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Tbx3 and Nr5α2 improve the viability of porcine induced pluripotent stem cells after dissociation into single cells by inhibiting RHO-ROCK-MLC signaling

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ABSTRACT

Porcine induced pluripotent stem cells (piPSCs) had been reported during the past 5 years, but there were few reports on how the cell signaling works in piPSCs. In order to clarify the signaling work that dominated the characteristic difference of two types of piPSCs which were derived from *Oct4*, *Sox2*, *Klf4* and *c-Myc* (termed 4F piPSCs) and *Oct4*, *Sox2*, *Klf4*, *c-Myc*, *Tbx3* and *Nr5α2* (termed 6F piPSCs) respectively, we performed this study. 4F piPSCs and 6F piPSCs were cultured in medium with or without the ROCK inhibitor Y27632 after dissociating into single cells, the efficiency of a single cell colony and the number of AP positive colonies were assessed. The total RhoA and GTP-bind RhoA were detected in 4F piPSCs and 6F piPSCs before and after digestion into single cells. To explore the relationship between RHO-ROCK-MLC signaling pathway and the two factors Tbx3 and Nr5α2, the 4F piPSCs were infected with lenti-virus Tbx3 and Nr5α2 (termed 4F+TND). Results showed that the viability of cells could be enhanced by Y27632 and the RHO-ROCK-MLC signaling pathway was activated after dissociation into single cells in 4F piPSCs but not in 6F piPSCs. And, the 4F+TND piPSCs could be passaged and keep in high viability after dissociation into single cells, though the morphology of colonies did not change. These results indicated that the Tbx3 and Nr5α2 can improve the viability of piPSCs after dissociation into single cells by inhibiting the RHO-ROCK-MLC signaling pathway. And this provides useful information for establishing porcine pluripotent cells in future study.

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1. Introduction

The porcine embryonic stem cells (pESCs) are considered to be useful in generating genetically modified animal models for human diseases. However, no authentic pESCs were obtained in despite of the 20 years of effort. Induced pluripotent stem cells (iPSCs) provided an alternative approach to get porcine pluripotent stem cells. Several porcine induced pluripotent stem cell (piPSC) lines have been established since the first report on piPSCs 5 years ago [1–3]. In general, these reported piPSCs showed two types of characteristics, one type looks like mouse embryonic stem cells (mESCs) and the other type looks like human embryonic stem cells (hESCs). In our laboratory, we also obtained two types of piPSCs: morphologically hESCs like 4F piPSCs induced by the classical four factors *Oct4*, *Sox2*, *Klf4* and *c-Myc* and morphologically mESCs like 6F piPSCs induced by *Oct4*, *Sox2*, *Klf4*, *c-Myc*, *Tbx3* and *Nr5α2* [4]. Both of them have normal karyotypes, the expression of pluripotent markers such as OCT4, SOX2 and NANOG, and could generate the teratoma containing three germ layers. However, there were also some differences between them, such as the morphology of colonies, the dependence on LIF signaling, the activation status of the XX chromosome and the expression of stage-specific embryonic markers. As the piPSCs reported by other groups that possess characteristics similar to our 4F piPSCs and 6F piPSCs [3,5–8], hESCs like and mESCs like piPSCs differ in the viability after single-cell passaging, which is very important for complicated genetic manipulation such as knock-in or out [9]. But there were few reports on the reason causing the difference between the two types of piPSCs, except some clues derived from reports on hESCs, that indicated that the activation of RHO-ROCK-MLC signaling pathway may be responsible for the viability of pluripotent stem cells after dissociation into single cells [10,11].

Rho family GTPases are essential for the regulation of mitosis, polarity, migration and adhesion between cells by processing signal input into mechanical forces [12,13]. Rho is one of the family members and shares the same chemical characteristic of GTPase

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domain cycling between the GTP-bound active form and the GDP-bound inactive form [12,14]. ROCK, the downstream mediator kinases of Rho, can be activated by GTP-bound active form Rho [13,15]. In human ESCs, it had been proved that the activated ROCK can promote the phosphorylation of the myosin light chain (MLC) [16–20]. The RHO-ROCK-MLC rendering E-cadherin reconstruction failure was proved to be the direct reason for the low survival rate in hESCs after dissociation into single cells [11]. In accordance to these findings, the viability of hESCs could be improved by inhibiting the ROCK and myosin activation through adding inhibitory chemical molecules or knockdown the *Rock1*/*2* and *Myh9* [11,18,19,21]. The similar passaging characteristics between the 4F piPSCs and the hESCs suggest that RHO-ROCK-MLC might work in piPSCs in a same way as in the hESCs, but there are no reports on it yet.

