



Retinoic acid induces myoblasts transdifferentiation into premeiotic Stra8-positive cells

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Abstract

Spermatogonia and sperm-like cells can be derived *in vitro* via the addition of RA (retinoic acid) to pluripotent ES and EG cells. At present, however, these cells have not been derived from unipotent cells. Here, we have generated premeiotic Stra8-positive cells from C2C12 myoblasts following treatment with 10 μ M all-*trans*-RA for 8 days. The differentiated C2C12 cells exhibited spherical morphology similar to spermatogonia, and they expressed gene markers of premeiosis, meiosis and postmeiosis. In addition, some of the transdifferentiated Stra8-positive cells had a tail-like phenotype. Flow cytometry results indicated that up to 20% of RA-induced C2C12 cells were Stra8-positive. Mvh (mouse vasa homologue) protein, a germ cell-specific ATP-dependent RNA helicase and Prm1 (protamine 1) were detected in transdifferentiated cells. The DNA content in induced C2C12 cells showed that Stra8-positive cells were diploid, suggesting that the myoblast transdifferentiation was in the premeiotic stage of spermatogenesis. The derivation of Stra8-positive cells from C2C12 myoblasts has important implications for studying unipotent cell differentiation. Furthermore, C2C12 myoblasts may provide a useful *in vitro* cell model to study signal transduction and transdifferentiation during RA treatments.

Keywords: C2C12 myoblast; embryonic carcinoma cell; retinoic acid; spermatogenesis; Stra8; transdifferentiation

1. Introduction

Spermatozoa are formed in seminiferous tubules where they are derived from spermatogonia that undergo meiotic (spermatocytes) and then meiotic (spermatids) division. ES (embryonic stem cells) and EG (embryonic germ cells) are embryonic in nature and thus are able to differentiate into spermatozoa (Geijsen et al., 2004; Aflatoonian and Moore, 2006; Nayernia et al., 2006b). Recent reports show that adult skin-derived stem cells and bone marrow-derived stem cells are also able to differentiate into gametes (Dyce et al., 2006; Nayernia et al., 2006a). One strategy for the establishment of male gametes from stem cells is to culture cells in the presence of RAs (retinoic acids). RA is required for vertebrate reproduction, and in the male, it plays a role in the maintenance of normal testicular structure and function. RA deficiency, on the other hand, leads to the termination of spermatogenesis and to the degeneration of seminiferous tubules (Chung and Wolgemuth, 2004; Zhou et al., 2008b).

The Stra8 (stimulated by retinoic acid gene 8) gene product is restricted in location to the developing male gonad, where it plays a required role for spermatogenesis (Oulad-Abdelghani et al., 1996; Miyamoto et al., 2002; Zhou et al., 2008a; Tedesco et al., 2009). This gene is activated during various stages of development by the stimulation of RA. For instance, Stra8 is expressed at the early stage of spermatogenesis in premeiotic cells, as well as in prenatal oogonies right before their entry into meiotic prophase. This gene is also expressed in postnatal spermatogonial stem cells and

proliferating spermatogonia. To examine the mechanism of ES cell-derived male gamete development, the Stra8 promoter has been used to select male gametes derived from ES cells and mesenchymal stem cells (Giullini et al., 2002; Nayernia et al., 2006a, 2006b). In 2006, Nayernia et al. reported that mice sperm could be derived from Stra8-positive ES cells. In addition, viable mice offspring were produced using oocytes microinjected with ES-derived sperm (Nayernia et al., 2006b). This demonstrated that ES cells could be induced to differentiate into functional sperm. Thus, this provided new insight regarding the regulation of spermatogenesis *in vitro*.

C2C12 myoblasts that have the mononuclear in standard culture media are a useful model for studying the differentiation of precursor cells to skeletal muscle cells (Moran et al., 2002). In addition, they possess the ability to differentiate into other structures, such as into osteoblasts following treatment with BMP (bone morphogenetic protein)-2 (Katagiri et al., 1994). Several studies from our laboratory and others indicate that RA is capable of efficiently inducing the differentiation of stem cells into male gametes. RA can also activate Stra8 gene expression (Li et al., 2007; Zhou et al., 2008c). Here, we show that RA induction can convert C2C12 myoblasts into Stra8-positive cells. These cells express male gamete gene markers as well as exhibiting morphological changes. Our results indicate that unipotent cells can be reprogrammed when they are stimulated with RA. Our experiments have enabled us to study the molecular mechanisms of RA regulation in the development of early stage spermatogenesis.

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Abbreviations: Acr, acrosin; DMEM, Dulbecco's modified Eagle's medium; EG cells, embryonic germ cells; ES cells, embryonic stem cells; FCS, fetal calf serum; Fgf5, fibroblast growth factor 5; Mvh, mouse vasa homologue; NF-68, neurofilament 68; PGCs, primordial germ cells; PI, propidium iodide; Prm1, protamine 1; RA, retinoic acid; Rbm, RNA-binding motif gene on Y chromosome; Scp3, synaptonemal complex protein 3; Stra8, stimulated by retinoic acid gene 8; Tp2, transition protein 2.

2. Materials and methods

2.1. Plasmid construction

To generate the germ cell-specific expression vector, pStra8-EGFP, a 1.4-kb fragment of Stra8 promoter was amplified from mouse P19 genomic DNA with primers (forward: AAGCTTAAACTTGCCCTCCAAGGGGGTAAG and reverse: CCGCGGCGACTGCCCGTCGCAGAATAA). The amplified 1.4-kb fragment in pMD18-T vector was subcloned into HindIII/SacII sites of a pEGFP-1 vector (BD Biosciences), and this was used to construct pStra8-EGFP. The same fragment was also inserted into SacI/HindIII sites of the pGL3-basic vector (BD Biosciences) in order to generate the pStra8-Luc construct.

