Original Article Wnt7a promotes muscle regeneration in branchiomeric orbicularis oris muscle

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Abstract: Background: The orbicularis oris muscle exhibits a deficiency in cleft lip patients. Compared with the somite-derived limb muscles, the regeneration performance of the branchiomeric orofacial muscle has seldom been investigated. Objective: This study aims to explore the possibility of augmenting the orbicularis oris muscle through the stimulus of Wnt7a. Methods: Adult rat orbicularis oris muscle and tibialis anterior muscle were injected with recombinant human Wnt7a protein. The muscles were harvested at different time points after Wnt7a delivery. Muscle regeneration-related activity, including cell proliferation, stem cell proportion, myofiber plasticity, and total fiber number, was examined. Results: Adult rat orbicularis oris muscle and tibialis anterior muscle exhibit similar regeneration-related activities after Wnt7a administration. Recombinant human Wnt7a administration resulted in enhanced cell proliferation, stem cell expansion, and fiber type remodelling in rat orbicularis oris muscle. In addition, newly formed myofibers were detected, contributing to an increased total fiber number. Conclusion: Wnt7a induces vigorous regeneration in rat orbicularis oris muscle. This study helps lay a foundation for developing biotherapies to combat orofacial muscle deficiency.

Keywords: Skeletal muscle, cell proliferation, myosin heavy chain, regeneration

Introduction

The orbicularis oris muscle (OOM), circling around the oral fissure, plays an important role in maintaining facial aesthetics. In cleft lip patients, however, the OOM exhibits a deficiency, posing a substantial challenge for the surgeons to reconstruct a full and symmetrical upper lip [1]. This decreased muscle volume persists even after primary cleft repair as muscle fibers in the reconstructed area tend to be scarce and hypoplastic [2-5]. Current surgical interventions to increase the muscle bulk include myomucosal flap and autologous fat grafting [6-9]. Nevertheless, both methods address the issue in a rather indirect way: by borrowing tissue from the unaffected area. Taking consideration of the fact that skeletal muscle tissue has a high capacity for regeneration [10], biotherapies to promote OO muscle regeneration in situ should be promising in lip augmentation.

Unlike the typical somite-derived limb muscles, orofacial muscles, including the OO muscle,

originate from the branchial arch [11]. This special developmental origin enabled the transition from passive filter feeding seen among invertebrates to an active predatory lifestyle in vertebrates [12, 13]. Consequently, branchiomeric muscles are distinct from somite-derived muscles in many aspects. Myogenic regulatory factors like Pitx2 and Tbx1 modulate the developmental trajectory in branchiomeric muscle, but barely participate the process in somitederived muscles [14]. Furthermore, in vitro studies revealed that muscle stem cells isolated from branchiomeric muscles proliferate more and differentiate later than those from somite-derived muscles [15-17]. In addition, the susceptibility to dystrophic muscle diseases differs in the two muscle groups: there tends to be a much higher vulnerability for somite-derived muscles in Duchenne Muscular Dystrophy [18]. Since skeletal muscle regeneration studies have been mostly done on somitederived limb muscles, it remains to be tested whether these muscle growth factors are equally effective in branchiomeric orofacial muscles.

Recent progress on skeletal muscle regeneration has put forward a novel Wnt ligand, Wnt7a, that can act as a potent muscle growth factor [19]. Exerting pleiotropic effects both on the muscle stem cell and the myofiber. Wnt7a can vigorously boost limb muscle regeneration [19-21]. On the one hand, Wnt7a promotes satellite cell expansion to maintain the stem cell reserve; meanwhile, Wnt7a enhances satellite cell mobility to facilitate myofiber repair [19, 20]. On the other hand, Wnt7a works on the myofiber to increase fiber diameter [21]. The administration of Wnt7a has also been proved to ameliorate muscular dystrophy by increasing myotube diameter and inducing a shift towards protective fiber types in cultured human myoblasts [22].

In order to explore biotherapies to augment the OO muscle, we implement an animal study here to test whether the potent limb muscle growth factor, Wnt7a, could efficiently regenerate the orofacial muscle in a similar way.

