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Original Article

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Notch signaling leads to a slower progression of embryonic myogenic differentiation in Landrace than in Langtang pigs

Shufang Cai[1,2], Bin Hu[1], Qi Zhu[2], Tianqi Duo[2], Xiaoyu Wang[2], Xian Tong[2], Xiaorong Luo[2], Renqiang Yuan[2], Yaosheng Chen[2], Jing Wang[3], Chenglong Luo[1,*], Baosong Xing[3,*], and Delin Mo[2,*]

[1]State Key Laboratory of Livestock and Poultry Breeding, Guangdong Public Laboratory of Animal Breeding and Nutrition, Guangdong Key Laboratory of Animal Breeding and Nutrition, Institute of Animal Science, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China, China, [2]State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510006, China, and [3]Henan Key Laboratory of Farm Animal Breeding and Nutritional Regulation, Institute of Animal Husbandry and Veterinary Science, Henan Academy of Agricultural Sciences, Zhengzhou 450002, China

*Correspondence address: Tel: +86-20-87576210; E-mail: luochenglong@gdaas.cn (C.L.) / Tel: +86-371-65737641; E-mail: bsxing@126.com (B.X.) / Tel: +86-20-39332991; E-mail: modelin@mail.sysu.edu.cn (D.M.)

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Abstract

Delving into porcine embryonic myogenesis is the key to elucidate the complex regulation of breed-specific differences in growth performance and meat production. Increasing evidence proves that pigs with less meat production show earlier embryonic myogenesis, but little is known about the underlying mechanisms. In this study, we examine the longissimus dorsi muscle (LDM) by immunohistochemistry and confirm that the differentiation of myogenic progenitors is increased ($P<0.05$) in Lantang (LT, fatty) pigs compared with that in Landrace (LR, lean) pigs, which results in more ($P<0.001$) differentiated myoblasts ($Pax7^{-}/MyoD^{+}$) and less ($P<0.001$) myogenic progenitors ($Pax7^{+}/MyoD^{-}$) in LT pigs at 35 days post-conception (35dpc). Additionally, embryonic myogenic progenitors isolated from LT pigs show greater ($P<0.001$) differentiation capacity with earlier expression of MyoD compared with those from LR pigs. Moreover, Notch signaling is more active ($P<0.05$) in LR pig myogenic progenitors than in LT pig myogenic progenitors. Inhibition of Notch signaling in LR myogenic progenitors suppresses Pax7 expression and increases MyoD expression, thus promoting myogenic differentiation. Consistently, the process of myogenic progenitors differentiating into myoblasts in *ex vivo* embryo limbs is accelerated when Notch signaling is inhibited. These results indicate that Notch signaling facilitates the maintenance of myogenic progenitors and antagonizes myogenic differentiation by promoting Pax7 expression and preventing MyoD expression in LR pigs.

Key words myogenic progenitor, MyoD, Pax7, embryonic myogenesis, differentiation

Introduction

Porcine skeletal muscle growth is predominantly determined by the total number of myofibers formed during prenatal skeletal muscle development, including two successive generations of myofibers [1,2]. Primary (embryonic) myogenesis takes place between 35- and 55-days post-conception (dpc), forming the first appeared multinucleated myofibers which are officially called primary myofibers and establish the scaffold of skeletal muscles. Following this, secondary (fetal) myofibers that form around each primary myofiber from 50 to 90dpc are important for muscle growth and maturation [3,4]. After birth, muscle growth depends entirely on hypertrophy of myofibers [5,6]. Therefore, skeletal muscle development during the embryonic stage has a profound impact on the long-term growth potential and muscle mass of adult pigs, and thus on livestock meat production [7].

Myogenic progenitors, originated from cells of the dermomyotome, labeled by the paired box transcription factors Pax3 and Pax7, are responsible for muscle development [8]. During embryonic

muscle formation, a fraction of muscle progenitors, referred to as embryonic myogenic progenitors, proliferate, differentiate, and fuse into myotubes. This progress is under the cooperative control of the muscle regulatory factors (MRFs), including Myf5, MyoD, MyoG, and MRF4 [9]. Among them, Myf5 and MyoD, as myogenic commitment factors, contribute to myoblast specification and differentiation, while MyoG and MRF4 are differentiation factors, inducing myogenic terminal differentiation [10,11].

Molecular mechanisms concerning the determination and differentiation of myoblasts during porcine embryonic development remain to be further explored. Previous studies in mouse reported that Notch and Wnt signaling pathways are involved in this process. Notch signaling pathway is implicated as an important regulator of proliferation and differentiation of myogenic progenitors [12,13]. In addition, it is also very important to maintain satellite cell quiescence [14,15]. Notch signaling activation requires physical interaction between a ligand (delta1/4 or jagged 1/2) and one of the four Notch receptors (Notchs 1–4). This interaction leads to the release of the Notch intracellular domain (NICD), which then translocates into the nucleus where it binds to the Rbpj transcription factor and induces downstream effectors, such as the Hes/Hey family [16,17]. Wnt signaling has been demonstrated to be crucial for the maintenance of fetal muscle progenitors in mouse [18,19], and also has been shown to play a important role in the expansion of satellite cells [19,20]. In addition, AKT/mTOR signaling also regulates the differentiation of myoblasts [21].

Increasing evidence proved that pigs with less meat production show more intense embryonic myogenesis. For example, the myofiber density and diameter are significantly higher in Meishan pigs (a Chinese indigenous breed) than in Large White pigs (a lean breed) at 35dpc [22]. In our previous study, we found that Wuzhishan pigs (a miniature pig breed) show earlier myoblast differentiation than Landrace (LR, lean) pigs in embryonic stages [23]. Primary muscle fibers appear earlier in Lantang (LT) pigs than that in LR pigs, at 35dpc and 42dpc respectively [24]. These findings suggest that the progression of embryonic myogenic differentiation is faster in pig breeds with less meat mass, which results in earlier myofiber formation.

In the present study, to verify this possibility, we compared the dorsal myogenesis progression of LT and LR pigs at 35dpc which was the time when myogenic cells rapidly differentiate and primary myofibers begin to appear. We also compared the differentiation capacities of their myogenic progenitors *in vitro*, and revealed for the first time that their differences are associated with Notch signaling.

Materials and Methods

Animals and tissues

Six LT sows and six LR sows were artificially inseminated with semen from the same breed boars, respectively. The pigs were allowed free access to food and water *ad libitum* and were housed under identical conditions. Sows were sacrificed at 35dpc, and embryos were collected. For each embryo, the longissimus dorsi muscle (LDM) tissues were isolated and digested to isolate embryonic muscle progenitors or fixed to prepare paraffin sections of tissue, or frozen in liquid nitrogen for further use. For each experiment, embryonic LDM tissues from three sows were used as biological replicates. Experimental animal procedures and protocols we used were approved by the Animal Care and Use Committee of Guangdong Province, China. The approval ID or permit numbers are SCXK (Guangdong) 2011-0029 and SYXK (Guangdong) 2011-0112.