2.2. Cells culture and generation of stable transfected cell lines

Mouse P19 EC (embryonic carcinoma) cells, C2C12 myoblasts and NIH3T3 fibroblasts were cultured in DMEM (Dulbecco's modified Eagle's medium, Gibco, Life Technologies) supplemented with 10% (v/v) FCS (fetal calf serum, Hyclone), 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Cells were grown at 37°C in the presence of 5% CO₂ and were subcultured (1:5) every 2–3 days. To perform cell differentiation, cells at 50% confluence were cultured in an induction medium (DMEM with 10% FCS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 10 μM RA), with the media being changed every 2–3 days.

To test the cellular specificity of the mouse Stra8 promoter, P19, C2C12 and 3T3 cells were seeded on 24-well plates at 3×10^4 cells/ml. Transfections were conducted using Lipofectamine[®] 2000 (Invitrogen) and vectors containing either pStra8-EGFP or pStra8-Luc. The transfected cells were incubated with the induction medium for different periods of time. For EGFP expression, cells were analysed using fluorescence microscopy, while luciferase activity was measured with a Dual-Luciferase Reporter (Vigorous) Assay System according to the manufacturer's instructions. To obtain stable transfected cells, C2C12 cells were grown in a 35-mm plate until they were 80% confluent. Cells were washed with PBS and then incubated with a mixture of 4 μg of DNA, 10 μl of Lipofectamine[®] 2000 and 500 μl of OptiMEM that was prepared as described by the manufacturer. Following 6 h incubation, the medium was replaced with regular culture medium that was supplemented with 400 μg/ml of G418. After 4 weeks of selection, G418-resistant colonies were collected and maintained in media with 40 μg/ml of G418.

2.3. Immunocytochemistry and Western blotting

Cells at 80% confluence were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature. Fixed cells were washed twice with ice-cold PBS, incubated with PBS containing 0.1% Triton X-100 for 10 min and then washed three times with PBS. Cells were blocked with BSA-blotting buffer (1% BSA, 0.1% Tween 20 in PBS) for 30 min and then incubated with BSA-blotting buffer plus anti-Mvh (ab13840, Abcam), anti-tubulin (sc-5286,

Santa Cruz Biotechnology, Inc) and anti-Prm1 (protamine 1) (sc-30174, Santa Cruz Biotechnology, Inc) antibody (1:1000), respectively. Cells were incubated in a humidified chamber overnight at 4°C. An anti-mouse secondary antibody conjugated with FITC (or TRITC) was applied for 2 h. For nuclear staining, fixed cells were incubated for 5 min with 5 mg/ml fluorescent PI (propidium iodide) or Hoe33342 (H342). The mitochondria of C2C12 cells were stained with 500 nM MitoTracker[®] Red CM-H2XRos (cat. no. M7513) for 5 min.

To examine the expression level of Mvh, cells were collected for Western blot analysis. The total cell lysates were prepared by solubilizing cells in $2 \times$ SDS sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris, pH 6.8, 10% glycerol, 10 mM NaF and Bromophenol Blue), at approximately 5×10^6 cells/ml. The extracts were heated in a boiling water bath for 5 min and then electrophoresed on 12% SDS/PAGE. The proteins were transferred to a PVDF membrane and incubated for 1 h at room temperature in blocking solution (5% non-fat dry milk in blotting buffer). The membrane was incubated overnight at 4°C in blocking solution containing anti-Mvh antibody (1:2000 ab13840, Abcam) and then washed at room temperature for 30–60 min with five changes of blotting buffer (25 mM Tris, pH 7.4, 0.15 M NaCl, 0.1% Tween[®] 20). The membrane was incubated with HRP-conjugated rabbit anti-mouse antibody for 1 h at room temperature. After washing with five changes of blotting buffer, the membrane was detected with Chemiluminescent Detection Substrate. The mouse anti-GAPDH antibody was used as an internal control.

2.4. RNA isolation and RT-PCR reaction

Total cellular RNA was isolated by Trizol method (Invitrogen), and the quality of RNA samples was determined by measurement of 260:280 ratio. Only samples with a 260:280 ratio of 1.8 or higher were used for reverse transcription. RNA samples were also treated by RNase-free DNase to avoid the genomic DNA contamination. One microgram of RNA from each sample was reverse-transcribed to cDNA using oligo-dT primers and RevertAid[™] reverse transcriptase (Fermentas). PCR reactions were performed using specific primers for 30 cycles at 94°C 30 s, 55–58°C 30 s and 72°C 45 s (Supplementary Table S1 at <http://www.cellbiolint.org/cbi/035/cbi0350365add.htm>). PCR products were separated on 1.0% agarose gel with ethidium bromide. Non-template negative controls were also performed to monitor non-specific reactions. RNA isolated from 1 g of mouse adult testis tissue was used as the positive control. For semiquantitative RT-PCR reaction, an equal amount of RNAs was used in each RT-PCR reaction, and GAPDH used as the internal control was amplified at the same time. PCR products were separated on agarose gel and documented by Genius Bio-Imaging System (Syngene). The relative mRNA contents were calculated based on the intensity of digitized gel bands calibrated by GAPDH. Software used for the quantification was Bio Image Intelligent Quantifier.