Materials and methods

Animals

A total of 42 adult male Sprague-Dawley rats (8 weeks, 270-280 g) were bought from Dashuo Biological Technology Company, Chengdu, China. All rats were raised on a 12-h light/dark cycle, in a humidity-controlled (53 ± 2%) and temperature-controlled (23 ± 2°C) facility. All experimental procedures on animals were in accordance with National Institute of Health Guidelines for the Care and Use for Laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC, protocol number: WCHSIRB-D-2019-067) at Sichuan University. The study was conformed with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines for preclinical animal studies and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Intramuscular recombinant human Wnt7a delivery and muscle dissection

A combination of 50 mg/kg Zoletil (Virbac, Nice, France) and 0.05 mg/kg atropine (Kangbaotai, Hubei, China) were applied by intramuscular injection to anesthetize the rats. The tibialis anterior (TA) muscle was accessed as previously reported [23]. An amount of 25 µl recombinant human Wnt7a (rh-Wnt7a, R&D systems, Minneapolis, MN, U.S.A., 100 µg/ml) was injected into the right TA muscle in each rat (n = 6). The contralateral TA muscle of the same animal was injected with 25 µl phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) as control (n = 6). For the injection of Wnt7a into the OOM, the following procedures were carried out. The wet mucosa of the rat's upper lip was exposed with forceps. The port of entry was 6 mm medial to the oral commissure (Figure 1A. 1B). The injection was made with a micro-syringe (B. Braun Melsungen AG, Melsungen, Germany), with a stopper 6 mm from the needle tip, tunnelled into the OOM all the way to the oral commissure. Retrograde infiltration was performed beneath the wet vermillion, working progressively toward the entry port. In the experimental group (n = 6), 25 μ l rh-Wnt7a was delivered into the right OOM in each rat. In the control group (n = 6), the OOM was injected with 25 µl PBS containing BSA as a control. The rats were randomly allocated to experimental or control groups. Rats were euthanized with diethyl ether (Nanjing Reagent, Jiangsu, China) inhalation, followed by decapitation, at different timepoints after Wnt7a delivery (Figure 1C). The entire TA muscle was harvested, and the OOM 6 mm medial to the oral commissure was dissected for further histologic examination.

Immunofluorescence

Muscle samples were processed for cryosections and subsequent immunofluorescence according to previously described methods [24]. Briefly, freshly isolated muscles were embedded in Tissue-Tek optimal cutting temperature compound (SAKURA, Japan) and cooled in liquid nitrogen-cooled isopentane (Macklin, Shanghai, China), Cryosections were made at 10 µm and fixed in ice-cold acetone (KESHI, Chengdu, China). The sections were blocked with blocking serum (5% bovine serum albumin and 5% goat serum) prior to incubation with primary antibodies at 4°C overnight. The secondary antibodies were incubated for 1 h at room temperature, then the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured with an Olympus BX63 fluorescence microscope (Oly-



Figure 1. Rh-Wnt7a delivery to rat OOM. A, B. Top and lateral view of the injection field. The yellow dot indicates the port of entry, and the yellow dotted line indicates the oral commissure. The port of entry was 6 mm medial to the oral commissure. C. Schematic illustration of the study rationale. The TA muscle was harvested at three weeks after Wnt7a administration. The OOM was harvested at two, three, and five weeks after Wnt7a administration.

mpus Corporation, Tokyo, Japan). The antibodies used in this study are listed in **Table 1**.

Quantification and statistical analyses

Quantification of cell numbers and myofiber diameter were performed using ImageJ, with three fields of view selected for each sample. All data were analysed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Data distribution was tested with one-sample Kolmogorov-Smirnov test. A two-tailed independent Student's t-test was used to evaluate statistical differences between PBS-treated and rh-Wnt7a-treated muscles at each timepoint. Results are presented as mean \pm SEM. *P* < .05 was considered significant.