Embryonic muscle progenitor isolation and culture condition

Embryonic muscle progenitors were isolated from the LDM of embryos of two breeds at 35dpc. The isolated LDM was cleaned free of connective tissues, minced and digested with 0.2% collagenase type I (Sigma, Shanghai, China) solution in a water bath at 37°C for 2 h to get sufficient cells. Mixed cells were pre-plated 2 h in 5.0 mL growth media on a culture dish to remove fibroblasts and then transferred to a new culture dish for attachment. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO, New York, USA) supplemented with 20% (v/v) fetal bovine serum (FBS; GIBCO), 1% penicillin-streptomycin antibiotics (MesGenBiotech, Shanghai, China) and 0.5% chicken essential extract (growth medium, GM). For experiments, muscle progenitors were sub-cultured onto 12-well plates at densities of 1×10^4 cells per well. Cells were switched into DMEM with 2% horse serum (differentiation medium, DM) after reaching 100% confluence to induce differentiation.

C2C12 cells purchased from American Tissue Culture Collection (ATCC; Manassas, USA) were cultured in DMEM with 10% FBS, and 1% penicillin-streptomycin (GM) at subconfluent density. To induce differentiation, cells were switched into DM when cells reached 100% confluence. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

In vitro Notch activation or inhibition

For Notch activation, C2C12 cells were treated with the Jagged1 peptide (CDDYYYGFGCNKFCRPR) or a control scrambled peptide (RCGPDCFDNYGRYKYCF) (GenScript, Piscataway, USA) (20 μg/mL) for 48 or 72 h in proliferation or myogenic differentiation assay [25,26]. For Notch inhibition, embryonic muscle progenitors cultured in GM were treated with DAPT [20 μM,dissolved in dimethyl sulfoxide (DMSO)] for 60, 72, 84, 96, 108, 120 or 132 h according to different assays; embryonic muscle progenitors cultured in DM were treated with DAPT for 144 h; and control cells were treated with carrier (DMSO) only [27].

Explant

Forelimbs from LR 35dpc embryos were cultured in 12-well plates in serum-free BGJb medium (Life Technologies, Carlsbad, USA) supplemented with 200 μg/mL ascorbic acid (Life Technologies) and 1% penicillin-streptomycin antibiotics. For Notch inhibition, forelimbs originated from the same embryo were immediately treated with 20 μM DAPT or DMSO carrier for 30 h. Then, the treated forelimbs and control forelimbs were cleaned and collected for further analysis.

Western blot analysis

Protein extracts were obtained from cultured cells, LDM or forelimbs homogenates using lysis buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, pH 8.0) supplemented with protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Thermo Scientific, Waltham, USA) on ice until protein was released completely. Total extracts were separated by SDS-PAGE on 10% (w/v) polyacrylamide gels, transferred onto 0.45-μm PVDF membrane (Roche, Basel, Switzerland). After being

blocked in 4% bovine serum albumin (BSA) for 1–2 h, the membranes were incubated with specific primary antibodies at 4°C overnight, then incubated with secondary antibodies for 1 h at room temperature. Blots were visualized using an Enhanced chemiluminescence (ECL) detection kit (FDbio, Hangzhou, China). β-Tubulin or GAPDH were used as loading controls. Antibodies are listed in Supplementary Table S1.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cultured cells, LDM or forelimbs using Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions, then cDNA was synthesized from 1 μg total RNA using a reverse-transcription kit (Promega, Beijing, China). The real-time quantitative PCR (qPCR) was performed using a SYBR Green qPCR kit (Genestar, Beijing, China), and detected on the LightCycler 480 II system (Roche). The primers used for qPCR are listed in Table 1. *GAPDH* was used as internal control and all reactions were run in triplicate.

Immunofluorescence microscopy

Cultured cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 15–20 min. After being blocked with 4% BSA in Tris-buffered saline with Tween (TBST) for 1 h, the cells were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000 in PBS). Antibodies used are listed in Supplementary Table S1. Immunostaining images were obtained with a fluorescence microscope (Nikon, Tokyo, Japan).

Immunohistochemistry

LDM or forelimbs were fixed in 4% paraformaldehyde for 19 h at 4°C, then dehydrated using gradient alcohol and embedded with paraffin. Paraffin embedded samples were cut into 5-μm sections. The paraffin sections were placed in an oven at 64°C for 30 min and immediately moved to xylene for dewaxing. The sections were rehydrated in gradient alcohol and antigen retrieval was performed using citrate antigen retrieval solution. Finally, immunofluorescence staining was performed using IHC kit (Abcam, Cambridge, UK) according to the manufacturer's instructions, and immunostaining images were obtained with a fluorescence microscope (Nikon).

Statistical analysis

The fusion index was calculated as the percentage of nuclei in fused myotubes out of the total nuclei, and counted by the counting function with Adobe Photoshop CS5. Data are presented as the mean ± SEM. Statistical differences between groups were tested using an unpaired two-tailed Student's *t*-test with GraphPad Prism software. $P < 0.05$ was considered as statistically significant.

Results

Myogenesis progression is more intense in LT than that in LR at 35dpc

To investigate early embryonic myogenesis, the expression of embryonic myosin heavy chain (eMyHC, a marker for fully differentiated myocytes) was analyzed in LDM of LT and LR pigs at 35dpc. The results showed that there were more eMyHC$^+$ cells in LT than in LR (Figure 1A,B; $P < 0.05$). To define the progression of myogenic differentiation, dual immunostaining of Pax7 and MyoD was conducted. It was found that the percentage of differentiated myoblasts (Pax7$^-$/MyoD$^+$; $P < 0.001$) was higher in LT than in LR (Figure 1C,D), while the percentage of undifferentiated progenitors (Pax7$^+$/MyoD$^-$) was higher in LR than in LT ($P < 0.001$). Accord-

