Tenocyte-derived exosomes induce the tenogenic differentiation of mesenchymal stem cells through TGF- β

Tianpeng Xu · Menglei Xu · Jiaxiang Bai · Jiayi Lin · Binqing Yu ·
Yu Liu · Xiaobin Guo · Jining Shen · Houyi Sun · Yuefeng Hao ·
Dechun Geng

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Abstract Mesenchymal stem cells (MSCs) hold great potential to treat tissue damage based on their multipotent property, and are also considered as suitable cell resources to create tissue-engineered grafts for tendon repair. However, the clinical application of MSCs is still limited by the lack of efficient methods to induce tenogenic differentiation. In this study, by performing the experiments in transwell system, we found that paracrine factors from tenocytes could induce MSCs to undergo the tenogenic differentiation. We further verified that tenocytes could secrete exosomes and these tenocyte-derived exosomes efficiently initiated the tenogenic differentiation of MSCs. Finally, we revealed that the TGF- β existing in tenocyte-derived exosomes mediated the process, as the inhibition of TGF- β signaling

abolished the effects of tenocyte-derived exosomes on MSCs. By investigating the effects of tenocytes on MSCs, we found that tenocytes-derived exosomes can induce tenogenic differentiation of MSCs in a TGF- β dependent manner. These studies provided critical information about the multipotency of MSCs and suggested potential strategies for clinical translation.

Keywords Mesenchymal stem cells · Tenocytes · Exosomes · Tenogenic differentiation

Introduction

Tenocytes are important cells that constitutes the tendon, the connective tissues that play critical roles in maintaining the normal physiological function of joint (Nourissat et al. 2015). Due to the limited proliferative ability of tenocytes, the self-recovery of tendon is poor upon the tendon injuries and lead to the destruction of tendon histology and function (Liu et al. 2017; Sharma and Maffulli 2005; Yang et al. 2013). Tissue-engineering grafts consist of tenocytes and acellular scaffolds thus are considered as a promising strategy for the treatment of massive tears in tendon, such as large and complex rotator cuff tears that larger than 3 cm (Longo et al. 2012; McClinton et al. 1982; Smith et al. 2004; Strickland 2005; Van Eijk et al. 2004; Zumstein et al. 2017). Importantly, the suitable seeding cells could enhance the extracellular matrix formation

Tianpeng Xu, Menglei Xu and Jiaxiang Bai have contributed equally to this work.

T. Xu · M. Xu · Y. Hao (✉)
Orthopedics and Sports Medicine Center, Suzhou
Municipal Hospital (North District), Nanjing Medical
University Affiliated Suzhou Hospital, 242, Guangji
Road, Suzhou 215006, People's Republic of China
e-mail: 13913109339@163.com

J. Bai · J. Lin · B. Yu · Y. Liu · X. Guo ·
J. Shen · H. Sun · D. Geng (✉)
Department of Orthopedics, The First Affiliated Hospital
of Soochow University, 188, Shizi Road, Suzhou 215006,
People's Republic of China
e-mail: szgengdc123@163.com

and collagen fibrils regeneration (Van Eijk et al. 2004; Zumstein et al. 2017). The best choice of the seeding cells is tenocytes, while it is impractical to collect so much autologous tenocytes directly from the patients (Van Eijk et al. 2004). The limited resource of seeding cells is still the major obstacles to improve the efficiency of grafts in treating tendon injuries (Liu et al. 2017). Thus generating tenocytes from stem cells to fulfill the requirement of seeding cells is in great demand, and mesenchymal stem cells are desirable resources.

Mesenchymal stem cells (MSCs) are multipotent stem cells that exists in almost all tissues (Nombela-Arrieta et al. 2011; Uccelli et al. 2008). Due to their self-renewal potential and the abilities to differentiate into several cell types, MSCs are considered to hold great promises for the treatment of tissue damages including tendon and ligament injuries (Dimarino et al. 2013; Shi et al. 2010; Wu et al. 2014). There have been several studies revealed that MSCs, administered by orthotopic injection or delivered by scaffolds, can favor the tendon repair and promote the tissue regeneration (Chen et al. 2016; Leong and Sun 2016; Teng et al. 2016). Moreover, recent studies found that pretreating MSCs to determine their differentiation commitment before their application into tissue repair can largely promote their directional differentiation efficiency *in vivo* and guarantee their therapeutic effects in animal models (Cai et al. 2015; Marquass et al. 2011). Thus, it is reasonable to hypothesize that pretreating MSCs to undergo tenogenic differentiation will also promote their beneficial effects on tendon injuries. However, the tenogenic differentiation of MSCs are still largely unknown and the efficient methods merit further investigations.