Results

OOM and TA exhibit similar regeneration-related activities after Wnt7a administration

At three weeks after Wnt7a delivery, cell proliferation, muscle satellite cell proportion and new fiber formation were examined in both

OOM and TA muscle. The results revealed that Wnt7a stimulated vigorous cell proliferation in the two muscle groups, as evidenced by significantly increased Ki67-positive cells (Figure 2A-D, 2M). Similarly, the proportion of Pax7-positive cells also significantly increased in the Wnt7a group compared to the BSA control group (Figure 2E-H, 2N). Furthermore, emb-MyHC was designated to measure the newly formed myofibers. The emb-MyHC positive myofibers were barely noticeable in the BSA control groups, but significantly increased after Wnt7a stimulation (Figure 2I-L, 20). Taken together, these results suggested that OOM could regenerate in a similar way to the TA muscle after Wnt7a delivery.

Wnt7a induces OOM resident cell proliferation

Next, we aimed to investigate the temporal changes of the

Wnt7a-stimulated muscle regeneration in the OOM. At first, OOM resident cell proliferation was evaluated. The amount of Ki67 positive cells was normalized to the total cell count in the field. The results revealed that the percentage of Ki67-positive nuclei in Wnt7a-treated groups was significantly higher than the BSA control group at the three timepoints (all P < .05, **Figure 3A-M**). As for the centrally-nucleated myofibers, they were barely noticeable in the control group at every timepoint examined (**Figure 3G-I, 3N**). In the Wnt7a-treated group, however, they increased to almost ten-fold higher (**Figure 3J-L, 3N**).

Wnt7a mediates satellite cell expansion in vivo

Satellite cells are the resident muscle stem cell wedged between the sarcolemma and basal lamina [25]. As a specific marker, Pax7 was used to map the satellite cell population in the OOM. The amount of Pax7 positive cells was normalized to the total cell count in the field. At two weeks, the proportion of satellite cells in the Wnt7a-treated group was almost 4-fold

Primary or secondary	Antibody	Supplier	Species	Dilution	Buffer solution	Incubation time	Temperature (°C)
Primary	Laminin (L9393)	Sigma-Aldrich (St. Louis, Missouri, U.S.A.)	Rabbit	1:1000	3% BSA	overnight	4
Primary	Ki67 (Ab16667)	Abcam (Cambridge, England)	Rabbit	1:500	3% BSA	overnight	4
Primary	Pax7 (Pax7)	DSHB (Iowa City, U.S.A.)	Mouse	1:5	3% BSA	overnight	4
Primary	MyHC-1 (A4.480)	DSHB (Iowa City, U.S.A.)	Mouse	1:20	3% BSA	overnight	4
Primary	MyHC-2A (SC-71)	DSHB (Iowa City, U.S.A.)	Mouse	1:20	3% BSA	overnight	4
Primary	MyHC-2X (6H1)	DSHB (Iowa City, U.S.A.)	Mouse	1:5	3% BSA	overnight	4
Primary	MyHC-2B (BF-F3)	DSHB (Iowa City, U.S.A.)	Mouse	1:10	3% BSA	overnight	4
Secondary	TRITC (ZF-0313)	ZSGB-BIO (Beijing, China)	Anti-Mouse	1:100	3% BSA	1 h	23
Secondary	FITC (ZF-0311)	ZSGB-BIO (Beijing, China)	Anti-rabbit	1:100	3% BSA	1 h	23
Secondary	TRITC (ZF-0316)	ZSGB-BIO (Beijing, China)	Anti-rabbit	1:100	3% BSA	1 h	23

Table 1. Antibodies used in this study



Figure 2. Regeneration-related activities in OOM and TA muscle. A-D. Immunofluorescence staining of Ki67 (red) and DAPI (blue) in BSA-treated and Wnt7a-treated muscle groups. White arrows indicate Ki67-positive cells. E-H. Immunofluorescence staining of Pax7 (red), laminin (green), and DAPI (blue) in BSA-treated and Wnt7a-treated muscle groups. White arrows indicate Pax7-positive nuclei. I-L. Immunofluorescence staining of emb-MyHC (red), laminin (green) and DAPI (blue) in BSA-treated and Wnt7a-treated muscle groups. White arrows indicate emb-MyHC (red), laminin (green) and DAPI (blue) in BSA-treated and Wnt7a-treated muscle groups. White arrows indicate emb-MyHC positive myofibers. M-O. Quantification of Ki67 positive nuclei, Pax7-positive nuclei and emb-MyHC positive myofiber. Scale bar = 50 μm.For each group, n = 6. *P < .05; **P < .01; ***P < .001.

