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Upregulations of *Gata4* and Oxytocin Receptor Are Important in Cardiomyocyte Differentiation Processes of P19CL6 Cells

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Abstract Oxytocin induces P19 cells to differentiate into cardiomyocytes possibly through the oxytocin/oxytocin receptor system. We added oxytocin to the growth medium of P19CL6, a subline of P19, but they did not differentiate into cardiomyocytes as indicated by RT-PCR and Western blotting results. During the cardiac commitment time of P19CL6 cells, the mRNA expression levels of the oxytocin receptor were upregulated by the addition of oxytocin as well as DMSO, but an upregulation of *Gata4* expression levels was only observed for the cells induced by DMSO. The in silico analysis of the upstream sequence of the oxytocin receptor predicted putative binding sites for Gata4 and Nkx2.5. These results suggest that upregulations of the oxytocin receptor and *Gata4* are important for cardiomyocyte differentiation processes. J. Cell. Biochem. 100: 629–641, 2007. © 2006 Wiley-Liss, Inc.

Key words: oxytocin; oxytocin receptor; P19CL6; cardiomyocytes; cardiac commitment; DMSO

Heart is the first organ to become fully functional during the development of an organism. Its muscles are called cardiomyocytes, and once these muscles mature, they contract spontaneously and continue to beat throughout the life of an organism. Through researches using model cell lines and transgenic animals, we now know that there are a number of genes involved in cardiomyocyte differentiation processes. Among them, there is a group of genes called "early cardiac marker genes," which are often used as marker genes to indicate the beginning of the cardiomyocyte differentiation processes: GATA binding protein 4 (*Gata4*), myocyte enhancer factor 2C (*Mef2c*), NK2 transcription factor related, locus 5 (*Nkx2.5*), and T-box 5 (*Tbx5*). *Gata4* is a member of the GATA family, whose members are evolutionary-conserved transcriptional factors for regulating development. It regulates the expression of genes critical for the cardiomyocyte differentiation [Durocher et al., 1997], and its overexpression enhances the cardiomyocyte differentiation of embryonic carcinoma (EC) cells [Grepin et al., 1997]. *Mef2c* is a member of the MEF2 family that is involved in the cardiac, skeletal, and smooth muscle developments [Skerjanc et al., 1998]. During cardiomyocyte differentiation processes, MEF2 proteins act as cofactors for GATA proteins [Morin et al., 2000]. *Nkx2.5* is the homeodomain-containing transcription factor and is highly expressed during the cardiac development (as early as embryonic day 7.5 in mouse), which continues through adulthood [Komuro and Izumo, 1993; Lints et al., 1993]. It is suggested that BMP signaling may induce a cardiac-specific *Nkx2.5* expression through the activation of the composite enhancer by *Smads* and *Gata4* [Akazawa and Komuro, 2005]. *Tbx5* is a member of the T-box transcription factor family and is known to be involved in the heart tube morphogenesis and the chamber formation [Stennard and Harvey, 2005]. In human, mutations in *Tbx5* are known to cause the Holt-Oram

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syndrome, which is a genetic syndrome characterized by upper limb malformations and cardiac septation defects [Basson et al., 1997; Li et al., 1997]. Tbx5 associates with Nkx2.5 and directly binds to the promoter of the gene for cardiac-specific natriuretic peptide precursor type A (*Nppa*) to promote the cardiomyocyte differentiation [Hiroi et al., 2001]. These early cardiac marker genes interact with each other to drive cardiomyocyte differentiation processes. However, our understanding about the regulatory networks of cardiomyocyte differentiation processes still remain to be solved [Brand, 2003].

Oxyotocin (OT) is a nonapeptide hormone that is involved in a variety of physiological responses, such as the stimulation of the uterine smooth muscle contraction during parturition, the milk ejection during lactation, and the establishment of complex social and bonding behaviors related to the reproduction and care of the offspring [Gimpl and Fahrenholz, 2001; Zingg and Laporte, 2003]. All of these actions of OT are mediated through one type of receptor, that is, the oxytocin receptor (*OTR*). *OTR* is a G protein-coupled receptor and contains seven transmembrane domains. It is expressed in brain, decidua, endometrium, epididymis, heart, kidney, mammary gland, myometrium, ovary, testis, thymus, and vas deferens [Kimura et al., 2003]. Due to its organ- and tissue-specific expression patterns, it is suggested that *OTR* is regulated largely at the gene transcription level [Shojo and Kaneko, 2000].

P19 cells are EC cells and can differentiate into cell types of all three germ layers [McBurney, 1993; Skerjanc, 1999]. Because of their capability of differentiating into cardiomyocytes, they are often used as a model cell line to study cardiomyocyte differentiation processes. Paquin et al. [2002] reported that the addition of OT to the culture medium induced the cardiomyocyte differentiation of aggregated P19 cells via the oxytocin/oxytocin receptor (OT/OTR) system. Furthermore, the appearance of beating cell colonies was earlier than those cultured in the medium-containing dimethyl sulfoxide (DMSO). Moreover, an addition of oxytocin antagonist not only blocks the oxytocin-induced cardiomyocyte differentiation of P19 cells but also the DMSO-induced differentiation. Thus, it is suggested that DMSO acts via the OT pathway [Paquin et al., 2002]. To validate this model, we used a subline of P19 cells, called "P19CL6" [Habara-Ohkubo, 1996]. This cell line was established from P19 cells after culturing them for a long-term (about 6 months) under mesodermal differentiation conditions. According to Habara-Ohkubo [1996], they are morphologically similar to P19 cells. However, unlike undifferentiated P19 cells, P19CL6 cells express the mesodermal marker gene *Brachyury* but not the stage-specific embryonic antigen-1 (SSEA-1), which is a cell surface embryonic antigen whose loss of expression characterizes the differentiation of murine EC and embryonic stem (ES) cells. Another difference between P19 and P19CL6 cells is that under the condition for the neural differentiation, the outgrowth of neurofilament is very poor compared to those of P19 cells [Habara-Ohkubo, 1996]. Taken all these facts together, Habara-Ohkubo [1996] suggested that P19CL6 cells are not committed to the mesoderm but represent a developmental stage closer to differentiated cardiomyocytes than P19 cells. In terms of culturing conditions, unlike P19 cells, which require the formation of aggregates in the suspension culture, P19CL6 cells can differentiate into cardiomyocytes efficiently under the adherent condition and upon the induction by 1% DMSO. Because of their high differentiation efficiency rate (more than 70% in the area of a dish compared to 10%–15% for P19 cells), the inclusion of other cell types (e.g., neuroectoderms) can be minimized to yield more homologous cell types than P19 cells. With this cell line, a novel muscle-specific transcription factor called "myocytic induction/differentiation originator (*Midori*)" was identified [Hosoda et al., 2001].

