Chapter 7

Analysis of Histone Deacetylase 7 (HDAC7) Alternative Splicing and Its Role in Embryonic Stem Cell Differentiation Toward Smooth Muscle Lineage

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Abstract

Histone deacetylases (HDACs) have a central role in the regulation of gene expression, which undergoes alternative splicing during embryonic stem cell (ES) cell differentiation. Alternative splicing gives rise to vast diversity over gene information, arousing public concerns in the last decade. In this chapter, we describe a strategy to detect HDAC7 alternative splicing and analyze its function on ES cell differentiation.

Key words HDAC7, Alternative splicing, Plasmid construction, Luciferase assay

1 Introduction

Alternative splicing is a specific feature in eukaryotic species, which has been classified as intron retention, exon skipping, alternative 5′ splice sites or alternative donor sites, and alternative 3′ splice sites or alternative acceptor sites [1]. Through alternative splicing, different mRNA species can be derived from a single gene, giving rise to different protein variants with different even opposite functions, which gives rise to vast diversity over gene information.

Histone deacetylases (HDACs), known as epigenetic regulators of gene transcription, are a family of enzymes that remove acetyl groups from lysine residues of histone proteins, which plays indispensable roles in nearly all biological processes [2]. Mammalian HDACs family, including 18 members, are grouped into four classes according to their homology with yeast histone deacetylases [3]. HDAC7, belonging to Class IIa. There are eight and four transcript variants in mouse and human HDAC7, respectively, which are derived from alternative transcription and splicing. Our previous study revealed that one of the mouse HDAC7 mRNA variants had two ATG codons separated by about 120 nucleotides which included three sequential stop codons. Our studies demonstrated

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that this HDAC7 mRNA underwent further splicing during embryonic stem (ES) cell differentiation towards a smooth muscle cell (SMC) lineage which is essential in maintaining the cardiovascular system in embryonic [4]. Recent study also showed that stem/progenitor cell differentiation toward SMC contributed to neointima formation [5]. Therefore, targeting on HDAC7 splicing may be beneficial for vascular disease intervention.

In this chapter, we describe the strategy that we used to detect HDAC7 alternative splicing and analyze its function. The strategy includes the identification of the existence of different HDAC7 splicing isoforms, the cloning of the different HDAC7 cDNAs and transfection, luciferase reporter analysis, analysis of different HDAC7 isoforms cellular localization by immunofluorescence staining and western blot assay.

2 Materials

2.1 Cell Culture and ES Cell Differentiation

1. Mouse ES cells (ES-D3 cell line, CRL-1934; ATCC, Manassas, VA), HEK293 cells (ATCC).
2. Mouse collagen IV (5 μg/ml).
3. ES culture medium: Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 ng/ml recombinant human leukemia inhibitor factor (LIF), 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin.
4. Differentiation medium (DM): MEM alpha medium supplemented with 10%, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin.
5. HEK293 culture medium: DMEM, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.
6. Phosphate-buffered saline (PBS): 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, no Calcium, no magnesium and a pH of 7.4.
7. 0.25% trypsin–EDTA.

2.2 PCR and Western Blot

1. RNeasy Mini Kit: For purification of total RNA from cells.
2. QIAshredder: For simple and rapid homogenization of cell lysates.
3. Improm-II RT kit: Reverse-transcribe RNA templates starting with total RNA into first-strand cDNA in preparation for PCR amplification.
4. RNase inhibitor: For inhibiting eukaryotic RNases.
5. Taq DNA polymerase: Synthesizes DNA from single-stranded templates in the presence of dNTPs and a primer.

6. Hypotonic Buffer: 10 mM Tris–HCl, pH 7.5; 10 mM KCl; 1 mM EDTA; protease inhibitor.
7. High Salt Buffer C: 10 mM Tris–HCl, pH 7.5; 420 mM KCl; 1 mM EDTA; 30% Glycerol; protease inhibitor.
8. Then ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0)
9. SYBR® Green PCR Master Mix: For PCR amplification.
10. Mouse anti-α-tubulin (Clone B-5-1-2, T 5168), rabbit-anti-HDAC7 (KG-17, H 2662), mouse anti-HA (Clone HA-7, H9658), mouse anti-FLAG (clone M2, F1804), rabbit anti-H4 (SAB4500311) and rabbit anti-MEF2C (SAB2103534) antibodies, and normal mouse IgG (I5381), normal donkey serum (D9663).
11. Protein G-agarose bead.

2.3 Plasmid Construction

1. pGL3-luc basic vector.
2. Renilla luciferase substrate.
3. 1× reporter lysis buffer.
4. Opti-MEM medium.
5. pShuttle2 and Adenoviral expression system 1.

2.4 Transient Transfection and Nucleofection

1. Fugene-6-Reagent: Multicomponent formulation for the transfection of eukaryotic cells.
2. HDAC activity assay kit (colorimetric): Detects HDAC activity.