2.5. FACS

FACS analysis was used to detect haploidization of induced C2C12 cells. Cells were dissociated with 0.25% trypsin/EDTA,

neutralized with DMEM with 10% FBS, washed twice with PBS and then resuspended in PBS containing 70% ethanol. The treated cells were incubated in an ice-cold water bath for 30 min, followed by washing three times with FACS buffer (PBS with either 1% BSA or 5% FBS, 0.05% NaN₃). Cells were then re-suspended in 20 ml of FACS buffer and 10 ml of PI solution (stock solution 10 mg/ml) and stored for future use. Approximately 1×10^6 cells/ml were used for sorting in a flow cytometer (Beckman Coulter). To determine the ratio of EGFP-positive cells, C2C12/pStra8-EGFP cells were treated with RA for different times, and 1×10^6 cells/ml were sorted by FACS.

3. Results

3.1. Stra8 gene expression in P19 and C2C12 cells

To monitor myoblast transdifferentiation, we made a pStra8-EGFP reporter vector that contained a 1.4-kb mouse Stra8 promoter sequence and an EGFP reporter gene. P19 and C2C12 cells were transfected with the construct, and the transfected cells were treated with 10 μ M *all-trans*-RA for different periods of time. In 24 h after the RA induction, approximately 30% of P19 cells were EGFP positive, indicating that the Stra8 promoter was activated in P19

cells (Figures 1A 1–1A 2). Unlike in P19 cells in which Stra8 was expressed endogenously, the Stra8 promoter was inactive in the transfected C2C12 cells, since no EGFP fluorescence was detected (Figures 1A 3–1A 4). However, after RA treatment for 3 to 8 days, EGFP-positive cells were observed (Figures 1A 5–1A 8). Thus, this suggests that RA treatment can activate Stra8 gene expression in C2C12 cells.

To quantitatively determine the conversion ratio, C2C12 cells were stably transfected with pStra8-EGFP and then induced with RA for 3 and 8 days. Cells were then sorted by the flow cytometry (Figure 1B). A low percentage of EGFP-positive cells (3.5%) were detected in non-induced C2C12/pStra8 cells, which might be due to non-specific spontaneous differentiation. For 3- and 8-day RA-treated C2C12/pStra8 cells, $20.37 \pm 1.85\%$ and $17.53 \pm 3.21\%$ of cells were positive for EGFP, respectively (Figure 1C). There was no significant statistical difference between 3- and 8-day treatments. These results indicate that RA treatment can induce up to 20% of C2C12 cells to express Stra8.

3.2. Stra8 promoter activation in RA-treated cells

To quantitatively measure Stra8 activation in C2C12 myoblasts, cells were transiently transfected with a pStra8-Luc plasmid. Luc activity assays showed that increasing the RA dose could significantly promote Stra8 activation in C2C12 cells (Figure 2A).

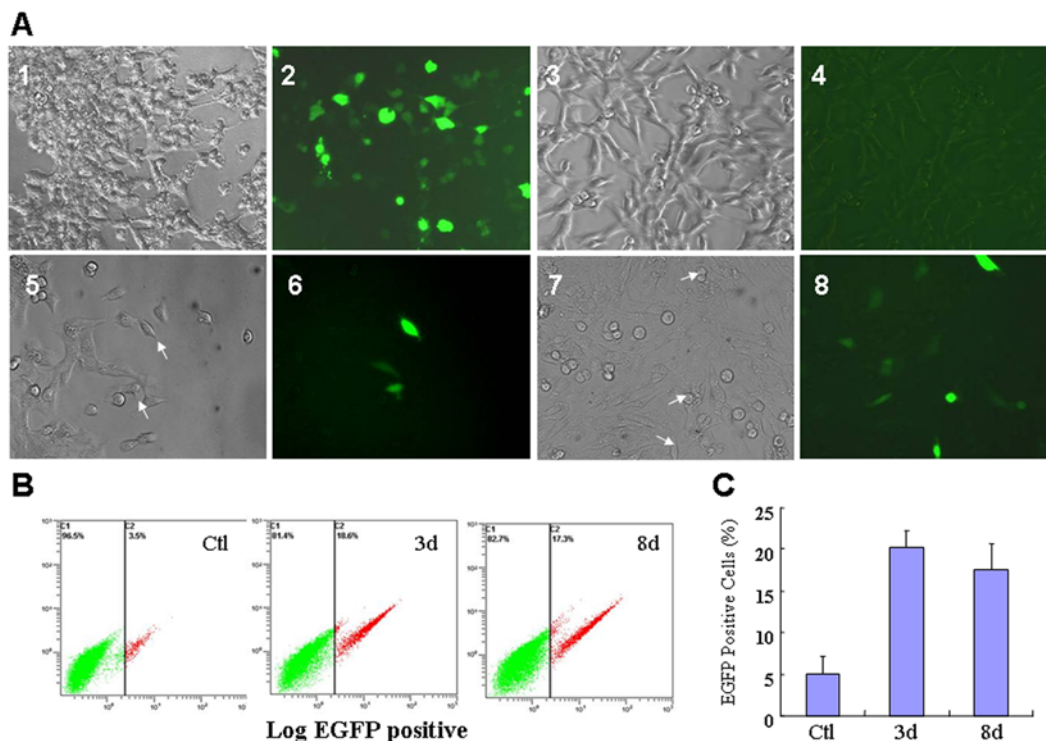


Figure 1 Stra8-EGFP expression in P19 and C2C12 cells

(A) Cells transiently transfected with pStra8-EGFP were induced by 10 μ M *all-trans*-RA. Stra8-EGFP-positive cells were observed using fluorescent microscopy. (1–2) One-day-induced P19 cells. (3–4) Non-induced C2C12 cells. (5–6) Three-day-induced C2C12 cells. (7–8) Eight-day-induced C2C12 cells. 1, 3, 5, 7 are phase-contrast images. 2, 4, 6, 8 are fluorescent images. Arrows indicate cells that produce fluorescence. The magnification is $\times 200$. (B–C) C2C12 cells stably transfected with pStra8-EGFP were induced by RA for 3 and 8 days. EGFP-positive cells were sorted by flow cytometry. (B) Sorting of non-induced C2C12 cells and cells induced for 3 and 8 days. Ctl, non-induced C2C12 cells. (C) Quantification of Stra8-EGFP-positive C2C12 cells was based on flow cytometry sorting ($n=3$).