Table 1. Sequence of primers used for qRT-PCR

Gene	Primer sequence (5′→3′)	
p-GAPDH	F: AGGTCGGTGTGAACGGATTTG	R: TGTAGACCATGTAGTTGAGGTCA
p-Pax7	F: GCGAGAAGAAAGCCAAGCAC	R: CTCGCGGGTGTAGATGTCTG
p-MyoD	F: CCACTCCGGGACATAGACTTG	R: AAAAGCGCAGGTCTGGTGAG
p-Notch1	F: CACCCAGTATACGCCTGTGG	R: CCTGGGTTTGCTCTGAGGAG
p-Notch3	F: TAGCCGTGTACCCTGTCAGA	R: ACAGTAGTGAGAGCCGTCCT
p-Rbpj	F: CAATTCAAGCCAAGTGCCCC	R: ACACGACTGTTGCTGTCGAT
p-Jagged1	F: CTGCTCGAAGGTGTGGTGTG	R: ATGTTTGCGCAGTTGTCCTG
p-Hes1	F: TCAACGCCATGACCTACCCT	R: GAAGCCGCCGAATACCTTTG
p-HeyL	F: GGCGGGACAGGATTCTTTGA	R: TGCATAGCTGTTGAGGTGGG
p-Hey1	F: ATCGGAGTTTGGGGTTTCGG	R: TTCAGATGCGAGACCAGTCG
m-GAPDH	F: AGGTCGGTGTGAACGGATTTG	R: TGTAGACCATGTAGTTGAGGTCA
m-Pax7	F: TCTCCAAGATTCTGTGCCGAT	R: CGGGGTTCTCTCTCTTATACTCC
m-MyoD	F: CCACTCCGGGACATAGACTTG	R: AAAAGCGCAGGTCTGGTGAG
m-MyoG	F: GAGACATCCCCCTATTTCTACCA	R: GCTCAGTCCGCTCATAGCC
m-MyHC	F: AAAAGGCCATCACTGACGC	R: CAGCTCTCTGATCCGTGTCTC
m-CKM	F: CTGACCCCTGACCTCTACAAT	R: CATGGCGGTCCTGGATGAT
m-Myf5	F: AAGGCTCCTGTATCCCCTCAC	R: TGACCTTCTTCAGGCGTCTAC
m-Hes1	F: CCAGCCAGTGTCAACACGA	R: AATGCCGGGAGCTATCTTTCT
m-HeyL	F: CAGCCCTTCGCAGATGCAA	R: CCAATCGTCGCAATTCAGAAAG
m-Hey1	F: GCGCGGACGAGAATGGAAA	R: TCAGGTGATCCACAGTCATCTG

p=pig; *m*=mouse.

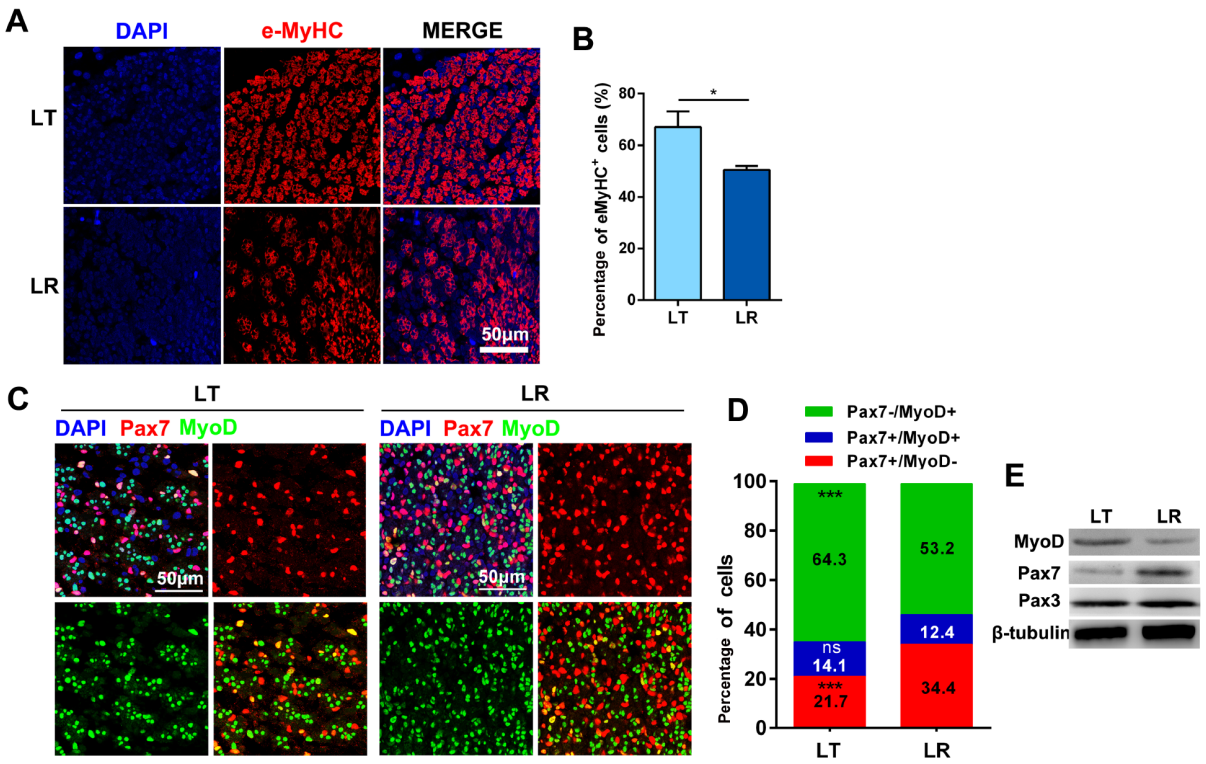

Figure 1. Myogenesis process is more intense in Lantang (LT) pigs than in Landrace (LR) pigs at 35 days post-conception (35dpc) (A) Immunofluorescence staining of embryonic myosin heavy chain (eMyHC) was performed on longissimus dorsi muscle (LDM) cross section of LT and LR pigs at 35dpc. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar = 50 μm. (B) Percentages of eMyHC-positive cells in (A) were calculated from six repeated experiments (six embryos) for each group. (C) Immunofluorescence staining for the paired box transcription factor Pax7 (a marker of muscle progenitor cells) and MyoD (a marker of differentiated myogenic cells) on cross sections described as (A). Scale bar = 50 μm. (D) Percentages of three cell populations in (C) were calculated from six embryos for each group. (E) The protein levels of Pax7, Pax3 and MyoD were detected by western blot analysis in LT and LR pig LDM. β-Tubulin was used as a loading control. Data are presented as the mean ± SEM, $n = 6$ per group. $^{*}P < 0.05$, $^{***}P < 0.001$.

ingly, higher Pax7 protein level and lower MyoD protein level were found in LR compared to those in LT (Figure 1E). The protein level of Pax3, an important upstream regulator of embryonic myogenesis, did not show any difference between the two pig breeds (Figure 1E). Altogether, these results demonstrated that myogenesis process is more advanced in LT than that in LR at 35dpc, as demonstrated by increased commitment cells and higher expression levels of differentiation markers in LT muscle compared with those in LR muscle.