3 Method

3.1 Detection and Verification of HDAC7 Alternative Splicing

3.1.1 ES Cell Differentiation

1. ES cells are normally maintained in gelatin-coated flask (0.04 % gelatine/PBS for 2h at room temperature) in ES medium, and subcultureed at ratio of 1:6 every other day.
2. For differentiation, ES cells are maintained in collagen IV-coated flasks or plates (5 μg/ml in PBS for 2 h at room temperature) in DM for time indicated. Medium is refreshed every other day.

3.1.2 RNA Extraction

HDAC7 alternative splicing is detected by routine RT-PCR, in which the total RNAs from differentiating cells at various time points are extracted using the RNeasy Mini Kit:

1. The cells are detached by scratching off using a cold plastic cell scraper in the presence of medium and pellet by centrifuging at 6400 × *g* at room temperature for 5 min. The pellet is resuspended in appropriate volume of Buffer RLT.

2. The cell lysates are applied to a QIAshredder column and spun down at 21130 × *g* at room temperature (RT) for 2 min. This step helps to shear chromosomal DNA.
3. One volume of 70% ethanol is added to the sheared lysate, and mixed well by pipetting.
4. Up to 700 μl of the sample is transfered to an RNeasy spin column placed in a 2 ml collection tube.
5. The tube lid is closed gently, and centrifuged at 20000 × *g* at RT for 1 min. Discard the flow-through.
6. Remaining mixed lysates are applied, and repeat **steps 4** and **5**.
7. 700 μl Buffer RW is added to the RNeasy spin column. The tube lid is closed gently, and centrifuged at 20000 × *g* at RT for 1 min. The flow-through is discarded.
8. 500 μl Buffer RPE is added to the column. The tube lid is closed gently, and centrifuged at highest speed at RT for 1 min. The flow-through is discarded. Repeat once.
9. The tube is spun down at 20000 × *g* at RT for 2 min.
10. The RNeasy spin column is placed in a new 1.5 ml collection tube (supplied).
11. 30–50 μl RNase-free water is directly added to the spin column membrane. The tube lid is closed gently, and centrifuged at highest speed at RT for 1 min to elute the RNA.
12. The RNA concentration is measured using NanoDrop machine.

3.1.3 Reverse Transcriptase-Polymerase Chain

1. 2 μg RNA is reverse-transcribed into cDNA with random primer by MMLV reverse transcriptase (RT).
2. The RNA is heated at 70 °C for 5 min and then chilled on ice. A mixture of RT reaction is added to the denatured RNAs, giving to a 20 μl reaction containing 1× RT buffer, 1 mM dNTPs, 3 mM $MgCl_2$, 50 ng random primers, 1 U/μl RNasin plus, and 100 U MMLV-RT.
3. The RT reaction is conducted by a protocol of 25 °C for 5 min, 37 °C for 60 min, and 70 °C 5 min. PCR is performed according to standard procedures.
4. Briefly, a 25 μl volume contains 1× PCR buffer, 0.2 mM dNTPs, 2 mM MgCl2, 10 pmol of primers, 1 U Taq DNA polymerase, and 50 ng cDNA (relative to RNA amount).
5. PCR protocol: 95 °C 5 min to pre-denature cDNAs, 35 cycles of three steps of amplification at 94 °C 1 min, 55 °C 1 min, and 72 °C 1 min, and final extension at 72 °C 5 min.
6. PCR product is run on 2% agarose gel containing 0.5 μg/ml ethidium bromide and image is taken under the BioSpectrum AC Imaging System.

With the primer set of HDAC7-s-1 versus HDAC7-s-2 (Table 1), we have demonstrated that HDAC7 mRNA underwent further splicing during ES cell differentiation toward SMC lineage in vitro (Fig. 1).

3.1.4 Expression Plasmid Construction

To verify the splicing events, we cloned the partially and fully spliced HDAC7 via PCR amplification into a modified pShuttle2-FLAG-HA vector, in which FLAG tag is inserted into downstream of the first ATG codon while HA tag is inserted into the upstream of stop codon in the second open reading frame. A short HDAC7-HA vector (the second open reading frame) was created by mutagenesis. An illustration of the 5′ terminal HDAC7 mRNA sequence is shown in Fig. 2a, in which primer positions are indicated. Figure 2b shows the illustration of the three vectors. If transfected into target cells, fully spliced HDAC7 plasmid will produce FLAG-HDAC7-HA double tagged protein, while partially spliced and short HDAC7