higher than that of the BSA control group (P < .001; Figure 4A, 4D, 4G, 4J, 4M). At three weeks, the percentage of Pax7⁻ positive cells was still significantly higher than that in the control group, but to a lesser extent (P < .001; Figure 4B, 4E, 4H, 4K, 4M). When examined at five weeks, the number of satellite cells were similar between the two groups (P = 0.308; Figure 4C, 4F, 4I, 4L, 4M). In accordance with the elevated cell proliferation and the proportion of centrally-nucleated myofibers, the increased satellite cell number in the Wnt7a-treated group suggested vigorous OOM regeneration.

Wnt7a drives fiber type switch in OOM

Typically in skeletal muscle regeneration, fiber type remodelling occurs following satellite cell

activation [26]. Skeletal muscle fibers can be classified into slow fibers and fast fibers according to MyHC expression. Fast fibers can be further divided into MyHC-2B, MyHC-2X, and MyHC-2A fibers, whereas slow fibers are also termed MyHC-1 fibers [27]. From type 1 to type 2B fibers, muscle contraction speed increases and the susceptibility to muscle fatigue decreases [28]. At three weeks, an increase in the proportion of MyHC-1-positive slow fibers was observed in OOM (P < .05, Figure 5A, 5E, 5Q). Meanwhile, the ratio of three types of fast fibers significantly decreased (P < .05; Figure 5B-D, 5F-H, 5Q), indicating a fast-to-slow fiber type switch. Nevertheless, at five weeks, the percentage of MyHC-2A positive myofibers declined slightly (P < .05; Figure 5J, 5N, 5R), while MyHC-1 positive slow fibers,



Figure 3. Wnt7a induces muscle resident cells' proliferation in OOM. A-F. Immunofluorescence staining of Ki67 (red) and DAPI (blue) in BSA-treated and Wnt7a-treated muscle groups. G-L. Immunofluorescence staining of laminin (green) and DAPI (blue) in BSA-treated and Wnt7a-treated muscle groups. White arrows indicate centrally-nucleated myofibers. M. Quantification of Ki67-positive nuclei, normalized by total cell count per field. N. Quantification of centrally-nucleated myofibers, normalized by total fiber number per field. Scale bar = 50 μ m. For each group, n = 6. *P < .05; **P < .01.

MyHC-2X positive and MyHC-2B positive fast fibers were restored to the levels observed in

the BSA control group (Figure 5I, 5K-M, 5O, 5P, 5R).



Wnt7a promotes myofiber hyperplasia

Based on the overall enhanced muscle regeneration and myofiber remodelling activity, we next interrogated whether Wnt7a could exert an effect on the final myofiber number and diameter. During adult skeletal muscle regeneration, the formation of embryonic isoform of myosin heavy chain (emb-MyHC) can be seen as a hallmark of newly formed myofiber, which is a critical process before final myofiber reconstruction. The emb-MyHC positive fibers were barely



Figure 5. Wnt7a induces fiber type switch in OOM. A-P. Immunofluorescence staining of MyHC-1/MyHC-2A/MyHC-2X/MyHC-2B (red), laminin (green) and DAPI (blue) in BSA-treated muscle groups and Wnt7a-treated groups. Q. Quantification of the proportion of MyHC-1, MyHC-2A, MyHC-2X and MyHC-2B positive myofibers in the OOM at 3 wk after injection. R. Quantification of the proportion of MyHC-1, MyHC-2A, MyHC-2A, MyHC-2A, MyHC-2X and MyHC-2B positive myofibers in the OOM at 3 wk after injection. Scale bar = $50 \ \mu$ m. For each group, n = $6 \cdot P < .05$.

noticeable in the control groups, but significantly increased at two weeks and three weeks (P < .05, Figure 6A, 6B, 6D, 6E, 6G). At five weeks, the proportion of newly formed fibers was still higher, but not significantly different, compared to the control group (P = 0.103; Figure 6C, 6F, 6G). As for the fiber number, the myofiber density in Wnt7a-treated groups significantly increased at the three timepoints examined (all P < .05, **Figure 6A-F**, **6H**). Taken together, rh-Wnt7a delivery induced substantial hyperplasia in the OOM.