In this study, we used P19CL6 cells to observe the differentiation ability of OT to add knowledge to the existing model of cardiomyocyte differentiation processes. By adding or not adding inducers, we found that unlike the parental cell line P19, P19CL6 cells can be differentiated into beating cardiomyocytes upon the induction by DMSO but not by OT, which are indicated by the RT-PCR results for cardiac and skeletal muscle marker genes, and Western blotting experiment for cardiac troponin I (cTnI). However, the real-time RT-PCR results for *OTR* during the cardiac commitment time do indicate that the mRNA expression levels of P19CL6 cells induced by OT were upregulated compared to those of undifferentiated cells. In addition, cells induced by DMSO

showed a continuous upregulation for *OTR*. To explain this differentiation difference between P19 and P19CL6 cells induced by OT, the coding regions of *OTR* of both cell lines were sequenced, but there was no difference between them. The in silico analysis of the upstream sequence of *OTR* showed the putative binding sites for early cardiac marker genes, such as *Gata4* and *Nkx2.5*. Combined with the results of real-time RT-PCR experiments for *Gata4*, *Nkx2.5*, and *OTR* for P19 and P19CL6 cells, we conclude that the upregulations of *Gata4* and *OTR* are important for the cardiomyocyte differentiation processes of P19CL6 cells, and the reason for the failure of OT to induce P19CL6 cells to differentiate into cardiomyocytes is due to the lack of an upregulation of *Gata4*. Thus, we proposed a new model for the differentiation processes initiated by inducers, such as OT and DMSO.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

P19 and P19CL6 cells were grown in a 100-mm culture grade dish (Falcon) supplemented with a growth medium that contains α-MEM (Sigma), 10% FBS (P19CL6 (Sigma); P19 & P19CL6 (HyClone)), and the antibodies (Nacalai Tesque) penicillin (44 U/ml) and streptomycin (44 μg/ml).

Before reaching confluence, the cells were passaged once and plated in either a 60-mm Low Cell Binding Dish (Nunc) or a 60-mm tissue culture grade dish (Falcon) for P19 and P19CL6, respectively. For P19, 2.5×10^5 cells were used for each dish with 5 ml of the growth medium supplemented with the appropriate concentration of an inducer (1% DMSO (Nacalai Tesque) or 10^{-7} M OT (Bachem)) or no inducers (Normal). After 4 days, all the cells and the medium were transferred to a 50-ml centrifuge tube, centrifuged for 2 min at 700*g*, then resuspended with the growth medium. The latter two steps were repeated once. Then, 10 ml of the growth medium were added, gently pipetted, and 1 ml of the solution was added to each well of a 24-well plate to observe for the presence of beating cells. In the case of collecting RNA, 5 ml of the growth medium were added after two centrifugations, pipetted gently, and plated onto a 60-mm tissue culture grade dish (Falcon).

For P19CL6, 3.7×10^5cells were plated with 5 ml of the growth medium. Twenty-four hours later, the medium was replaced with that containing an inducer with an appropriate concentration (1% DMSO, 10^{-6} M OT, 10^{-7} M OT, or 10^{-8} M OT) or that containing no inducers (Normal). In both cell lines, the medium was replaced with a fresh one every 2 days.

RNA Preparation and First-Strand cDNA Synthesis

To extract total RNA from P19 and P19CL6 cells, TRIzol Reagent (Invitrogen) was used after the cells were washed three times by an ice-cold PBS. For organ parts, hearts and skeletal muscles of legs, three 10-week-old male C57BL/6J mice were sacrificed and pooled. The organ parts were frozen once by the liquid nitrogen and homogenized with TRIzol Reagent.

To purify the collected total RNA, RNeasy Mini Kit (Qiagen) was used by following the manufacturer's protocol. Two micrograms of the purified RNA were reverse transcribed using SuperScript III First-Strand Synthesis System (Invitrogen) to synthesize the first-strand cDNA by following the manufacturer's protocol.

RT-PCR and Real-Time RT-PCR

Synthesized first-strand cDNA (0.5 μl) were used for each PCR reaction. For RT-PCR, Taq DNA Polymerase (Promega) and GeneAmp PCR System 9700 (Applied Biosystems) were utilized. The following thermal profile was used for all PCR experiments: 95°C for 2 min, and then appropriate cycles at 94°C for 30 s, annealing (temperature see Table I) for 30 s, and 72°C for 30 s, and terminated by a final extension at 72°C for 7 min. The amplified PCR products were separated in 2.5% agarose gel and visualized using a 302 nm UV light.

In real-time RT-PCR experiments, SYBR Premix Ex Taq (Perfect Real Time) (Takara) and Mx3000P QPCR System (Stratagene) were utilized. The same thermal profile as the one used for RT-PCR was used for 20 s, instead of 30 s, for annealing. The PCR products were subjected to a melting curve analysis and to a conventional agarose electrophoresis to exclude a synthesis of unspecific products. To quantify each experiment, first, a Ct value was quantified using a standard curve for the specific gene and relatively quantified using β-actin as an internal reference gene. Finally, it was normalized to the average expression levels of the undifferentiated samples. All the normalized

