.

Cell Viability during TSC Differentiation in the Presence of SUAM-14746

To test the cytotoxicity of SUAM-14746, I checked the TSC viability by MTT assay. After the differentiation induction for 4 days and 6 days with 10 μM or 30 μM SUAM-14746, I treated the cells with MTT and solubilized them. By measuring the absorbance at 550 nm with the sample, I compared the cell viability. The absorbance was not significantly different between the cells with 10 μM or 30 μM SUAM-14746 and the control cells (Fig. 1-5), demonstrating that SUAM-14746 at these concentrations did not affect the viability during TSC differentiation.

TSC Differentiation with Another POP-Specific Inhibitor, KYP-2047

Finally, I examined the effect of another POP-specific inhibitor, KYP-2047, on TSC differentiation. I used 60 μM KYP-2047 as a concentration high enough to inhibit POP activity in various cells and tissues (Jalkanen et al. 2011, Myöhänen et al. 2012, Savolainen et al. 2015). I induced the TSC differentiation, cultured the cells with 60 μM KYP-2047 for 6 days, and collected total RNAs. To see the effect on the differentiation into SpTs and TGCs, I investigated the expression of *Tpbpα* and *Pl1* in those cells as well as in undifferentiated cells (day 0). Unexpectedly, *Tpbpα* and *Pl1* expression levels were not different between the control cells and TSCs with 60 μM KYP-2047 (Fig. 1-6A and B), while this inhibitor efficiently decreased POP activity in TSC extracts (Fig. 1-6C and D).

This discrepancy might be attributed to different effects between SUAM-14746 and KYP-2047 on the POP protein conformation. To briefly assess the POP conformation, I conducted native-PAGE using the recombinant POP protein mixed with SUAM-14746, KYP-2047, or DMSO (control). As a result, the control experiment resulted in the appearance of two bands that probably corresponded to the oligomer and open monomer as reported (Fig. 1-6E, Savolainen et al., 2015). In contrast, the mixture of recombinant POP with KYP-2047 resulted in one band at a lower position, corresponding to the compact monomer (Savolainen et al. 2015), and that with SUAM-14746 showed a similar pattern (Fig. 1-6E). Therefore, I could not detect a conformational difference between POP bounds by SUAM-14746 and KYP-2047, which suggested the existence of other difference between the two inhibitors.

DISCUSSION

The TSC culture system has been widely used for studying mechanisms of placental differentiation, and the cells differentiate into three types of placental cells typically by the culture for 4-8 days after the differentiation induction. During the differentiation, expression of a SynT marker gene is induced at an early time point, and markers for TGC and SpT are simultaneously increased at later points (Hughes et al. 2004, Natale et al. 2009, Parast et al. 2009, Takao et al. 2012, Tanaka et al. 1998). My current data showed that *Gcm1* expression reached a peak at day 2 and *Tpbp*α and *Pl1* expression was the highest at day 6 (Fig. 1-1), which is consistent with the previous reports. In addition, a marker for undifferentiated TSCs was down-regulated immediately after the induction in both previous studies and my results (Fig. 1-1A). These clearly indicate that the TSC differentiation was successful in my culture system.

During TSC differentiation, POP mRNA was significantly increased at day 4, whereas the POP protein amount and enzymatic activity were fairly constant (Fig. 1-2). In addition, I observed a slight but significant increase in POP activity at day 4, while the POP protein amount was constant during TSC differentiation. This kind of discrepancy was reported in several studies (Bellemère et al. 2004, García-Horsman et al. 2007, Irazusta et al. 2002, Myöhänen et al. 2008a,b), and is considered to be due to the stability of POP mRNA, the translational efficiency, rapid turnover of POP protein, or the existence of endogenous POP inhibitors (Salers 1994, Yoshimoto et al. 1982). Thus, such post-transcriptional regulation of the POP gene may be present during TSC differentiation.

I detected the POP protein in both cytoplasm and membrane fractions but not in the nuclei in TSCs before and after differentiation (Fig. 1-2E). This is consistent with a previous

report which showed that the POP protein is localized in the cytoplasm and membrane of the mouse placenta (Matsubara et al., 2011). During differentiation of mouse cerebellar granule cell and flesh fly imaginal disc, POP is localized to nuclei at early stages, and the nuclear POP is suggested to play some roles in controlling the differentiation (Moreno-Baylach et al. 2008, Ohtsuki et al. 1994). However, POP is present in the cytoplasm during most period of the cerebellar granule cell differentiation (Moreno-Baylach et al. 2008), suggesting that the cytoplasmic POP is involved in the regulation of cell differentiation. In addition, no studies have reported the relation of membrane-bound POP to cell differentiation. Taken together with my data showing that most POP was present in the cytoplasm of TSC, the cytoplasmic POP is likely to function in TSC differentiation.

Of particular interest is that SUAM-14746 had no effects on the initiation of TSC differentiation (Fig. 1-3B and F), while TSCs did not differentiate into TGCs and SpTs (Fig. 1-3D and E). SynTs were normally differentiated at day 2, but most cells at day 6 were not multinuclear (Fig. 1-3C). These indicate that most cells are neither undifferentiated cells nor any of TGCs, SpTs, and SynTs. One thing to notice is that the cells seemed to proliferate well as evidenced by the microscopic image showing more number of cells in the presence of the inhibitor than the control (Fig. f1-3A, compare the middle panel and the right panel). The cells might be in the middle state of differentiation into placental cells or they might be cell types unrelated to the placenta.

The expression of a SynT marker gene, *Gcm1*, was significantly decreased by the addition of 5-30 μM SUAM-14746 at day 6 (Fig. 1-3C and G). I suppose that these results do not reflect the specific inhibition of SynT differentiation by the following reasons. The *Gcm1* decrease at day 6 by 5-30 μM SUAM-14746 is probably due to the decrease of the percentage of SynT in the entire cell population. As I discussed above, in the presence of SUAM-14746,

TSCs is not in the undifferentiated state but continued to proliferate, which results in the increase of the total cell number. However, SynTs do not generally proliferate (Simmons & Cross 2005), and their absolute number in a dish should be similar between the cells with SUAM-14746 and the control. This should lower the relative percentage of SynTs, which results in lower levels of *Gcm1* expression. Therefore, I think that SUAM-14746 does not specifically inhibit the SynT formation, and POP is unlikely to be involved in TSC differentiation into SynTs. The detailed mechanism of SynT differentiation will require further investigation.

In contrast, the TSC differentiation into SpTs and TGCs was specifically inhibited by the addition of SUAM-14746 at day 6 as shown in Fig. 1-3. The dose-dependent decrease of *Tpbpα* and *Pl1* expression supports the specific effect of SUAM-14746. Previous data demonstrating the POP localization at higher levels in SpTs and TGCs in the mouse placenta are also consistent with its role in the differentiation into these cells (Matsubara et al., 2011). In addition, POP has been suggested to be involved in cell differentiation by different studies (Ohtsuki et al. 1994, Matsubara et al. 1998, Moreno-Baylach et al. 2008), providing the possibility that it is associated with the differentiation of broader types of cells. Collectively, it is strongly suggested that POP plays an important role in the regulation of placental differentiation into TGCs and SpTs, and SUAM-14746 is an inhibitor which efficiently inhibits the process.

Then, by what kind of mechanism does POP control the TSC differentiation? It is notable that PI3K-Akt pathway leads to activation of the *Mash2* gene, which encodes a transcription factor essential to SpT differentiation (Guillemot et al. 1994, Tanaka et al. 1997). Because POP activates the PI3K-Akt pathway (Takao et al. 2012), it may be involved in SpT differentiation through controlling this pathway. In case of TGC differentiation, IP_3 may be a

key molecule. The focal adhesion kinase activity, which leads to the accumulation of IP_3, was reported to be decreased during TSC differentiation (Parast et al. 2001). This suggests that IP_3 production needs to be suppressed for TGC differentiation, and POP was actually reported to inhibit the accumulation of IP_3 (Harwood 2011, Williams et al. 1999). Therefore, POP may be involved in TGC differentiation through controlling IP_3. Considering that POP is known to function as an interacting molecule (Brandt et al. 2005, Di Daniel et al. 2009, Lambeir 2011, Matsuda et al. 2013), it possibly controls TSC differentiation through interaction with other proteins. Further studies will be required to reveal the mechanism by which POP regulates the differentiation into SpTs and TGCs, but our current data indicate that POP plays a role in TSC differentiation.