Based on this dose–response experiment, we selected a concentration of 10 μM RA for our following experiments, in order to avoid the cytotoxic effects of RA. When cells were induced with 10 μM RA for 3 days, luciferase activity in C2C12 cells was increased 3.3-fold, compared with non-induced cells (Figure 2B). Similar results were observed for NIH3T3 fibroblasts, where luciferase activity was increased 3.4-fold in induced cells (Figure 2C). In addition, the relative activity of luciferase was increased 45-fold in RA-induced P19 cells, compared with non-induced cells (Figure 2D). These observations indicate that the Stra8 gene activation is not limited to pluripotent stem cells such as P19 and testis (Oulad-Abdelghani et al., 1996; Giulli et al., 2002). Indeed, this gene can also be activated in RA-treated unipotent cells such as C2C12 myoblasts and NIH3T3 fibroblasts.

3.3. RA-induced C2C12 myoblasts change morphology during transdifferentiation

Previous reports showed that RA treatment could initiate spermatogenesis, while ES cells were undergoing differentiation into male gametes (Bowles et al., 2006; Nayernia, et al. 2006b). To further study the RA-induced differentiation of C2C12 myoblasts, we screened for phenotypic changes in RA-treated C2C12 cells. Normal C2C12 morphology is considered to be a fibroblastoid phenotype (Figure 3A, 1). However, after RA induction for 3 days, some cells assumed a spherical morphology (Figure 3A, 2). When cells were induced for 8 days, a greater number of round cells were observed. In addition, some round cells formed a tail-like structure similar to the male gametes, although the proportion of these cells was low (Figure 3A, 3). When pStra8–EGFP was transiently transfected into RA-induced cells, we found that most

EGFP-positive cells had the spherical morphology (Figures 1A, 5–1A, 8), and some of those EGFP-positive cells showed the initiation of a tail-like structure (Figures 3A, 5–3A, 6). Occasionally, we observed an EGFP-positive cell with round head and long tail (Figures 3A, 7–3A, 8). When RA induction was extended to 12 days, more tail-like cells were monitored (Figure 3B). The tail-like cells was also detected by the labelling of mitochondria and tubulin staining, showing the morphological similarity with male gametes (Supplementary Figure S1 at <http://www.cellbiolint.org/cbi/035/cbi0350365add.htm>). In addition to changing the cell morphology, the Stra8-positive cells also exhibited alterations in cell surface features. We found that Stra8–EGFP-positive cells were able to form very tight cell clusters, similar to the formation of embryoid bodies that occur when cells are cultured in suspension conditions (Figure 3A, 4).

3.4. Characterization of Stra8-positive cells derived from C2C12

To characterize changes in gene expression in transdifferentiated C2C12 cells, we first examined several germ layer markers. RT-PCR results showed that non-induced C2C12 cells only expressed the mesoderm marker Brachyury (T) (Figure 4A). When cells were induced by RA, however, ectoderm markers such as Fgf5 (fibroblast growth factor 5) and NF-68 (neurofilament 68) were detected in both 3- and 8-day-induced cells. Thus, this suggests that RA-induced C2C12 cells might transdifferentiate from the mesoderm to the ectoderm (Figure 4A). To monitor if muscle cell markers remained in the differentiated C2C12 cells, we did immunocytochemistry and semiquantitative RT-PCR analysis. The immunocytochemistry analysis showed that MyoD protein

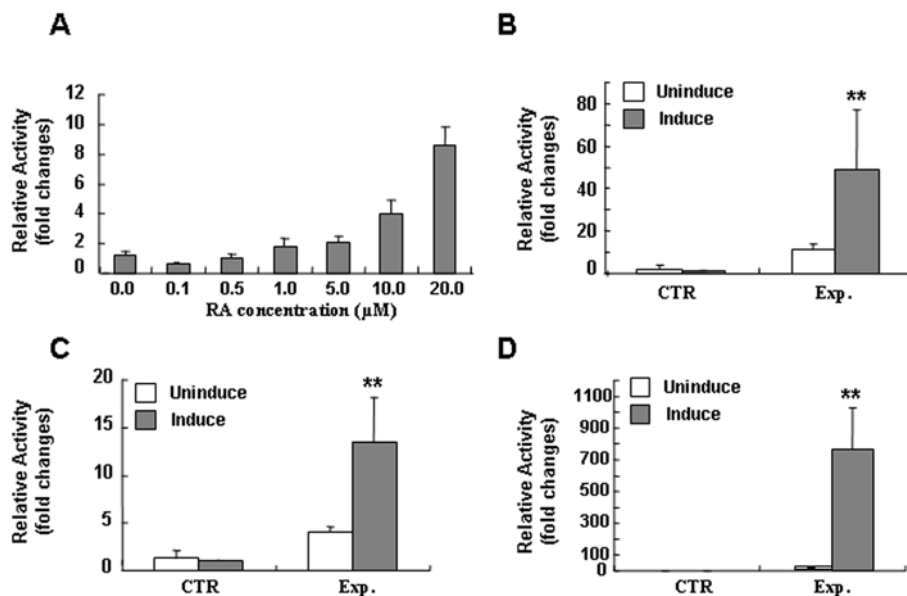


Figure 2 Stra8 promoter activation in EC and mouse somatic cells

Cells transiently transfected with pStra8–Luc were induced with RA for 3 days. Luciferase activity was measured using a Luminometer and following the protocol described in the Materials and methods section ($n=6$). (A) Dose-dependent activation of the Stra8 promoter in C2C12 cells induced with 0.0–20.0 μM RA. (B) C2C12 myoblasts. (C) NIH3T3 fibroblasts. (D) P19 cells. Ctl, cells transfected with the promoter-less vector pGL3-basic; Exp., cells transfected with pStra8–Luc. ** $P<0.01$ statistical analyses by Student's t test.