Table 1
Primers for HDAC7 cloning and splicing

Gene	Name of primer	Sequence	Length (bp)	Acc No.
HDAC7-c	HDAC7-c1	5′>cga tct ggt acc tgg atg cac agc c <3′ 5′>gtc agc tct aga ctg aca tca gag acg agg<3′	2603	AK036586
HDAC7-1	HDAC7-1-1 HDAC7-1-2	5′>gcc ggg gct gtg cat cca gg<3′ 5′>gcg ggc tgc cct gcc ctc cag<3′	2546	AK036586
HDAC7-2	HDAC7-2-1 HDAC7-2-2	5′>gcg ggc tgc cct gcc ctc cag<3′ 5′>gtc tcc cta tag tga gtc<3′	2531	AK036586
HDAC7-s	HDAC7-s-1 HDAC7-s-2	5′>cga tct ggt acc tgg atg cac agc c<3′ 5′>gct acg gca ctt cgc ttg ctc<3′	u-371 s-314	AK036586

HDAC7-c Primers for cloning. *HDAC7-1* Primers for Spliced HDAC7, *HDAC7-2* Primers for short HDAC7, *HDAC7-s* Primers to distinguish unspliced and spliced isoforms, *u* unspliced, *s* spliced.

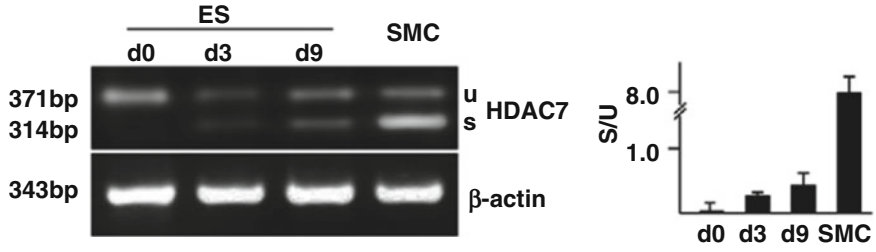

Fig. 1 HDAC7 undergoes further splicing during ES cell differentiation into SMCs. RT-PCR shows HDAC7 splicing in both undifferentiated and differentiated ES cells. The ratio of spliced to unspliced in the *right panel. U* unspliced isoform, *s* spliced isoform. The data presented are representative or means (± s.e.m.) of three independent experiments

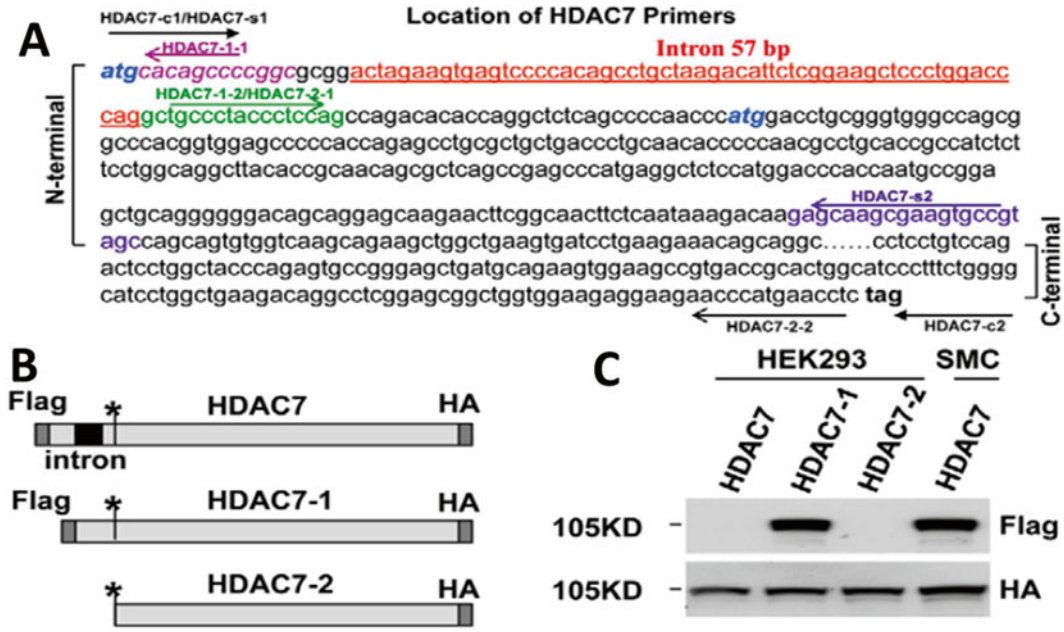

Fig. 2 Cloning of different HDAC7 isoforms. (**a**) A schematic illustration of the location of HDAC7 primers. (**b**) A schematic illustration of the cloned HDAC7 splicing isoforms. (**c**) Western blot confirmation of the HDAC7 isoform clones. HEK293 was transfected with pShuttle2-HDAC7, pShuttle2-HDAC7-1, and pShuttle2-HDAC7-2. SMCs are infected with ad-HDAC7 virus

plasmids only produce HDAC7-HA protein. If partially spliced HDAC7 undergoes further splicing, both HDAC7-HA and FLAG-HDAC7-HA will be detected by Western blot analysis.