TABLE I. Primers Used in RT-PCR and Real-Time RT-PCR

Gene	5′Forward Primer3′	5′Reverse Primer3′	Size (bp)	Anneal. Temp.	Ref.
Mlc2a (BC061100)	^{47}aggctgcagccaccaagcaggcaca^{71}	^{306}cttctcccgaagagtgtgaggaag^{282}	260	64	NA
α-MHC (NM_010856)	^{1010}tggtcaccaacaacccatacgact^{1033}	^{1164}tgtcagcttgtagacaccagcctt^{1141}	155	64	Pattyn et al. [2003]
β-MHC (NM_080728)	^{5806}gccaacaccaacctgtccaagttc^{5829}	^{6008}tgcaaaggctccaggtctgagggc^{5984}	203	62	Robbins et al. [1990]; Xu et al. [1999]
Myf5 (NM_008656)	^{685}ctccaactgctctgacggcatgcc^{708}	^{962}gatagataagtctggagctgggagg^{939}	278	64	NA
MyoD (NM_010866)	^{673}aggctctgctgcgcggacc^{690}	^{1161}tgcagtcgatctctcaaagcacc^{1139}	489	60	Floss et al. [1997]
Myog (NM_031189)	^{364}cacctgtctcaaccccaac^{382}	^{808}cagcccacttaaaagccc^{790}	445	60	Ekmark et al. [2003]
GAPDH (BC083149)	^{556}accacagtccatgccatcac^{575}	^{725}catgccagtgagctttcccgt^{704}	168	60	NA
Pou5f1 (NM_013633)	^{327}aggcaggagcacgagtggaaag^{348}	^{482}gctggcaaactgttctagctcc^{460}	156	60	Goldin and Papaioannou [2003]
Nanog (XM_132755)	^{404}cctccattctgaacctgagc^{423}	^{550}tgctgggatactccactggt^{531}	147	60	Rozen and Skaletsky [2000]
Brachyury (NM_009309)	^{1064}actggttctagcctcggagtg^{1083}	^{1175}ccattgctcacagaccagag^{1156}	112	60	Rozen and Skaletsky [2000]
Gata4 (NM_008092)	^{1513}ggttcccaggcctcttgcaatgcgg^{1537}	^{1666}agtggcattgctggagttaccgctg^{1642}	154	60	Papadimou et al. [2005]
Nkx2.5 (NM_008700)	^{447}caagtgctctccctgctttcc^{466}	^{580}ctttgtccagctccactgc^{562}	134	58	Rozen and Skaletsky [2000]
Midori (AF338872)	^{4659}cacagatctgacaggagctgac^{4680}	^{4806}catgtcacagtaggtgttacactg^{4783}	148	60	NA
Tbx5 (NM_011537)	^{1603}gagcacagccaaatttacca^{1622}	^{1730}tagccgagcgatagaaggt^{1711}	128	60	Rozen and Skaletsky [2000]
OTR (D86599)	^{1433}ctttcttcgtgcagatgtgg^{1452}	^{1549}gtagatccatgggttgcag^{1530}	117	60	Rozen and Skaletsky [2000]
B-actin (X03672)	^{833}caacgagcggttccgatg^{850}	^{899}gccacaggattccatacca^{880}	67	60	NA
Mef2a (BC061128)	^{926}ttgagcactacagacctcacg^{946}	^{1033}tgcaccagtatttccaatcaa^{1013}	108	60	Kim et al. [2003]
Tbp (NM_013684)	^{592}atggtgtgcacaggagccaag^{612}	^{788}tcatagctactgaactgctg^{769}	197	60	Almeida et al. [2004]
CDS-1 (D86599)	^{444}agtgccagagtgtcccca^{461}	^{972}gtcgagcgacatcagcaa^{955}	529	58	NA
CDS-2 (D86599)	^{900}tcgtctggtcaaatactttgca^{920}	^{1412}aaggccagaacaatgatgaag^{1392}	513	58	NA
CDS-3 (D86599)	^{1333}gcacgggtcagtagtgtcaa^{1352}	^{1817}ccatatacgctccatgcaca^{1798}	485	58	Rozen and Skaletsky [2000]

In the reference column ("Ref."), "NA" indicates that these primers designed at our laboratory.

ratios were log-transformed (logarithm of base 2) to indicate up- (>0) and down-regulations (<0).

Western Blotting

The cells were washed twice with ice-cold PBS, scrapped, and transferred to a centrifuge tube. After centrifuging at 700*g* for 2 min, the cells were washed with ice-cold PBS. The previous step was repeated twice. Then, the cells were immersed in the liquid nitrogen for 30 s and store at −80°C.

After thawing on ice, the cells were lysed using 0.25 M Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol. The lysates were sonicated and centrifuged at 14,000*g* for 20 min at 4°C. The supernatants were carefully removed to a new tube. The protein concentration was determined by using the DC Protein Assay (BioRad). The proteins (150 μg) were subjected to a 12% SDS–polyacrylamide gel electrophoresis and subsequently transferred to the PVDF membranes. The membranes were incubated 2 h at room temperature in a blocking buffer (TBS-T with 3% non-fat powdered milk). They were reacted with the primary antibody anti-cTnI (MAB1691; Chemicon; diluted 1:500 in TBS-T) for 2 h at room temperature. After three washes, the secondary antibody biotinylated rabbit anti-mouse immunoglobulin (Vector BA-2000; Vector Laboratories; diluted 1:5,000 in TBS-T) was added for 1 h at room temperature. Protein bands were made visible using the Vectastain ABC Kit (Vector Laboratories).

DNA Sequencing

The fragments-containing coding regions of the mouse *OTR* gene were amplified from the cDNA of undifferentiated cells of P19 and P19CL6 by PCR with oligonucleotide primers CDS-1, -2, and -3 (Table I), where coding regions are located from 565 to 1731 (D86599). The following thermal profile was used for all PCR experiments: 95°C for 2 min, and then 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and terminated by a final extension at 72°C for 7 min. The amplified fragments were visualized by ethidium bromide staining under UV light after an electrophoresis in 2% agarose gels. A maximum of three fragments of each allele was cut out from the gels and purified. The fragments were sequenced directly by the dideoxy chain termination method with BigDye Terminator v3.1 Cycle Sequencing Kit and ABI

PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

In Silico Experiments

The upstream sequence of *OTR* was obtained from Ensembl (ENSMUSG00000049112). To search for putative transcription factor binding sites, Match (version 2.7) [Kel et al., 2003] distributed with the TRANSFAC Professional database (version 9.4) (BIOBASE Biological Databases) was used. The settings used for Match were: "muscle-specific" for "Profiles" and "minimize the sum of both error rates" (which gives an optimal number of false positives and false negatives) for "Cut-off selection."