Embryonic muscle progenitors from LT express MyoD earlier and have stronger differentiation capacity *in vitro* than those from LR

To further investigate myogenic potentials of embryonic muscle progenitors in these two pig breeds, we isolated them from LDM by collagenase digestion combined with differential adherent purification. Identification of isolated cells by immunofluorescence staining showed that the Pax7-positive cells and Desmin-positive cells accounted for more than 80% of total cells respectively, both for LT and LR (Figure 2A–C). *In vivo* study demonstrated that muscle progenitors in LT showed a stronger myogenic differentiation tendency than muscle progenitors in LR, implying that MyoD expression in LR progenitors might be slowed down, which subsequently blocks myogenic differentiation. To test this hypothesis, newly isolated LT and LR progenitors were cultured in growth medium, and time course expression of MyoD was tested by immunofluorescence staining (Figure 2D). With the extension of culture time, the number of MyoD$^+$ cells was gradually increased both in LT and LR progenitors. More MyoD$^+$ cells appeared earlier in LT progenitors than in LR progenitors (Figure 2E). Then, immunofluorescence staining for eMyHC was performed to compare the differentiation ability of these two kinds of progenitors at day 6 after differentiation. There were significantly more and larger eMyHC$^+$ myotubes generated from LT progenitors than from LR progenitors (Figure 2F). The statistical results indicated that LT progenitors showed a higher fusion index than LR progenitors (Figure 2G; $P < 0.001$). Consistent with these findings, protein levels of MyoD and MyoG were higher in LT progenitors than in LR progenitors (Figure 2H). Collectively, these results indicated that embryonic muscle progenitors from LT have earlier expression of MyoD and stronger differentiation capacity *in vitro* than embryonic muscle progenitors from LR

Notch signaling is more active in LR than in LT myogenic progenitors

To explore the molecular mechanism of distinct differentiation capacity between LT and LR progenitors, we detected Wnt, AKT/mTOR and Notch signaling pathways. The results showed that the total protein level and active protein level of β-catenin, an important mediator of canonical Wnt signaling pathway, were comparable between LT and LR progenitors (Figure 3A). To examine the activity

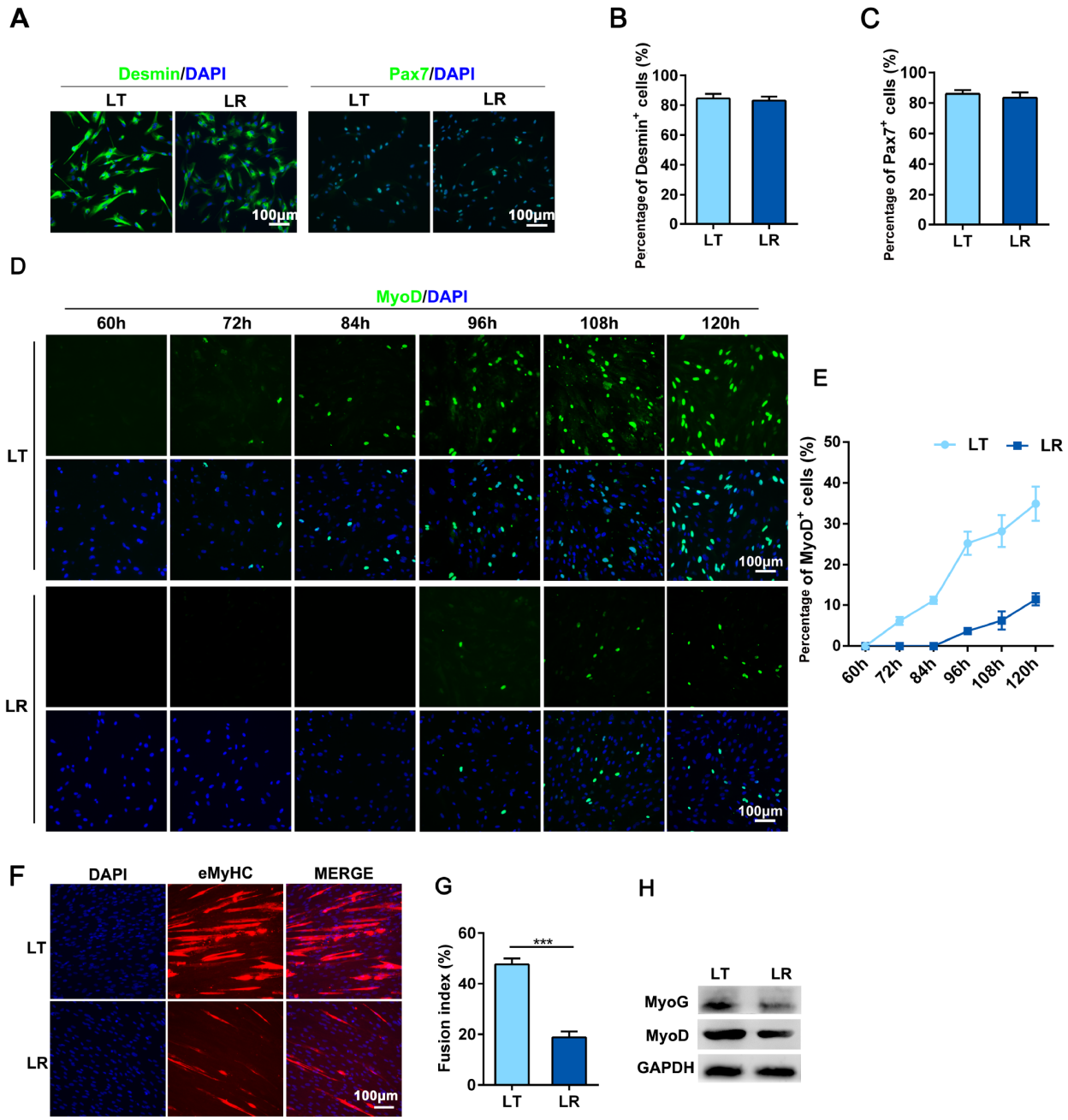

Figure 2. Embryonic muscle progenitors from LT pigs express MyoD earlier and have stronger differentiation capacity than that from LR pigs *in vitro* (A) Immunofluorescence staining of Desmin (a marker of myogenic cells) and Pax7 was performed to identify the isolated cells from LT and LR pig LDM. The cell nuclei were stained with DAPI. (B) Percentages of Desmin-positive cells in (A) were calculated from six repeated experiments for LT and LR pigs, respectively. (C) Percentages of Pax7-positive cells in (A) were calculated from six repeated experiments for LT and LR pigs, respectively. (D) Immunofluorescence staining for MyoD in muscle progenitors cultured in growth medium (GM) for indicated time. (E) Percentages of MyoD-positive cells in (D) were counted from six repeated experiments for each group. (F) Immunofluorescence staining for eMyHC in muscle progenitors at 6 day (d) after differentiation induction. (G) The fusion index (the percentage of nuclei in fused myotubes out of the total nuclei) in (F) was calculated. For each group, statistical data were obtained from six repeated experiments. (H) Western blot analysis was used to detect the protein levels of MyoG and MyoD in cells described as (F). GAPDH was used as a loading control. Scale bar = 100 μm. Data are shown as the mean ± SEM, $n = 6$ per group. ***$P < 0.001$.

of the AKT/mTOR pathway, the expression levels of total and phosphorylated protein of AKT and S6K1 were measured. It was found that the levels of AKT, pAKT, S6K1 and pS6K1 showed no difference between two breeds (Figure 3B,C). Interestingly, analysis of qPCR data revealed that Notch genes, including ligand (*Jagged1*, $P < 0.0001$), Notch receptors (*Notch1* and *Notch3*, $P < 0.01$) and downstream effectors (*Hey1*, $P < 0.01$; *HeyL*, $P < 0.01$ and *Hes1*, $P < 0.05$) had higher mRNA levels in LR progenitors than in LT progenitors (Figure 3D). In line with these results, results of western blot analysis demonstrated that the protein levels of Jagged1, Hey1 and Hes1 were higher in LR than in LT (Figure 3E). Immunofluorescence staining showed that there were more Jagged1 protein