Since in contrast to 4F piPSCs, 6F piPSCs could be single-cell passaged as mESCs, in this study to further explore the role of Rho-ROCK-MLC signaling in piPSCs, the cell viability, the abundance of total RhoA and GTP-bind RhoA of 4F piPSCs and 6F piPSCs in the medium with and without ROCK inhibitor Y27632 were detected after they were dissociated into single cells. Then to further confirm the function of *Tbx3* and *Nr5α2*, which are unique to 6F iPSCs, on regulating the RHO-ROCK-MLC signaling, the 4F piPSCs were infected with *Tbx3* and *Nr5α2* and the passaging characteristics of the resulting cell lines were assessed. Results from these studies indicated that the two transcription factors *Tbx3* and *Nr5α2* can improve the viability of piPSCs after dissociation into single cells by inhibiting the RHO-ROCK-MLC signaling pathway. Our finding also provided useful information for establishing bona fide porcine pluripotent cells.

2. Materials and methods

The reagents and media used in the research were purchased from Life Technologies, R&D, Millipore, Abcam, Beyotime and Bioind unless otherwise stated.

2.1. Animals

ICR mice used for mouse embryonic fibroblast cells (MEF) were purchased from the Beijing Vital River Company.

All research procedures are consistent with the Northeast Agriculture University of Biological Sciences Guide for the care and use of laboratory animals.

2.2. Cell culture

Both the 4F piPSCs and 6F piPSCs previously obtained in our lab were cultured in the modified X (mX) medium which consisted of 38% knockout Dulbecco's modified eagle medium (DMEM), 24% DMEM/F12, 24% Neurobasal, 10% knockout serum replacement, 1 mmol/L L-glutamine, 0.05 mmol/L β-mercaptoethanol, 0.5% MEM nonessential amino acids, 1% penicillin–streptomycin, 0.25% N2, 0.5% B27, 0.25 mg/mL BSA 1000 U/ml LIF (human) and 8 ng/ml bFGF. The 4F+TND piPSCs were also cultured in the mX medium. And, the 293T cells used for the lentivirus package were cultured in the FBS medium which consisted of 86% high glucose DMEM, 10% FBS, 1% NEAA, 2 mmol/L L-glutamine, and 1% penicillin–streptomycin. The MEFs used for the feeder layer were also cultured in the FBS medium.

2.3. Lentivirus production

293T cells were transfected with the TetO-FUW plasmid containing *Tbx3*, *Nr5a2* and *Dsred* together with packaging plasmids PMD2G and PSPAX. The transfection reagent was Lipofectamine LTX and Plus Reagent. Supernatants were collected at 24 h and 48 h after transfection, and filtered by 0.45 μm filter. FUW-M2rtTA was also packaged with the above method.

2.4. Alkaline phosphatase (AP) staining

piPSCs were fixed with 4% paraformaldehyde at room temperature for 1 min, and washed three times with PBS, and then performed with BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime) following the manufacturer's instructions.

2.5. Western blot

piPSCs were lysed with cell lysis buffer for western and IP (Beyotime). Lysates were separated in SDS–PAGE for the routine western blot assay. The primary antibodies used for incubation were RhoA (Abcam, ab68826), RhoA-GTP (Abcam, ab41435), MLC (Abcam, ab11082), pMLC (Abcam, ab2480), E-cadherin (Abcam, ab15148) and β-actin (Santacruz, sc47778). At last, the membranes were visualized by DAB Horseradish Peroxidase Color Development Kit (Beyotime).

2.6. Quantitative realtime PCR

Total RNA was extracted from piPSCs using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using PrimeScript® RT Reagent Kit (TaKaRa, Perfect Real Time) according the manufacturer's instructions. For quantitative realtime PCR, reactions were performed using SYBR® Premix ExTaqTM II (Perfect Real Time, TaKaRa) and conducted with a 7500 Real-Time PCR System (Applied Biosystems). The primers used were listed in the Table 1.

