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Type 3 iodothyronine deiodinase is expressed in human induced pluripotent stem cell derived cardiomyocytes

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ABSTRACT

Aims: Type 3 iodothyronine deiodinase (D3), which converts thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3) to 3,3′,5′-triiodothyronine (rT_3) and 3,3′-diiodothyronine (T_2), respectively, inactivates thyroid hormones. We investigated the expression and regulation of D3 in human cardiomyocytes which were differentiated from human induced pluripotent stem cells (hiPSCs).

Main methods: We characterized D3 activity using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. D3, myosine heavy chain α and β (MHCα and β, respectively), sarcoplasmic reticulum calcium ATPase (SERCA), and phospholamban (PLB) mRNA levels were analyzed by quantitative real-time PCR (qPCR) in hiPSC-derived cardiomyocytes (hiPS-CMs).

Key findings: We identified enzyme activity that catalyzes the conversion of T_3 to T_2 in both hiPS-CMs and hiPSCs, which showed characteristics compatible with those for D3. D3 mRNA was identified in these cells using qPCR analysis. T_3 and hypoxia mimetics such as $CoCl_2$ and DFO, increased the D3 mRNA level in both hiPS-CMs and hiPSCs. Addition of iopanoic acid, a competitive inhibitor of iodothyronine deiodination, in the culture medium of hiPS-CMs, increased the mRNA levels such as MHCα and β, SERCA, and PLB induced by T_3.

Significants: Our findings indicate that D3 is expressed in hiPS-CMs, and may decrease the intracellular T_3 concentration, and may decrease the expression of cardiac genes such as MHCα and β, SERCA, and PLB in hiPS-CMs.

1. Introduction

Thyroid hormones regulate cardiac function through the direct effects on cardiac myocyte as well as the indirect effects on the peripheral vasculature [1]. The prohormone thyroxine (T_4) is converted to the active hormone 3,5,3′-triiodothyronine (T_3) by type 1 and 2 iodothyronine deiodinases (D1 and D2, respectively) [2,3]. On the other hand, type 3 iodothyronine deiodinase (D3) inactivates both T_4 and T_3 to 3,3′,5′-triiodothyronine (rT_3) and 3,3′-diiodothyronine (T_2), respectively [4]. Since D1, D2 and D3 are differentially expressed in individual tissues, T_3 concentration is regulated in tissue-specific manner.

The biological action of thyroid hormone occurs by the entry of T_4 and T_3 into the cell by transporters such as monocarboxylate transporter 8 (MCT8) and the binding of T_3 to specific receptors in the nucleus such as thyroid hormone receptor α and β (TRα and TRβ), which regulate the transcription of target genes such as myosine heavy chains (MHCs), sarcoplasmic reticulum calcium ATPase (SERCA) and phospholamban (PLB) in the heart [5].

Although D3 expression was negligible in healthy heart, increased cardiac D3 expression has been observed in animal models of hypertrophic, dilated and ischemic cardiomyopathy [6–10]. Fetal-type cardiac genes such as atrial natriuretic peptide are abundantly expressed in fetal heart, down-regulated in the adult heart, and re-expressed in the diseased heart. The expression profile of D3 is consistent with the idea that D3 is one of such fetal-type cardiac genes. However, information about deiodinases expression in human cardiomyocytes has been few so far [11].

To investigate the thyroid hormone actions in human cardiomyocytes, availability of primary human cardiomyocytes is severely limited. Human cardiomyocytes may be differentiated from human embryonic stem (ES) cells *in vitro* [12], although the use of human ES cells is ethically and legally limited. Human induced pluripotent stem cells (hiPSCs) are generated from other cell types by introducing four transcription factors, and are similar to human ES cells in morphology, proliferative capacity, and gene expression profiles [13,14]. Recently, we have succeeded in the efficient generation of hiPSC-derived

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cardiomyocytes (hiPS-CMs) which showed structural and functional cardiomyocyte features such as clear sarcomere formation, self-beating and action potentials resembling ventricular and pacemaker cells [15]. To understand the regulation of thyroid hormone actions in human cardiomyocytes, we investigated the expression and regulation of iodothyronine deiodinases, TRs and MCT8 in hiPSCs and hiPS-CMs.