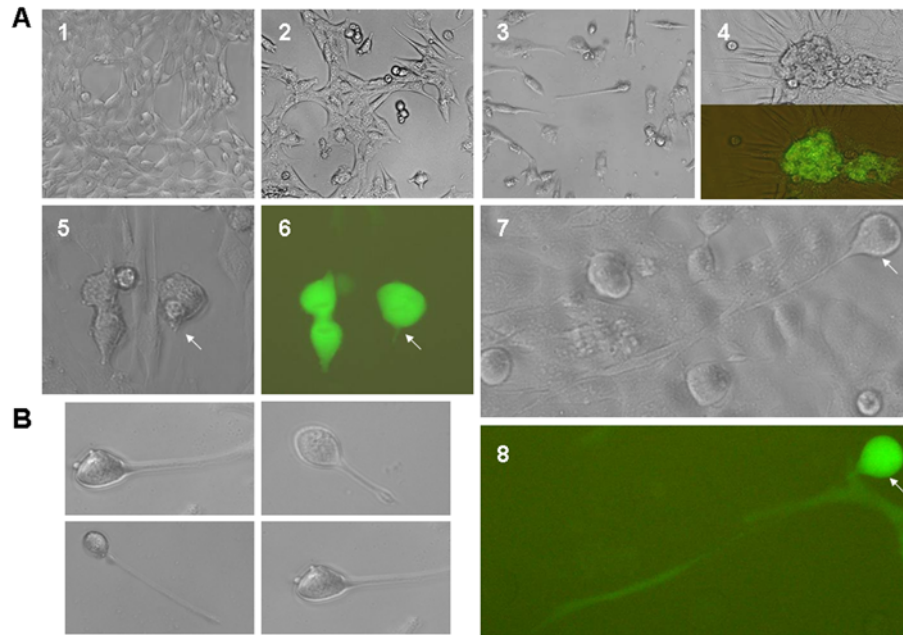


Figure 3 Morphology variation of RA-treated C2C12 myoblasts

(A) C2C12 cells were induced with 10 μ M RA for different lengths of time. (1) Non-treated C2C12 cells; (2) RA treated for 3 days; (3) RA treated for 8 days. (4) pStra8–EGFP stable transfected C2C12 cells were cultured in suspension and formed cell clusters during RA treatment for 3 days. Upper images are phase contrast; lower images are fluorescent cells. (5–6) pStra8–EGFP-transfected C2C12 cells were induced for 8 days with RA; arrows indicate the initiation of sperm tail-like morphology. (7–8) Sperm tail-like morphology of a Stra8–EGFP-positive cell (arrow) induced by RA for 8 days. 7, phase-contrast image; 8, fluorescent image. 1–3 images are at $\times 100$; 4–8 images are at $\times 200$. (B) Images show more sperm tail-like cells from separate experiments that were induced by RA for 12 days. Image magnifications are at $\times 200$.

expression was down-regulated along with the term of RA treatment (Supplementary Figure S2A at <http://www.cellbiolint.org/cbi/035/cbi0350365add.htm>). RT-PCR analysis showed that in RA-treated C2C12 cells, the expression level of muscle markers including MyoG, Myf5 and MyoD were all significantly reduced, although the low-level RNA signals (<10%) could still be detected in 8-day-induced cells (Figure S2, B–C). This result suggested that during the transdifferentiation, the expression of myogenic genes in RA-treated C2C12 cells were down-regulated.

We then screened for the expression of the mouse vasa homologue protein (Mvh), a protein that is essential for male gametogenesis. Indirect immunofluorescence analysis indicated that Mvh was primarily localized to the cytoplasm in RA-induced cells, but it was not detected in non-induced cells (Figures 4C, 1–4C, 4). Western blotting analyses also confirmed Mvh expression in RA-induced C2C12 cells (Figure 4B). Next, we examined the Prm1, a protein that is transcribed in postmeiotic spermatids, protein expression in the differentiated cells. After the induction for 8 days, some differentiated cells did express Prm1 (Figures 4C, 5–4C, 8). These observations indicate that RA treatment can promote C2C12 cell transdifferentiation towards male gamete-like cells.