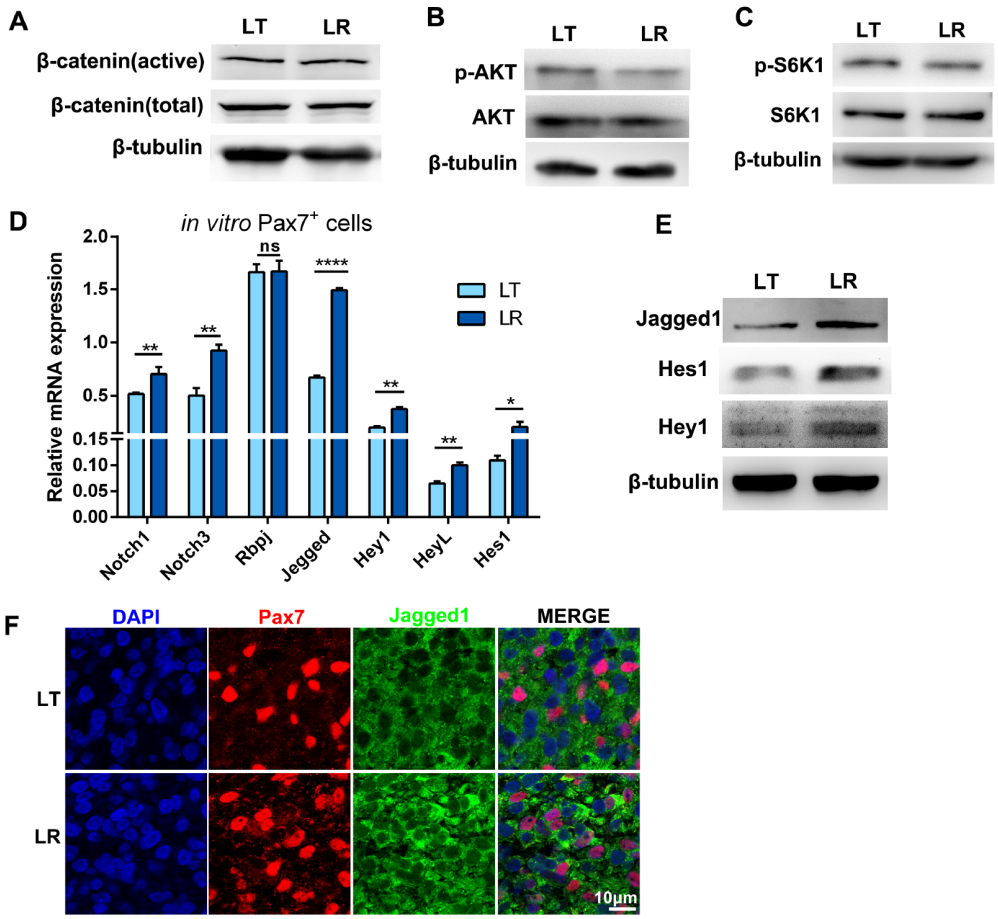

Figure 3. Notch signaling is more active in LR myogenic progenitors than in LT myogenic progenitors (A-C) Western blot analysis was used to detect the protein levels of active β-catenin, total β-catenin, protein kinase B (AKT) and phosphorylated (p)-AKT, ribosomal protein S6 kinase 1 (S6K1) and p-S6K1 in LT and LR pig muscle progenitors cultured in GM. β-Tubulin was used as the loading control. (D) Real-time quantitative PCR (qPCR) was performed to analyze the mRNA levels of Notch genes. (E) Protein levels of Jagged1, Hes1 and Hey1. (F) Immunofluorescence staining for Pax7 and Jagged1 was performed on LDM cross section from LT and LR pigs at 35dpc. Scale bar = 10 μm. Data are shown as the mean ± SEM, $n = 6$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

expressed in LR dorsal cells than in LT dorsal cells, whether it is a $Pax7^{+}$ cell or a $Pax7^{-}$ cell (Figure 3F). Collectively, Notch signaling is more active in LR myogenic cells than in LT myogenic cells.

Inhibition of Notch signaling promotes the differentiation of embryonic myogenic progenitors *in vitro*

Given that Notch signaling is over-activated in LR progenitors and it has an inhibitory effect on myoblast differentiation, we speculated that this signaling may contribute to the difference of differentiation ability between progenitor cells originated from two different pig breeds. LR progenitors were cultured in GM containing 20 μM γ-secretase inhibitor DAPT (an inhibitor of Notch signaling) to inhibit Notch activity. As expected, the expressions of Notch effectors Hey1, HeyL and Hes1 were decreased after DAPT treatment (Figure 4A,B; $P < 0.05$). In addition, inhibition of Notch signaling led to the decrease of Pax7 mRNA expression ($P < 0.05$) and increase of MyoD mRNA expression (Figure 4C,D; $P < 0.05$). Furthermore, time-course expression of MyoD was tested by immunofluorescence staining and the results showed that there were more $MyoD^{+}$ cells in the DAPT group at each indicated time points (Figure 4E,F). Immunofluorescence staining and western blot analysis for eMyHC at day 6 after differentiation indicated a lower cell fusion index ($P < 0.05$) together with a decreased eMyHC protein level in DAPT-treated groups (Figure 4G–I) compared with the control group. Taken together, these findings revealed that Notch inhibition inhibited Pax7 expression and promoted MyoD expression and myogenic differentiation.

Boosted Notch signaling prevents C2C12 myoblast differentiation

To further clarify the regulatory function of Notch signaling in myogenic differentiation, C2C12 cells were treated with the peptide of Jagged1, a Notch ligand known to activate Notch signaling in skeletal muscle cells. After 2 days of Jagged1 treatment in GM, the expression of Pax7 was up-regulated while MyoD was down-regulated (Figure 5A). In addition, the percentages of $Pax7^{+}/MyoD^{+}$ ($P < 0.05$) and $Pax7^{+}/MyoD^{-}$ ($P < 0.05$) cells were increased, while the percentage of $Pax7^{-}/MyoD^{+}$ ($P < 0.001$) cells were decreased (Figure 5B,C), which implied that Notch signaling prevents myogenic differentiation. Then, C2C12 cells were induced to differentiate in DM supplemented with Jagged1 peptide, followed by differentiation assays. It was found that the percentage of $MyoG^{+}$ cells was significantly reduced after 1 day of treatment (Figure 5D,E;