2. Materials and methods

2.1. Materials

Human basic fibroblast growth factor (hbFGF) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Recombinant human Activin A, human bone morphogenetic protein 4 (BMP-4), and human Dkk-1 were purchased from R&D Systems (Minneapolis, MN). Accumax was purchased from Innovative Cell Technologies (San Diego, CA). Anti-vascular cell adhesion molecule 1 (VCAM1) antibody conjugated with allophycocyanin (APC) was purchased from BioLegend (San Diego, CA). Deferoxamine (DFO) was purchased from Abcam (Cambridge, MA). Thyroid hormone standards were purchased from the following vendors:T_4, T_3, and 3,3′-diiodothyronine (3,3′-T_2) from Sigma-Aldrich (St. Louis, MO), rT_3 from Calbiochemical (La Jolla, CA), T_2 from Tokyo Kasei (Tokyo, Japan). $CoCl_2$ and iopanoic acid (IOP) were purchased from Nakarai Tesque (Kyoto, Japan). [^{125}I]T_4 and [^{125}I]rT_3 were purchased from Perkin Elmer (Waltham, MA).

2.2. Cell culture

hiPSC line 201B6 was previously described [14]. 201B6 was used as the human pluripotent cell representative in all experiments. These cells were adapted and maintained in mouse embryonic fibroblast conditioned medium (MEF-CM) supplemented with 4 ng/mL hbFGF as described previously [15].

Cardiomyocyte differentiation was induced as described previously [15]. In brief, cells were seeded in MEF-CM plus 4 ng/mL hbFGF for 2–3 days before induction. To induce cardiac differentiation, we replaced MEF-CM with RPMI + B27 medium (RPMI1640, 2 mM L-glutamine, ×1 B27 supplement without insulin) supplemented with 100 ng/mL of human Activin A for 24 h, followed by 10 ng/mL human BMP-4 and 10 ng/mL hbFGF for 4 days with no culture medium replacement. The culture medium was subsequently replaced with RPMI + B27 supplemented with 100 ng/mL of human Dkk1 for 2 days. At day 7, the culture medium was changed to RPMI + B27 without supplementary cytokines; culture medium was refreshed every 1–2 days. Beating cells appeared at day 8–9 and were harvested by day 11.

2.3. Cell sorting

After differentiation, cells were dissociated by incubation with Accumax. For magnetic activated cell sorting (MACS; Miltenyi), cells were stained with anti-VCAM1 antibody conjugated with APC followed by anti-APC microbeads (Miltenyi). We used VCAM1-positive cells by MACS as hiPS-CMs in the present study.

2.4. Measurement of deiodinases activities

Cells were sonicated in 0.1 M potassium phosphate, pH 6.9, and 1 mM EDTA. D1 and D2 activity assays were performed as previously described using [^{125}I]rT_3 and [^{125}I]T_4, respectively [16]. D3 activity was assayed as previously described using 1–100 μg cellular protein, 30 mM dithiothreitol (DTT), several concentrations of T_3 in the presence or absence of 1 mM 6-propyl-2-thiouracil (PTU) or 1 mM IOP [17]. After incubation at 37 °C for 2 h, reaction was stopped by the addition of same volume of cold methanol. The mixture was centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was decanted into a new tube and was stored at −25 °C. The products of deiodination were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The protein concentration was measured according to the method of Bradford using bovine serum albumin as a standard. The deiodinating activity was calculated as femtomoles of T_2 produced/mg protein/min from T_3.

2.5. Liquid chromatography-tandem mass spectrometry

A stock solution of each thyroid hormone standard was prepared at 1 mg/ml using 40% ammonium hydroxide (v/v) in methanol. Every time a batch of samples was injected into the LC-MS/MS instrument, the calibration standards, ranging from 0.1 to 100 ng/ml, were freshly prepared from the stock solution through dilution with methanol.

An API 3200 tandem mass spectrometer system (AB Sciex, MA) equipped with Shimadzu HPLC system and ZORBAX Extend-C18 chromatographic column (Agilent) was used for measurement of thyroid hormones. The negative ion multiple reaction monitoring (MRM) mode was used. Nitrogen was used as both curtain and collision gas. MS/MS parameters were optimized for every thyroid hormone standard, by infusion of 1 μg/ml standard solution as described previously [18]. The mobile phase was 0.01% (v/v) ammonium hydroxide in methanol and deionized water; the gradient parameters were described previously [18].