2.7. Statistical analysis

Differences of data (mean ± SEM) were analyzed by SPSS statistical software. Data of the number of AP positive colonies, the efficiency of colony formation and the gene expression level were compared by a one way ANOVA. For all analyses, differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. The viability of 4F piPSCs could be improved by Y27632 after dissociation into single cells

In order to detect whether the Rho-ROCK-MLC signaling works in the piPSCs, two lines of 4F piPSCs and 6F piPSCs were dissociated into single cells and were seeded in a 6-well plate with or without the ROCK inhibitor Y27362. Two days later, AP staining was carried out in all the groups (Fig. 1A). Our results showed that the number of AP positive colonies in the 4F piPSCs with the Y27632 group was significantly higher than the 4F piPSCs without the Y27632 group (Fig. 1B). However, there was no difference between the 6F piPSCs with and without Y27632 groups (Fig. 1B). The efficiency of a single cell colony formation was considered as one of the most critical standards for detecting the cell viability after dissociation into single cells. Thus, two lines of 4F piPSCs and 6F piPSCs were dissociated into single cells and seeded 1 cell/well in a 96-well plate with or without Y27362. Four days later, the number of single cell colonies was counted. Our results showed that the efficiency of single cell colony formation in the 4F piPSCs with the Y27632 group was significantly higher than the 4F piPSCs without the Y27632 group (Fig. 1C). But, there was still no difference between the 6F piPSCs with or without Y27632 groups (Fig. 1C). All these results

Table 1
Primer sequence for realtime PCR.

Gene name	Forward primers	Reverse primers
Abr	GTTCTTGCTGCTGCTCAATTC	TTCTCTCCACTCTGACCTCTC
RhoA	GATGAAGCAGGAGCCAGTAAA	CCTCACTCCATCTTTGGTCTTT
RhoB	ATCGAAGTGGACGGCAAG	GTCCACCGAGAAGCACATAA
RhoC	CTACTCCTGTTGCCTTGGTC	TGTGAGCCAGACGTGTATAAAG
ROCK1	TACTGACAGGGAGGTGAGATTA	CACAGTGTCTCGGAGTGTTT
ROCK2	AGCGCAGTTTGAGAAGCA	TCCGCATATCTGTGTCATTACC
RAC	GAAGCCCTACCTCTTTGGTAAG	CTCAAAGGAGCCACCCTAAAT
MLC1	GGAGGCATTTCTCCTCTTTGA	CTGCATTGGTGGGATTTGTG
MLC2	GAACAGGGATGGCTTCATAGAC	CCTGGAGCTTCCTTGATCATTT
MLC3	CTGACAGAAGACGAGGTAGAGA	GCCATGATGTGCTTGACAAAT
MYH9	GCGGACCTTCCATATCTTCTAC	TTGGACAGGAAGCGGTATTT
MYH10	AAGGACCTGGAAGCTCAAATC	TTCACGTTGGTAGTCCTTCATC
E-cadherin	GCTGCTCCTGCTCCTTATT	CTTCTCCACCTCCTTCTTCATC
GAPDH	GCAAAGTGGACATTGTCGCCATCA	TCCTGGAAGATGGTGATGGCCTT

together indicated that the Rho-ROCK-MLC signaling might influence the viability of the 4F piPSCs but not the 6F piPSCs after dissociation into single cells.

3.2. The RHO-ROCK-MLC signaling was activated in 4F piPSCs after dissociation into single cells

In order to further explore the function of RHO-ROCK-MLC signaling in the piPSCs, the activation status of the signaling pathway was detected by western blot assay. At first, the GTP-bound RhoA and total RhoA were detected in both 4F piPSCs and 6F piPSCs. Results showed that the level of active RhoA increased significantly when the level of total RhoA did not change in the 4F piPSCs after enzyme treatment (Fig. 2A). While, there were no significant changes in both the level of the active RhoA and total RhoA levels in the 6F piPSCs after dissociation treatment (Fig. 2A). And then, the phosphorylation level of nonmuscle myosin light chain (MLC), which was one of most important downstream molecules in the RHO-ROCK-MLC signaling, was detected. Results showed that the level of phosphorylated MLC was elevated in the 4F piPSCs when the total MLC level did not change. But the increase in the level of phosphorylated MLC in 4F piPSCs could be inhibited by Y27632 after dissociation treatment (Fig. 2B). However, the level of RhoA-GTP and pMLC were both not changed in 6F piPSCs (Fig. 2B). At last, E-cadherin level was detected by western blot and realtime PCR. Results of western blot showed that regardless of the presence of Y27632, in the 4F piPSCs newly synthesized E-cadherins appeared 1 h after dissociation into single cells and significantly reduced after 4 h, but the level of E-cadherin of 4F piPSCs with the Y27632 group is higher than that of 4F piPSCs without the Y27632 group at the same time points after dissociation treatment (Fig. 2C). However, still regardless of the presence of Y27632, in the 6F piPSCs the level of E-cadherin increased from 1 h to 4 h after dissociation treatment and the Y27632 did not affect the E-cadherin level (Fig. 2C). Interestingly, the realtime PCR results