Statistical Analyses

The data are expressed as mean $\pm$ SEM of three different samples. To derive statistical comparisons, first, a F-test for uniformity of variances was performed. $P < 0.05$ was used as a threshold value. Next, t-test for equal or unequal variances on the mean difference between targets (DMSO or OT) and control (Normal) was performed. All these statistical calculations were performed through R [Ihaka and Gentleman, 1996].

RESULTS

Differentiation Ability of OT

To test the differentiation ability of P19 and P19CL6 cells, OT was added to the growth media for both cell lines. In P19 cells induced by 10^{-7} M OT, the presence of embryoid bodies was evident under a microscope only after a day of plating. Then, at the time of medium change, day 2, embryoid bodies were visible by naked eyes. On day 4, the embryoid bodies were transferred to a tissue culture grade dish. After transferring the embryoid bodies to the culture grade dish, as early as the next day (day 5), some cells induced by OT or 1% DMSO contracted. On day 6, beatings were observed for all the wells (10 wells in the 24-well plate) tested for cells induced by OT. For cells induced by DMSO, another day (day 7) was needed for contractions by all the wells.

In contract, P19CL6 cells reached confluence on day 3, and many floating cells were observed. Although three different conditions (Normal, DMSO, and OT) were tested in this study, there was no significant difference among them up to day 5. The difference among them became clearly visible starting around day 6, where the amount of floating cells increased for DMSO compared to the other two conditions. Then, starting on day 8, the morphologies of cells cultured with DMSO changed significantly, where ring-shaped, large clusters of cells were observed. On day 10, beating cells were observed only for cells induced by DMSO. The cells were cultured to day 16, but those induced by 10^{-7} M OT did not differentiate into beating cells. Similar results were observed for higher (10^{-6} M) and lower (10^{-8} M) concentrations of OT.

Characterization of Differentiated Cells

To characterize the differentiated cells, the total RNA was extracted from P19 and P19CL6 cells on day 10 and 16, respectively. Then, RT-PCR experiments for cardiac and skeletal muscle marker genes were performed (Fig. 1A). As shown by the results of *Mlc2a*, *α-MHC*, and *β-MHC*, in P19CL6 cells, only those induced by DMSO differentiated into cardiomyocytes. The results of the RT-PCR experiments were supported by the Western blotting experiment for cTnI, where only P19CL6 cells induced by DMSO expressed cTnI (Fig. 1B).

Determination of Cardiac Commitment Time and Real-Time RT-PCR Experiments

Peng et al. [2002] reported that the first 4 days of DMSO treatment were sufficient for P19CL6 cells to differentiate into beating cardiomyocytes. In their study, a DMSO-containing medium was replaced to a DMSO-free medium on day 0, 2, 4, 6, or 10. Interestingly, this "day 4" corresponds to the fact that 4 days of the induction by an inducer are needed for P19 cells to differentiate into beating cardiomyocytes. Thus, we hypothesized that the first 4 days of treatment by an inducer may have a special meaning; in other words, some irreversible events, namely "cardiac commitment (cell commitment)", occur. To test our hypothesis, we performed more detailed analyses for the requirement of DMSO treatment to specify the time of the cardiac commitment. Figure 2A shows the results of this withdrawal. As it can be inferred from these results, at least 3.5 days of the DMSO treatment were necessary for P19CL6 cells to become beating cardiomyocytes.

Next, we extracted RNA from P19CL6 cells induced by OT and DMSO during the cardiac

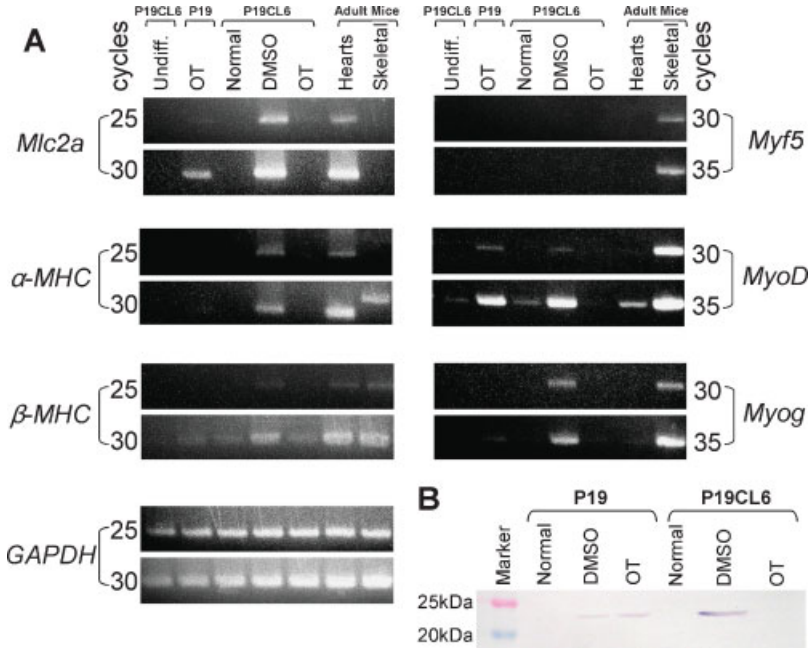

Fig. 1. Characterization of differentiated P19 and P19CL6 cells. **A**: RT-PCR results for cardiac and skeletal muscle marker genes. For cardiac marker genes, *Mlc2a*, *α-MHC*, and *β-MHC* were used, and for skeletal marker genes, *Myf5*, *MyoD*, and *Myog* (*Myogenin*) were used. *GAPDH* was used as an internal control. P19CL6 undifferentiated cells (**Lane 1**); P19 cells induced by OT (**Lane 2**); P19CL6 cells cultured under the growth medium (**Lane 3**), induced by DMSO (**Lane 4**), induced by OT (**Lane 5**); adult mouse hearts (**Lane 6**); and skeletal muscles (**Lane 7**). Differentiated samples were collected on day 10 for P19 (Lane 2) and on day 16 for P19CL6 (Lane 3–5). **B**: Western blotting experiment for cTnI. All samples were collected on day 14. P19 with no inducers (growth medium) (Lane 2), induced by DMSO (Lane 3), induced by OT (Lane 4); P19CL6 cells cultured under the growth medium (Lane 5), induced by DMSO (Lane 6), and induced by OT (Lane 7). The bands (approximately 22.5 kDa corresponding to troponin I) were observed for Lane 3, Lane 4, and strongly for Lane 6. For both results (A & B), only the representative results of three biologically independent samples used for each condition were shown.