While SUAM-14746 efficiently inhibited the TSC differentiation into SpTs and TGCs, another POP-specific inhibitor, KYP-2047, had no effect (Fig. 1-6). Each inhibitor blocks the POP active site to prevent the protease from digesting substrates (Jarho et al. 2004, Saito et al. 1991), and I actually confirmed the inhibiting activity of both inhibitors (Fig 1-4 and Fig 1-6). In addition, I did not observe any difference between their effects on the POP protein conformation by native-PAGE (Fig. 1-6C). There are several possibilities why KYP-2047 exhibited different effects on TSC differentiation from SUAM-14746. For example, the ability to penetrate into cells, the time to form the POP-inhibitor complex, and the speed to be excreted might be different. In fact, two POP-specific inhibitors, KYP-2047 and JTP-4819, were reported to show different penetration and sustainability in rat brain (Jalkanen et al. 2011), in spite of the resemblance in their chemical structure. Also, some POP-specific inhibitors induced deleterious phenotypes in cultured schistosomula, while SUAM-14746 did not (Fajtová et al. 2015). In addition, several inhibitors for another serine protease belonging to the POP family, dipeptidyl peptidase IV, are known to exhibit different physiological

effects (Nauck et al. 2007). Therefore, the two inhibitors, SUAM-14746 and KYP-2047, probably have different properties, and SUAM-14746 inhibits TSC differentiation into SpTs and TGCs.

In conclusion, a POP-specific inhibitor, SUAM-14746, specifically impaired the TSC differentiation into SpTs and TGCs, which strongly suggests that POP 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 POP in placental differentiation.

CHAPTER 2

MOUSE PROLYL OLIGOPEPTIDASE CONTROLS TROPHOBLAST STEM CELL DIFFERENTIATION INTO SPONGIOTROPHOBLAST VIA REGULATING THE MASH2 GENE EXPRESSION

ABSTRACT

POP is a serine protease possessing unique enzymatic activity and expressed in the mouse placenta at a high level. In chapter 1, I indicated that POP plays a role in TSC differentiation into TGC and SpT, and here I explored the molecular mechanism of the regulation of SpT formation. Interestingly, I found the significant downregulation of a SpT master regulator gene, *Mash2*, by a POP-specific inhibitor, SUAM-14746, during TSC differentiation. Because both POP and *Mash2* are related to PI3K-Akt signaling, I added a PI3K inhibitor, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002) to the TSC differentiation culture, but this inhibitor did not change the expression patterns of any placental marker genes or *Mash2*. These data suggested that POP regulated the *Mash2*-mediated SpT differentiation independent of the PI3K-Akt pathway.

INTRODUCTION

POP is a protease that can digest the -Pro-X- bond within peptides less than 30 amino acids (Moriyama et al. 1988, Wilk 1983). It is recognized as a multifunctional serine protease acting not only as a protease but also as an interacting protein. So far, various physiological roles of POP have been proposed, such as regulation of synapse in hippocampus (D'Agostino et al. 2013), secretion of insulin and glucagon in pancreas (Kim et al. 2014), sperm motility and spermatogenesis in testis (Dotolo et al. 2016), and α-synuclein dimerization related to Parkinson's disease (Savolainen et al. 2015). However, while the POP expression and activity were known to be high during development (Matsubara et al. 2010), its function in embryo or placenta was unclear. Thus, I analyzed the function of POP in the mouse placenta using the TSC culture system in chapter 1, and derived a conclusion that POP plays a role in TGC and SpT differentiation. Here, I attempted to elucidate the molecular mechanism of placental differentiation regulated by POP.

The placental differentiation is controlled by the expression of a master gene for each cell type. *Gcm1* is a SynT master gene, which encodes a transcription factor with a gcm-motif, and essential for the formation of SynTs in the labyrinth layer of mouse placenta (Cross et al. 2000, Schreiber et al. 2000). *Hand1* is a basic helix-loop helix (bHLH) transcription factor, which is essential for TGC differentiation (Firulli et al. 1998, Riley et al. 1998, Scott et al. 2000) and for invasive activity (Hemberger et al. 2004). *Mash2* is another bHLH transcription factor, known as a SpT master gene. *Mash2*-deficient embryos die by around E10, because of the absence of spongiotrophoblast in the mutant placenta (Guillemot et al. 1994, Tanaka et al. 1997). These suggest that *Hand1* and/or *Mash2* are involved in the regulation of differentiation into TGC and SpT by POP.

Takao et al. reported that a maternally imprinted gene, *Tssc3*, regulated *Mash2* transcription in TSCs through the PI3K-Akt signaling pathway (Takao et al. 2012). On the other hands, specific inhibition of POP led to inactivation of cellular PI3K and Akt, suggesting the regulation of PI3K-Akt activation by POP in pancreatic cancer cells (Duan et al. 2014). These data enabled me to hypothesize that POP might control *Mash2* activation through regulating the PI3K-Akt signaling pathway.

In this chapter, I examined the expression of *Mash2* during TSC differentiation in the presence of a POP-specific inhibitor, SUAM-14746 and a PI3K-specific inhibitor, LY294002. While SUAM-14746 significantly inhibited *Mash2* expression, the supplement of LY294002 was not effective on the differentiation. These findings suggest that POP plays a role in the SpT differentiation by regulating the *Mash2* expression irrespective of the PI3K-Akt pathway.

MATERIALS AND METHODS

TSC Culture, Induction of Differentiation, and Treatment with Chemicals

TSC culture and induction of differentiation were performed as described in chapter 1. For treatment of TSCs with a POP-specific inhibitor, SUAM-14746 (Saito et al. 1991), or a PI3K-specific inhibitor, LY294002 (Wako Pure Chemical) (Vlahos et al. 1994), I supplemented 10 μM inhibitor or an equal volume of DMSO as a control, when FGF4 and heparin were removed from the culture system. The cells were cultured for 6 days, and the medium was changed every other day.

qRT-PCR Analysis

Preparation of cDNA sample and qRT-PCR were conducted as described in chapter 1. The *Mash2* primers used for PCR were 5'-TCTCTCGGACCCTCTCTCAG-3' and 5'-GGACCCCGTACCAGTCAAG-3' for *Mash2*. The expression of *Aip, Cdx2, Gcm1, Tpbpα* and *Pl1* genes was assessed by using the primers described in chapter 1 (Table 1). All the data were normalized to *Aip* and the highest value in each data set was set to 1.0.

Statistical Analysis

Results were represented as the average ± S.D., and their statistical significance was analyzed by one-way ANOVA followed by Tukey's post hoc test. P value less than 0.05 was considered statistically significant.

RESULTS

Expression of *Mash2* during TSC Differentiation with SUAM-14746

To clarify the mechanism by which the TSC differentiation into SpT was regulated by POP, I focused on a master control gene of SpT, *Mash2*. I first investigated the *Mash2* mRNA expression during TSC differentiation without any inhibitors. I collected total RNAs from undifferentiated cells as the sample at day 0 and from those at 1, 2, 4, and 6 days after the induction. By qRT-PCR using *Aip* as an internal control, the *Mash2* mRNA level was significantly increased immediately after the induction and reached a peak at day 2, and decreased thereafter (Fig. 2-1A). This expression pattern of *Mash2* was consistent with the data in previous reports (Hughes et al. 2004, Kibschull et al. 2004, Takao et al. 2012, Tanaka et al. 1998). Then, I supplemented a POP-specific inhibitor, 10 μM SUAM-14746. Simultaneously with the induction of the TSC differentiation by removing FGF4 from the culture medium, the SUAM-14746 or DMSO (control) was added. I examined *Mash2* expression at day 2 as it was the peak and at day 6 as the cells were terminally differentiated. By qRT-PCR, *Mash2* expression was significantly decreased in TSCs cultured with SUAM-14746 at day 2 compared to the control (Fig. 2-1B). These suggested that POP-regulated TSC differentiation into SpT was mediated by the *Mash2* gene.