HDAC7 Expression Plasmid Construction

1. Total RNAs of 3 day pre-differentiated ES cells are extracted using the RNeasy Mini Kit.
2. Full-length mouse HDAC7 cDNA fragment is obtained by RT-PCR amplification with the primer set HDAC7c-1 and HDAC7c-2 (Table 1).
3. The HDAC7 PCR product is digested with KpnI and XbaI and cloned into KpnI/XbaI sites of the modified pShuttle2-Flag-HA vector, designated pShuttle2-HDAC7. The fully spliced HDAC7, designated as pShuttle2-HDAC7-1 (Flag and HA tagged full length HDAC7) and the short HDAC7 (HA tagged), designated as pShuttle2-HDAC7-2 isoforms are created by PCR-based mutagenesis with specific primer sets (Table 1).
4. All the constructs are verified by DNA sequencing.

Adenoviral Vector Construction

To increase expression efficiency, HDAC7, HDAC7-1, and HDAc7-2 fragments are subcloned into adenoviral expression system 1 vector.

1. pShuttle2-HDAC7, pShuttle2-HDAC7-1, and pShuttle2-HDAC7-2 plasmid DNAs are digested with PI-Sce and I-Ceu.
2. The fragments are ligated to PI-Sce/I-Ceu digested and dephosphorylated adenoviral arm vector.
3. The resulting Ad-HDAC7, Ad-HDAC7-1, and Ad-HDAC7-2 viral DNA vectors are linearized with Pac I digestion and transfected into HEK293 cells for viral particle production, according to the protocol provided.

3.1.5 Transient Transfection and Adenoviral Infection

To verify HDAC7 isoforms, HEK is transfected with pShuttle2-HDAC7-1, and pShuttle2-HDAC7-2 vectors, while SMCs are infected with Ad-HDAC7 virus.

Transient Transfection

1. HEK293 cells are seeded at 1×10^5 cell/well in 6-well plate for 24 h and treated with serum-free DMEM for 1 h prior to transfection.
2. 1 μg HDAC7 isoform plasmids is diluted in 50 μl of Opti-MEM medium and mixed with 3 μl of Fugene 6 reagent in 50 μl of Opti-MEM medium, incubated at room temperature for 30 min and then added to one well of the 6-well plates in serum-free DMEM. Five hours later, one volume of DMEM containing 20 % FBS is added and incubated for 24 h, followed by refreshing medium and further incubation for 24 h.
3. After 48 h, the cells are subjected to Western blot assays.

Adenoviral Infection

SMCs are seeded at 1×10^5 cell/well in 6-well plate for 24 h prior to virus infection. Upon infection, the medium is removed, and 1 ml fresh growth medium containing 1×10^6 viral particle [at 10 multiplicity of infection (MOI)] is added and incubated for 6 h, and then cultured in normal growth medium for 24 h. The cells are then subjected to Western blot assays.

3.1.6 Western Blot Analysis

1. The cell culture plates, or flasks are placed on ice and washed with ice-cold PBS.
2. The cells are scratched off using a cold plastic cell scraper, and transferred into a precooled microcentrifuge tube.
3. The ice-cold lysis buffer 0.5 ml per 5×10^6 cells/60 mm dish/75 cm^2 flask is added, supplemented with protease inhibitors and 0.5 % Triton X-100, and lysed by sonication. Constant agitation is maintained for 30 min at 4 °C.
4. The cell lysates are spun at $16{,}000 \times g$ for 5 min in a 4 °C precooled centrifuge.
5. The tube is gently removed from the centrifuge and placed on ice. The supernatant is transfered to a fresh tube kept on ice, and the pellet is discarded.
6. The protein concentration is determined.

7. To reduce and denature: 5× SDS is added and each cell lysate in sample buffer is boiled at 100 °C for 5 min and aliquot. Lysates are stored at −20 °C.
8. Equal amounts of protein are loaded into the wells of the SDS-PAGE gel, along with molecular weight markers. 40 μg of total protein is loaded from cell lysate.
9. Samples are run on the SDS-PAGE gel for 1.5 h at 160 V.
10. The separated protein is transferred to nitrocellulose transfer membranes.
11. The membranes are blocked with TBS containing 5% dry milk and 0.1% Tween 20 on a shaker at room temperature for 1 h.
12. The membranes are incubated with appropriate dilutions of primary antibody in 5 or 2% blocking solution overnight at 4 °C or for 2 h at room temperature.
13. The membranes are washed in three washes of TBST, 5 min each.
14. The membranes are incubated with the appropriate primary antibodies at 4 °C overnight.
15. The membranes are washed in three washes of TBST, 5 min each.
16. The membranes are incubated with the labeled secondary antibody (1:1000) in 5% blocking buffer in TBST at room temperature on a shaker for 1 h.
17. The membranes are washed in three washes of TBST, 5 min each, then rinse in TBS.
18. The membranes are developed. Blots are stripped and re-probed with α-tubulin as loading control.