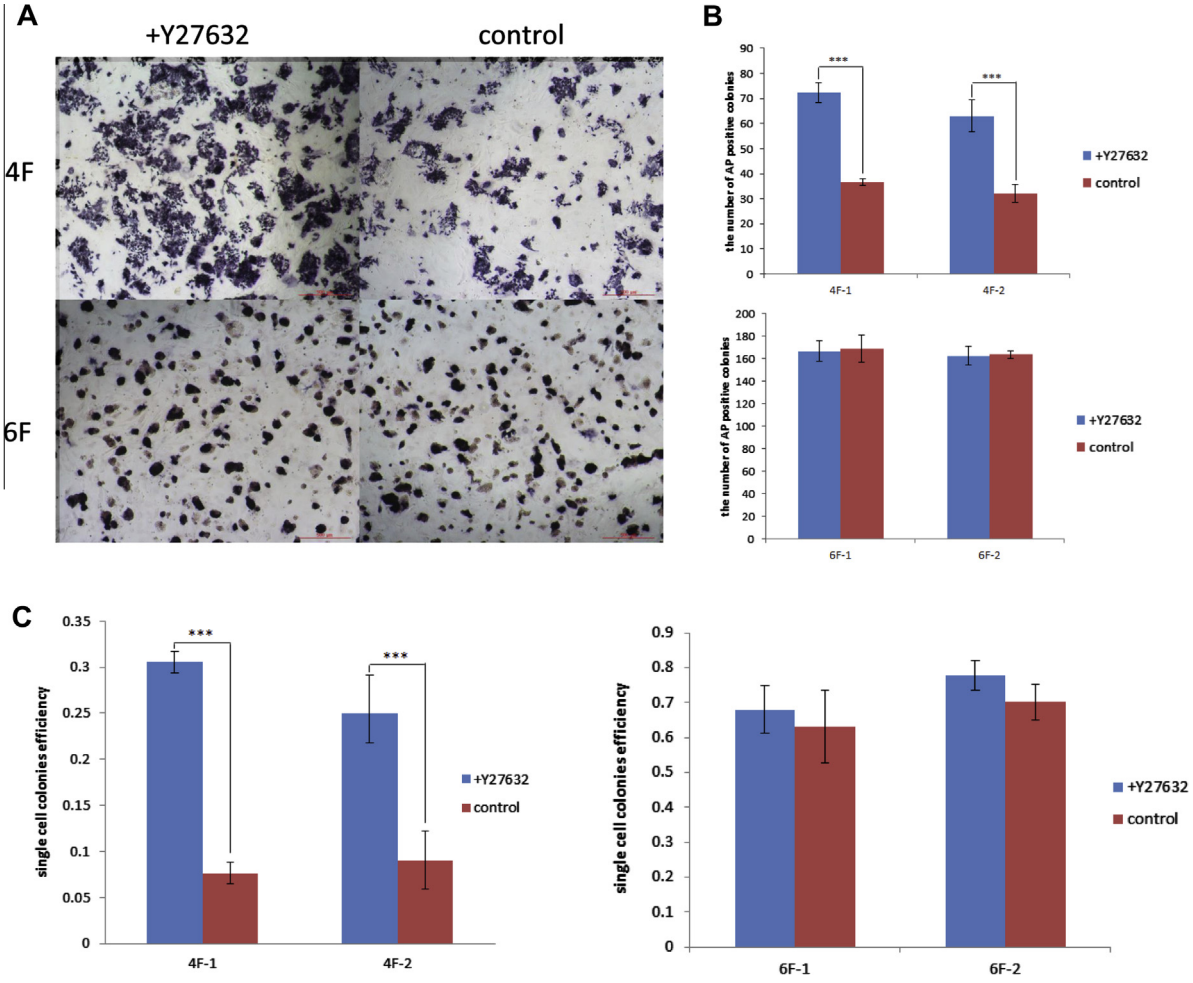

Fig. 1. The viability of 4F piPSCs could be improved by Y27632 after dissociation into single cells. (A) AP staining for counting the positive colonies 2 days after seeding the single cell. +Y27632 represents adding Y27632 to the medium after seeding cells; control represents adding nothing; 4F represents the piPSCs were obtained from *Oct4*, *Sox2*, *Klf4* and *c-Myc*; 6F represents the piPSCs were obtained from *Oct4*, *Sox2*, *Klf4*, *c-Myc*, *Tbx3* and *Nr5α2*. (B) The number of AP positive colonies from 4F piPSCs and 6F piPSCs with or without Y27632. (C) The single cell efficiency of 4F piPSCs and 6F piPSCs with or without Y27632.

revealed comparable amounts of E-cadherin transcripts from 1 h to 4 h after digesting treatment in both 4F piPSCs and 6F piPSCs, suggesting that changes in E-cadherin protein level along time may be not due to the altered transcriptional activity, but likely the post-transcriptional regulation by the Rho-ROCK-MLC signaling (Fig. 2D).

3.3. *Tbx3* and *Nr5α2* could improve the viability of 4F piPSCs after dissociation into single cells

Tbx3 and *Nr5α2* are the two additional factors used in the induction of 6F piPSCs compared with the 4F piPSCs. So, in order to further explore the relationship between the two pluripotent factors (*Tbx3* and *Nr5α2*) and RHO-ROCK-MLC signaling, some important genes related to the RHO-ROCK-MLC signaling were detected in these two types of piPSCs by a realtime PCR assay. Results showed that the levels of *Abr*, *RhoB*, *RhoC*, *MLC1*, *MLC3*, *MYH9* and *MYH10* were significantly lower in the 6F piPSCs than in the 4F piPSCs, suggesting that the *Tbx3* and *Nr5α2* may repress the Rho-ROCK-MLC signaling (Fig. 3A). Then, we wanted to know whether the viability of 4F piPSCs would be improved when *Tbx3* and *Nr5α2* were ectopically expressed in the 4F piPSCs after dissociation into single cells. So, the dissociated 4F piPSCs were infected with lentivirus carrying *Tbx3*, *Nr5α2* and Ds-red (TND). Eight days later, a few colonies appeared in the TND group but not in the uninfected group (Fig. 3B). Then we tried to pick them up and carry out single-cell passaging. Interestingly, the morphologically hESC like TND piPSCs colonies could sustain this kind of single-cell passaging (Fig. 3B). Moreover, regardless of the presence of Y27632, TND piPSCs could maintain comparable high cell viability and the number of AP positive colonies after dissociation into single cells (Fig. 3C and D). At last, using western blot assay, we also found that the phosphorylation level of RhoA and MLC had no difference between the TND piPSCs with and without Y27632 after digesting treatment (Fig. 3E).

4. Discussion

There were two types of piPSCs which are morphologically similar to mESCs and hESCs respectively. Differences between