The Effect of a PI3K Inhibitor, LY294002, on TSC Differentiation

Previous reports suggested that POP was involved in the regulation of PI3K-Akt signaling (Duan et al. 2014) and that the *Mash2* gene expression was controlled by PI3K-Akt pathway (Takao et al. 2012). So I investigated the involvement of PI3K in the POP-regulated TSC differentiation into SpT by adding a PI3K inhibitor, 10 μM LY294002, to the culture

system. I collected the cells at days 2 and 6 to perform qRT-PCR for placental marker genes and *Mash2*. *Cdx2* expression was the highest in undifferentiated cells and decreased at days 2 and 6, and no difference was observed between the control cells and the cells with LY294002 (Fig. 2-2A). Similarly, *Gcm1* expression was peaked at day 2 in both the cells with and without the inhibitor (Fig. 2-2B), and the expression levels of *Tpbpa* and *Pl1* were greatly increased at day 6 in both conditions (Fig. 2-2C and D). These results showed that the supplement of 10 μM LY294002 did not affect the TSC differentiation into any of SynT, SpT and TGC. Indeed, the expression of *Mash2* showed similar patterns between the control cells and LY294002-treated cells (Fig. 2-2E). These findings indicated that POP controlled the SpT differentiation by regulating *Mash2* gene expression, in which the PI3K-Akt pathway is not involved.

DISCUSSION

Here, I demonstrated that the POP-specific inhibitor, SUAM-14746, significantly decreased *Mash2* expression during TSC differentiation at day 2 (Fig. 2-1B). This indicates that POP controls SpT differentiation by regulating the *Mash2* gene at an early stage. In the normal formation of placenta, *Mash2* is mainly expressed in chorion and EPC, and EPC differentiates into TGC and SpT (Guillemot et al. 1994, Nakayama et al. 1997). *Mash2*-deficient placentas were lack of SpTs, and embryos were dead until E10 (Guillemot et al. 1994, 1995). This defect in SpTs was reported to be due to smaller size of EPC, and therefore, POP might control EPC formation.

Although I tested a hypothesis that POP controlled *Mash2* expression through the PI3K-Akt pathway, it was not the case. Then, what kind of pathway does POP regulate? In intestinal tumor, *Mash2* was reported to be upregulated mostly by the Wnt signaling pathway (Herbst et al. 2014, Jubb et al. 2006, Saran et al. 2013, Schuijers et al. 2015). During gestation, *Mash2* was also reported to be controlled by the Notch-signaling (Nakayama et al. 1997) and the hippo-signaling (Du et al. 2014). These studies led to the presumption that POP was related to these pathways to control *Mash2* expression. It is also interesting that AP2γ contributes to activation of the *Mash2* gene through the ERK pathway in placenta (Sharma et al. 2016) and that POP is suggested to be regulated by this transcription factor (Matsubara et al. 2013). In combination with a report showing that AP2γ controls not only PI3K-Akt but also ERK pathways, AP2γ possibly activates POP expression which may in turn activate the *Mash2* gene through the ERK pathway. Further analyses will be necessary for determining the detailed regulatory mechanism of SpT differentiation by POP.

SUAM-14746 decreased *Mash2* expression by approximately 60% at day 2, but the

expression was not completely diminished (Fig. 2-1B). One may suspect whether this level of downregulation really causes the almost complete loss of SpTs in the TSC culture system. However, at day 2, precursor cells for TGC and SpT, corresponding to EPC, were presumably present in a dish, and *Mash2* might be down-regulated solely in the cells to become SpT but not those for TGC. Therefore, I assume that the downregulation of *Mash2* by SUAM-14746 is critical to SpT differentiation.

In conclusion, the POP-specific inhibitor, SUAM-14746, significantly decreased the expression of *Mash2* at day 2, but this regulation was not through the PI3K-Akt pathway, which suggests that POP plays a role in the SpT differentiation by regulating the *Mash2* expression independent of the PI3K-Akt pathway.

GENERAL DISCUSSION

In this thesis, I clarified the POP's role in placenta, a positive effect on the differentiation into TGC and SpT, by experiments with POP-specific inhibitors in the TSC culture system (chapter 1). In addition, I suggested the POP-regulated SpT differentiation via *Mash2* expression (chapter 2), by demonstrating that the inhibition of POP decreased the expression of *Mash2*, a master gene of SpT differentiation. Figure 3 shows a model of the role of POP in TSC differentiation proposed on the basis of findings in this thesis.

Generally, the placental differentiation involves several regulatory pathways. For example, *Gcm1*, a master gene of SynT, regulates the formation of labyrinth layer and a branching morphogenesis through the control of *Syncytin-1*, *Syncytin-2*, and *Cebpα* (Cross et al. 2000, Schubert et al. 2008). In addition, the cyclic adenosine monophosphate (cAMP)-dependent protein kinase cascade activates *Gcm1* expression (Chang et al. 2005) and the cAMP-Epac1-Calcium/calmodulin-dependent protein kinase I (CaMKI) signaling cascade promotes the desumoylation of Gcm1 protein to activate it (Chang et al. 2011). Peroxisome proliferator-activated receptor γ (PPARγ) also plays a role in differentiation of labyrinthine trophoblast lineages (Barak et al. 1999, Parast et al. 2009).

A TGC master gene, *Hand1*, is enhanced by *Sox15* to promote TGC differentiation in rat choriocarcinoma (Rcho-1) cells (Yamada et al. 2006). In the same system, the involvement of FAK signaling pathway in TGC differentiation is also pointed out because the phosphorylation of FAK increases during Rcho-1 differentiation (Parast et al. 2001). In case of SpT differentiation, *Mash2* is a master gene. In the placenta, *Mash2* is a potential target of Notch signaling (Gasperowicz & Otto 2008), while in intestinal tumor, it is a famous target of the Wnt signaling pathway to be regulated by binding of β-catenin-TCF complex to its promoter (Herbst et al. 2014, Jubb et al. 2006, Schuijers et al. 2015). These suggest that any of such signaling pathways are related to the control of TGC and SpT differentiation by POP

in the placenta. Interestingly, *Hand1* and *Mash2* are known to function in a competitive manner; *Hand1* inhibits SpT differentiation, and *Mash2* inhibits TGC differentiation. Thus, the differentiation into TGC and SpT may be independently controlled by different signals.

Mash2 is known as a paternally imprinted gene (Guillemot et al. 1995), but its promoter is known to be resistant to DNA methylation (Caspary et al. 1998, Tanaka et al. 1999), so the transcription is presumably regulated by histone modifications or some other factors. Indeed, a long noncoding RNA was reported to regulate the activation of a human orthologue of *Mash2* by mediating the changes in chromatin structure in colorectal cancer (Giakountis et al. 2016). In any case, it is sure that SUAM-14746 affected a signaling pathway leading to *Mash2* expression. Then, what was the signaling pathway controlled by POP? In chapter 2, I showed that the PI3K-Akt pathway was not related, so other signaling pathways such as Wnt and ERK signaling pathways (Jubb et al. 2006) may be the one. In PI3K-Akt pathway, POP facilitates the phosphorylation of IRS1 in pancreatic cancer cells (Duan et al. 2014) and in *Dictyostelium*, it inhibits the dephosphorylation of IP_3 (Williams et al. 1999). These indicate that POP positively regulates the phosphorylation. Therefore, in the Wnt pathway, POP may facilitate the phosphorylation of LRP, which results in activation of the transcriptional activity of β-catenin. In the ERK signaling, POP may regulate the phosphorylation of ERK and/or MEK, ERK-targeted kinase. To determine the signaling cascade regulated by POP in placenta, further analyses will be required.

It is also interesting that both POP and *Mash2* genes are likely controlled by a placenta-specific transcription factor, AP2γ. Our group detected the binding of AP2γ at the POP promoter in mouse placenta, although the transcriptional activity was unclear (Matsubara et al. 2013). *Mash2* was reported to be down-regulated in the AP2γ-deficient placenta (Kaiser et al. 2015), but the actual binding to the promoter was not reported. These suggest that the

Mash2 gene is regulated by AP2γ through the POP gene; AP2γ may activate the POP gene by directly binding to the promoter but not to the regulatory region of *Mash2*, and it may be POP that activates the *Mash2* gene. AP2γ is a regulator critical for the placental formation, and one of the downstream gene, POP, may control the lineage of SpT differentiation via regulating *Mash2* expression.

As described above, *Hand1* and *Mash2* normally worked in an antagonistic manner by competing for E-factor binding (Scott et al. 2000), but both TGC and SpT were derived from a single cell lineage, EPC. As long as I know, there are no studies showing the phenotype lacking both TGC and SpT at the same time while maintaining the other structures. Therefore, my data show a rare example of the TSC differentiation, which provides precious information for the study of placental differentiation.