commitment time. To obtain a detailed transcriptional view during these time periods, samples were collected in 12-h intervals. Figure 2B shows the results of the real-time RT-PCR experiments for *OTR* as well as for "stemness" (*Pou5f1* and *Nanog*), mesoderm (*Brachyury*), and early cardiac marker genes (*Gata4*, *Midori*, *Nkx2.5*, and *Tbx5*). The results for the stemness marker genes clearly indicate that their expression levels were downregulated starting from day 3 for the DMSO-treated cells. When three conditions (Normal, DMSO, and OT) were compared, the profiles of expression changes for early cardiac marker genes, except *Nkx2.5*, show that those of Normal and OT seem very much alike; however, only those of DMSO indicate a continuous increase from day 3 to 5. The results for *Brachyury* further support this observation. In the case of *OTR*, student *t*-tests performed between treated (DMSO and OT) and untreated (Normal) cells clearly indicate that the expression levels of *OTR* for the OT-treated cells increased on day 4. However, unlike those of the DMSO-treated cells, the expression level of *OTR* decreased quickly on day 4.5.

Sequencing of *OTR* Coding Regions and In Silico Analysis for Upstream Sequence of *OTR*

The previous real-time RT-PCR results for *OTR* showed that the addition of OT to P19CL6

Fig. 2. Cardiac commitment time of P19CL6. **A**: Determination of cardiac commitment time. The days shown for "DMSO → Normal" are the time in which the DMSO-containing medium was changed to that without DMSO (growth medium). "Observation Time" is the time when dishes were observed for the presence of beating cardiomyocytes. The number shown for each box is the number of dishes that contains beating cardiomyocytes (a total of three dishes was used for each condition). **B**: Real-time RT-PCR results. The experiments were performed for "stemness" (*Pou5f1* and *Nanog*), mesoderm (*Brachyury*), early cardiac marker genes (*Gata4*, *Nkx2.5*, *Midori*, and *Tbx5*), and *OTR*. "log2(ratio)" was calculated by taking the logarithm of base 2 of the quantified value. Bars indicate the standard error of the mean values (n = 3).

A

DMSO->Normal	Observation Time						
	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16
Day 2							
Day 2.5							
Day 3						2	
Day 3.5					1	3	3
Day 4				3	2	3	3
Day 4.5				3	3	3	3
Day 5				3	3	3	3
Up to This Day	3	3	3	3	3	3	3

B

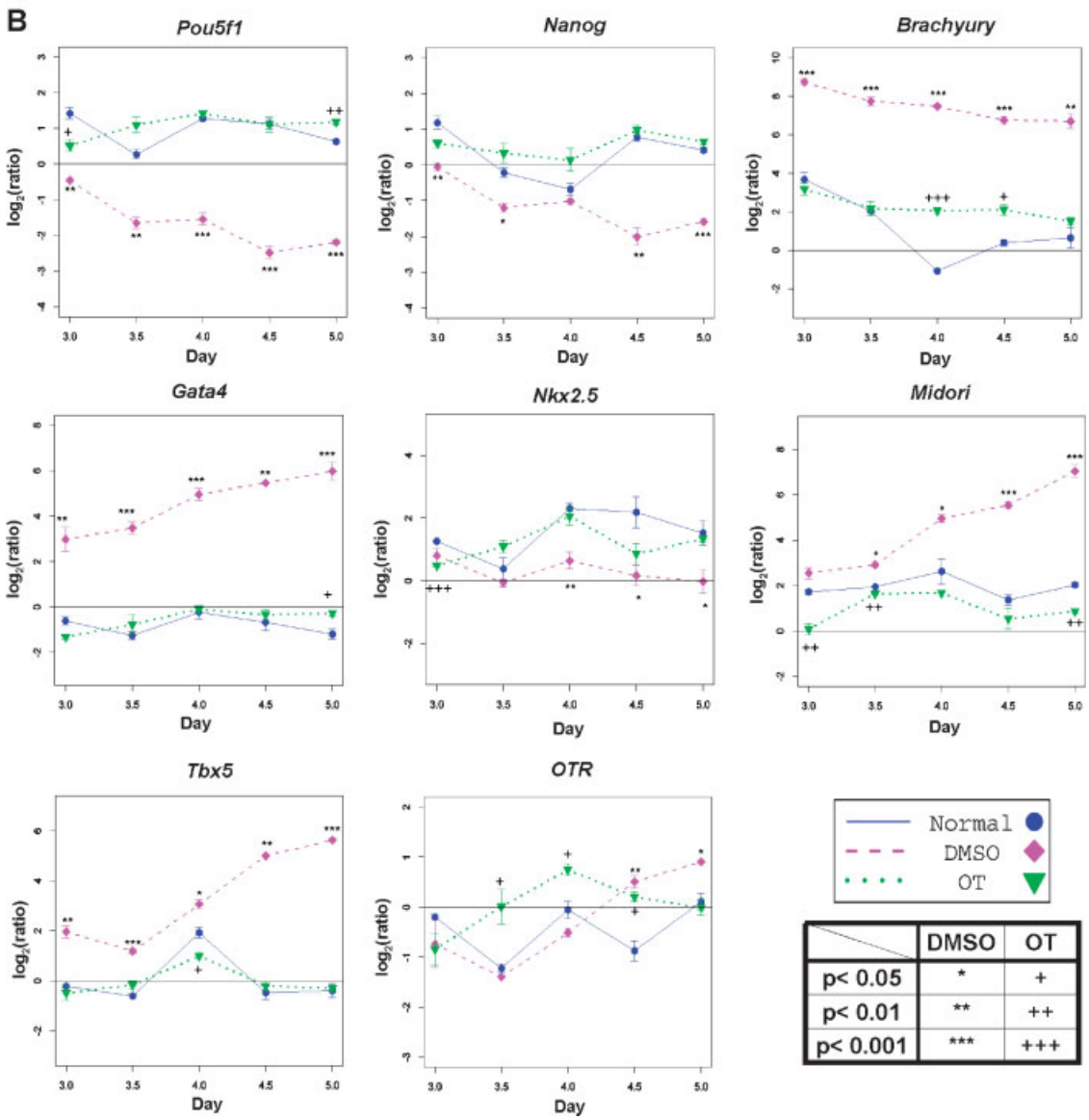

Fig. 2.