The transdifferentiation of Stra8-positive cells was also characterized by monitoring the expression of various gene markers characteristic of PGCs (primordial germ cells), as well as premeiotic, meiotic and postmeiotic spermatogonia. Samples were analysed by semiquantitative RT-PCR analysis and

compared with the positive control of testis tissue. Under these conditions, germ cell-related gene markers were undetectable in non-induced C2C12 cells (Figure 4D – C2C12). In contrast, following 3-day induction in C2C12 cells, several markers for the early stages of spermatogonial development were detected, including Dazl and Stra8 (Figure 4D – C2C12). When the induction was extended up to 8 days, additional premeiotic markers were detected, such as Tex18 (testis expressed gene 18) and Rbm (RNA-binding motif gene on Y chromosome). The 8-day-induced cells also expressed meiotic and postmeiotic markers, such as Scp3 (synaptonemal complex protein 3), Acr (acrosin), Tp2 (transition protein 2) and Prm1 (Figure 4D – C2C12). These experimental results indicate that RA can induce C2C12 myoblasts to reprogramme their gene expression profiles similar to that of male gametes. Interestingly, we noticed that RA-treated NIH3T3 fibroblasts did not express meiotic and postmeiotic markers, although the Stra8 gene was expressed following 8 days of RA treatment. In contrast to C2C12 cells, some premeiotic markers, such as Dazl and Rbm, were constitutively expressed in 3T3 cells (Figure 4D – 3T3). Thus, these observations indicate that both C2C12 and 3T3 cells express the Stra8 marker after 8 days of RA induction. However, only C2C12 cells are capable of transdifferentiation into male gamete-like cells.

Finally, to determine whether meiosis occurred in transdifferentiated C2C12 cells, we used flow cytometry to analyse the DNA content of RA-treated cells. For these experiments, we used mouse sperm as a control for haploid cells. Neither RA-treated nor