This study is the first report that a serine protease plays a role in placental differentiation. There are two possibilities of how POP functions in the placental differentiation, as a protease or as an interacting protein. As an enzyme, POP has been suggested to digest some kinds of hormones or neuropeptides *in vitro* (García-Horsman et al. 2007), such as substance P (Sastry et al. 1981), oxytocin (Nishimori et al. 1996), and GnRH (Khodr & Siler-Khodr 1980, Radovick et al. 1990), all of which are expressed in placenta. However, the only substrate that has been decided to be catalyzed *in vivo* is α-MSH (Perroud et al. 2009, Warden et al. 2009), and POP-knockout mice are not lethal while it is the only enzyme that can cleave -Pro-X- within a peptide. Thus, POP is considered to rather function as an interacting protein during mouse development. Consistently, two proteins, α-synuclein and Gapdh, that were reported to interact with POP are also expressed in the placenta (Rada et al. 2014, Uéda et al. 1993). These proteins may interact with the placental POP to control the SpT and/or TGC differentiation. Alternatively, some other proteins may bind to POP, because each tissue

appears to contain each specific interaction counterpart of POP. The identification of such a protein in the TSC culture system will be required.

POP is evolutionarily conserved from bacteria to mammals, and therefore has been thought to play some fundamental roles in biological activity. Because POP expression is high in undifferentiated cells and cancerous cells (Goossens et al. 1996, Liu et al., Yamakawa et al. 1994), its major role would be the regulation of cell proliferation or differentiation. Given that the development is the repeat of differentiation, POP is a significant molecule in embryogenesis or morphogenesis. So far, any of POP-deficient mice exhibited the embryonic lethal phenotype, presumably due to compensation by other proteins, but there is a POP-deficient strain, on the KOMP (https://www.komp.org/) database, which showed the growth retardation. This suggests that POP is indispensable for embryo development in some conditions. Here, I indicated the extreme significance of POP in TSC differentiation, and suggested the regulation of some cell signaling pathways by POP. Such signaling cascades are functioning in various types of cells, so POP possibly controls many signal molecules, which may result in different phenotypes depending on the cell type. My study revealed a novel function of POP in placental differentiation, but it must be based on some fundamental roles of this protease. Therefore, I expect that my data improve our understanding of basic functions of POP that can be applied to further research of POP as well as of other proteases or regulatory molecules.

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TABLE and FIGURES

Table 1 Primers used in this study.

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

Fig. 1-1

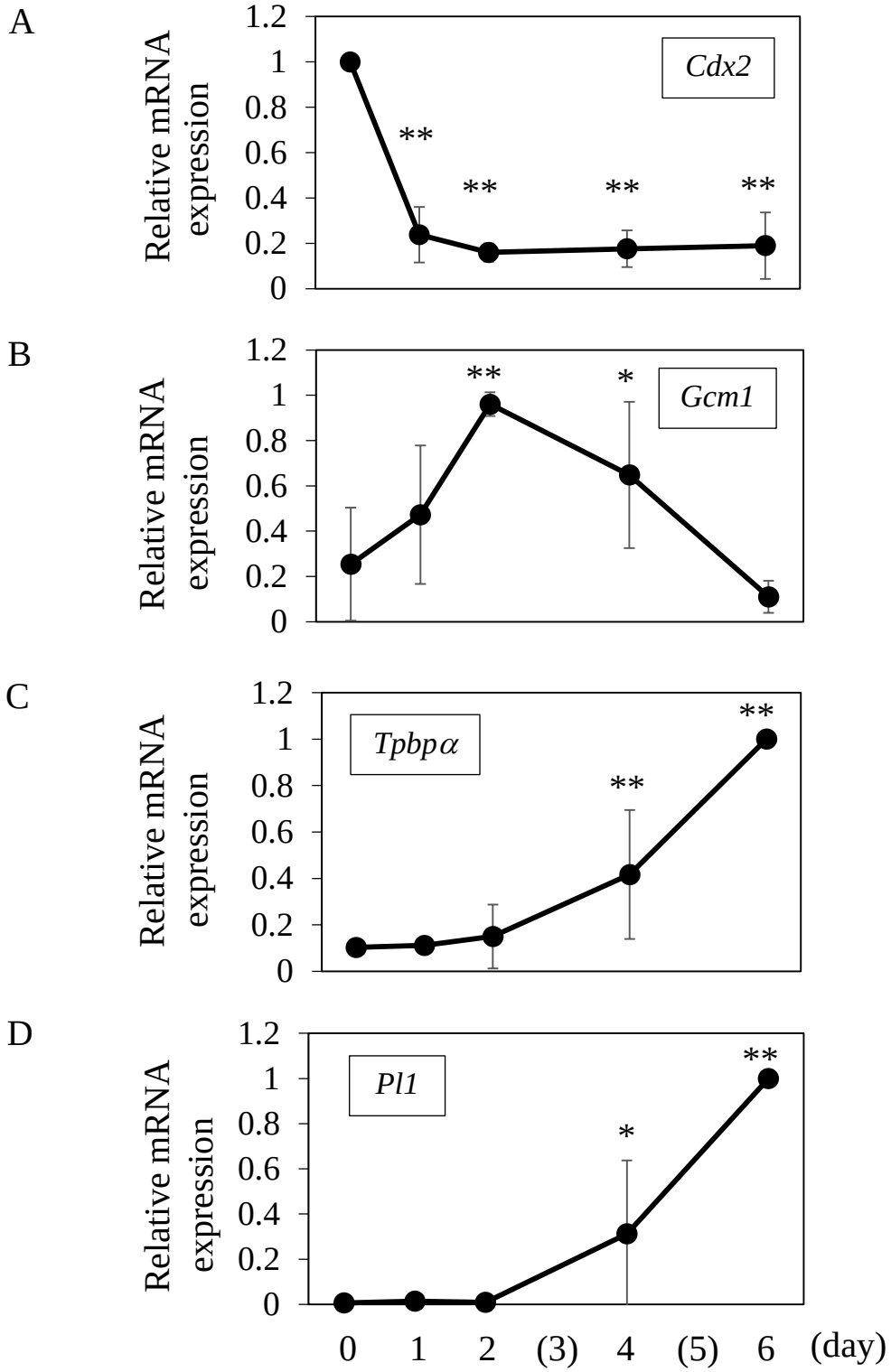

A
B
C
D
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
Cdx2
Gcm1
Tpbpα
Pl1
**
*
0
1
2
(3)
4
(5)
6
(day)

Figure 1-1. The expression pattern of marker gene for each type of placental cell.

2×10^5 TSCs were seeded on a 35-mm dish with FGF4, and on the next day, TSC differentiation was induced by removing FGF4. Total RNAs were prepared from the cultured cells at 0, 1, 2, 4, and 6 days after the induction, and treated with Turbo DNase. cDNA was synthesized by reverse transcription with the oligo(dT) primer and 500 ng of each RNA, and was used for quantitative PCR using the ABI Prism 7300 real-time PCR system. 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 at the indicated day after the differentiation induction. 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 < 0.01 compared with day 0.

A: *Cdx2* for the undifferentiated cell, B: *Gcm1* for SynT, C: *Tpbpα* for SpT, and D: *Pl1* for TGC.