2.6. Quantitative real-time polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen). One microgram of RNA was reverse transcribed into cDNA with Superscript III Reverse Transcriptase SuperMix (Invitrogen). Quantitative real-time PCR (qPCR) was performed on a Rotor-Gene Q (Qiagen). All experiments were performed with TaqMan Gene Expression Master Mix (Applied Biosystems). All TaqMan probes listed in Table 1 were purchased from Applied Biosystems. Expression level was calculated by the ddCt method and normalized to ribosomal 18S RNA.

2.7. Statistical analysis

Data are expressed as mean ± SD. The Student's *t*-test was used to compare differences between two groups. One-way ANOVA was used to compare more than two groups, followed by the Student-Newman-Keuls *post hoc* test to detect differences between groups.

3. Results

3.1. Characteristics of iodothyronine deiodinases activity in hiPSCs and hiPS-CMs

After incubation hiPSCs and hiPS-CMs sonicates with T_3 in the presence of 30 mM DTT, LC-MS/MS analysis revealed only 2 clear peaks that corresponded to T_3 and T_2. Monodeiodination of T_3 was dependent on the protein concentration and incubation period for up to 2 h. Incubation at 0 °C or preheating the cell sonicate at 56 °C for 30 min

Table 1
TaqMan probe list.

Gene symbol	Probe id
D3	Hs00956431_s1
MCT8	Hs00185140_m1
MHCα	Hs01101425_m1
MHCβ	Hs00293096_m1
PLB	Hs01848144_sl
SERCA	Hs00544877_ml
TRα	Hs00268470_m1
TRβ	Hs00230861_m1
18S	Hs99999901_s1

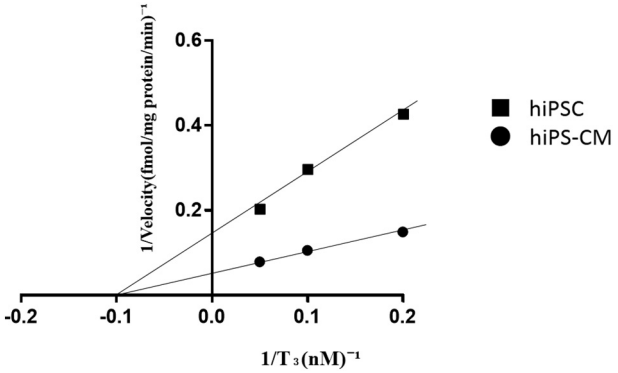

Fig. 1. Characterization of D3 activity in hiPSCs and hiPS-CMs. Deiodinating activity was measured in hiPSCs and hiPS-CMs sonicates. Cell sonicates were incubated for 2 h with various T_3 concentrations in the presence of 30 mM DTT. Using the double reciprocal plot of T_3 deiodination, the kinetic constants for T_3 were calculated as K_m = 8.5 (hiPS-CMs) and 11.1 nmol/L (hiPSCs), respectively, and V_{max} = 17.9 (hiPS-CMs) and 7.5 fmoles T_2 produced/mg prot/min (hiPSCs), respectively.

completely abolished the deiodination. The deiodinating activity was not influenced by 1 mM PTU, but it was completely inhibited by 1 mM IOP. Using a double reciprocal plot, we calculated the T_3 kinetic constants in hiPSCs and hiPS-CMs and found that K_m values were equal to 8.5 (hiPS-CMs) and 11.1 nmol/L (hiPSCs), respectively, and that the maximum velocity (V_{max}) values were equal to 17.9 (hiPS-CMs) and 7.5 fmoles T_2 produced/mg protein/min (hiPSCs), respectively (Fig. 1). These results indicate that T_3 deiodinating activity is present in both hiPS-CMs and hiPSCs and the characteristics of this activity were compatible with D3.

On the other hand, neither D1 nor D2 activities were detected in both hiPSCs and hiPS-CMs.

3.2. D3 mRNA expression in hiPSCs and hiPS-CMs

qPCR demonstrated D3 mRNA expression in both hiPSCs and hiPS-CMs (Fig. 2). The D3 mRNA level in hiPS-CMs was approximately 2.4-fold higher than that in hiPSCs.

