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Original Article

Pax3 preserves myoblasts undifferentiated by suppressing MyoD and enhancing cell cycle regulators

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Abstract: Pax3 is a member of the paired-box (Pax) family and plays a critical role in the initial stage of skeletal muscle development. However, its precise function in myogenesis *in vitro* and *in vivo* remains unknown. In particular, how Pax3 affects the expression of myogenic regulatory factors (MRFs) is fundamental and has not yet been clarified. To address these issues, we performed Pax3 cDNA transfection experiments to the C2C12 cell line. We analyzed the effects of Pax3 gene transfer by cell counting, immunostaining, Western blot, microarray, and real-time PCR analyses. In Pax3 transfected C2C12 cells, the proliferation of myoblasts were enhanced, whereas the formation of myotubes was suppressed compared to non-transfected cells. In addition, the expression of MyoD was exclusively reduced among MRFs by Pax3 transfection. Moreover, we found that the cell cycle regulators such as cyclin A, cyclin B, and CDK1 were significantly upregulated by Pax3. Taken together, these results indicate that Pax3 preserves myoblasts undifferentiated by suppressing the expression of MyoD and enhancing the expression of the cell cycle regulators.

Keywords: Pax3, MyoD, cell proliferation

Introduction

Pax3 is a member of the Pax gene family, which consists of nine transcription factors characterized by the presence of a common paired-box (Pax) domain. Each member of this family is essential for organogenesis during embryonic and fetal development. PAX proteins are involved in a wide variety of cellular processes, including cell proliferation, differentiation, migration, self-renewal, and apoptosis [1]. Among the Pax family, Pax3 and the closely related Pax7 are known to regulate myogenesis. Both have distinct but overlapping roles in the initial stage of skeletal muscle development [2].

The development of skeletal muscle, which is called myogenesis, involves a complex interplay between the downregulation of non-muscle genes and the upregulation of muscle-specific genes. It has been established that this interplay is controlled by Pax3, Pax7, and myogenic regulatory factors (MRFs), such as Myf5, MEF2, MRF4, MyoD, and myogenin [3, 4]. At present, it is considered that Pax3 controls embryonic myogenesis through Pax7 and these MRFs. Nevertheless, how Pax3 regulates the expression of Pax7 and MRFs has not yet been clarified. Previously, we studied the effect of TSA, one of the most potent histone deacetylase inhibitors, on myogenesis using the C2C12 skeletal muscle cell line. We examined how the expression of Pax3, Pax7, and each MRF changes by TSA and found that the change in Pax3 expression is not directly correlated with other Pax7 and MRFs [5].

In the present study, to investigate the role of the transcriptional factor Pax3 in myogenesis and its effect on the expression of Pax7 and MRFs, we performed Pax3 cDNA transfection experiments to the C2C12 cell line. We analyzed the effect of Pax3 transfection by cell counting, immunostaining, Western blot, microarray, and real-time PCR analyses. We found that the proliferation of myoblasts was increased, whereas the formation of myotubes was decreased in the Pax3 transfected C2C12 cells compared to non-transfected cells. We also found that Pax3 does not alter the expression of Pax7, Myf5, and myogenin expression, whereas it significantly suppresses MyoD expression

in Western blot and microarray analysis. Furthermore, functional annotation analysis revealed that the majority of the groups of genes upregulated by Pax3 transfection were related to cell cycle. In particular, the expression of cell cycle regulators such as cyclin A, cyclin B, and CDK1 were most significantly upregulated by Pax3 transfection. These effects of Pax3 on gene expression were further confirmed by real-time PCR. Our findings indicate that Pax3 inhibits myogenic differentiation by suppressing the expression of MyoD and enhancing the expression of the cell cycle regulators. Thus, Pax3 is considered to play a role in preserving muscle cell precursors undifferentiated.