Fig. 1-2

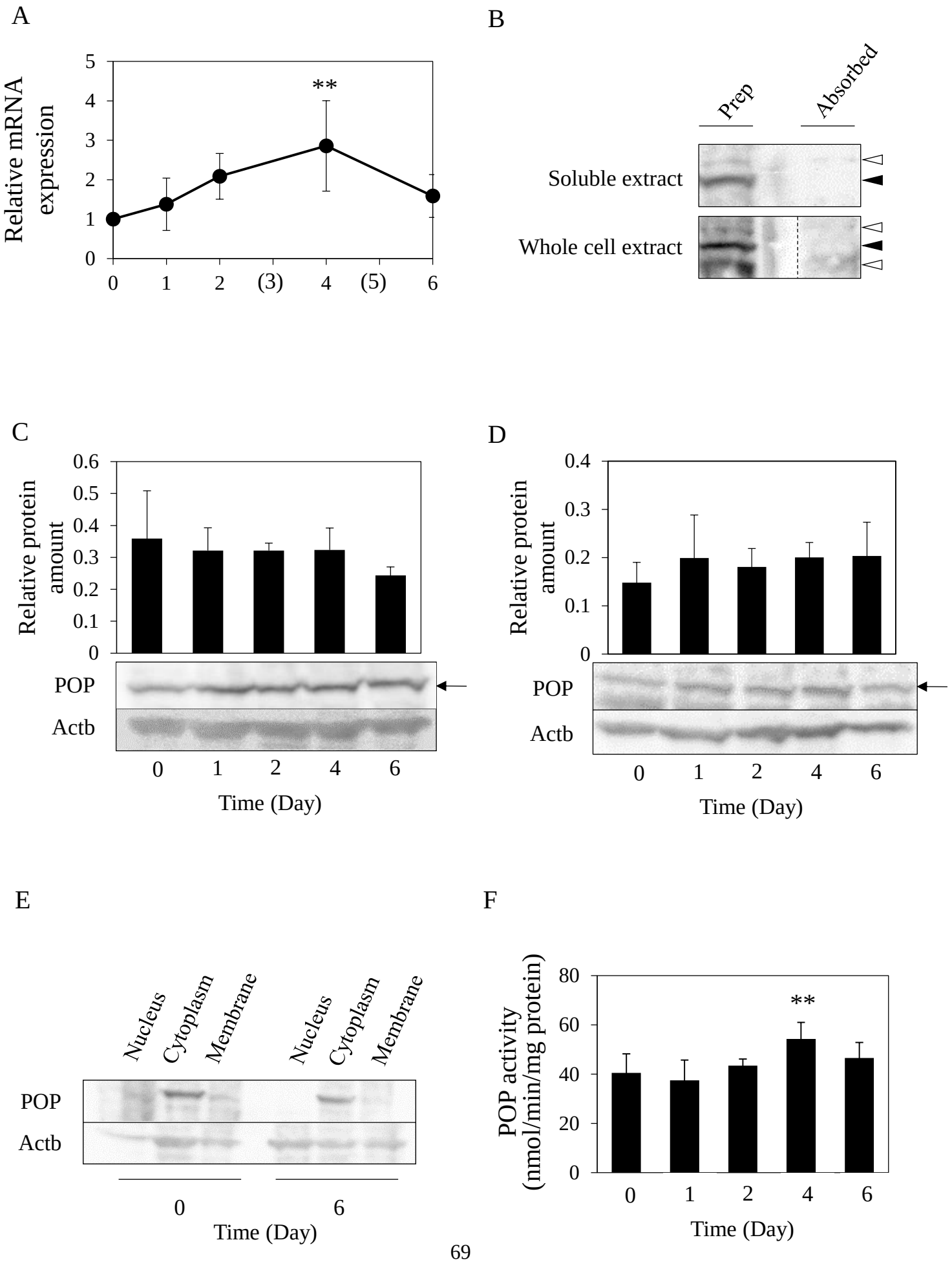

A
Relative mRNA expression
5
4
3
2
1
0
**
0
1
2
(3)
4
(5)
6
B
Prep
Absorbed
Soluble extract
Whole cell extract
C
Relative protein amount
0.6
0.5
0.4
0.3
0.2
0.1
0
POP
Actb
0
1
2
4
6
Time (Day)
D
Relative protein amount
0.4
0.3
0.2
0.1
0
POP
Actb
0
1
2
4
6
Time (Day)
E
Nucleus
Cytoplasm
Membrane
Nucleus
Cytoplasm
Membrane
POP
Actb
0
6
Time (Day)
F
POP activity (nmol/min/mg protein)
80
60
40
20
0
**
0
1
2
4
6
Time (Day)

Figure 1-2. POP expression and enzymatic activity during TSC differentiation.

A: POP mRNA expression by qRT-PCR. The POP 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. POP mRNA expression significantly increased after the induction, reached a peak at day 4, and decreased thereafter.

B: Western blot analysis for specificity of the anti-POP antibody. The anti-POP antibody was pre-absorbed with recombinant POP 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 POP band is indicated by black arrowheads, and the other bands by white arrowheads.

C, D: Western blot analysis for analyzing the POP protein amount. 10 μg of soluble extract (C) or whole cell extract (D) from TSCs at indicated days were used. Specific POP bands are indicated by arrows. The signal intensity was measured using Image J software, and the POP signal was normalized to Actb. The relative POP 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 POP levels did not significantly change during TSC differentiation.

E: Western blot analysis with cytoplasmic, membrane, and nuclear fractions of TSCs. In both TSCs at day 0 and day 6, the POP signal was mainly detected in the cytoplasmic fraction and at a lower level in the membrane but not in the nucleus.

F: POP 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. 1-3

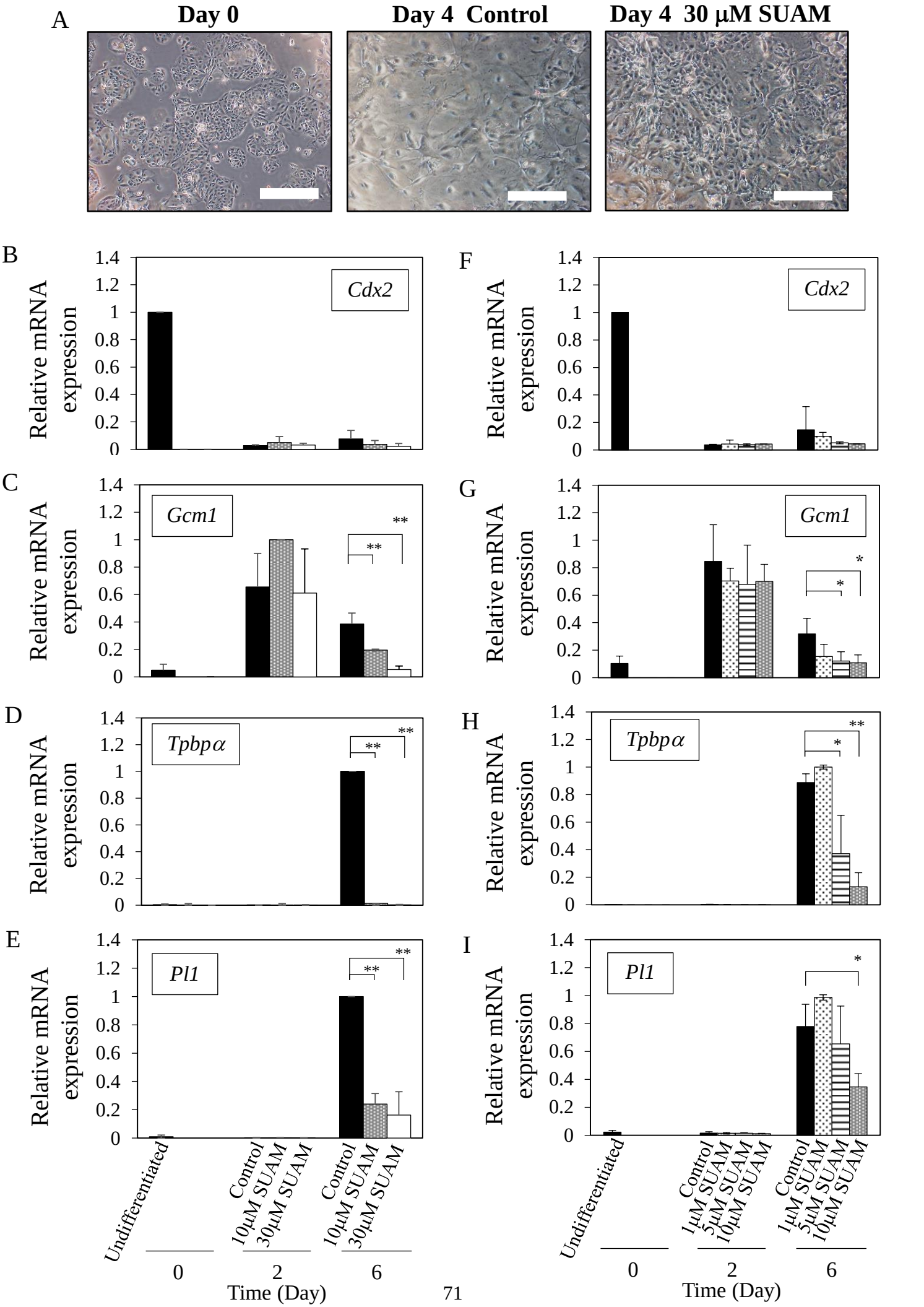

A
Day 0
Day 4 Control
Day 4 30 μM SUAM
B
C
D
E
F
G
H
I
Relative mRNA expression
Cdx2
Gcm1
Tpbpα
Pl1
Undifferentiated
Control
10μM SUAM
30μM SUAM
1μM SUAM
5μM SUAM
10μM SUAM
0
2
6
Time (Day)
71

Figure 1-3. 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). *Tpbpa* 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, compared to the cells with DMSO.