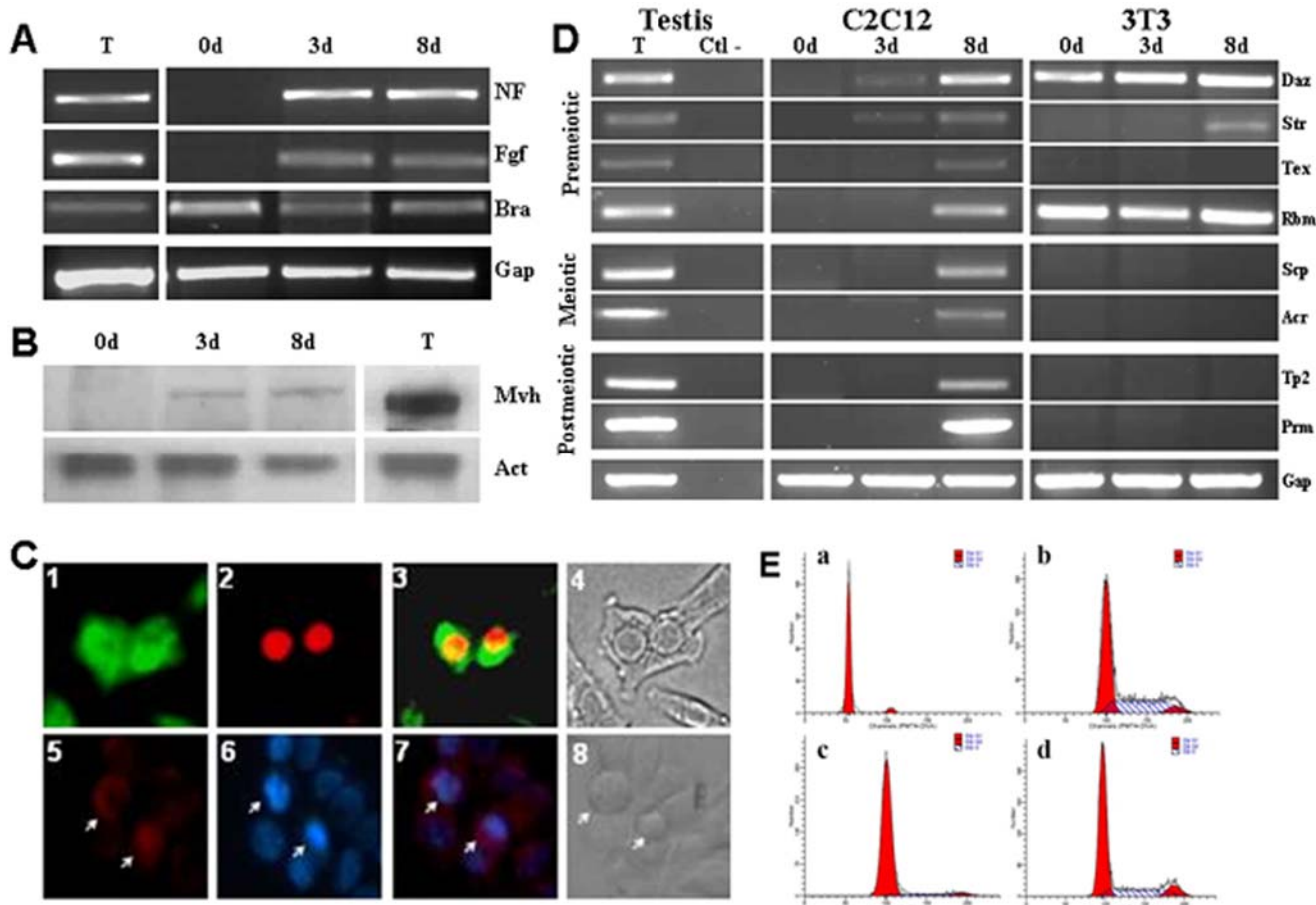


Figure 4 The characterization of gene expression in induced C2C12 cells

(A) RT-PCR analysis of germ layer markers in C2C12 cells induced for 3 and 8 days. (B) Western blotting analysis of Mvh expression in C2C12 cells induced for 3 and 8 days. Mvh is anti-Mvh antibody; Act is anti-actin antibody which was used as an internal control. (C) Immunofluorescent images of RA-treated C2C12 cells. 1, anti-Mvh antibody; 2, PI staining; 3, overlaid image; 4, phase-contrast image; 5, anti-Prm1 antibody; 6, H342 staining; 7, overlaid image; 8 phase-contrast image; arrows indicate the protamine-positive cells. Magnification is at $\times 200$. (D) RT-PCR analysis of premeiotic, meiotic and postmeiotic gene markers. (E) Determination of DNA content of C2C12 cells by flow cytometry analysis. (a) The haploid standard from mouse sperm cells; (b) non-induced C2C12 cells; (c and d) C2C12 cells treated with RA for 3 and 8 days, respectively. Abbreviations: T, mouse testicle tissue, used as the positive control; Gap, GAPDH used as internal control; Ctl-, non-template control; Ctl, non-induced C2C12 or 3T3 cells; Daz, deleted in azoospermia-like gene; Str, Stra8; Tex, testis-expressed gene 18; Rbm, RNA-binding motif gene on Y chromosome; Scp, synaptonemal complex protein 3; Acr, acrosin; Tp2, transition protein 2; Prm1, protamine 1; NF, neurofilament-68 for ectoderm markers; Fgf, fibroblast growth factor-5; Bra, Brachyury (T) gene for mesoderm marker.

untreated C2C12 cells produced a haploid cell population (Figure 4E). This result suggests that the Stra8-positive cells were arrested in the mitotic phase and, as such, they did not undergo meiosis. This occurred even though RA induced C2C12 to produce many of the gene markers of male gametes.

It has been reported that BMP4/ALK3/SMAD5 signalling pathway regulates spermatogonia differentiation (Matsui and Hayashi, 2007). To test if this signaling pathway is activated when C2C12 was treated by RA, we analysed Smad5 gene expression with semiquantitative RT-PCR. The preliminary result showed that after RA treatment for 3 and 8 days, Smad5 expression was significantly increased (>6 -fold), suggesting that RA-induced C2C12, differentiating into tail-like cells, may be regulated through BMP4/ALK3/SMAD5 signalling pathway (Supplementary Figure S3 at <http://www.cellbiolint.org/cbi/035/cbi0350365add.htm>).

4. Discussion

Unipotent cells such as myoblasts and fibroblasts are capable of differentiating into other cell lineages. Graf's laboratory recently reported that NIH3T3 cells could transdifferentiate into macrophage-like cells (Feng et al., 2008) following treatment with transcription factor PU.1 and C/EBPa/b. On the other hand, C2C12 myoblasts can convert from the myoblast differentiation pathway into an osteoblast lineage when exposed to BMP-2 (Katagiri et al., 1994; Okada et al., 2009). In the present study, we found that C2C12 cells changed their morphology from a fibroblastoid shape to a spherical shape, and they expressed Stra8 signal when incubated with RA. Some of these Stra8-positive C2C12 cells showed a tail-like structure similar to the morphology

of sperm, although the cell was larger than a mature sperm. In addition to these morphological changes, the differentiated cells expressed multiple gene markers such as *Mvh* and *Prm1*, which are typically only detected in germ cells. In contrast to C2C12 cells, RA-treated 3T3 fibroblasts did not exhibit changes in morphology, and they did not express the meiotic and postmeiotic gene markers. However, *Stra8* gene expression was detected following 8 days of RA induction. This observation suggests that C2C12 retains some degree of multipotency. Along these same lines, several premeiotic markers such as *Dazl* and *RbmY* could be detected in both induced C2C12 and 3T3 cells, although the expression of meiotic and postmeiotic markers such as *Scp3*, *Acr*, *Tp2* and *Prm1* (Xing et al., 2003) were only detected in C2C12 cells at 8-day induction. The ectoderm-specific markers *Fgf5* and *NF-68* were also detected in RA-differentiated C2C12 cells. Although these markers are always expressed in gametes, they are not thought to be expressed in mesoblasts like C2C12. Meanwhile, the expression of muscle cell-specific markers, such as *MyoG*, *Myf5* and *MyoD* were all significantly down-regulated in RA-treated C2C12 cells. Thus, these results suggest that RA signalling might induce gene reprogramming in C2C12 myoblasts, thereby initiating transdifferentiation.

Both *Mvh* and *Prm1* genes are specifically expressed in migratory PGCs. This occurs in the region of the gonadal ridge in both genders and from the spermatocyte stage to the round spermatid stage in male spermatogenesis (Toyooka et al., 2000). In our experiments, we found that *Stra8*-positive C2C12 cells also expressed these two markers, suggesting that induced C2C12 cells could be reprogrammed to an early stage of spermatogenesis. Although the differentiated cells exhibited a male gamete-like morphology and expressed meiotic and postmeiotic gene markers, there was no haploid cell population detected in the *Stra8*-positive cells. Therefore, this suggests that these cells might arrest at the mitotic spermatogonia or primary spermatocyte stage. There are several possible reasons for this result: (i) the population of derived haploid cells was too low to be selected by flow cytometry; (ii) RA induction time was too short, and as such, the differentiated cells stayed in the mitotic stage. Along these lines, we noticed that more sperm tail-like cells could be observed when extending the induction time from 8 to 12 days (Figure 3B); (iii). In addition to the RA treatment, C2C12 myoblasts may need additional inducers, such as growth factors or transcription factors, in order to promote differentiation to the meiotic stage. Although these ideas are speculative, they should be the subject of future research studies.

BMP-activated Smads are known to play an essential role in PGCs generation and localization in the early mouse embryo (Chang and Matzuk, 2001; Tremblay et al., 2001). Both receptors of *Alk3* and *Smad5* are expressed in migratory PGCs and the germ cell compartment of male fetal gonad and that their expression is maintained in the postnatal life in mitotic spermatogonia, thus suggesting that *Alk3* and *Smad5* mediate BMP signalling throughout the development of mitotic germ cells (Pellegrini et al., 2003). In our preliminary study, we found that the expression of *Smad5* was significantly enhanced in RA-treated C2C12 cells. This observation suggested that RA-activated BMP4/*Alk3*/*Smad5* pathway may be involved in C2C12 cells differentiation into germ

cells. Additional studies are needed to reveal the detailed pathway of C2C12 differentiation towards spermatogonial cells via RA induction.