Materials and methods

Cell culture and Pax3 transfection

C2C12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (growth medium [GM]). Pax3 transfection was performed using Effectene® Transfection Reagent according to the manufacturer's instructions (QIAGEN, USA) with some modifications [6]. Pax3 containing OmicsLink™ Expression-Ready ORF cDNA Clone (GeneCopoeia, USA) (0 (mock), 0.4 or 0.8 μg) was transfected to the C2C12 cell line in GM. The cells were cultured for 48 h in GM and the confluent cells were then differentiated by lowering the serum concentration to 2% horse serum (differentiation medium [DM]). All cells were grown in a humidified 37°C incubator with 5% CO_2 and 95% air [7].

Cell count assay

Cell count assay of cell proliferation was performed using a Cell Counting Kit-8 (Dojindo, Japan), a modification of MTT assay [8]. C2C12 cells were plated at 100 mL/well in 96-well plates at a density of 2×10^5 cells/mL. The assay was carried out at 4, 24, and 48 h after Pax3 transfection. The optical density (OD) was then quantified at a wavelength of 450 nm using a Benchmark Plus microplate reader (Bio-Rad, USA). The measurement for each group was triplicated.

Histology and immunohistochemistry

C2C12 cells were observed using a phase contrast microscope (Nikon, Japan). For immunohistochemistry, C2C12 cells at 72 h after the medium switch from GM to DM were fixed, permeabilized, and stained with anti-MHC (MF20) (Developmental Studies Hybridoma Bank (DSHB), Iowa, USA) for 60 min according to the mouse on mouse (M.O.M) procedure (Vector Laboratories, USA). After washing with phosphate buffered saline (PBS), the cells were incubated for 30 min with Alexa Fluor 488-conjugated anti-mouse IgG antibody (Invitrogen, USA). The slides were mounted with VECTASHIELD plus DAPI (Vector Laboratories) [9]. The fluorescence images were captured using a FSX100 microscope (Olympus, Japan) and analyzed with FSX-BSW software (Olympus). The fusion index was calculated as previously reported [5].

Western blotting

Western blot was carried out as previously reported [10]. The blots were incubated with agitation at room temperature in the presence of a mouse monoclonal anti-Pax7 (DSHB), Myf5 (sc-302, Santa Cruz, USA), MyoD (sc-304, Santa Cruz), myogenin (F5D, DSHB), and β-actin (Santa Cruz) antibodies (diluted 1:100 in 0.1% Tween 20 in TBS). The signals were developed by enhanced chemiluminescence (ECL kit, Amersham Biosciences, USA) and images were captured with an LAS100 plus (FUJIFILM, Japan). Densitometry of the Western blot was performed using NIH ImageJ Software (NIH, USA) [11]. The density of the β-actin bands was used to standardize the density of MRFs protein expression [12].

DNA microarray

DNA microarray analysis was performed on two independent cultures. C2C12 cells were cultured in GM with or without Pax3 transfection for 48 h. Total RNA was isolated from each group using TRIzol reagent (Invitrogen) [13]. Microarray analysis was performed using Whole Mouse Genome Microarray (4 × 44 K) v2 (Agilent Technology, USA). Microarray data from Pax3-transfected and non-transfected C2C12 cells were extracted from scanned images and analyzed.

Real-time PCR

Real-time PCR was performed using a 7300 real-time PCR system (Applied Biosystems, USA) as previously described [5]. Each cDNA