Fig. 1-4

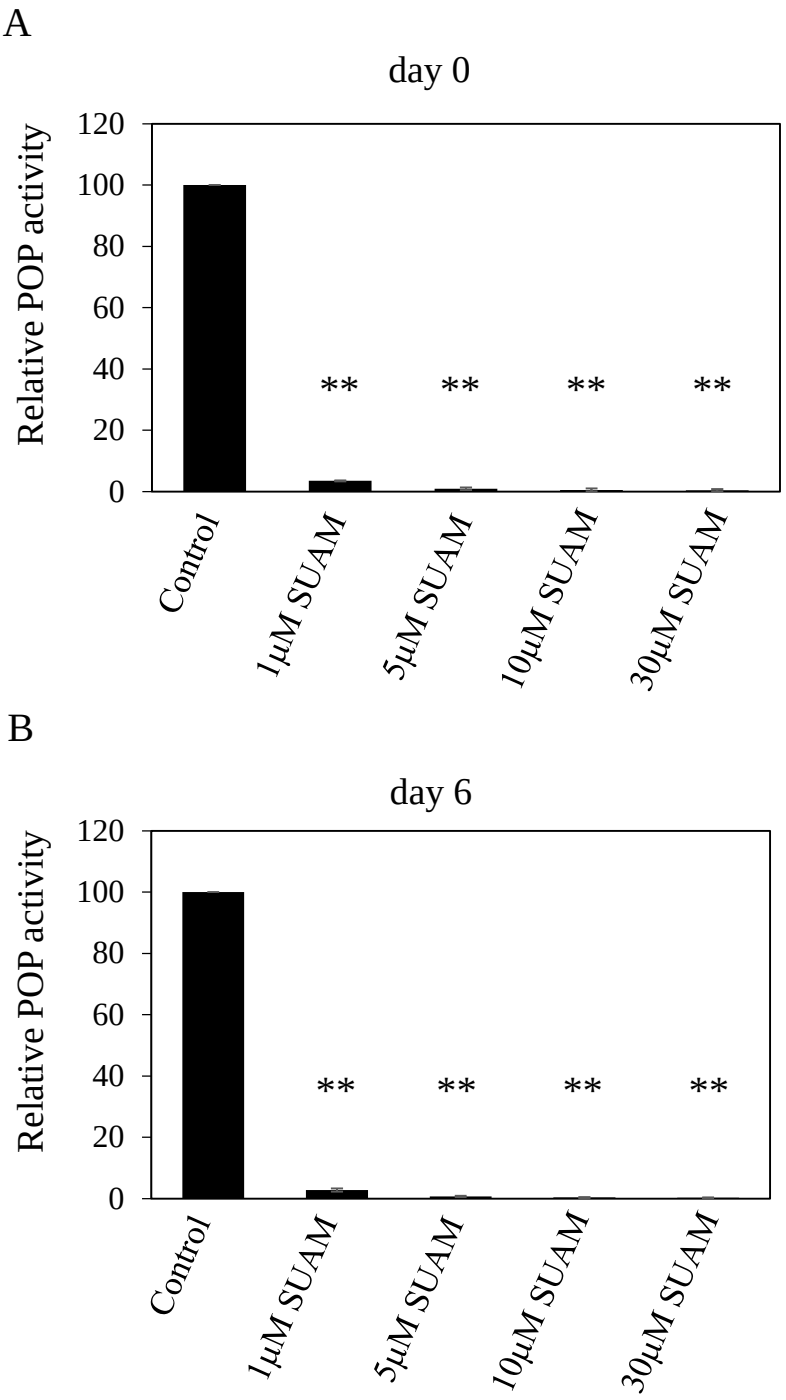


Figure 1-4. The inhibitory effects of SUAM-14746 on POP in TSCs.

Soluble extracts of TSCs at (A) day 0 and (B) day 6 were incubated with 30 μM, 10 μM, 5 μM, 1 μM SUAM-14746, or DMSO (control), and POP enzymatic activity was measured. The value of control was set to 100 in both data sets, and relative POP enzymatic activity is shown. All concentrations of SUAM-14746 efficiently inhibited POP 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 < 0.01 compared to the control.

Fig. 1-5

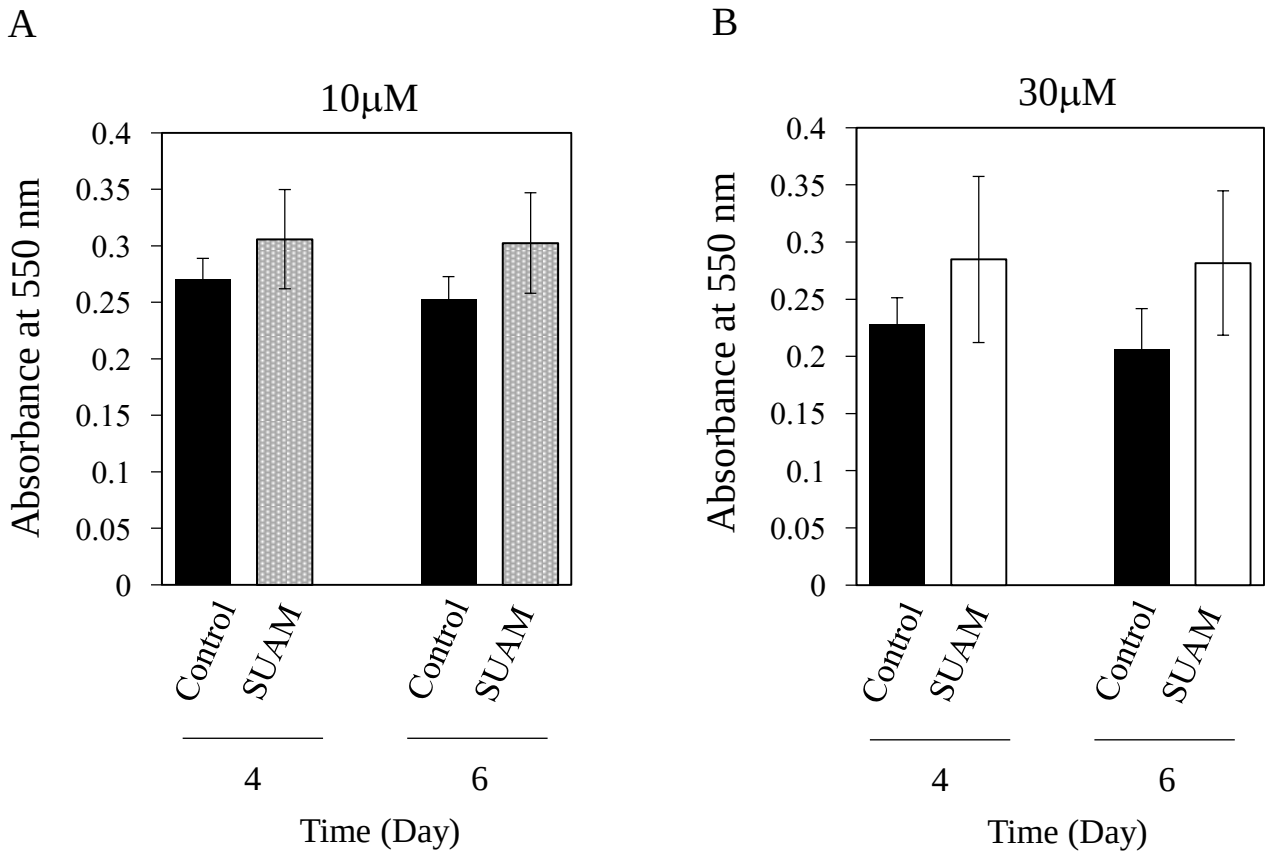


Figure 1-5. The effects of SUAM-14746 on TSC viability.