3.3. Effect of T_3 on the expression of D3 mRNA

We investigated the effect of T_3 on the D3 mRNA level in the hiPS-CMs. Since the culture medium of hiPS-CMs with B27 supplement contains 10^{-9} M T_3, cells were incubated without B27 supplement in the absence (hiPS-CMs − T_3) or the presence of 10^{-8} M T_3 (hiPS-CMs + T_3) for 48 h. D3 mRNA level in hiPS-CMs + T_3 was approximately 2.5-fold higher than that in hiPS-CMs − T_3 (Fig. 3).

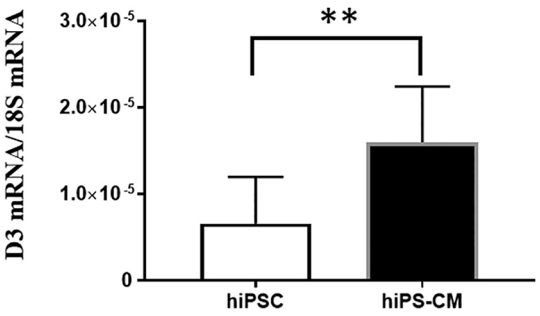

Fig. 2. Comparison between D3 mRNA levels in hiPSCs and hiPS-CMs. D3 mRNA levels are represented by the mean ± SD of 9 wells. $^{**}p < 0.01$.

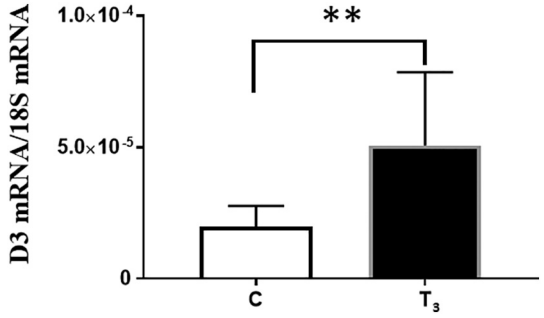

Fig. 3. Effect of T_3 on D3 mRNA level in hiPS-CMs. hiPS-CMs were incubated without B27 supplement in the absence (hiPS-CMs − T_3) or the presence of 10^{-8} M T_3 (hiPS-CMs + T_3) for 48 h. D3 mRNA are represented by the mean ± SD of 9 wells. $^{**}p < 0.01$.

3.4. Effect of hypoxia mimetics on the expression of D3 mRNA

Hypoxia-inducible factor (HIF) is up-regulated *in vitro* by hypoxia mimetics such as $CoCl_2$ and DFO, which inhibit HIF ubiquitination and lead to its accumulation under normoxic conditions [19]. We investigated the effects of $CoCl_2$ and DFO on D3 mRNA levels in both hiPSCs and hiPS-CMs. Cells were incubated with 10^{-4} M $CoCl_2$ or 10^{-4} M DFO for 24 h. D3 mRNA levels in both hiPSCs and hiPS-CMs cultured with $CoCl_2$ or DFO were significantly higher than those in the cells without hypoxia mimetics (Fig. 4).

3.5. Effect of T_3 on the mRNAs of MHCα, MHCβ, SERCA, and PLB in hiPS-CMs in the presence or absence of IOP

We next sought to determine whether the endogenous D3 could decrease the intracellular T3 concentration in hiPS-CMs and alter the expression of the T_3 responsive cardiac genes. To test this, we exposed hiPS-CMs to IOP, a competitive inhibitor of iodothyronine deiodination, or vehicle control (DMSO). hiPS-CMs, which were exposed to 20 μM of IOP or DMSO, were incubated without B27 supplement in the absence (hiPS-CMs − T_3) or the presence of 10^{-8} M T_3 (hiPS-CMs + T_3) for 48 h. The IOP concentration used (20 μM) was optimized in prior experiments to inhibit cellular deiodination by whole-cell *in situ* deiodination assay [20].