Exosome are endosomal original extracellular vesicles (Carretero-Gonzalez et al. 2018; Colombo et al. 2014). The internal endosomes contains intraluminal vesicles with the diameter among 30–100 nm, upon the fusion of these endosomes with the plasma membrane, the intraluminal vesicles will be secreted as exosomes (Carretero-Gonzalez et al. 2018; Colombo et al. 2014). Different from the microvesicles (150–1000 nm diameter) that direct budded from the plasma membrane, the composition of exosomes are closer to internal endosomes than plasma membrane (Colombo et al. 2014). Such extracellular vesicles are found to be secreted by various kinds of cells (Raposo and Stoorvogel 2013; Schneider and

Simons 2013; Simons and Raposo 2009; Thery et al. 2002). Exosomes carry various kinds of proteins and micro-RNAs and can serve as important messengers between different cells (Raposo and Stoorvogel 2013; Schneider and Simons 2013; Simons and Raposo 2009; Thery et al. 2002). It has been proved that MSCs can obtain special properties after the education by the exosomes produced by other cells types (Takeda and Xu 2015). Likewise, tumor cells derived exosomes can endow MSCs the properties to modulate tumor immune microenvironment and thus promote tumor progression (Lin et al. 2016). While exosomes form neuron cells affect the neuronal differentiation potency of MSCs (Takeda and Xu 2015). Recent investigations also discovered that direct co-culture of MSCs and tenocytes will affect the gene expression and some other properties of MSCs (Kraus et al. 2013; Schneider et al. 2011). These observations indicated that tenocytes may also secret exosomes and these extracellular vesicles may influence MSCs.

In this study, we mainly focused on the mechanisms that underlying the effects of tenocytes on the tenogenic differentiation of MSCs. We hypothesized that the tenocyte-secreted exosomes played critical roles in inducing MSCs to undergo the tenogenic differentiation. These investigation provides better understanding of the interaction between MSCs and tenocytes and will help to improve the related clinical strategies.

Materials and methods

Cell isolation and culture

Adult Sprague–Dawley (SD) rats were maintained in Animal Center of Soochow University and all operations have been approved by the care of Experimental Animals Committee. The 2-month-old rats were euthanized to aspirate the bone marrow from thigh-bones and shinbones 10 mL syringe containing low glucose DMEM medium (10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin). MSCs were then isolated through density gradient centrifugation with percoll (1.073 g/ml) at 2500 rpm/min for 20 min. The cells were resuspended in low glucose DMEM medium and cultured in standard incubators. MSCs within 3–8 passages were used in the experiments.

The Achilles tendons were removed from rats for the isolation of tenocytes. The tendons were cut into 1-mm pieces and put into 15 cm dishes with 10 ml low glucose DMEM medium. The medium was replenished every 2 days. Colonies can be seen after about 6 days, and then removed the tissue mass. The tenocyte colonies were digested by trypsin at about 10 days and reseeded into 10 cm dishes, which counted as passage 1 tenocytes. Tenocytes within 3–5 passages were used in the experiments.

The treatment of MSCs with tenocytes and tenocyte-derived exosomes

Some MSCs (5×10^4) were directly co-cultured with tenocytes (5×10^4 or 1×10^5) labeled with red fluorescent lipid dye PKH26 (purchased from Sigma) in the wells of 6-well plate for 14 days. After the co-culture, the mixed cells will be digested by trypsin and suspended with PBS that containing DNase I (400 U/ml) and 2% FCS. Then the MSCs will be isolated for further analysis by flow cytometry through the deletion of PKH26 labeled tenocytes. Some of MSCs (5×10^4) were co-cultured with tenocytes (5×10^4 or 1×10^5) in the wells of 6-well transwell plate for 14 days. Other MSCs (1×10^5) are seeded in the wells of 6-well plate and treated with tenocyte-derived exosomes for 14 days.

Exosomes isolation

Tenocyte derived exosomes were isolated as reported previously (Baglio et al. 2015; Shabbir et al. 2015). Briefly, tenocytes were cultured for 48 h and the conditional medium was collected, then the cells are removed through $500g \times 5$ min centrifugation. The supernatant was then centrifuged at $1000g$ for 5 min to further remove the cell. Then the supernatant were collected and centrifuged at $10,000g$ for 60 min to remove cell debris. The resultant supernatant was centrifuged at $100,000g$ for 90 min and the pellet was exosomes. The pellet was suspended with PBS and repeated the centrifugation at $100,000g$ for 90 min to wash the exosomes. Exosomes-depleted medium was used to resuspend the exosomes pellet and these exosomes are stored for further experiments.