cells upregulate the expression of *OTR* during the cardiac commitment time. However, our RT-PCR and Western blotting results for OT-treated cells indicated that the addition of OT did not induce P19CL6 cells to differentiate into cardiomyocytes. In order to explain this differentiation ability of OT, the coding regions (Exon 3 and 4) of *OTR* were sequenced (data not shown). *OTR* of both P19 and P19CL6 cells were sequenced, but there was no difference for the coding regions of *OTR* between P19 and P19CL6.

Next, we hypothesized that the binding of OT to *OTR* is not the only regulatory pathway of the expression of *OTR*. To test this hypothesis, an in silico analysis was performed to characterize the upstream sequence of *OTR* computationally. The mouse *OTR* gene contains four exons and three introns [Kubota et al., 1996]. Exon 1 and 2 correspond to the 5′-untranslated region, followed by exons 3 and 4 encoding the amino acids of the receptor. To predict which regulatory elements bind to the upstream sequence of *OTR*, the 2 kb-upstream sequence from the transcription start site (Ensembl ENSMUS G00000049112) was obtained. Then, a weight matrix-based tool for searching putative transcription regulator binding sites in DNA sequence, "Match" [Kel et al., 2003], was applied. Since we are interested in cardiomyocyte differentiation processes, the search was limited to a "muscle-specific" profile, which is designed to search for potential binding sites within regulatory regions of muscle-specific genes, provided by Match. The search found 19 putative binding sites for four types of transcription regulators (Fig. 3). These were Gata4, Mef2a, Nkx2.5, and Tbp.

Characterizing the Relationships Among Predicted Transcriptional Regulators and *OTR*

Since real-time RT-PCR experiments for *Gata4* and *Nkx2.5* were performed previously (Fig. 2B), where a large difference in the expression levels of *Gata4* among different culture conditions was observed, RT-PCR experiments were performed to observe expression changes of *Mef2a* and *Tbp*. As shown in Figure 4A, there was no visible difference for *Mef2a* and *Tbp* among different culture conditions. Next, to further elucidate the transcriptional relations among *Gata4*, *Nkx2.5*, and *OTR*, we performed real-time RT-PCR experiments for P19 cells during the 4 days of inductions. In accordance with P19CL6 cells, RNA samples were collected in 12-h time periods from day 1 to 4. Figure 4B shows the time-course expression changes of the non-induced and induced P19 cells cultured under

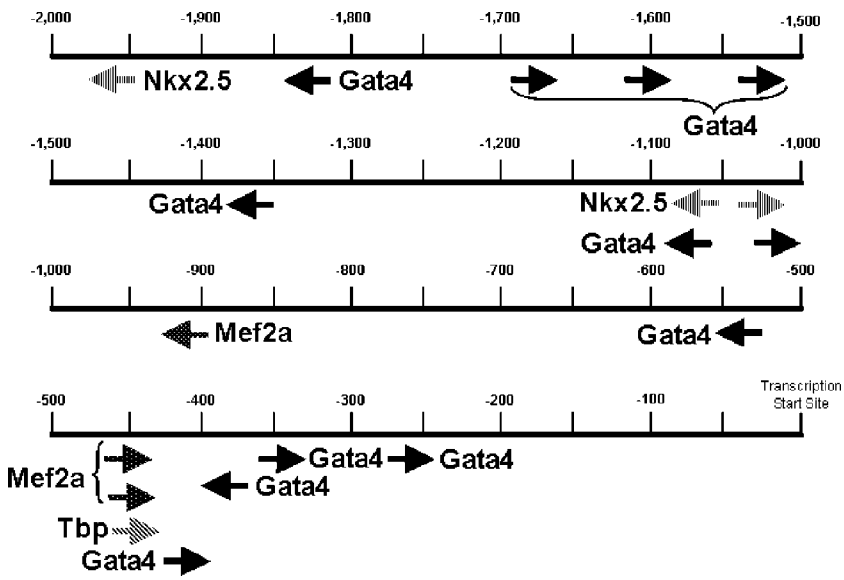

Fig. 3. Upstream sequence analysis. **A**: The 2 kb upstream sequence from the transcription start site was analyzed computationally. Arrows indicate the putative ''muscle-specific'' transcriptional regulator binding sites. There are a total of 19 binding sites consisting of four types of transcriptional regulators: Gata4, Mef2a, Nkx2.5, and Tbp.