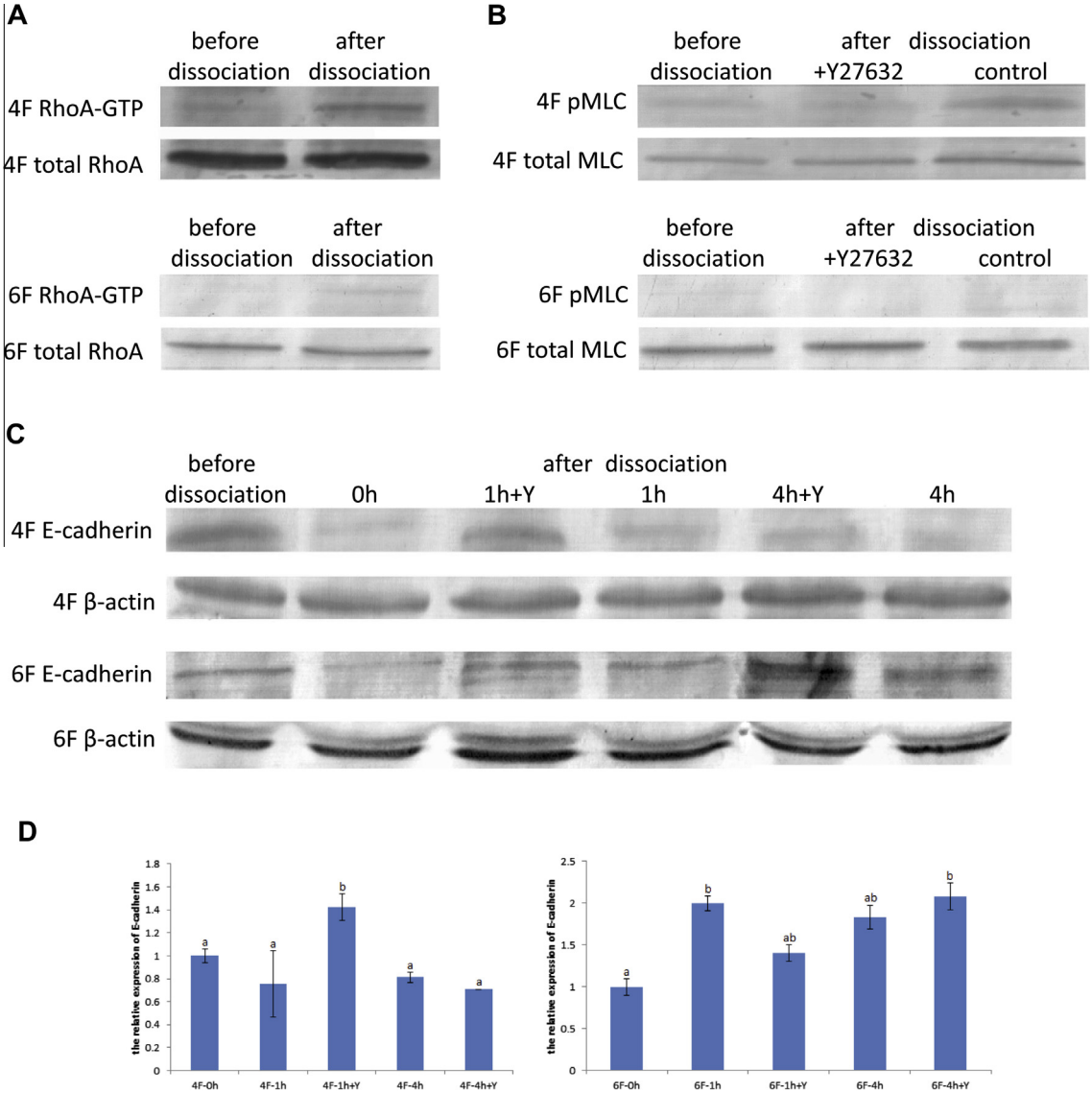

Fig. 2. The RHO-ROCK-MLC signaling was activated in 4F piPSCs after dissociation into single cells. (A) Detection of active RhoA and total RhoA in both 4F piPSCs and 6F piPSCs by western blot. (B) Detection of phosphorylation of MLC and total MLC in both 4F piPSCs and 6F piPSCs by western blot. (C) Detection of E-cadherin in both 4F piPSCs and 6F piPSCs by western blot. β-Actin was an internal standard. 0 h, 1 h and 4 h represents the time after enzyme treatment. (D) Quantitative RT-PCR for *E-cadherin* in both 4F piPSCs and 6F piPSCs. The expression of *E-cadherin* was relative to the expression of *GAPDH*.