From the experiments described above, HDAC7-1 is detected by both anti-FLAG and anti-HA antibodies, while HDAC7 and HDAC7-2 are detected only by HA as expected (Fig. 2c). As shown in Fig. 1, SMCs shows higher HDAC7 splicing. Indeed, Ad-HDAC7 infection in SMCs produced both FLAG and HA bands, and the FLAG band is much stronger (Fig. 2c), confirming further splicing event in SMCs.

3.2 To Analyze the Potential Role of HDAC7 Splicing in SMC Differentiation

As described above, HDAC7 undergoes further splicing during ES cell differentiation toward to SMC lineage. We wondered whether this splicing was involved in this process. We used luciferase reporter system to assess the effect of overexpression of HDAC7 isoforms on SMC marker gene expression like SM22 and SMA.

3.2.1 Report Gene Plasmid Construction

The DNA fragments corresponding to mouse SM22 (*AH003214*) and SMA (*M57409*) gene promoter are amplified from mouse genomic DNA with primer set as follows:

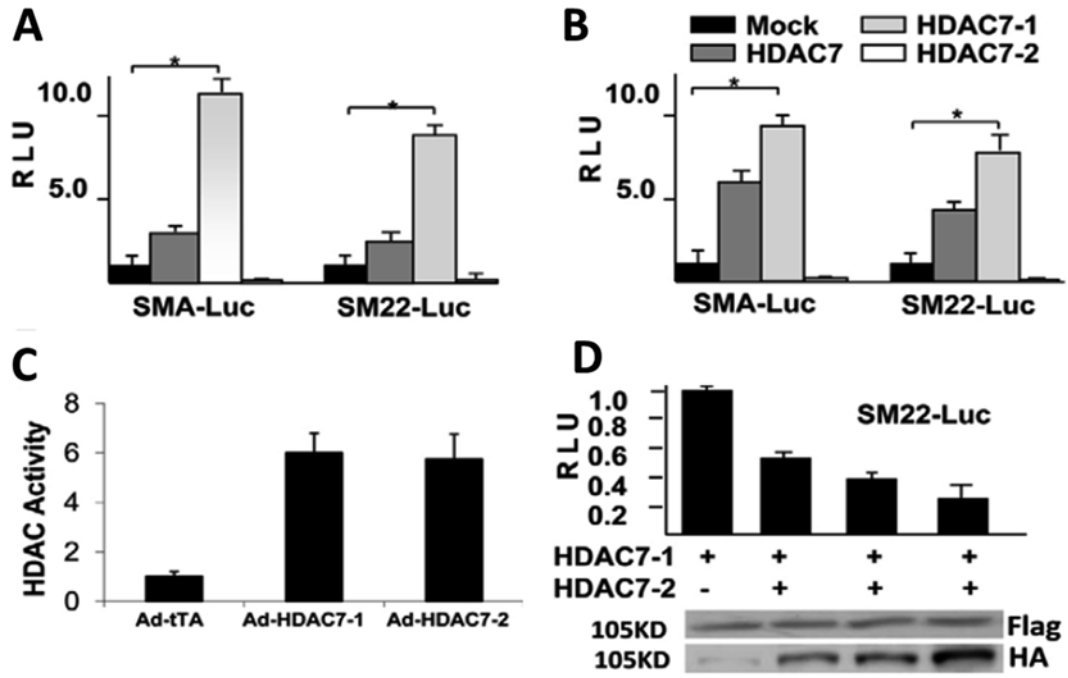

Fig. 3 Different HDAC7 isoforms exert different effects on SMC differentiation. (**a**, **b**) Luciferase reporter analysis shows the different effect of HDAC7 isoforms on SMA-Luc and SM22-Luc reporters in differentiated ES cells (**a**) and mature SMCs (**b**), respectively. *: $p < 0.05$. (**c**) Both HDAC7 isoforms possess similar deacetylase activity. Differentiated ES cells were infected with ad-HDAC7-1 and ad-HDAC7-2 viruses and incubated for 48 h, followed by HDAC activity assays. Ad-tTA was included as control. (**d**) HDAC7-2 suppresses HDAC7-1-induced SM22 reporter gene expression in a dose-dependent manner in ES cells. ES cells were cotransfected with 0.5 μg pShuttle2-HDAC7-1, and 0, 0.5, 1.0, 1.5 μg pShuttle2-HDAC7-2, pShuttle2 empty vector was included to compensate the total plasmid amount. HA and Flag represent the expression of the short and spliced forms, respectively. *RLU* relative luciferase unit. The data presented are representative or means (± s.e.m.) of nine independent experiments for (**a**), (**b**), (**d**) and three for (**c**)

SM22-P, 1350 bp, 5′-ttcaggacgtaatcagtg-3′ (nt 4-21) and 5′-agcttcggtgtctgggctg-3′ (nt 1371-1353); SMA-P, 1113 bp, 5′-tgcatgagccgtgggag-3′ (nt 16-32) and 5′-acttaccctgacagcgac-3′ (nt 1128-1111). The PCR product of SM22 and SMA gene promoter sequences are cloned into pGL3-luc basic vector, designated pGL3-SM22-Luc and pGL3-SMA-Luc, respectively. All the plasmids are verified by DNA sequencing. Reporter analysis has revealed that different HDAC7 isoforms had different effect on SM22 and SMA expression in ES or SMC cells (Fig. 3a, b, and d).