The mRNA levels of MHCα, MHCβ, SERCA, and PLB in the hiPS-CMs + T_3 in the absence of IOP were significantly higher than those in the hiPS-CMs − T_3 in the absence of IOP (hiPS-CMs − T_3 − IOP), respectively (Fig. 5A–D). Although the mRNA levels of MHCα, MHCβ, SERCA, and PLB in the hiPS-CMs − T_3 in the presence of IOP (hiPS-CMs − T_3 + IOP) were decreased to approximately 70% (MHCα, MHCβ and SERCA) or 30% (PLB) of these mRNA levels in the hiPS-CMs − T_3 − IOP, respectively, the mRNA levels of MHCα, MHCβ, SERCA, and PLB in the hiPS-CMs + T_3 in the presence of IOP were

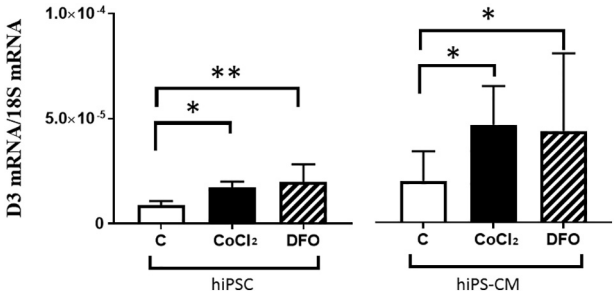

Fig. 4. Effects of hypoxia mimetics on D3 mRNA levels in hiPSCs and hiPS-CMs. hiPSCs and hiPS-CMs were incubated with 10^{-4} M $CoCl_2$ or 10^{-4} M DFO for 24 h. D3 mRNA levels are represented by the mean ± SD of 4 wells. $^{*}p < 0.05$, $^{**}p < 0.01$.