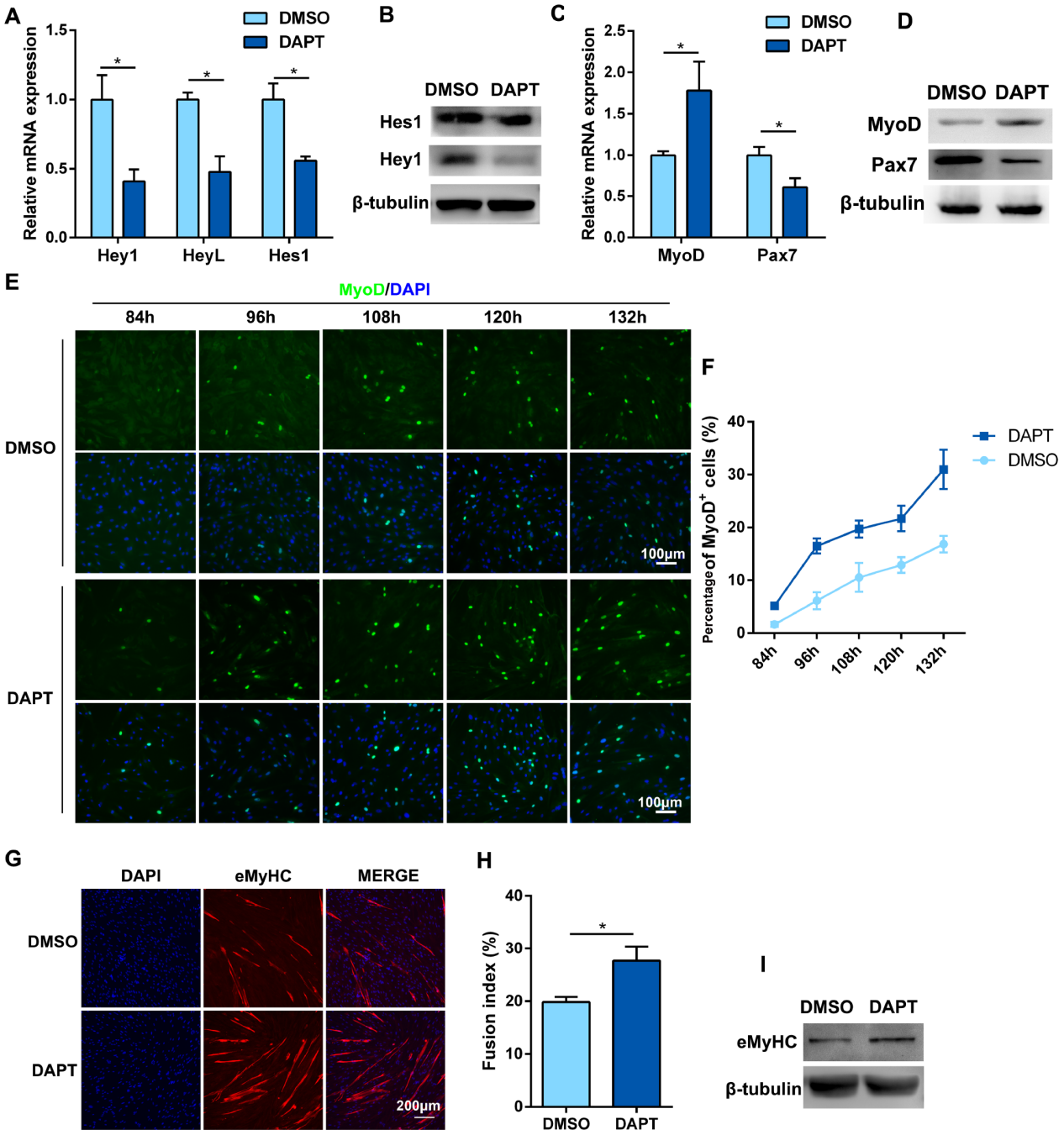

Figure 4. Inhibition of Notch signaling promotes the differentiation of embryonic myogenic progenitors *in vitro* (A) The mRNA levels of *Notch* genes in control [dimethyl sulfoxide (DMSO)-treated] and DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S -phenylglycinet-butyl ester)-treated LR muscle progenitors cultured in GM for 3 d. (B) The protein levels of Notch genes in cells described as (A). (C) The mRNA levels of *Pax7* and *MyoD* in control and DAPT-treated cells cultured in GM for 3 d. (D) The protein levels of Pax7 and MyoD in control and DAPT-treated cells cultured in GM for 3 d. (E) Immunofluorescence staining for MyoD in control and DAPT-treated muscle progenitors cultured in GM for indicated time. Scale bar = 100 μm.(F) Percentages of MyoD-positive cells in (E) were calculated from six repeated experiments for each group. (G) Immunofluorescence staining for eMyHC was performed in treated muscle progenitor cells at 6 d after differentiation induction. Scale bar = 200 μm. (H) The fusion index of differentiated progenitor cells in (G) was calculated. (I) Western blot analysis was used to detect the protein level of eMyHC in cells described as (G). Data are shown as the mean ± SEM, $n = 6$ per group. *$P < 0.05$.

$P < 0.05$). When being induced to differentiate for 3 days, as expected, cells treated with jagged1 formed fewer myotubes (Figure 5F), which was proved by a decreased fusion index (Figure 5G; $P < 0.05$). In line with this, the expressions of MyoG and MHC were down-regulated, indicating that there was a differentiation defect in Jagged1-treated myoblasts (Figure 5H).

Notch signaling slows down myogenic differentiation of muscle progenitors in embryo limbs