3.2.2 Transient Transfection

1. ES cells are seeded at 2×10^4cell/well in collagen IV-coated 24-well plates in DM 48 h and pretreated with serum free alpha-MEM (500 μl/well) for 1 h prior to transfection. SMCs are seeded at 2×10^4cell/well in gelatin-coated 24-well plates in DMEM 24 h and pretreated with serum free DMEM (500 μl/well) for 1 h prior to transfection.

2. One microgram pGL3-SM22-Luc or pGL3-SMA-Luc reporter plasmid, 1 μg HDAC7 isoform plasmids, plus 0.2 μg Renilla-Luc reporter plasmid are diluted in 50 μl of Opti-MEM medium and mixed with pre-diluted 7 μl of Fugene 6 in 50 μl of Opti-MEM medium, incubated at room temperature for 30 min and then added to three wells of the 24-well plates and incubated for 5 h. After that, the medium is removed and complete growth medium is added and incubated for 48 h, followed by luciferase activity assays.

3.2.3 Luciferase Reporter Assay

1. Cells are transfected with reporter plasmids and overexpression plasmids as described above in Subheading 3.3.
2. After 48 h transfection, the cells are washed once with PBS.
3. The cells are lysed with 200 μl/well 1× reporter lysis buffer.
4. All assay components (reagent and sample) are allowed to equilibrate to room temperature prior to assay.
5. 30 μl cell lysate is mixed with 100 μl firefly luciferase.
6. After at least 10 min, detect luciferase activity.
7. The relative luciferase activity unit (RLU) is defined as the ratio of firefly luciferase activity to that of Renilla luciferase activity with that of control group set as 1.0.

Data presented in Fig. 3a, b, and d revealed that both HDAC7 and HDAC7-1 plasmids increased, while HDAC7-2 decreased SM22 and SMA reporter gene expression in ES cells (Fig. 3a) and SMCs (Fig. 3b). HDAC7-2 exerted suppressive effect on HDAC7-1-induced SM22 reporter gene expression in ES cell in a dose-dependent manner (Fig. 3d).

3.3 To Explore the Underlying Mechanisms

From the data described above, we have found that fully spliced HDAC7 (HDAC7-1) promoted SMC differentiation while the short one (HDAC7-2) exerted suppressive effect. As HDAC7 is a class-II HDAC, its deacetylase activity is largely derived from associated class I HDACs [6]. As HDAC7-2 lacks the first 22 amino acids as compared to HDAC7-1, we wonder whether this shortage decreases its interaction with class I HDACs, therefore possessing less deacetylase activity. To test this, HDAC activity assays were performed.

3.3.1 Histone Deacetylase Activity Assay

1. ES cells are seeded on collagen-IV-coated dishes and cultured in DM for 3 days (70% confluence).
2. Ad-tTA (empty vector control), Ad-HDAC7-1, Ad-HDAC7-2 virus are added at 10 MOI in DM.
3. After 6 h, the virus solution is removed and fresh DM medium is added and incubated for 2 days.
4. The cells are lysed using the same procedure for Western blot **steps 1–6**.

5. 50 μg cell lysate is added to 96-well plate and subjected to HDAC activity assay.

Data presented in Fig. 3c shows that overexpression of HDAC7-1 and HDAC7-2 via adenoviral gene transfer significantly increases HDAC activity. There is no difference between these two isoforms concerning its HDAC activity. So the suppressive effect of HDAC7-2 seems not derived from HDAC activity.

HDAC7 is a shuttle protein, located in both cytoplasm and nucleus [7]. So the next step is to assess whether HDAC7-1 and HDAC7-2 differ in cellular location. To address this issue, immunofluorescence staining and cellular fraction assays were performed.