them included not only the morphology, but the proliferative capacity, the surface maker and passaging characteristics [2,22,23]. However, there were few reports on the mechanism responsible for the differences existing between them. In this study, we explored the mechanism that the 4F piPSCs could not be passaged by dissociation into single cells. In the previous reports Y27632, an inhibitor for Rho-ROCK-MLC signaling, had been proved to be functional for improving the viability of hESCs after dissociation into single cells [10]. Results of the present study showed that Y27632 could significantly improve the viability of 4F piPSCs after dissociation into single cells, indicating that in 4F piPSCs the Rho-ROCK-MLC signaling works in a similar way as in hESCs. In contrast, Y27632 did not affect the viability of 6F piPSCs, and further results showed the RHO-ROCK-MLC signaling was inactivated in the 6F piPSCs. According to these results, we hypothesized that the pluripotent factors (*Tbx3* and *Nr5α2*) might somehow repress the Rho-ROCK-MLC signaling. Some reports showed that knockdown of important genes in Rho-ROCK-MLC signaling such as *ROCk1|2* and *Myh9* would improve the viability of hESC after digesting treatment [18,19,21]. Our results also showed these important genes in the Rho-ROCK-MLC signaling were expressed at a lower level in 6F piPSCs than 4F piPSCs, and the 4F piPSCs could be passaged by dissociation into single cells after the introduction of *Tbx3* and *Nr5α2*, although their morphology did not change.