Proliferation assay

MSCs treated with tenocytes or tenocyte-derived exosomes are seeded in wells of 96-well plated (1×10^3 per well). The culture medium will be removed and replaced with 100 μ l fresh medium before the assay. Then 10 μ l CCK-8 was added into the medium and cultured for 2 h in the standard incubator. Finally, read the absorbance at 450 nm. Cell number was tested at 0, 24, 48 and 72 h after cell seeding.

Western blot

Cells or exosomes were lysed with lysis buffer containing protease inhibitor. Then total protein extracts (10 μ g) from tenocytes or their exosomes were fractionated by SDS-polyacrylamide gel electrophoresis. After transferring proteins onto nitrocellulose membranes, incubated the membranes with specific primary antibodies and followed by the incubation with secondary antibodies. Wash the membranes with TBST and then determined the expression of proteins with enhanced chemiluminescence mix. The antibodies information: Anti- α -Tubulin, #2144, CST; Anti-p-ERK1/2, #4695, CST; Anti-Shc, #2432, CST; Anti-Integrin beta 1, ab179471; Anti-tenomodulin, ab203676; Anti-Collagen I, ab34710; Anti-Collagen III, ab7778; Anti-CD9, ab2215; Anti-CD61, Invitrogen # 17-0619-41; Anti-TSG101, Invitrogen # MA1-23296; Anti-CYC1, Invitrogen # PA5-25257; Anti-Grp94, Invitrogen # MA3-016; Anti-TGF- β , #3711, CST; Anti-p-SMAD3, Invitrogen # PA5-36877; Anti-SCX, Invitrogen # PA5-23943; Anti-TnC, #12221, CST.

Statistics

All experiments were performed for at least three times. Data was analyzed with two-tail *t* test using Excel software. The results are shown as the mean $\pm$ SEM and analyzed by unpaired two-tailed Student's *t* test, the differences were considered significant when $p < 0.05$.

Result

Direct co-culture with tenocytes induce the tenogenic differentiation of MSCs

To investigate the effects of tenocytes on MSCs, we first co-cultured MSCs with tenocytes directly for 14 days and then we evaluated the tenogenic differentiation of MSCs by western blot analysis. We found that the tenocyte co-culture did significantly enhanced the expression of tendon specific markers, including collagen I (Coll I), collagen III (Coll III) and tenomodulin (TNMD) in MSCs (Fig. 1a). Recent studies have reported the involvement of MAPK signaling in tendon development (Liu et al. 2017), we

also tested the expression of related proteins Shc and phosphorylated ERK 1/2 (p-ERK 1/2). The results showed that the direct interaction with tenocytes could largely promote the Shc and p-ERK 1/2 expression of MSCs (Fig. 1a). Interestingly, β -integrin, which can be enhanced upon mechanical stimulation, was also observed in MSCs co-cultured with tenocytes but not in normal MSCs (Fig. 1a). To test how long it is needed to achieve the effect of tenocytes on MSCs, we co-culture MSCs and tenocytes for 10 days, 14 days and 18 days. We found that the expression of Coll I, TNMD and β -integrin in MSCs is nearly the same at day 14 and day 18, which both higher than the expression at day 10, suggesting 14 days is enough for tenocytes to regulate the differentiation of MSCs

Fig. 1 MSCs undergo tenogenic differentiation upon the direct co-culture with tenocytes. MSCs with or without tenocytes co-culture were collected for the western blot analysis, tenocytes alone were used as positive control and MSCs alone were used as negative control. **a** The protein levels of Coll I, Coll III, TNMD, Shc, p-ERK 1/2 and β -integrin in MSCs were determined. **b** The protein levels of Coll I, TNMD and β -integrin

in MSCs were determined at different time of co-culture. **c** The proliferation rates of MSCs with or without tenocytes co-culture were tested by CCK-8 assay at 0, 24, 48 and 72 h after cell seeding. Teno tenocytes. Data are shown as Mean $\pm$ SEM and analyzed by unpaired two-tailed Student's *t* test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ns no significance

(Fig. 1b). These results revealed that cell–cell contact with tenocytes could induce MSCs to undergo tenogenic differentiation.

We also noticed that the direct communication from tenocytes affected the proliferation of MSCs. Thus we tested the proliferation rate of MSCs with or without tenocyte treatment and found that, compared to normal MSCs, those co-cultured with tenocytes exhibited a much higher proliferation rate (Fig. 1c).