3.3.2 Indirect Immunofluorescence Staining

1. SMCs are infected Ad-HDAC7-1 or Ad-HDAC7-2 virus at 10 MOI for 6 h and incubated in complete growth medium for 24 h.
2. The cells are detached from the flasks by trypsin and seeded onto gelatin-coated slides in growth medium and incubated for 24 h.
3. The cells on slides are washed once with PBS quickly to remove medium and fixed with 4% paraformaldehyde solution at room temperature for 15 min.
4. After three times of PBS washing with 5 min each, the cells are permeabilized by using 1% Triton X-100/PBS at room temperature for 10 min.
5. After three times of PBS washing with 5 min each, the permeabilized cells are incubated with 5% donkey serum in PBS at room temperature for 30 min (blocking procedure to prevent nonspecific intracellular antibody binding).
6. Diluted mouse anti-HA antibody (1:100 in 5% donkey serum/PBS) is applied to the slides and incubated at 37 °C in humidified box for 1.5 h, followed by three washes with PBS, 5 min each.
7. The cells are washed with PBS three times.
8. Alexa Fluor 546 donkey anti-mouse IgG (Dako, 1:1000 in 5 % donkey serum/PBS) was applied and incubated at 37 °C in humidified box for 40 min.
9. After three washes with PBS, 5 min each, the slides are counterstained with DAPI for 3 min, followed by PBS washing three times.
10. Coverslip is applied together with fluorescent mounting medium and the staining is observed and images are taken under a confocal microscope.

As shown in Fig. 4a, HDAC7-1 was located in both cytoplasm and nucleus, while HDAC7-2 was mainly located in the cytoplasm.

3.3.3 Cellular Fraction

1. SMCs are infected with Ad-tTA or Ad-HDAC7-2 virus at 10 MOI for 48 h.

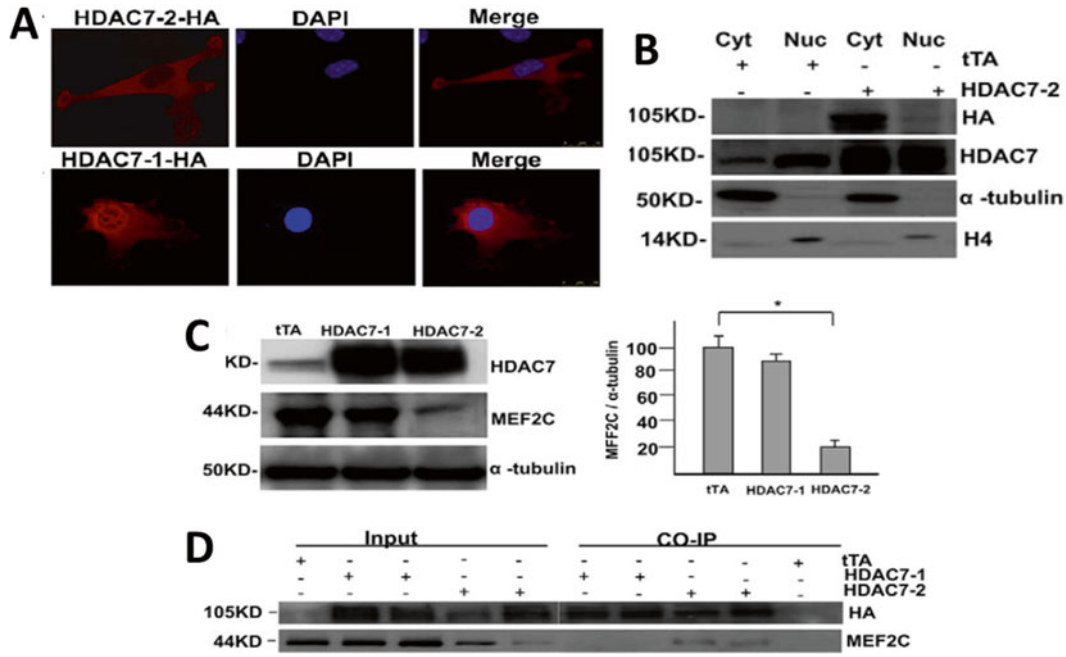

Fig. 4 Different HDAC7 isoforms show different cellular location and exert different effect on MEF2C. (**a**, **b**). HDAC7 isoforms shows different cellular location. SMCs were infected with ad-HDAC7-1, Ad-HDAC7-2 for 48 h, followed by immunofluorescence staining with anti-HA antibody (**a**) or cellular fraction and Western blot (**b**). Ad-tTA was included as control. Note that HDAC7-2 is mainly located in the cytoplasm. Scale bars: 50 μm. (**c**) HDAC7-2 decreased MEF2C protein level in SMCs as revealed by Western blot analysis. The *right panel* shows the relative amount of MEF2C against tubulin loading control. *: $p < 0/05$. € HDAC7-2 physically interacts with MEF2C as revealed by immunoprecipitation assays. Data presented are representative images or mean of three independent experiments

2. The cells are washed with ice-cold PBS containing 5% FBS, and then scratched off from the flask with rubber policemen.
3. The cells are pelleted by spinning at 6400 × *g* at 4 °C for 5 min.
4. Cell pellet is resuspended in 1 ml cold PBS, and transfered into 1.5 ml centrifuge tube.
5. Cells are spun down at 10,000 × *g* at 4 °C for 10 s, and the supernatant is thoroughly decanted.
6. The cell pellet is resuspended in 200 μl/75 ml-flask of hypotonic buffer, and incubated on ice for 15 min with vortexing briefly every 5 min.
7. 12.5 μl of 10% NP-40 is added and vortexed immediately at 20,000 × *g* for 10 s.
8. The mixture is spun down at 20,000 × *g* at 4 °C for 10 s.
9. The supernatant is transferred to a fresh tube; this is the cytoplasm fraction.