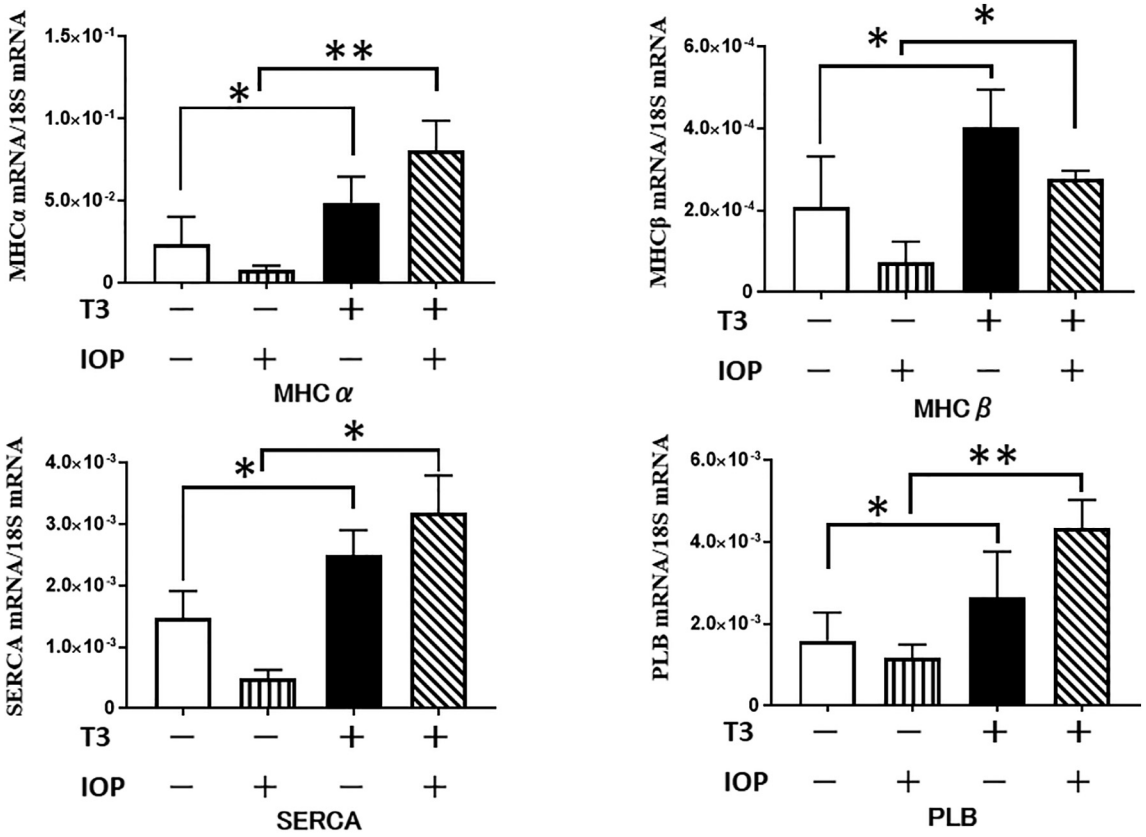

Fig. 5. Effect of T_3 on the mRNA levels of MHCα and β, SERCA, and PLB in the absence or presence of IOP in hiPS-CMs. hiPS-CMs, which were exposed to 20 μM of IOP or DMSO, were incubated without B27 supplement in the absence (hiPS-CMs − T_3) or the presence of 10^{-8} M T_3 (hiPS-CMs + T_3) for 48 h. mRNA levels are represented by the mean ± SD of 4 wells. $^*p < 0.05$, $^{**}p < 0.01$.

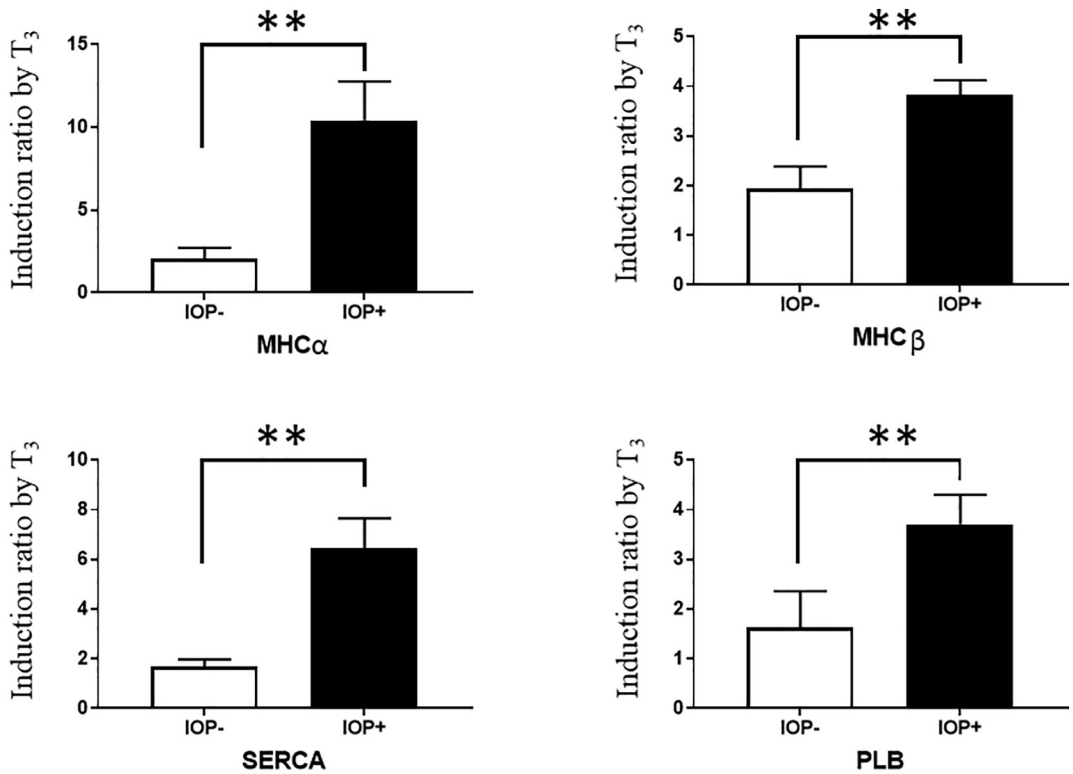

Fig. 6. Comparisons between the mRNA induction ratio by T_3 in hiPS-CMs cultured with IOP and that without IOP. hiPS-CMs, which were exposed to 20 μM of IOP or DMSO, were incubated without B27 supplement in the absence (hiPS-CMs − T_3) or the presence of 10^{-8} M T_3 (hiPS-CMs + T_3) for 48 h. Induction ratios by T_3 in hiPS-CMs cultured with or without IOP are represented by the mean ± SD of 4 wells. $^{**}p < 0.01$.