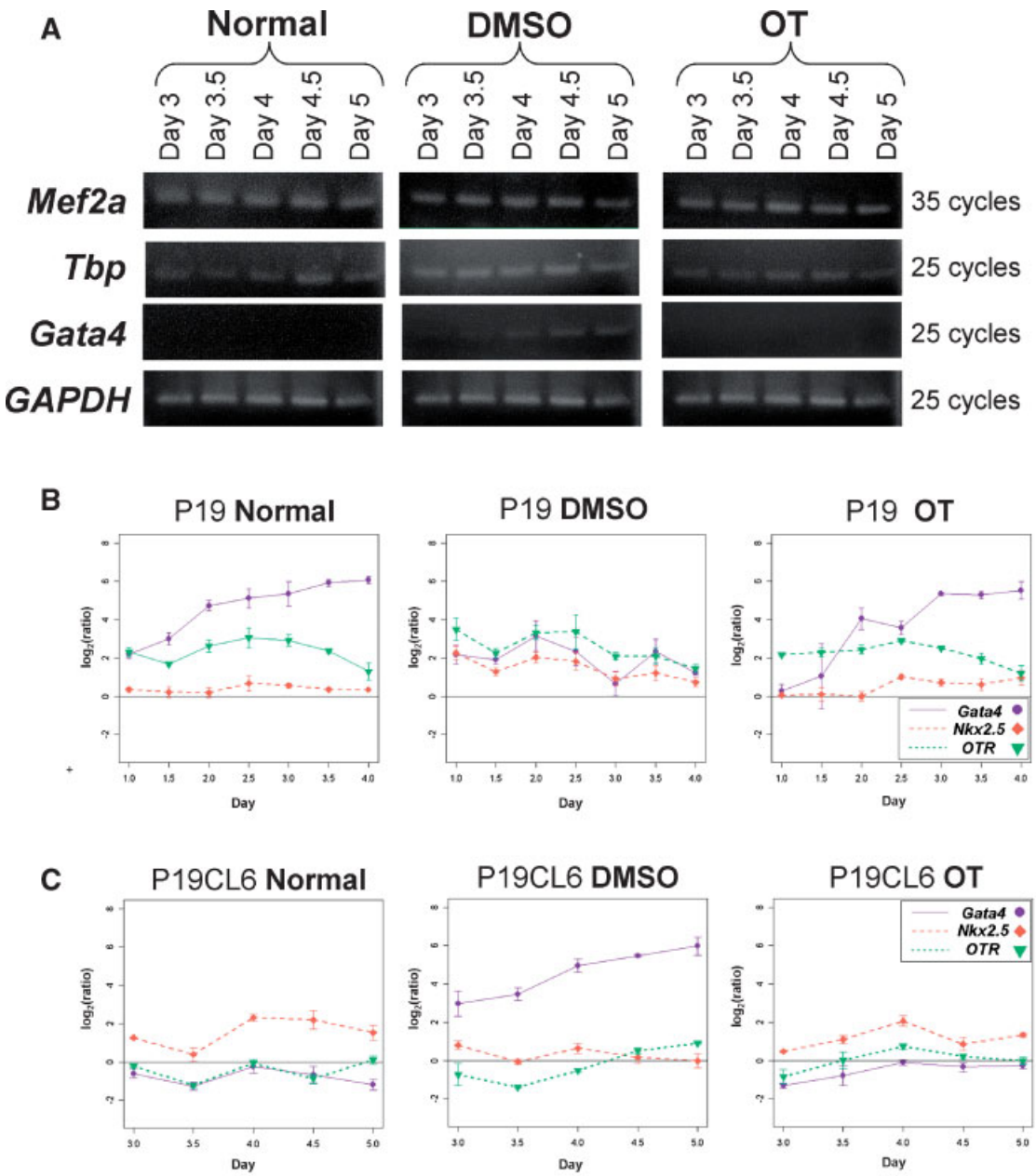

Fig. 4. Expression profiling of putative transcriptional regulators. **A**: RT-PCR results of *Mef2a* and *Tbp* for P19CL6 cells. *Gata4* was used as a positive control. **B**: Real-time RT-PCR results of *Gata4, Nkx2.5,* and *OTR* for P19 cells. **C**: For P19CL6 cells, ''log2(ratio)'' was calculated by taking the logarithm of base 2 of the quantified value.

the non-adherent condition. There are large differences for expression changes of *Gata4* and *Nkx2.5*. In the case of non-induced and OT-induced cells, the expressions of *Gata4* were upregulated in prior to the upregulation of *Nkx2.5*. This trend was unobservable for the DMSO-induced cells. However, the expression changes of *OTR* among different culture conditions seem similar. Thus, no definite conclusion about the relationships among predicted transcriptional regulators and *OTR* can be drawn from these results.

Next, to illustrate the relationships among *Gata4*, *Nkx2.5*, and *OTR*, the real-time RT-PCR results for P19CL6 cells were re-plotted in the same manner as Figure 4B. Unlike the results of P19 cells, there is a clear upregulation of *Gata4* in prior to *OTR* for the DMSO-induced P19CL6

cells, which were the only cells that differentiated into cardiomyocytes (Fig. 4C).

DISCUSSION

In this study, the differentiation ability of OT was tested using P19CL6 cells. Unlike P19 cells, OT did not induce P19CL6 cells to differentiate into beating cardiomyocytes as indicated by the RT-PCR results for cardiac and skeletal marker genes (Fig. 1A), and the Western blotting experiments (Fig. 1B). As shown in the figure, the RT-PCR and Western blotting results of P19 cells induced by OT for cardiac muscle marker genes are different from the DMSO-induced P19CL6 cells. The reason for this can be explained by the fact that the efficiency rate for the differentiation of P19 cells is not very high compared to that of P19CL6 cells. Although OT did not induce P19CL6 cells to differentiate into cardiomyocytes, the real-time RT-PCR results during the cardiac commitment time for OT-treated P19CL6 cells showed the upregulation of *OTR* on day 4 (Fig. 2B). To explain this consequence, we sequenced the coding regions of *OTR* to search for the difference between P19 and P19CL6 cells, but no substitution was found. This, together with the real-time RT-PCR results for *OTR* during the cardiac commitment time, show that *OTR* of P19CL6 cells are functional.

From the first identification of *OTR* in the rat mammary gland [Soloff and Swartz, 1973], various studies have been conducted to understand the OT/OTR system and to identify transcriptional regulators that control the expression of *OTR* [Inoue et al., 1994; Rozen et al., 1995; Kubota et al., 1996; Bale and Dorsa, 1997; Burger et al., 1999; Hoare et al., 1999; Robinson et al., 2001; Schmid et al., 2001; Telgmann et al., 2003; Hasbi et al., 2004]. In the case of murine *OTR*, the promoter region lacks an apparent TATA box but contains multiple putative interleukin-response elements and estrogen responsive elements [Kubota et al., 1996]. The same authors also performed comparative studies on the upstream sequences of human, mouse, rat, and bovine *OTR* genes. Among these species, they found the homologous region between −1,671 and −1,581 in the 5′-flanking region (−1,080 to −990 from the transcription start site), but they could not identify any binding sites for known transcriptional factors in the region [Kubota et al., 1996]. With the recent advances in computational methods and informatics techniques, we now know more about transcription regulators and their binding sites. Thus, we performed a follow-up study on this matter. By limiting our search to "muscle-specific" transcriptional regulators, we found two binding sites for Gata4 (−1,052 and −1,028) and two binding sites for Nkx2.5 (−1,051 and −1,035), which falls under the previously reported homologous region.