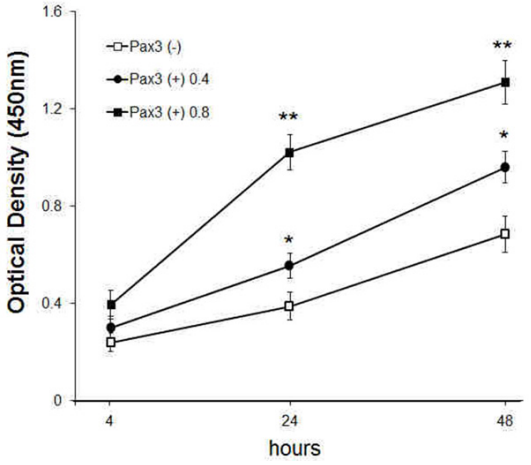

Figure 1. Pax3 promotes myoblast proliferation in C2C12 cells. C2C12 cells were transfected with Pax3 (0.4, 0.8 μg, or mock) and cultured in GM. The optical density was measured at 4, 24, and 48 h after transfection. Data from 3 cultures with triplicates were expressed as mean ± standard deviation (SD) (*$P < 0.05$, **$P < 0.05$). GM, growth medium.

from Pax3 transfected or non-transfected C2C12 cells was mixed with 0.2 μM forward and reverse primers, and 10 μl of SYBR Green Master Mix (Applied Biosystems).

Sequences of the primers sets used in this experiment are as follows: Pax7, 5'-GACCTTGACCAGAACACCATCG-3' and 5'-GCTGTATTCCCGACCGTTGAAC-3'; Myf5, 5'-GACCTTGACCAGAACACCATCG-3' and 5'-GCTGTATTCCCGACCGTTGAAC-3'; MyoD, 5'-GACCTTGACCAGAACACCATCG-3' and 5'-GCTGTATTCCCGACCGTTGAAC-3'; myogenin, 5'-GACCTTGACCAGAACACCATCG-3' and 5'-GCTGTATTCCCGACCGTTGAAC-3'; cyclin A2, 5'-TCGCTGCATCAGGAAGACCA-3' and 5'-GGCAGGCTGTTTACTGACTG-3' cyclin B2, 5'-TGACGCTCGTCGACTATGAC-3' and 5'-TGCTGCTGGCATACTTGTTC-3'; CDK1, 5'-CTCGGCTCGTTACTCCACTC-3' and 5'-TCCACTTGGGAAAGGTGTTC-3', GAPDH, 5'-GACCTTGACCAGAACACCATCG-3' and 5'-GCTGTATTCCCGACCGTTGAAC-3'.

Functional annotation analysis

Functional annotations among differentially expressed genes were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) bioinformatics resource [14]. The functional categories and biological pathways were annotated by the Gene ontology tool [15] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [16]. Annotations were considered significantly over-represented when the *p*-value of the Fisher's exact test in DAVID was < 0.001.

Statistical analysis

Statistical comparisons were performed using the unpaired two-tailed Student's t-test with a probability value $P < 0.05$ considered statistically significant.

Results

Pax3 promotes myoblast proliferation in C2C12 cells

To examine how Pax3 transfection affects the myoblast proliferation of C2C12 cells, we performed cell count assay. C2C12 cells cultured in GM were transfected with Pax3 and the number of cells was counted using a Cell Counting Kit-8 at 4, 24, and 48 h after transfection. The cell growth curves with or without Pax3 transfection were presented in **Figure 1**. From 24 h culture, higher OD values were obtained in the Pax3 transfected group (0.4, 0.8 μg) than the control group (mock) in a dose-dependent manner ($P < 0.05$). This result suggests that Pax3 transfection promoted the proliferation in C2C12 cells. Since the effect was prominent, we used 0.8 μg of Pax3 cDNA as the transfected group for further analysis.

Pax3 inhibits myotube formation in C2C12 cells

Next, we questioned whether or not Pax3 transfection promotes myotube formation. C2C12 cells were transfected with Pax3 cultured for 48 h in GM and the culture medium was then switched to DM. We observed C2C12 cells under a phase contrast microscope over time and found suppressed myotube formation in Pax3-transfected cells compared to non-transfected cells. We then immunostained C2C12 cells with an antibody against MHC and observed the same effect on the myotube formation of C2C12 cells (**Figure 2A**). To quantitate the fusion of C2C12 cells after transfection, we calculated the fusion index. It was determined as the number of nuclei in MHC-positive cells versus the total number of nuclei. The fusion index was 30.4 ± 5.1 in Pax3 non-