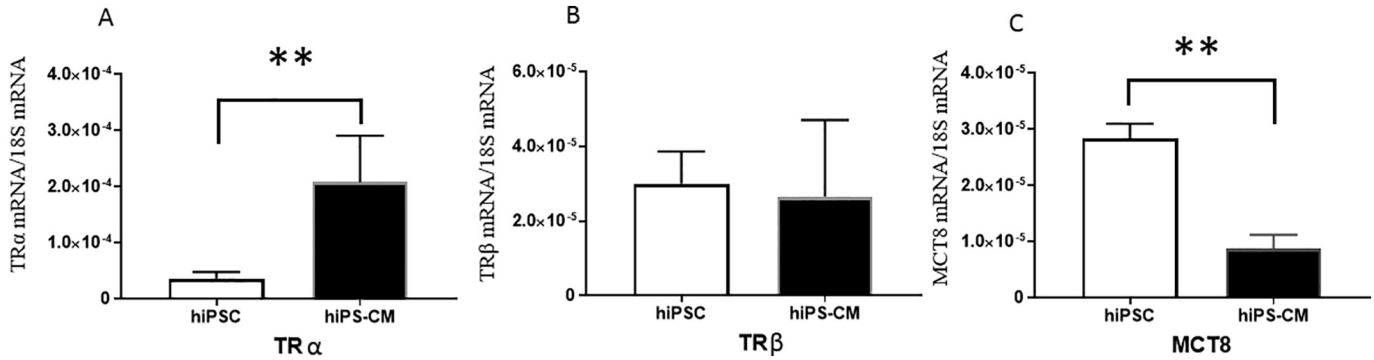

Fig. 7. Comparisons between mRNA levels of thyroid hormone receptors and thyroid hormone transporter in hiPSCs and hiPS-CMs. mRNA levels are represented by the mean ± SD of 7 wells. $^{**}p < 0.01$.

significantly higher than those in the hiPS-CMs − T_3 + IOP, respectively (Fig. 5A–D). Interestingly, the increments of the mRNA level of MHCα, MHCβ, SERCA, and PLB by T_3 in the presence of IOP were significantly higher than those values in the absence of IOP, respectively (Fig. 6A–D).

3.6. Thyroid hormone receptors and MCT8 mRNAs in hiPSCs and hiPS-CMs

qPCR demonstrated that both TRα and TRβ mRNAs were expressed in hiPSCs and hiPS-CMs (Fig. 7A, B). In hiPSCs, the mRNA level of TRα was not significantly different from that of TRβ. TRα mRNA level in hiPS-CMs was approximately 5.7-fold higher than that in hiPSCs. However, TRβ mRNA level in hiPS-CMs was not significantly different from that in hiPSCs.

qPCR also demonstrated that MCT8 mRNA was expressed in both hiPSCs and hiPS-CMs. The mRNA level of MCT8 in hiPS-CMs was approximately 30% of that in hiPSCs (Fig. 7C).

4. Discussion

We demonstrated that T_3 deiodinating activity was present in both hiPSCs and hiPS-CMs. T_3 deiodinating activity in these cells had a low K_m for T_3 and was not affected by 1 mM PTU. Therefore, the characteristics of T_3 deiodinating activity in hiPSCs and hiPS-CMs are compatible with those of D3 [2]. We also identified D3 mRNA in both hiPSCs and hiPS-CMs, which confirmed D3 expression in these cells.

The V_{max} value of D3 for T_3 in hiPS-CMs was approximately 2.4-fold higher compared with that in hiPSCs, though the K_m value in hiPS-CMs was similar with that in hiPSCs. The D3 mRNA levels in hiPS-CMs were approximately 2.4-fold higher compared with that in hiPSCs. The difference in the V_{max} values of D3 between hiPSCs and hiPS-CMs appears to be determined at the transcriptional level, as the increment of V_{max} value of D3 in hiPS-CMs was similar with that in D3 mRNA level.

D3 mRNA expression is regulated by multiple factors such as T_3, mitogen-activated protein kinase, transforming growth factor β, sonic hedgehog, HIF and microRNAs [8,20–24]. In the present study, we have shown that T_3 up-regulates D3 mRNA level in hiPS-CMs. We have also shown that hypoxia mimetics which up-regulated HIF-1 expression, increased the D3 mRNA levels in hiPS-CMs. Since HIF-1 expression is increased in human hearts with hypertrophic [25] and dilated cardiomyopathy [26], it is supposed that D3 expression may be induced in these human hearts.