Tenocytes induce the tenogenic differentiation of MSCs in a paracrine dependent manner

To verify whether the effects of the tenocytes on MSCs were dependent on the paracrine factors, we performed the co-culture experiments in the transwell system. After the 14 days co-culture, we performed the western blot experiments and found that, even without direct cell–cell contact, tenocytes could still upregulate the expression of Col I, Coll III, TNMD, Shc, p-ERK 1/2 and β -integrin in MSCs (Fig. 2a). We also found that co-culturing with tenocytes in transwell system could also induce the expression of

scleraxis (SCX), an important tenocytes marker, in MSCs (Fig. 2b). Moreover, in this condition, tenocytes could promote the proliferation rate of MSCs (Fig. 2c). These data indicated that tenocyte-derived paracrine factors played critical roles in initiating the tenogenic differentiation of MSCs.

Tenocyte-secreted exosomes regulate the tenogenic differentiation of MSCs

To evaluate the role of exosomes in tenocyte-induced tenogenic differentiation of MSCs, we isolated exosomes from tenocyte-conditional medium. Consistent with the definition of exosomes, tenocyte-derived exosomes expressed high levels of CD9, CD61 and TSG101, but low levels of intracellular proteins like CYC1 and Grp94 (Fig. 3a). Importantly, these tenocyte-secreted exosomes can significantly enhance the expression of Col I, Coll III, TNMD, Shc, p-ERK 1/2 and β -integrin in MSCs and promote the proliferation of MSCs, while the exosomes-depleted tenocyte conditional medium cannot exhibit the same effect (Fig. 3b, c). Furthermore, these exosomes modulate

Fig. 2 Tenocytes induce the tenogenic differentiation of MSCs through their secretome. MSCs co-cultured with tenocytes in a transwell system for 14 days were collected for the western blot analysis, tenocytes alone were used as positive control and MSCs alone were used as negative control. **a** The levels of Col I, Coll III, TNMD, Shc, p-ERK 1/2 and β -integrin in MSCs were evaluated. **b** The level of SCX in MSCs was evaluated. **c** The proliferation rates of control MSCs and MSCs from transwell system were tested by CCK-8 assay at 0, 24, 48 and 72 h after cell seeding. *Teno* tenocytes. Data are shown as Mean $\pm$ SEM and analyzed by unpaired two-tailed Student's *t* test. *p < 0.05; **p < 0.01; ***p < 0.001. *ns* no significance

Fig. 3 Exosomes mediate the effects of tenocytes on MSCs. **a** Tenocyte-derived exosomes were isolated and specific markers including CD9, CD61, TSG101, CYC1 and Grp94 were tested by western blot. **b** MSCs treated with tenocyte-derived exosomes or exosomes-free supernatant were collected for the analysis of Coll I, Coll III, TNMD, Shc, p-ERK 1/2 and β-integrin. Tenocytes alone were used as positive control and

MSCs alone were used as negative control. **c** The proliferation rates of MSCs with or without tenocytes-derived exosomes treatment were tested by CCK-8 assay at 0, 24, 48 and 72 h after cell seeding. *CL* cell lysis, *sup* supernatant. Data are shown as mean ± SEM and analyzed by unpaired two-tailed Student's *t* test. *p < 0.05; **p < 0.01; ***p < 0.001. *ns* no significance

the differentiation of MSCs in a dose dependent manner (Fig. 4). These results revealed that exosomes secreted by tenocytes was indispensable for tenocyte-mediated tenogenic differentiation of MSCs.

TGF-β mediates the effects of tenocyte-derived exosomes on MSCs

Recent studies reported that tenocytes can produce amount of TGF-β, which have been proved to be able to influence the tenogenic differentiation of MSCs. To determine whether TGF-β exists in tenocyte-derived exosomes, we tested the protein level of TGF-β in both tenocyte and tenocyte-derived exosomes. We found that compared to tenocytes, tenocyte-derived exosomes did contain higher level of TGF-β (Fig. 5a). Moreover, we also tested the TGF-β signaling in MSCs with or without tenocyte-derived exosomes treatment and found that, tenocyte-derived exosomes could promote the expression of phosphorylated

SMAD3 (p-SMAD3), an important regulator in TGF-β signaling pathway, in MSCs (Fig. 5b). To further examine the potential role of TGF-β in the process, we applied TGF-β signaling inhibitor (SB 431542) during the treatment of MSCs and found this inhibitor can abolished the effects of tenocytes-derived exosomes on MSCs (Fig. 5b). We also found that TGF-β signaling played critical roles in initiating the expression of SCX in MSCs during exosomes treatment (Fig. 5c). These data revealed that tenocyte-derived exosomes induced the tenogenic differentiation of MSCs in a TGF-β dependent manner.