(A) 30 μM and (B) 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. 1-6

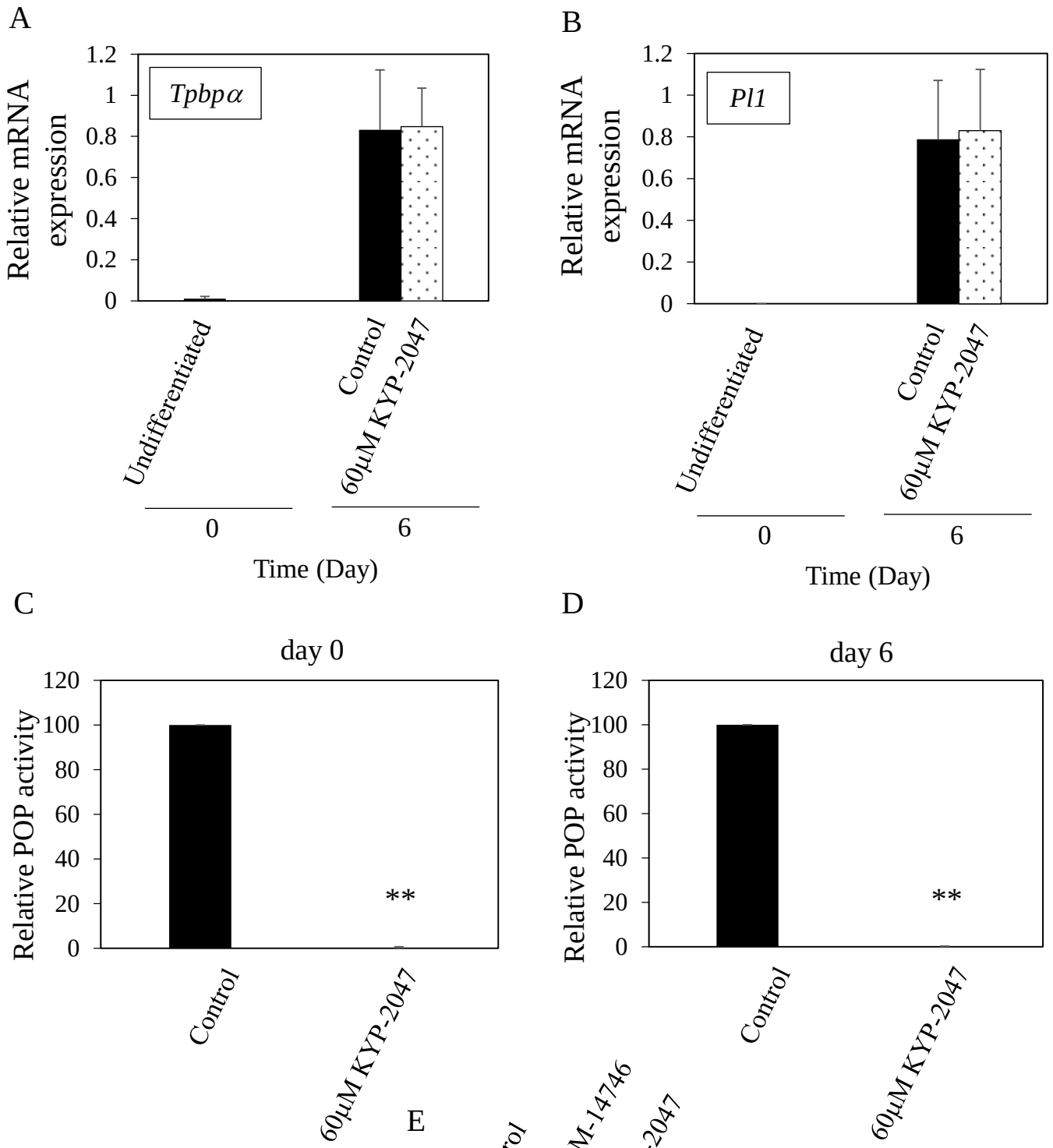

A
Relative mRNA expression
Tpbpα
1.2
1
0.8
0.6
0.4
0.2
0
Undifferentiated
Control
60μM KYP-2047
0
6
Time (Day)
B
Relative mRNA expression
Pl1
1.2
1
0.8
0.6
0.4
0.2
0
Undifferentiated
Control
60μM KYP-2047
0
6
Time (Day)
C
day 0
Relative POP activity
120
100
80
60
40
20
0
**
Control
60μM KYP-2047
D
day 6
Relative POP activity
120
100
80
60
40
20
0
**
Control
60μM KYP-2047
E
Control
SUAM-14746
KYP-2047
Oligomer
Open monomer
Compact monomer

Figure 1-6. The effect of 60 μM KYP-2047 on TSC differentiation.

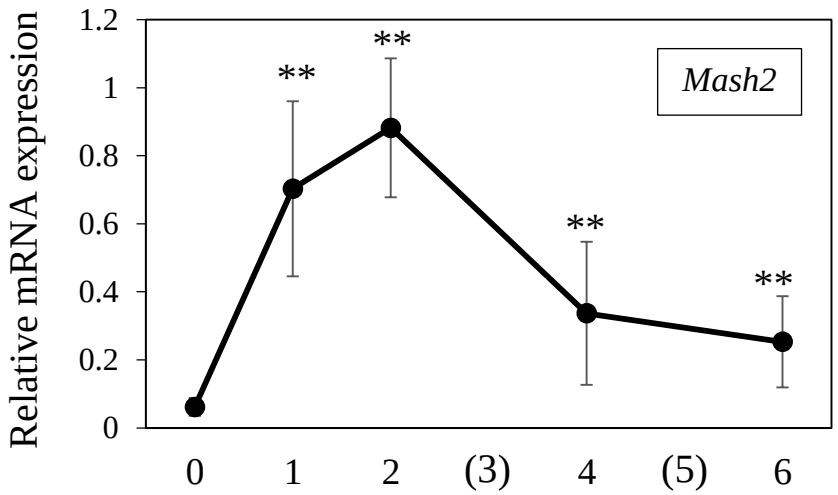
A, B: *Tpbpα* and *Pl1* expression in TSCs cultured for 6 days and in undifferentiated cells with 60 μM KYP-2047 or DMSO (control) by qRT-PCR. The data are averages from three independent experiments, and were analyzed by Student's *t*-test to compare the sample with 60 μM KYP-2047 to the control. Unlike 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 POP 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 < 0.01 compared to the control.

E: Native-PAGE analysis was conducted to investigate the conformation of POP protein with SUAM-14746, KYP-2047, and DMSO (control). Four microgram of recombinant POP protein was mixed with excess amount of an inhibitor or DMSO and electrophoresed in 10% acrylamide gel using the loading buffer without SDS and 2-mercaptoethanol. The gel was stained with CBB for 1 hr. Three forms of POP protein are indicated by arrows. In the control lane, two bands were detected as an oligomer and an open monomer, but with SUAM-14746 and with KYP-2047, a compact monomeric band was observed at a lower position. The image is a representative result in three independent experiments.

Fig. 2-1

A


Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
**
Mash2
0
1
2
(3)
4
(5)
6

B

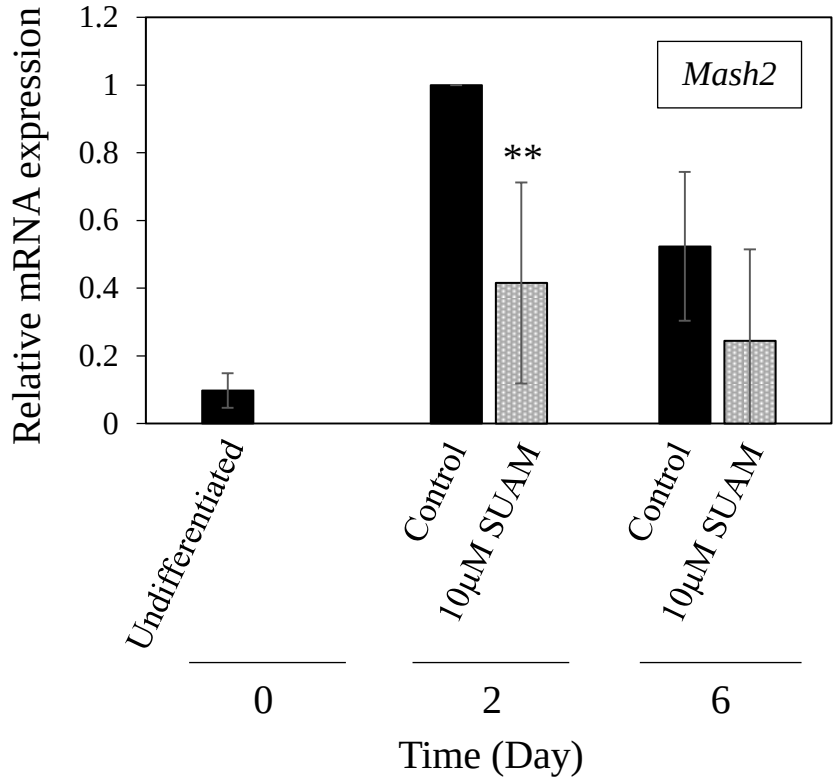

Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
**
Mash2
Undifferentiated
Control
10μM SUAM
Control
10μM SUAM
0
2
6
Time (Day)

Figure 2-1. The expression of *Mash2* gene during TSC Differentiation with SUAM-14746.