The mRNA levels of MHCα and β, SERCA, and PLB were increased during the differentiation into hiPS-CMs. On the other hand, it is considered that the expression of MHCα and SERCA are positively regulated by T_3, whereas the expression of MHCβ and PLB are negatively regulated by T_3 [1]. In the present study, T_3 increased the expression of not only MHCα and SERCA, but also MHCβ and PLB in hiPS-CMs. It has been reported that T_3 promotes the maturation of hiPS-CMs [27,28]. Therefore, we may suggest that the increase of the mRNA levels of MHCβ and PLB by T_3 in hiPS-CMs may be due to the greater effect of T_3 on the promotion of hiPS-CMs maturation than the direct T_3 effect on these gene regulation. To inhibit endogenous D3 activity, IOP was used in the present study. Interestingly, inhibition of endogenous D3 activity by IOP in hiPS-CMs increased the effects of T_3 on the expression of MHCα, MHCβ, SERCA and PLB, though IOP decreased these expressions in some degree by itself. These results suggest that endogenous D3 may decrease the intracellular T_3 concentration and suppress the expression of these T_3-responsive genes in hiPS-CMs.

In general, D3 expression is thought to be high in embryonic cells and decreased with differentiation. Therefore, our present results are unexpected. When transcriptionally compared to native human cardiac muscle cells, hiPS-CMs most closely resemble cells from embryonic or fetal heart [29]. It has been thought that the physical stimuli influencing hiPS-CMs through mechanical and bioelectrical transduction may be necessary to promote more developmentally mature cardiomyocytes [29]. The expression of D3 in hiPS-CMs may reflect the immature nature of hiPS-CMs as cardiac muscle cells. The physiological role of D3 during cardiomyocyte differentiation awaits further investigation.

The expression of D3 in human ES cells or hiPSCs has not been reported previously. The physiological significance of the D3 expression in hiPSCs is not clear at this time, though D3 activity of the hiPSCs determined in this study is equivalent to that of ECC-1 human endometrium carcinoma cell line [30].

Although D2 expression was demonstrated in human heart in the previous study [31,32], we could not detect D2 activity in hiPS-CMs in the present study. Nkx-2.5 and GATA-4 are among the main regulators of tissue-specific transcription in the heart [33]. Two sites which bind Nkx-2.5 were found in the human *Dio2* promotor [34]. It has been shown that Nkx-2.5 and GATA-4 synergistically played prime roles in human *Dio2* gene expression [34]. Since the Nkx-2.5 mRNA level in hiPS-CMs was approximately 30% of that of human fetal heart [35,36], reduced expression of Nkx-2.5 may explain the reasons why we could not detect the D2 activity in hiPS-CMs.

In mouse ES cells, TRα mRNA level was higher than TRβ [37]. Furthermore, TRαmRNA level was increased during differentiation into cardiomyocytes, although TRβ mRNA level was not increased [37]. In the present study, both TRα and β mRNA levels were equivalently expressed in hiPSC. Interestingly, TRα mRNA level in hiPS-CMs was significantly increased, whereas TRβ mRNA was not significantly different compared with that of hiPSCs. We suggest that the ontogeny of TR isoform in human cardiomyocytes may be similar with that in rodents.

MCT8 is an active and principal thyroid hormone transporter [38] and is expressed in the mouse and human heart [39,40]. It was reported that MCT8 expression was upregulated in a rat model of diabetic cardiomyopathy [41]. In the present study, MCT8 mRNA level decreased during differentiation of hiPSCs to hiPS-CMs. Further investigations are

necessary to clarify the regulation of MCT8 expression in hiPS-CMs, as the detailed information about it is scant at present [42].

5. Conclusion

In summary, our present results indicate that D3 is expressed in hiPS-CMs, and may decrease the intracellular T_3 concentration, and may decrease the expression of T_3-responsive genes such as MHCα and β, SERCA, and PLB in these cells. Further examinations are necessary to clarify the roles of D3 in hiPS-CMs and in differentiation of hiPSCs to mature hiPS-CMs.

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Author disclosure statement

The authors have nothing to disclose.

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