Collectively, our experiments highlight the critical roles of tenocyte-derived exosomes in regulating the tenogenic differentiation of MSCs. These information will provide a better understanding about the multipotency of MSCs and hold potential for the treatment of tendon injuries.

Fig. 4 Exosomes influence MSCs in a dose dependent manner. Tenocyte-derived exosomes were isolated for the treatment of MSCs. MSCs were treated with different amount of tenocyte-derived exosomes for 14 days and collected for the western blot analysis of Col I, Col III, TNMD, Shc, p-ERK 1/2 and β -integrin. MSCs alone were used as negative control

Discussion

Due to the limitation of self-regeneration, tendon and ligation injuries are still clinical challenges and the supply of sufficient autologous or stem cell-differentiated tenocytes will provide promising strategies for the diseases (Nourissat et al. 2015; Yang et al. 2013). However, the collection of autologous tenocytes is unpractical and the expansion of these tenocytes is limited (Van Eijk et al. 2004). Thus, generating tenocytes from stem cells becomes a desirable way to fulfill the requirement for clinical application.

Mesenchymal stem cells are a group of tissue stem cells holding multipotency and have been shown to differentiate into adipocytes, osteocytes, neurons and chondrocytes (Nombela-Arrieta et al. 2011; Uccelli

et al. 2008). However, the tenogenic differentiation of MSCs and the key modulators in this process are still largely unknown. Through co-culture tenocytes and MSCs in the transwell system, we found that tenocytes can induce tenogenic differentiation of MSCs in a paracrine manner. As previous studies reported, cell–cell contact between tenocytes and MSCs could also induce the tenogenic differentiation of MSCs, the comparison studies between these two systems will provide more information to further improve the differentiation efficiency of MSCs. Moreover, because of the lack of specific biomarkers for tenocytes and tenogenic differentiation, it is still difficult to study the tenogenic differentiation process of MSCs (Liu et al. 2017). In current studies, we access the tenogenic differentiation of MSCs by evaluating the expression of a series of proteins, which can be produced by tenocytes and supposed to be functional markers of tenocytes (Liu et al. 2017). The discovery of better and specific biomarkers for tenocytes will surely provide a more efficient way to study the biology of tenocyte and evaluate the tenogenic differentiation potential of stem cells.

Exosomes are 30–100 nm diameter microvesicles and can be secreted by many kinds of cells (Raposo and Stoorvogel 2013; Schneider and Simons 2013; Simons and Raposo 2009; Thery et al. 2002). In this study, we evaluated the effect of tenocytes-derived exosomes on MSCs. We found that tenocytes-derived exosomes alone could induce tenogenic differentiation of MSCs. We also found that TGF- β in these microvesicles takes responsibility for the differentiation of MSCs, as the TGF- β inhibitor could abolished the effects of these exosomes on MSCs. Several studies also showed that soluble TGF- β could influence MSCs towards tenogenic differentiation (Schneider et al. 2011; Yin et al. 2016). The different efficiency of soluble TGF- β and exosomes-carried TGF- β in promoting tenogenic differentiation of MSCs merits more investigations. Moreover, although the detailed mechanisms is not clear, these tenocyte-secreted exosomes could also promote the proliferation of MSCs and hold potential to fulfill the large demand of tenocytes in clinical trials. However, further in vivo studies by using animal models is still merited to verify the therapeutic function of MSCs that pre-treated with tenocytes or tenocyte-derived exosomes. Moreover, considering the difference that exists between human tenocytes and rat tenocytes,

Fig. 5 TGF- β plays critical roles in the effect of tenocyte-secreted exosomes on MSCs. Tenocyte-derived exosomes were isolated. **a** The concentration of TGF- β in tenocytes or tenocyte-derived exosomes was determined. **b, c** MSCs were treated with

tenocyte-derived exosomes with or without TGF- β inhibitor (SB 431542) and collected for the western blot analysis of p-SMAD3, Col I, TNMD, β -integrin, Tn-C and SCX

the studies using human cells will provide more direct evidence about the translational potential of these investigations.

Collectively, our study demonstrated that tenocytes secreted exosomes can initiate the tenogenic differentiation of MSCs in a TGF- β dependent manner. These results provide promising strategies for the treatment of tendon injuries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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