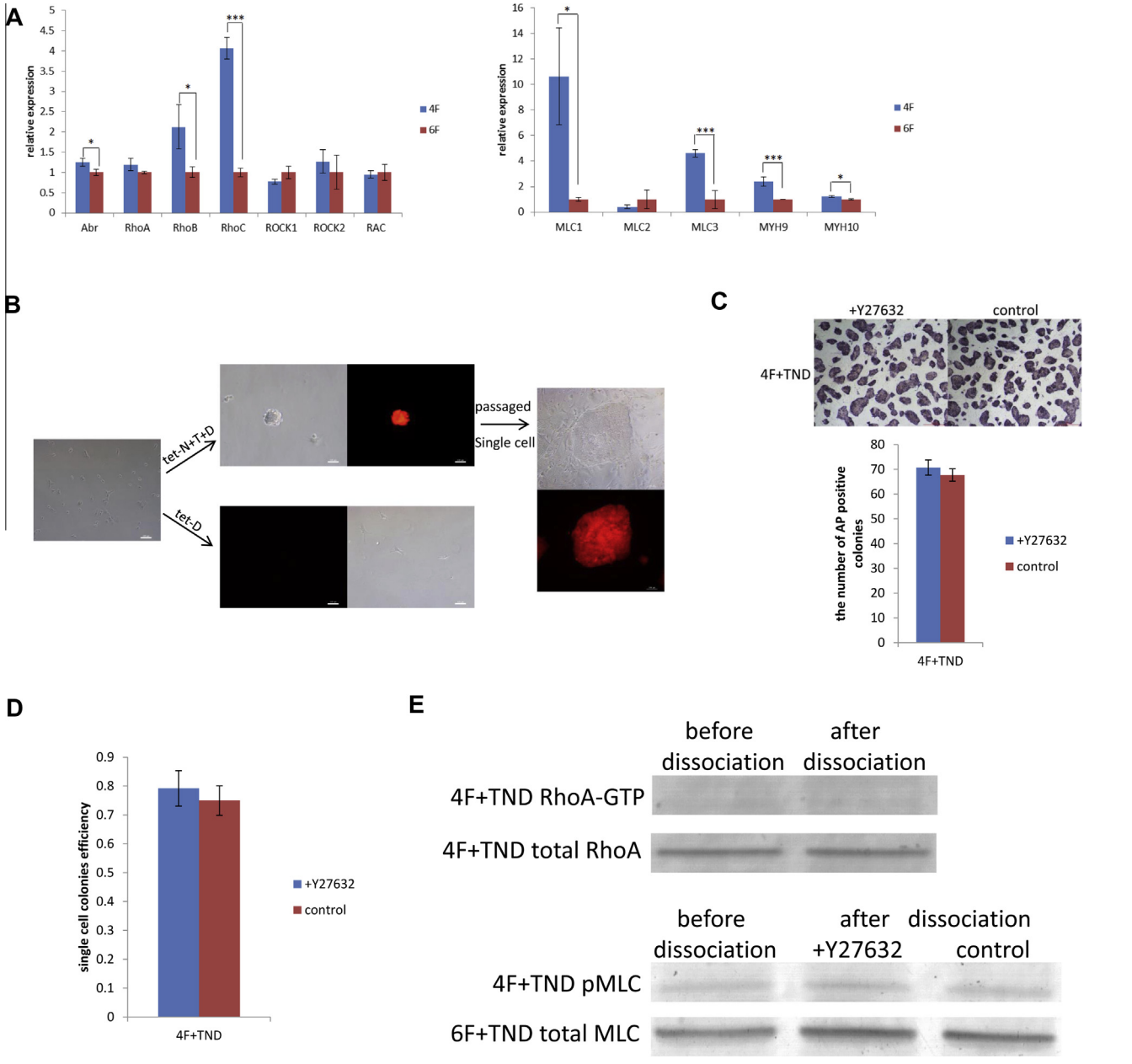

Fig. 3. *Tbx3* and *Nr5α2* could improve the viability of 4F piPSCs after dissociation into single cells. (A) Quantitative RT-PCR for *Abr*, *RhoA*, *RhoB*, *RhoC*, *ROCK1*, *ROCK2*, *RAC*, *MLC1*, *MLC2*, *MLC3*, *MYH9* and *MYH10*. The expression of these genes was relative to the expression of *GAPDH*. (B) The 4F piPSCs were infected with lenti-virus after dissociation into single cells. Colonies were picked up 8 days later and could be passaged by enzyme treatment. Tet-N+T+D represents the lenti-virus(*Nr5α2*, *Tbx3* and *Ds-red*) induced by doxycycline. Tet-D represents the lenti-virus *Ds-red* induced by doxycycline. (C) AP staining for counting the positive colonies 2 days after seeding the single cells. +Y27632 represents adding Y27632 to the medium after seeding cells; control represents adding nothing. 4F+TND represents the 4F piPSCs infected by the lenti-virus(*Nr5α2*, *Tbx3* and *Ds-red*). (D) The single cell efficiency of 4F+TND piPSCs and 6F piPSCs with or without Y27632. (E) Detection of phosphorylation of RhoA and MLC, and the total RhoA and MLC in 4F+TND piPSCs by western blot.

These results supported our hypothesis on the function of *Tbx3* and *Nr5a2*.

How could *Tbx3* and *Nr5α2* inhibit the RHO-ROCK-MLC signaling? There were few reports showing the interaction between the two pluripotent factors and RHO-ROCK-MLC signaling in stem cell research. But results from other fields suggested that *Nr5a2* and *Tbx3* can participate in the inhibition of Rho-ROCK-MLC signaling. Reports on cancer researches showed that *Nr5α2* can regulate the actin cytoskeleton and E-cadherin which are the downstream of RHO-ROCK-MLC signaling [24,25]. Results from pig fibroblast transdifferentiation researches indicated that there were relations between Nr5α2 and mesenchymal–epithelial transition (MET), which in turn could made ROCK pathway inactivation [26]. And, some reports showed that there were also some relationships between *Tbx3* and Rho-ROCK-MLC signaling. The expression of *Tbx3* could be inhibited by the RHO-ROCK-MLC signaling, and *Tbx3* could be activated by RAC1, an inhibitor to the ROCK phosphorylation [27].