In conclusion, our results indicate that RA treatment is able to transdifferentiate C2C12 myoblasts into *Stra8*-positive cells. These cells exhibit morphological changes, and they express various male gamete markers. Accordingly, C2C12 myoblasts possess a wider plasticity and may provide a useful cell model to study signal transduction and transdifferentiation during RA treatments.

Author contribution

Wenwen Jia designed and implemented all experiments, prepared all final figures and drafted this article. De Cheng participated in luciferase assay and Western blotting. Shuai Chen and Lei Lei carried out some of the immunocytochemistry. Huayan Wang participated in the experiment design and coordination and helped draft the manuscript. All authors read and approved the final manuscript.

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References

- Aflatoonian B, Moore H. Germ cells from mouse and human embryonic stem cells. *Reproduction* 2006;132:699–707.
- Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S et al. Retinoid signaling determines germ cell fate in mice. *Science* 2006;312:596–600.
- Chang H, Matzuk MM. *Smad5* is required for mouse primordial germ cell development. *Mech Dev* 2001;104:61–7.
- Chung SS, Wolgemuth DJ. Role of retinoid signaling in the regulation of spermatogenesis. *Cytogenet Genome Res* 2004;105:189–202.
- Dyce PW, Wen L, Li J. *In vitro* germline potential of stem cells derived from fetal porcine skin. *Nat Cell Biol* 2006;8:384–90.
- Feng R, Desbordes SC, Xie H, Tillo ES, Pixley F, Stanley ER et al. PU.1 and C/EBPalpha/beta convert fibroblasts into macrophage-like cells. *Proc Natl Acad Sci USA* 2008;105:6057–62.
- Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 2004;427:148–54.
- Giulii G, Tomljenovic A, Labrecque N, Oulad-Abdelghani M, Rassoulzadegan M, Cuzin F. Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. *EMBO Rep* 2002;3:753–9.
- Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T et al. Bone morphogenetic protein-2 converts the differentiation pathway

- of C2C12 myoblasts into the osteoblast lineage. *J Cell Biol* 1994;127:1755–66.
- Li W, Dou ZY, Hua JL, Wang HY. Activation of Stra 8 gene during the differentiation of spermatogonial stem cells. *Sheng Wu Gong Cheng Xue Bao* 2007;23:639–44.
- Matsui Y, Hayashi K. Epigenetic regulation for the induction of meiosis. *Cell Mol Life Sci* 2007;64:257–62.
- Miyamoto T, Sengoku K, Takuma N, Hasuike S, Hayashi H, Yamauchi T et al. Isolation and expression analysis of the testis-specific gene, STRA8, stimulated by retinoic acid gene 8. *J Assist Reprod Genet* 2002;19:531–5.
- Moran JL, Li Y, Hill AA, Mounts WM, Miller CP. Gene expression changes during mouse skeletal myoblast differentiation revealed by transcriptional profiling. *Physiol Genom* 2002;10:103–11.
- Nayernia K, Lee JH, Drusenheimer N, Nolte J, Wulf G, Dressel R et al. Derivation of male germ cells from bone marrow stem cells. *Lab Invest* 2006a;86:654–63.
- Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathack K, Drusenheimer N et al. *In vitro*-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. *Dev Cell* 2006b;11:125–32.
- Okada M, Sangadala S, Liu Y, Yoshida M, Reddy BV, Titus L et al. Development and optimization of a cell-based assay for the selection of synthetic compounds that potentiate bone morphogenetic protein-2 activity. *Cell Biochem Funct* 2009;27(8):526–34.
- Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P et al. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. *J Cell Biol* 1996;135:469–77.
- Pellegrini M, Grimaldi P, Rossi P, Geremia R, Dolci S. Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of BMP4 in spermatogonia differentiation. *J Cell Sci* 2003;116:3363–72.
- Tedesco M, La Sala G, Barbagallo F, De Felici M, Farini D. Stimulated by retinoic acid gene 8 (STRA8) shuttles between nucleus and cytoplasm and displays transcriptional activity. *J Biol Chem* 2009;284(51):35781–93.
- Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M, Noce T. Expression and intracellular localization of mouse vasa-homologue protein during germ cell development. *Mech Dev* 2000;93:139–49.
- Tremblay KD, Dunn NR, Robertson EJ. Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. *Development* 2001;128:3609–21.
- Xing W, Krishnamurthy H, Sairam MR. Role of follitropin receptor signaling in nuclear protein transitions and chromatin condensation during spermatogenesis. *Biochem Biophys Res Commun* 2003;312:697–701.
- Zhou H, Jin Z, Liu J, Yu S, Cui Q, Yi D. Mesenchymal stem cells might be used to induce tolerance in heart transplantation. *Med Hypotheses* 2008a;70:785–7.
- Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM et al. Expression of stimulated by retinoic acid gene 8 (Stra8) and maturation of murine gonocytes and spermatogonia induced by retinoic acid *in vitro*. *Biol Reprod* 2008b;78:537–45.
- Zhou Q, Nie R, Li Y, Friel P, Mitchell D, Hess RA et al. Expression of stimulated by retinoic acid gene 8 (stra8) in spermatogenic cells induced by retinoic acid: an *in vivo* study in vitamin A-sufficient postnatal murine testes. *Biol Reprod* 2008c;79(1):35–42.

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