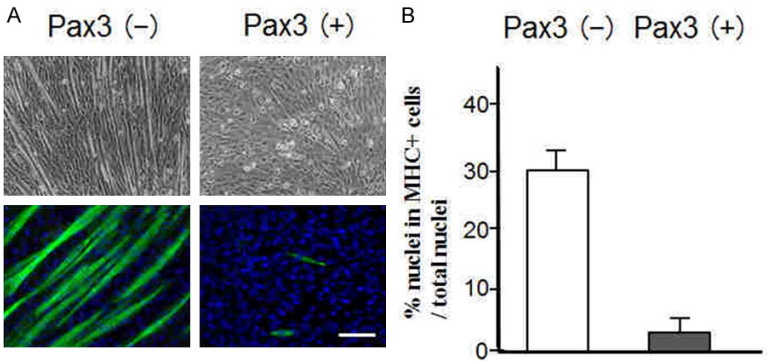

Figure 2. Pax3 inhibits myotube formation in C2C12 cells. A. C2C12 cells were transfected with Pax3 cultured for 48 h in GM and the culture medium was then switched to DM. Cells were cultured for 72 h after the medium switch and analyzed by immunocytochemistry using an anti-MHC antibody. Upper: phase contrast. Lower: MHC immunofluorescence and DAPI staining. Scale bar, 50 μm. B. Fusion index was 30.4 ± 5.1 in Pax3 non-transfected versus 4.2 ± 1.2 in Pax3-transfected cells. DM, differentiation medium.

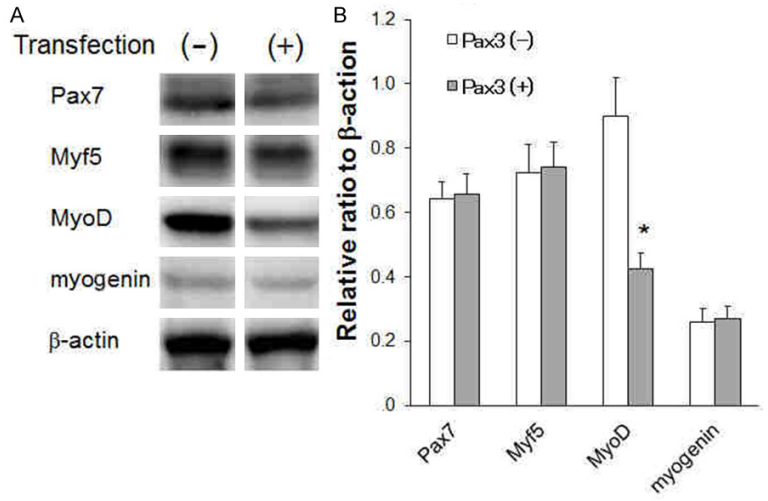

Figure 3. Pax3 transfection does not significantly alter Pax7, Myf5, and myogenin expression but it suppresses MyoD expression. A. C2C12 cells were transfected with Pax3 and cultured in GM for 48 h. Protein extracts from these C2C12 cells were subjected to Western blotting using anti-Pax7, Myf5, MyoD, and myogenin antibodies. B. Densitometric analysis of each MRF/β-actin ratio from both Pax3-transfected (filled bar) and non-transfected control (open bar) cells. Values are mean ± standard deviation (SD) of three individual experiments (*$P < 0.05$).

transfected versus 4.2 ± 1.2 in Pax3-transfected cells (**Figure 2B**). These results demonstrate that Pax3 inhibits the myotube formation in C2C12 cells.