Tbx3 and *Nr5α2* may have an important role in the establishment of porcine embryonic stem cells (pESCs). pESCs had been tried to be established for about 20 years, but no authentic cell lines were obtained [28,29]. These studies on pESCs showed that the porcine blastocysts can give rise to the outgrowth and it could be passaged for limited times, in fact no more than 20 passages in most of the cases [30,31]. In our study, the viability of 4F piPSCs could be improved by Y27632 in a single-cell passage, but the cell lines still could not sustain the succeeding single-cell passages more than 5 times (data not shown). However, when the 4F piPSCs were infected with *Tbx3* and *Nr5α2*, the resulting cell lines could be passaged for a long time through dissociation into single cells before we submitted this manuscript. Reports showed that *Nr5α2* could enhance the expression of Oct4 in epiblast and pluripotent stem cell [32,33]. *Tbx3* had also been proved to be useful for improving the germ-line competence of miPSCs [34]. All of these indicated that the activation of *Tbx3* and *Nr5α2* may be essential to the establishment of pESCs.

In summary, we found that Rho-ROCK-MLC signaling plays a negative regulatory role in the formation of 4F piPSCs colonies after dissociation into single cells. And, *Tbx3* and *Nr5α2* could inhibit Rho-ROCK-MLC signaling and improve the viability of piPSCs after dissociation into single cells. Our finding also provided new clues for the establishment of pESCs.

Conflict of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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