10. The nuclear pellet is washed once with ice-cold PBS and then resuspended in 70 μl of high salt buffer C and incubated on ice for 45 min with vortexing every 5 min.
11. After spinning down at 20,000 × *g* at 4 °C for 5 min, and the supernatant is recovered. This is the nuclear extract.
12. The cytoplasm and nuclear fractions are subjected to Western blot analysis.

As shown in Fig. 4b, HDAC7-2 was mainly located in the cytoplasm, confirming the observation by immunofluorescence staining (Fig. 4a).

MEF2C is reported to play an essential role in SMC differentiation [8]. We wondered whether HDAC7-2 affected MEF2C and therefore suppressed SMC differentiation. First, we detected the MEF2C protein levels by Western blot assays following HDAC7-1 and HDAC7-2 overexpression via adenoviral gene transfer. As shown in Fig. 4c, HDAC7-2 significantly decreased MEF2C protein level. Thereafter, we tested whether there was a direct interaction between HDAC7 and MEF2C by co-immunoprecipitation.

3.3.4 Co-immunoprecipitation

1. ES cells are cultured in collagen IV-coated flasks in DM for 3 days, and then infected with Ad-tTA, Ad-HDAC7-1 or HDAC7-2 at 10 MOI and cultured for 48 h.
2. The cells are lysed as described in Western blot **steps 1–6**.
3. 1 mg whole lysates are pre-cleared with 2 μg normal mouse IgG and 10 μl of Protein G-agarose bead (Sigma) by incubation at 4 °C on a rotator for 1 h, followed by spinning at 10,000 × *g* at 4 °C for 5 min to remove the beads.
4. The supernatant is incubated with 2 μg mouse anti-HA antibody at 4 °C on a rotator for 2 h, and 10 μl of Protein G-agarose beads is added and incubated for another 2 h.
5. The beads are pulled down by spinning at 400 × *g* at 4 °C for 3 min, and washed five times with 1 ml PBS buffer, 5 min each.
6. The precipitate is resuspended in 25 μl of 1× SDS loading buffer, and incubated at 70 °C for 30 min, followed by spinning at 400 × *g* at 4 °C for 3 min.
7. The supernatant is applied to Western blot probed with anti-MEF2C and HA. 50 μg cell lysate is included as input control.

As shown in Fig. 4d, HDAC7-2 but not HDAC7-1 binds to MEF2C, which might cause MEF2C degradation.

From the strategy and experiments described above, we have demonstrated that HDAC7 undergoes further splicing during ES differentiation toward SMC differentiation. The partially spliced HDAC7 mRNA produces a short HDAC7 isoform, which mainly

locates in the cytoplasm and interacts with transcription factor MEF2C. The interaction may retain MEF2C in the cytoplasm and increase MEF2C degradation, therefore suppressing SMC marker gene expression. Further splicing will alleviate the production of short HDAC7 isoform, therefore abolishing the suppressive effect and leading to SMC marker expression.

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References

1. Sun X et al (2015) SplicingTypesAnno: annotating and quantifying alternative splicing events for RNA-Seq data. Comput Methods Programs Biomed 119(1):53–62, PubMed: 25720307
2. Lee JH, Hart SR, Skalnik DG (2004) Histone deacetylase activity is required for embryonic stem cell differentiation. Genesis 38(1):32–38, PubMed:14755802
3. Grozinger CM, Hassig CA, Schreiber SL (1999) Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proc Natl Acad Sci U S A 96(9):4868–4873, PubMed: 10220385
4. Margariti A et al (2009) Splicing of HDAC7 modulates the SRF-myocardin complex during stem-cell differentiation towards smooth muscle cells. J Cell Sci 122(Pt 4):460–470, PubMed: 19174469
5. Tang Z et al (2012) Differentiation of multipotent vascular stem cells contributes to vascular diseases. Nat Commun 3:875, PubMed: 22673902
6. Haberland M et al (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet 10(1):32–42, PubMed: 19065135
7. Kato HI et al (2004) Histone deacetylase 7 associates with hypoxia-inducible factor 1alpha and increases transcriptional activity. J Biol Chem 279(40):41966–41974, PubMed: 15280364
8. High FA et al (2007) An essential role for Notch in neural crest during cardiovascular development and smooth muscle differentiation. J Clin Invest 117(2):353–363, PubMed: 17273555