A: *Mash2* mRNA expression during TSC differentiation without a POP inhibitor by qRT-PCR. The experiment was done and the results are presented as in Figure 1-1.

B: *Mash2* expression in TSCs cultured for 2 or 6 days with 10 μM SUAM-14746 or DMSO (control) and in undifferentiated cells by qRT-PCR. The inhibitor treatment was done and the data are indicated as in Figure 1-3. The graph shows that the *Mash2* expression was significantly decreased by SUAM-14746 at day 2. 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 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 to the control.

Fig. 2-2

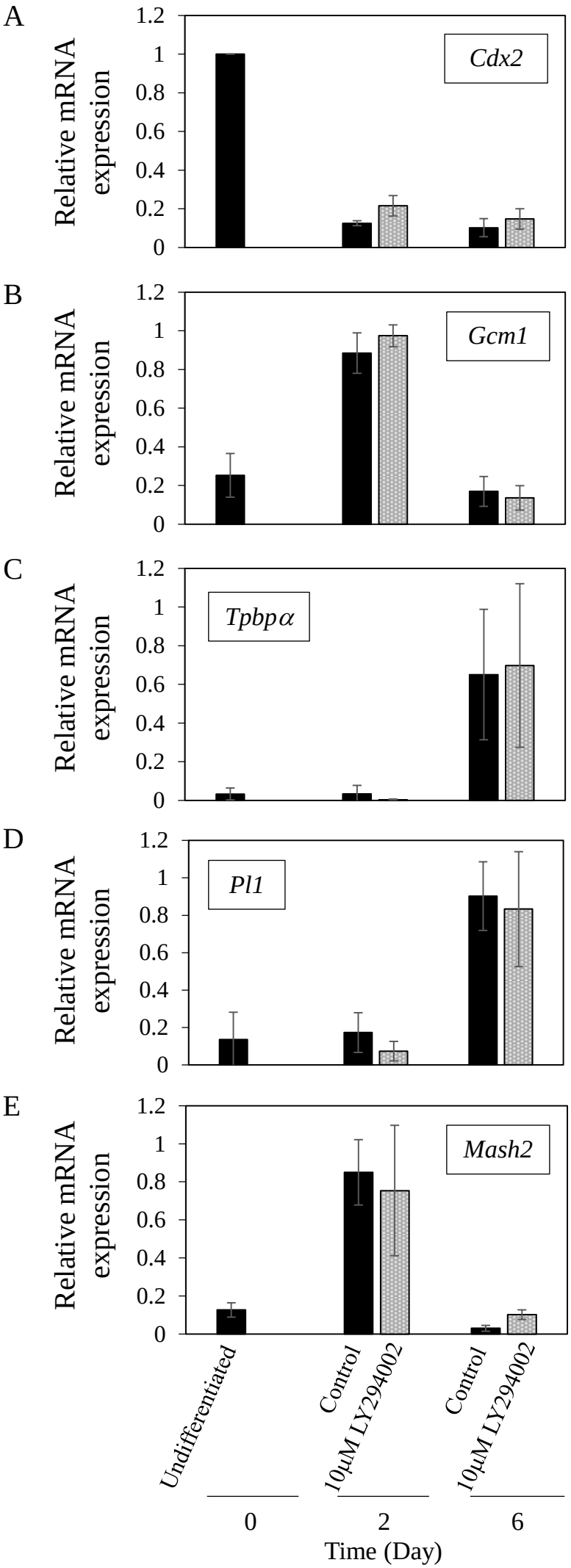

A
B
C
D
E
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
Cdx2
Gcm1
Tpbpα
Pl1
Mash2
Undifferentiated
Control
10μM LY294002
Control
10μM LY294002
0
2
6
Time (Day)

Figure 2-2. The expression of placental marker genes and *Mash2* gene in TSCs treated with 10 μM LY294002.

Samples treated with 10 μM LY294002 or DMSO (control) were collected at the indicated days and analyzed by qRT-PCR. There were no difference in any of the marker gene expression between LY294002 and control. The *Mash2* expression was not affected either. 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 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.

A: *Cdx2* for the undifferentiated cell, B: *Gcm1* for SynT, C: *Tpbpα* for SpT, D: *Pl1* for TGC and E: *Mash2*

Fig. 3

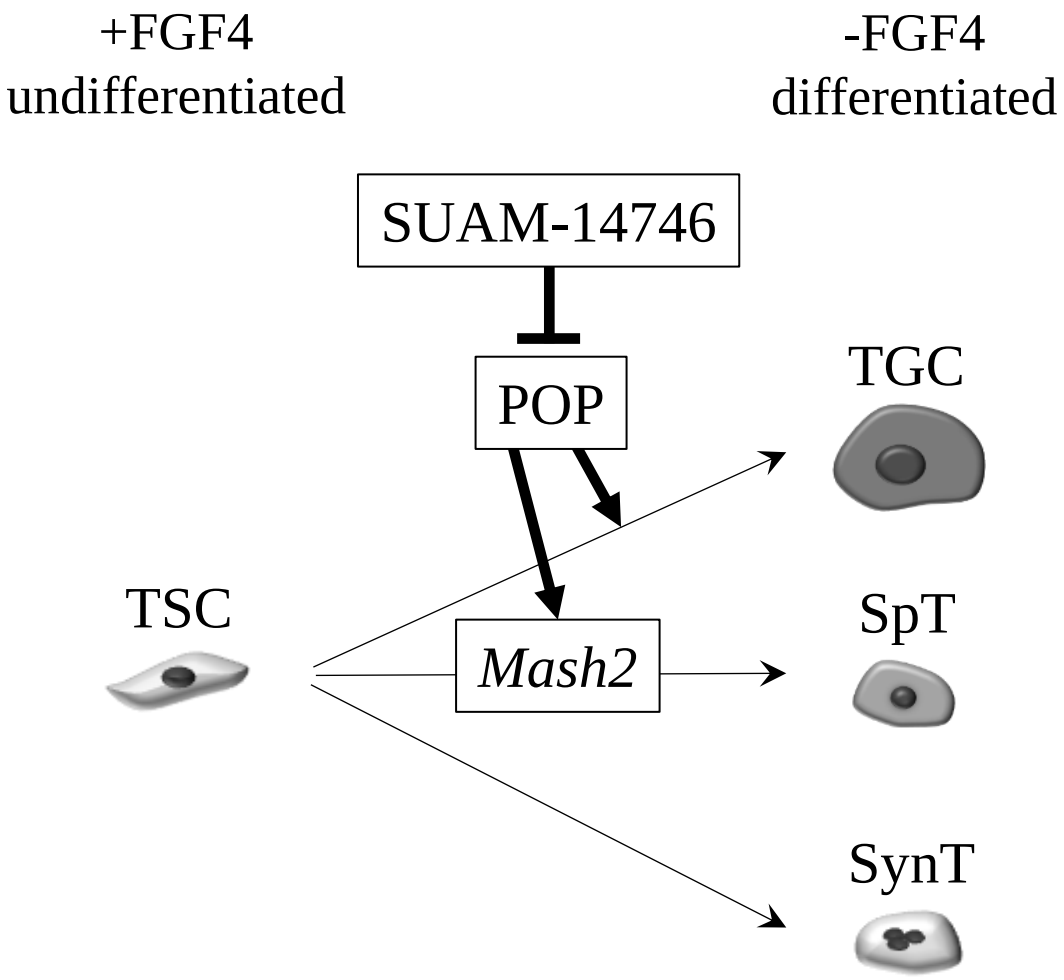


Figure 3. A model of POP's role in TSC differentiation.

TSCs normally differentiate into three types of cells, TGC, SpT, and SynT, by removing FGF4 from the culture medium. POP positively controls TGC and SpT differentiation, but POP is not involved in SynT differentiation. The POP-specific inhibitor SUAM-14746 impairs TGC and SpT differentiation. For SpT differentiation, POP regulates the expression of a master gene, *Mash2*.