In order to further confirm the correlation between earlier pro-

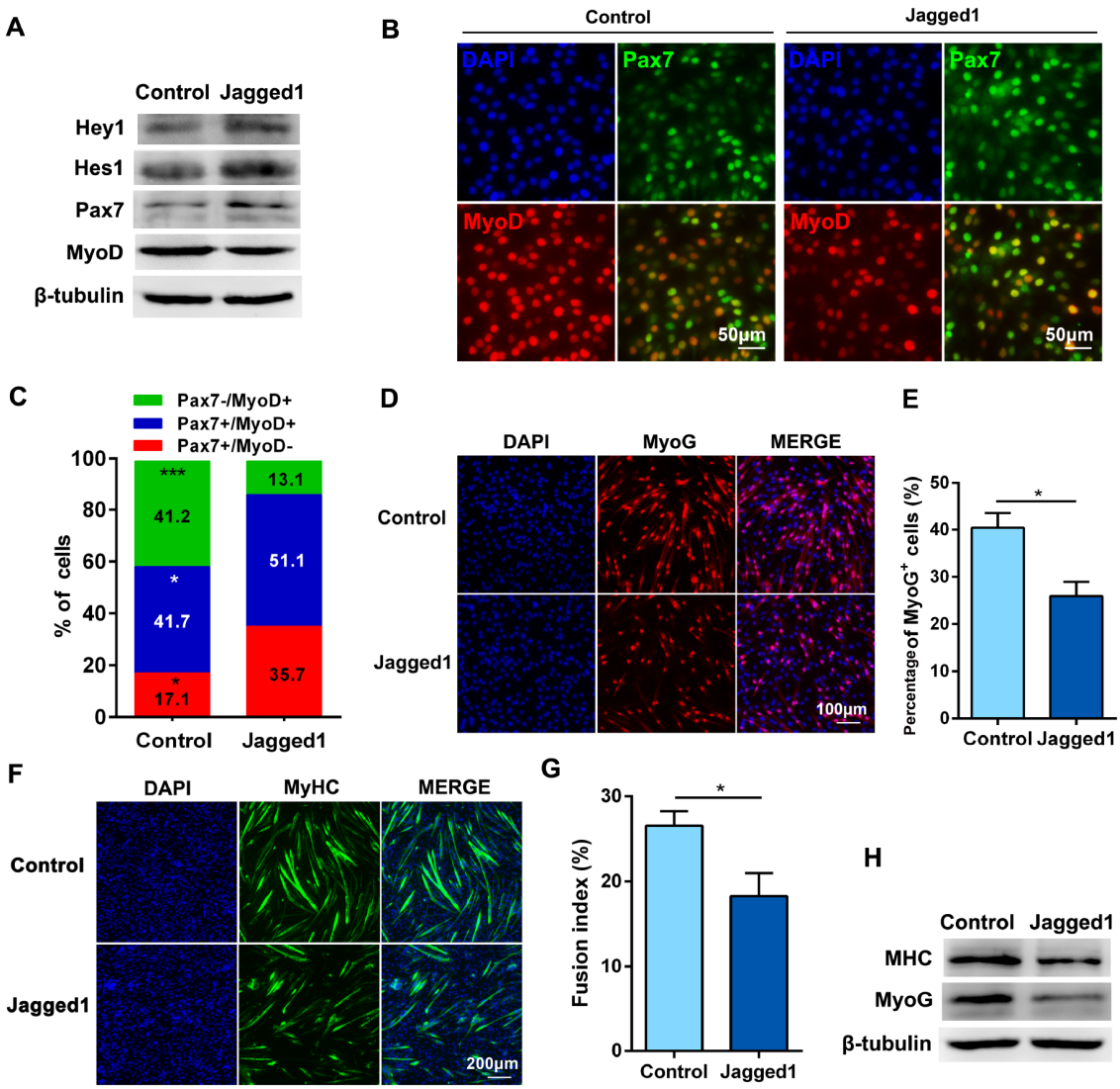

Figure 5. Boosted Notch signaling prevents C2C12 myoblast differentiation (A) Western blot was used to detect the protein levels of Hey1, Hes1, Pax7 and MyoD in control (ddH_2O-treated) and Jagged1-treated C2C12 cells cultured in GM for 2 d. (B) Immunofluorescence staining for Pax7 and MyoD in control and Jagged1-treated cells. Scale bar = 50 μm. (C) Percentages of three cell populations in (B), including Pax7$^+$/MyoD$^-$ progenitor cells, Pax7$^+$/MyoD$^+$ committed myoblasts and Pax7$^-$/MyoD$^+$ differentiated myoblasts, were calculated from six repeated experiments for each group. (D) Immunofluorescence staining for MyoG was performed in control and Jagged1-treated cells to examine myoblast differentiation at 1 d after differentiation induction. Scale bar = 100 μm. (E) Percentages of MyoG-positive cells in (D) were calculated in six microscopic fields for each group. (F) Immunofluorescence staining for MyHC was performed in control and Jagged1-treated cells at 3 d after differentiation induction. Scale bar = 200 μm. (G) The fusion index of differentiated myoblasts in (F) was calculated. (H) Western blot analysis was used to detect the protein levels of MyHC and MyoG in control and Jagged1-treated cells at 3 d after differentiation induction. Data are shown as the mean ± SEM, $n = 6$ per group. *$P < 0.05$, ***$P < 0.001$.

gression of myogenic differentiation and repressed Notch signaling in LT with less muscle, an *ex vivo* limb culture system was used [28]. Embryo forelimbs of pig were separated at 35dpc and cultured for 30 h, with or without 20 μM DAPT. qPCR analysis showed that Notch genes were successfully suppressed after DAPT treatment (Figure 6A,C; $P < 0.05$). In addition, inhibition of Notch signaling led to reduced numbers of Pax7$^+$/MyoD$^-$ cells ($P < 0.01$), whereas the MyoD$^+$ cell proportion was increased (Figure 6D,E; $P < 0.01$), confirming the robustness of our *ex vivo* model. Accordingly, we found decreased expression level of Pax7 ($P < 0.01$), increased expression level of MyoD ($P < 0.05$) and unchanged expression level of Pax3 in DAPT-treated samples (Figure 6B,C). Altogether, these results demonstrated that, in embryonic muscle progenitor cells, Notch signaling antagonizes myogenic differentiation by promoting Pax7 expression and preventing MyoD expression.

Discussion

Lean pigs and indigenous Chinese pigs have distinctly different characteristics in meat production [2,29]. As a typical lean pig breed, LR is characterized by high lean meat percentage, fast muscle growth, and high feed remuneration; while as a typical indigenous Chinese breed, LT has been proved to be superior in terms of perceived meat quality, high intramuscular fat content, but not in lean meat percentage and body weight [2,30]. Hence, understanding the