Pax3 does not alter Pax7, Myf5, and myogenin expression but suppresses MyoD expression

Next, we analyzed how Pax3 affects the expression of Pax7 and MRFs. C2C12 cells were transfected with Pax3 and cultured in GM for 48 h. We compared the expression of Pax7, Myf5, MyoD, and myogenin in Pax3-transfected and non-transfected cells by Western blot analysis. There were no apparent differences in the expression of Pax7, Myf5, and myogenin between Pax3-transfected and non-transfected cells. In sharp contrast, the expression of MyoD was significantly decreased in Pax3-transfected cells (**Figure 3A**). Densitmetric analysis revealed that the relative ratio of the expression of MyoD to β-action was 0.90 ± 0.12 in Pax3 non-transfected versus 0.43 ± 0.05 in Pax3-transfected cells. The relative ratio to β-action (in non-transfected versus transfected cells) of Pax7 was 0.64 ± 0.05 versus 0.66 ± 0.06, Myf5 0.72 ± 0.09 versus 0.74 ± 0.08, and myogenin 0.26 ± 0.04 versus 0.27 ± 0.04 (**Figure 3B**). These results indicate that Pax3 transfection does not alter Pax7, Myf5, and myogenin expression, but that it significantly suppresses MyoD expression.

To evaluate the effect of Pax3 on whole gene expression, we performed DNA microarray analysis of Pax3-transfected and non-transfected C2C12 cells. We purified mRNA from each group 48 h after transfection. Among 22,811 analyzed genes, 2,275 exhibited a more than 1.8-fold change of expression. The changes in gene expression of MRFs in Pax3-transfected cells compared with control cells are summarized in **Table 1**. Except for MyoD, the expressions of MRFs were not significantly changed by Pax3 transfection. There was a 1.27-fold change in Pax7, a -0.07-fold change in Myf5, a -0.63-fold change in MRF4, a 0.86-fold change in MEF2A, a 0.23-fold change in MEF2C, and a 0.87-fold

Table 1. Fold change of gene expressions of MRFs and muscle fiber componentsin Pax3 transfected cells compared with control cells

Function	Name	Symbol	Description	Fold Change
Myogenic regulatoryfactors	Pax7	Pax7	Paired Box Gene 7	1.27
	Myf5	Myf5	Myogenic Factor 5	-0.07
	MRF4	Myf6	Myogenic Factor 6	-0.63
	MEF2A	Mef2a	Myocyte Enhancer Factor 2A	0.86
	MEF2C	Mef2c	Myocyte Enhancer Factor 2C	0.23
	MyoD	Myod1	Myogenic Differentiation 1	-2.47
	Myogenin	Myog	Myogenin	0.87
Muscle fiber components	MHC adult	Myh14	Myosin, Heavy Polypeptide 14, Skeletal Muscle, Adult	-0.48
	Myosin, light polypeptide 4	Myl4	Myosin, Light Polypeptide 4	-1.24
	Troponin T	Tnnt1	Troponin T1, Skeletal, Slow	-2.52
		Tnnt3	Troponin T3, Skeletal, Fast	-0.72
	Troponin C	Tnnc1	Troponin C, Cardiac/Slow Skeletal	-0.62
		Tnnc2	Troponin C2, Fast	-0.54
	Troponin I	Tnni2	Troponin I, Skeletal, Fast 2	-1.21
	Desmin	Des	Desmin	-2.38

Table 2. Functional annotation clustering of DAVID IDs that were upregulated in Pax3 transfected cells vs non-transfected cells