To further understand the relationships among these early cardiac marker genes and *OTR*, real-time RT-PCR experiments were performed for P19 cells to compare them with those of P19CL6 cells (Fig. 4B,C). Since the expression changes of *OTR* among different culture conditions are similar, no relationships among *Gata4*, *Nkx2.5*, and *OTR* can be inferred from the results of P19 cells. In contrast, the results for P19CL6 cells show the upregulation of *Gata4*, not *Nkx2.5*, in prior to the upregulation of *OTR* for the DMSO-induced P19CL6 cells. These results are not an indication that the expression of *Nkx2.5* is not needed for P19CL6 cells to differentiate into cardiomyocyte because Monzen et al. [1999] showed that the simultaneous overexpressions of Nkx2.5 and Gata4, but not the overexpression of Nkx2.5 or Gata4 alone, rescued the differentiation ability of P19CL6noggin cells, which is a permanent P19CL6 cell line that overexpresses the bone morphogenetic protein antagonist noggin that does not differentiate into beating cardiomyocytes upon the treatment by 1% DMSO. However, in our real-time RT-PCR results, the upregulation of *Nkx2.5* is also recorded for the non-induced P19CL6 cells much earlier than for the DMSO-induced ones. Taken all of these results together, we conclude that the upregulation of *Gata4* is prior to that of *OTR*.

Based on the results provided from various studies, the actions of OT go well beyond the classical concepts of the induction of uterine contractions during parturition and milk ejection during lactation (reviewed extensively by Zingg and Laporte, 2003; Gimpl and Fahrenholz, 2001). Although the actions of OT range widely, all of them are mediated by OTR [Zingg and Laporte, 2003]. In this study, we provided evidences for the potential mechanistic aspects of the OT/OTR system; that is, its action during the cardiomyocyte differentiation. On this subject matter, Paquin et al. [2002] proposed that

OT triggers the cardiomyocyte differentiation processes of P19 cells through OTR. Furthermore, since previous studies indicated that the administration of DMSO in the cell culture almost instantaneously triggers the release of Ca^{2+} from intracellular stores and suspected that DMSO affects a pathway that has an extracellular component, Paquin et al. [2002] suggested that OT could be a serum-borne factor that is active during the cardiomyocyte differentiation processes induced by DMSO. Therefore, they placed DMSO in the upstream of the signaling pathways mediated by the OT/OTR system in the cardiomyocyte differentiation processes. However, their model does not fit for P19CL6 cells because the addition of OT to the culture medium did not induce P19CL6 cells to differentiate into cardiomyocytes as indicated in Figure 2. This initiated us to provide a model for the cardiomyocyte differentiation processes (Fig. 5). Our model is based on the model by van der Heyden and Defize [2003], which was titled "signal transduction cascades involved in cardiac differentiation of P19 embryonal carcinoma cells" and included the proposed model of Paquin et al. [2002]. The main distinction between the previous model [van der Heyden and Defize, 2003] and ours is that we separated the signaling pathways triggered by DMSO from the ones initiated by OT based on our results in this study. Furthermore, since OT acts as a paracrine and/or autocrine mediator, rather than endocrine as a circulating hormone, in the uterus, amnion, heart, and vessel [Shojo and Kaneko, 2000], we distinguished the actions of the OT/OTR system in these two aspects by adopting the concept of Hu et al. [2001]; that is, porcine luminal epithelial cells may act in an autocrine manner to bind to OTR and stimulate the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion from these cells, thereby obscuring any response to exogenous OT treatment. We considered the addition of OT to the culture medium to be paracrine and the secretion of OT from the cultured cells to be autocrine. Although neither Paquin et al. nor we have measured the levels of OT in the culture medium for P19 and P19CL6 cells, respectively, we considered Paquin's hypothesis of DMSO for stimulating the production of OT to be invalid, at least for P19CL6 cells. Thus, an arrow is placed directly from DMSO to OTR in our model. In addition, since our results indicated that the upregulation of *Gata4* is prior to that of *OTR*, the potential actions exerted by Gata4 were separated from those of Nkx2.5, Mef2c, Tbx5, and Midori, and directly linked to OTR. Moreover, in concert with the model by van der Heyden and Defize [2003], the actions of Midori were separated from those of other early cardiac marker genes. This is due to the fact that there is not enough evidence for its correct position on the signaling cascades for the cardiomyocyte differentiation.

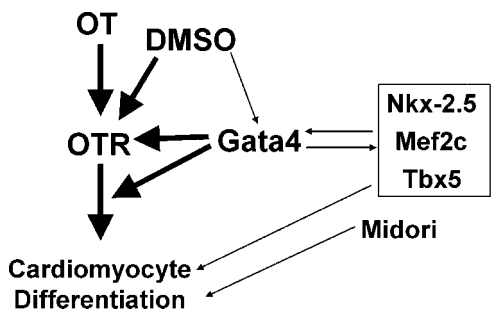

Fig. 5. Postulated the paracrine OT/OTR system for the cardiomyocyte differentiation.

As shown by our results as well as Paquin et al. [2002], DMSO upregulated *OTR* as well as early cardiac marker genes, especially *Gata4*. This phenomenon of *OTR* along with the generation of OT gene knockout mice (OTKO mice) [Nishimori et al., 1996; Young et al., 1996; DeVries et al., 1997; Wagner et al., 1997] suggest that the upregulation of *OTR* is necessary for the cardiomyocyte differentiation processes. A recent study on OTKO mice explained that the reason why OTKO mice can be fertile may be due to the phenomenon where vasopressin crosses over and binds to the *OTR* [Ragnauth et al., 2004]. Thus, OT is not the only peptide that binds to *OTR* and upregulates the expression of *OTR*.

In conclusion, we have provided an additional evidence for the importance of *OTR* in the cardiomyocyte differentiation processes and its possible interaction with Gata4 for P19CL6 cells. However, the generation of *OTR* gene knockout mice [Takayanagi et al., 2005] suggests that further studies are needed to elucidate the importance of *OTR* in cardiomyocyte differentiation processes in vivo.

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