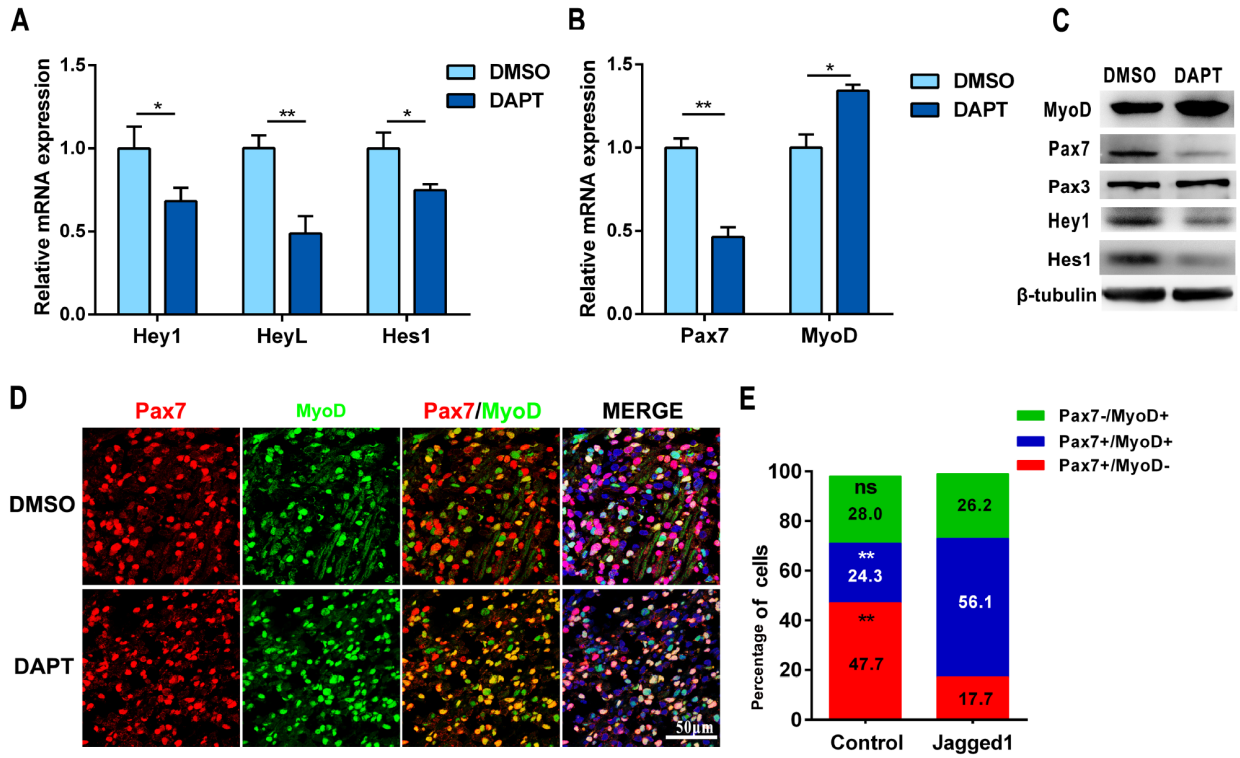

Figure 6. Notch signaling prevents myogenic differentiation of embryonic muscle progenitors (A,B) qPCR results for *Hey1*, *Hey L*, *Hes1*, *Pax7* and *MyoD* mRNA in control (DMSO-treated) and DAPT-treated *ex vivo* limb explants kept in culture for 30 h. (C) The protein levels of Hey1, Hey L, Hes1, Pax7, Pax3 and MyoD in *ex vivo* limb culture. (D) Immunofluorescence staining for Pax7 and MyoD on cross sections of control and DAPT-treated limb explants from LR 35 dpc embryos. The cell nuclei were stained with DAPI. Scale bar = 50 μm. (E) Percentages of three cell populations ($Pax7^+$/$MyoD^-$ progenitor cells, $Pax7^+$/$MyoD^+$ committed myoblasts and $Pax7^-$/$MyoD^+$ differentiated myoblasts) in (D) were calculated in six microscopic fields from six *ex vivo* limb explants for each group. $^*P < 0.05$, $^{**}P < 0.01$.

differences in the development of skeletal muscle between the two pig breeds will be beneficial for livestock genetic improvement, which also contributes to the understanding of human muscle regeneration and muscular atrophy due to the anatomical, physiological, pathological, and genomic similarities between pig and human [31].

In the present study, we found more eMyHC-positive cells in LT LDM at 35dpc, indicating precocious terminal differentiation of myoblasts in LT. This is fully consistent with our previous findings that the formation of primary myofibers in embryonic LT is earlier than that in LR [23]. MyoD, which can initiate the process of multiple non-muscle cell lineages into muscle cell lineages, is a crucial master switch in regulating muscle-specific gene transcription [32,33]. Its higher expression in LT LDM reflected that dermomyotome-derived muscle progenitors differentiate more rapidly, leading to a larger percentage of differentiating myoblast, and differentiated myocytes in LT compared with LR at the same embryonic stage. Consistently, MyoD-positive cells appear earlier in LT progenitors cultured *in vitro*, resulting in more eMyHC-positive myotubes with a higher fusion index. Collectively, these analyses suggested that, compared with LR muscle progenitors, LT muscle progenitors showed greater myogenic differentiation capacity at early embryonic stage, which is associated with earlier and higher MyoD expression.

It has been reported that the miRNA and mRNA expression profiles prepared for subsequent skeletal muscle developmental processes appear earlier in Wuzhishan pigs than in LR at the early embryonic stage [34]. Earlier demethylation of myogenic genes contributes to embryonic precocious terminal differentiation of myoblasts in Wuzhishan pigs [23]. Satellite cells from LT have a greater differentiation capacity than LR satellite cells, which is related to the mTOR signaling [35]. Transcriptome analysis showed that the expressions of the myogenesis-related genes were greater in early Duroc embryos than in early Pietrain embryos (14 to 49 d of gestation), whereas the opposite was found in late embryos (63 to 91 d of gestation), suggesting that the myogenesis process is more intense in early Duroc embryos than in Pietrain embryos [36]. These findings further demonstrate that there are differences in myogenic differentiation among pig breeds, and that the molecular mechanisms responsible for this are multifaceted.

Cell signals mediated by the Notch pathway are involved in the regulation of myogenic differentiation in vertebrate embryos and cultured cell lines [27,37,38]. Activation of Notch signaling in C2C12 myoblasts can repress MRFs as well as other muscle-specific genes expression, and block myotube formation [39–41]. In mouse embryos, the Notch ligand Delta1 (Dll1) controls both maintenance of myogenic progenitors and early differentiation of myoblasts [42]. However, overexpression of Dll1 in chick embryos does not affect early steps of myogenesis, but it blocks the differentiation of postmitotic myogenic cells [37]; and Notch in zebrafish embryos controls the segmental arrangement of myogenic cells, but it does not affect their commitment or differentiation [43–45]. These findings suggest that Notch signaling plays different roles in embryonic myogenesis in different vertebrate species. In this study, we de-

monstrated higher expression levels of Notch genes in LT LDM, suggesting that Notch signaling may function in porcine skeletal muscle development and contribute to the differences in embryonic myogenesis between pig breeds. By using *in vitro* Notch manipulation and an *ex vivo* limb culture system, we found that Notch signaling facilitates the maintenance of myogenic progenitor cells and antagonizes myogenic differentiation by promoting Pax7 expression but preventing MyoD expression.

Embryonic and fetal muscle development depends on a sufficient population of myogenic progenitors that are characterized by expressions of the paired-box transcription factors Pax3 and Pax7 [46,47]. By dual immunostaining of Pax7 and MyoD, we confirmed that the myogenic differentiation progression in LT embryos was more rapid than that in LR embryos, which resulted in more differentiated myocytes (eMyHC$^+$) at 35dpc but a serious depletion of progenitor cells (Pax7$^+$/MyoD$^-$). It has been reported that premature myogenic differentiation and depletion of progenitor cells cause severe muscle hypotrophy [12]. We speculate that the severe loss of muscle progenitors caused by intense myogenesis at early embryonic stage may be one of the reasons for less meat production of LT pigs because there are not sufficient progenitors differentiating into myofibers in the later stage of embryo, resulting in a small total number of myofibers.

In summary, we reveal here that myogenic differentiation is enhanced in LT compared with that in LR at 35dpc. In addition, embryonic muscle progenitors from LT have stronger differentiation capacity *in vitro*. Mechanistically, the stronger Notch signaling in LR myogenic progenitors facilitates the maintenance of myogenic progenitor cells and antagonizes myogenic differentiation by promoting Pax7 expression and preventing MyoD expression. The results presented here provide new insights into the mechanisms of the differences in skeletal muscle growth and meat production between pig breeds.

Supplementary Data

Supplementary Data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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