GO term	Category	No. of genes	*p*-value
M phase of mitotic cell cycle*	GOTERM_BP_FAT	16	2.1E-06
Mitosis*	SP_PIR_KEYWORDS	15	2.8E-06
Cell cycle process*	GOTERM_BP_FAT	22	8.0E-06
Mitosis*	GOTERM_BP_FAT	15	8.1E-06
Nuclear division*	GOTERM_BP_FAT	15	8.1E-06
Mitotic cell cycle*	GOTERM_BP_FAT	17	8.1E-06
Glycosylationsite: N-linked (GlcNAc...)	UP_SEQ_FEATURE	92	8.7E-06
Glycoprotein	SP_PIR_KEYWORDS	95	9.1E-06
Organelle fission*	GOTERM_BP_FAT	15	1.2E-05
Sushi/SCR/CCP	INTERPRO	8	2.5E-05
Complement control module	INTERPRO	8	2.8E-05
Cell cycle*	GOTERM_BP_FAT	27	3.7E-05
Extracellular region part	GOTERM_CC_FAT	30	5.1E-05
M phase*	GOTERM_BP_FAT	17	5.1E-05
CCP	SMART	8	5.1E-05
Kinetochore*	SP_PIR_KEYWORDS	8	8.3E-05
Cell cycle*	SP_PIR_KEYWORDS	21	1.0E-04
Cell division*	SP_PIR_KEYWORDS	15	1.5E-04
Cell division*	GOTERM_BP_FAT	16	1.6E-04
Signal peptide	UP_SEQ_FEATURE	75	1.7E-04

*Considered to be related to cell cycle.

change in myogenin expression. In contrast, Pax3 suppressed MyoD expression by approximately 2.5-fold. These results are consistent with those of Western blot analysis.

Pax3 enhances the expression of the cell cycle regulators

Next, we questioned what groups of genes were predominantly affected by Pax3 transfection. To identify the biological processes that are significantly enriched in Pax3 transfected cells, we performed functional annotation analysis using the DAVID bioinformatics resource. **Table 2** shows the top-twenty functional categories that were upregulated by Pax3 transfection. We identified that the majority of groups of genes (marked*) were related to cell cycle.

Therefore, we investigated the change in expression of cell cycle-related genes in DNA microarray analysis. In agreement with the

Table 3. Fold change of gene expressionsof cell cycle related moleculesin Pax3 transfected cells compared with control cells

Name	Symbol	Fold Change	Name	Symbol	Fold Change
Anillin, Actin Binding Protein	Anln	3.80	Cyclin-Dependent Kinase Inhibitor 3	Cdkn3	2.08
Protein Kinase Atr	ATR	-2.09	Chromatin Licensing And Dna Replication Factor 1	cdt1	1.89
Aurora Kinase A	Aurka	3.30	Cdc28 Protein Kinase 1B	Cks1b	2.13
Budding Uninhibited By Benzimidazoles 1 Homolog	Bub1b	2.38	Cdc28 Protein Kinase Regulatory Subunit 2	Cks2	2.10
Cyclin A2	Ccna2	2.63	Dna-Damage Inducible Transcript 3	Ddit3	-2.14
Cyclin B1	Ccnb1	3.35	Ect2 Oncogene	Ect2	2.85
Cyclin B2	Ccnb2	4.22	Mad2 Mitotic Arrest Deficient-Like 1	Mad2l1	2.59
Cyclin F	Ccnf	1.83	Antigen Identified By Monoclonal Antibody Ki 67	Mki67	2.37
Cell Division Cycle 20 Homolog	Cdc20	4.47	Nima-Related Expressed Kinase 2	Nek2	3.24
Cell Division Cycle Associated 3	Cdca3	4.21	Proliferating Cell Nuclear Antigen	Pcna	1.94
Cell Division Cycle Associated 5	Cdca5	1.82	Polo-Like Kinase	Plk1	3.25
Musmusculus Cell Division Cycle Associated 8	Cdca8	2.50	Polymerase (Rna) I Polypeptide A	Polr1a	-1.79
Cyclin-Dependent Kinase 1	Cdk1	1.77	Protein Regulator Of Cytokinesis 1	Prc1	2.82
Cyclin-Dependent Kinase 13	Cdk13	2.64	Sestrin 2	Sesn2	-1.81
Cyclin-Dependent Kinase 14	Cdk14	-2.40	Transforming Growth Factor, Beta 2	Tgfb2	2.15
Cyclin-Dependent Kinase 2	Cdk2	-1.75	Transformation Related Protein 53	Trp53	-1.89
Cyclin-Dependent Kinase Inhibitor 2D	Cdkn2d	1.59	Twist Homolog 1	Twist1	-1.97

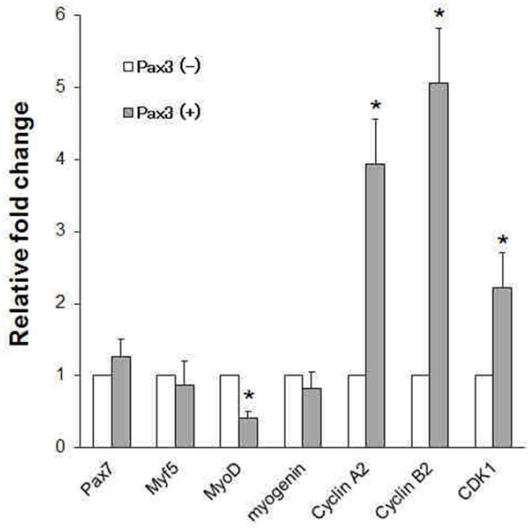

Figure 4. Effect of Pax3 transfection on the expression of Pax7, Myf5, MyoD, myogenin, cyclin A, cyclin B, and CDK. C2C12 cells were transfected with Pax3 and then cultured in GM for 48 h. Target gene expression levels from both Pax3-transfected (filled bar) and non-transfected control (open bar) cells were normalized by reference genes and the fold change was calculated by the $\Delta\delta C_T$ method. Values are mean ± SD of three individual experiments (*P < 0.05).

results of functional annotation analysis, genes related to the cell cycle were markedly affected by Pax3 transfection. Among the cell cycle-related genes, those which exhibited a change of more than approximately 1.8-fold are summarized in **Table 3**. In particular, we found that the expression of key cell cycle regulators such as cyclin A, cyclin B, and CDK1 were changed by around 2- to 4-fold (cyclin A2 2.63-fold, cyclin B1 3.35-fold, cyclin B2 4.22-fold, and CDK1 1.77-fold, respectively).

The effect of Pax3 on gene expression is recapitulated by real-time PCR

We performed real-time PCR to confirm the results obtained by DNA microarray analysis. The relative fold change of Pax3-transfected to non-transfected cells was decreased in MyoD (0.41 ± 0.10). It was not, however, significantly changed in Pax7 (1.27 ± 0.23), Myf5 (0.87 ± 0.33), and myogenin (0.83 ± 0.22). Conversely, the relative fold change was increased in cyclin A2 (3.94 ± 0.61), cyclin B2 (5.06 ± 0.76), and CDK1 (2.22 ± 0.48) (**Figure 4**). These results were consistent with those of DNA microarray analysis.

Discussion

As a family encoding transcription factors, Pax genes play critical roles in in tissue specification and organogenesis during embryonic development [1]. Among them, Pax3 and its

homologue Pax7 play distinct but overlapping roles in the initial stage of skeletal muscle development [2]. For instance, multipotent Pax3-positive cells of the dermomyotome generated numerous derivatives, including the skeletal muscle of the trunk and limbs [17]. The development of skeletal muscle undergoes a multistep process, which requires the coordinated expression of Pax3, Pax7, and various MRFs, such as Myf5, MEF2, MRF4, MyoD, and myogenin [3, 4]. Although it is considered that Pax3 plays a critical role for the control of myogenesis through these Pax7 and MRFs, its precise mechanism remains unclear.

In this report, we demonstrated that in the Pax3 transfected C2C12 cells, the proliferation of myoblasts was increased, whereas the formation of myotubes was decreased compared to non-transfected cells. Therefore, enforced expression of Pax3 inhibits myogenic differentiation of C2C12 cells. C2C12 myoblasts are one of the most commonly used models for studying myogenesis *in vitro*. The effect of Pax3 was visible not only in the kinetics of cell growth, but also in the morphological analysis. Pax3 transfected cells kept their originally circular shape to prevent forming a new, specific, elongate morphology, typical of muscle cell phenotype. It is important to specify that Pax3 promoted proliferation without accelerating cell differentiation. There have been several reports that studied the effects of Pax3 on myogenesis in C2C12 cells using retroviral vectors. Consistent with our results, some showed that Pax3 inhibits myogenic differentiation in C2C12 cells [18-20]. In primary myoblasts, conversely, Pax3 transfection was reported to have no effect [21] or to induce [19] myogenic differentiation. This discrepancy may be explained by the different source of culture cells and experimental conditions.

Despite the earlier Pax3 transfection studies mentioned above, little is known about how Pax3 regulates the expression of MRFs, or what groups of genes were predominantly affected by Pax3. To address these questions, we performed Western blot, microarray, and functional annotation analyses. We found that Pax3 does not alter Pax7, Myf5, or myogenin expression, whereas it suppresses MyoD expression. There was good agreement between histological and biochemical findings. It was reported that upregulation of several somite marker genes, particularly Paraxis and Meox1, which suggested that Pax3 drives the generation of an intermediate population with features similar to the epithelializing somite population. In addition, Pax3 expression resulted in the induction of Myf5, c-Met, Nr2f2, and Prrx1 but not MyoD or Myf6 in mESCs, indicating that Pax3 drives cell fate changes that mimic the events happening in the early myotome [22]. It indicated that Pax3 impact on gene expression was related to cell type. It is important to note that this suppression of the expression of MyoD is accompanied by inhibition of myogenic differentiation in C2C12 cells. MyoD is regarded as a master transcription factor for myogenic specification and terminal differentiation [23]. Therefore, these results provide evidence that Pax3 inhibits myogenic differentiation that is normally induced by MyoD in the C2C12 cell line.

In addition, we identified that the majority of groups of genes upregulated by Pax3 transfection were related to cell cycle using DAVID GO analysis. GO analysis is a classical method to annotate gene function but still inexact in some fields, based on which we can have a comprehensive understanding about interactions of genes, functions that they participate in and relations between up- and down-stream, and obtain genes involved in these significant items. This is the first report to demonstrate that Pax3 is involved in the expression of cell cycle regulators using the enforced expression method of Pax3. In particular, the expression of key cell cycle regulators such as cyclin A, cyclin B, and CDK1 [24] were most significantly upregulated by Pax3 transfection. These effects of Pax3 on gene expression were further confirmed by real-time PCR. These findings are consistent with the result of cell count assay. Taken together, it is suggested that the promoting effect of Pax3 on myoblast proliferation results from enhancing the expression of the cell cycle regulators.

Our data is insufficient to explain how the cell cycle is promoted by Pax3 transfection. However, we could put forth that the upregulated expression of cell cycle regulators is attributed to the suppression of MyoD activity. MyoD induces myogenic differentiation, and is considered to play a cooperative role in the down-regulation of cell cycle regulators, such as cyclins and cdks [25]. Interestingly, the overex-

pression of cyclins/cdks has been reported to inhibit the activity of MyoD and prevent myogenic differentiation [26]. This is the opposite condition from our experiment and supports the idea that MyoD and cell cycle regulators have mutual effects. Alternatively, Pax3 may directly control cell cycle regulators. To date, little attention has been paid to the interaction between Pax3 and cell cycle regulators. In this regard, it is noteworthy that Pax3 is reported to regulate the FGF signaling pathway [27]. This provides a possible mechanism fo r how Pax3 may influence cell division.

In summary, our results indicate that Pax3 promotes proliferation and inhibits differentiation in myogenesis by suppressing the expression of MyoD and enhancing the expression of the cell cycle regulators such as cyclin A, cyclin B, and CDK1. Pax3 is thus considered to play a role in preserving myoblasts undifferentiated. Further studies are needed to better understand the role of Pax3 in skeletal muscle development.

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Disclosure of conflict of interest

None.

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