In contrast, Wnt7a delivery led to a significant reduction of diameter in myofibers of recipient muscle. At two weeks and three weeks, the average Ferret's diameter of myofibers in Wnt7a group was significantly smaller than that of the



Figure 6. Wnt7a promotes myofiber hyperplasia. A-F. Immunofluorescence staining of emb-MyHC (red), laminin (green), and DAPI (blue) in BSA-treated and Wnt7a-treated muscle groups. White arrows indicate emb-MyHC positive myofibers. G. Quantification of emb-MyHC positive myofibers, normalized by total fiber number per field. H. Quantification of total fibermyofibers per field in BSA-treated and Wnt7a-treated muscle groups. I. Quantification of minimal fiber ferret in BSA-treated and Wnt7a-treated muscle groups. Scale bar = 50 μ m. For each group, n = 6. *P < .05; **P < .01; ***P < .001.

control group (P < .001 and P < .05, respectively, **Figure 6A**, **6B**, **6D**, **6E**, **6I**). At five weeks, the average Ferret's diameter was not significantly different from the BSA controls (P = 0.25; **Figure 6C**, **6F**, **6I**). These data suggested a trend toward myofiber hypertrophy with Wnt7a stimulus.

Discussion

The orbicularis oris (OO) muscle, the sphincter around the mouth, derives from the second branchial arch and plays an important role in facial expression, swallowing, and speaking [13]. OO muscle deficiency is frequently seen in congenital orofacial deformity such as cleft lip [2, 3, 5, 29]. However, the regenerative capacity of the OO muscle has seldom been studied. Our study here offered a fresh perspective of a previously unidentified notion: orofacial muscles can regenerate efficiently with the stimulus of the limb muscle growth factor Wnt7a. Analysed at different timepoints post Wnt7a delivery, the OO muscle exhibits robust regeneration, ranging from early signs such as increased cell proliferation, to muscle stem cell expansion and muscle fiber remodelling. The core event of skeletal muscle regeneration lies in the satellite cell activation [30]. As the resident stem cell, satellite cells can reconstitute the myofiber many fold: as few as seven satellite cells associated within one fibermyofiber can give rise to thousands of myogenic progenitors to replenish the injured or lost myonuclei [31]. The significantly increased Ki67positive cells and centrally-nucleated myofiber indicated the initial regenerative activity in response to Wnt7a injection. Further examination into these proliferative cells demonstrated that Pax7-positive satellite cells expand efficiently until 5 weeks post Wnt7a administration, which lays the foundation for the reestablishment of regenerated myofiber.

The specification of different muscle fiber types manifests in the myofiber maturation [25]. Fasttwitch fibers possess a higher contraction velocity and are thus poised for generally powerful actions like jumping and kicking; while slow-twitch fibers are more fatigue-resistant and found in muscles responsible for long-lasting and repetitive activities like respiration [26]. The major fiber type in the OO muscle is MyHC-2B, in contrast to the dominance of MyHC-2A and MyHC-2X in levator veli palatini muscle [22, 32], another orofacial muscle. The different anatomic location and functional requirements of these two muscles might account for the differences in their fiber type composition, further confirming the heterogeneity and complexity of head muscles [11]. Apart from fiber-type specification, myofibers can shift from one type to another under different conditions, which is called fiber-type plasticity [28]. Since muscle fiber types can have a profound effect on muscle diseases, such as Duchenne Muscular Disease (DMD) and muscle aging, manipulating this plasticity to modulate the fiber type emerges as a therapeutic avenue: inducing slow fibers to ameliorate DMD, or enhancing fast fiber growth to counteract aging effects [28]. Wnt7a administration induced a fast-to-slow fiber type switch at three weeks, but this transition somehow relapsed at five weeks. A possible explanation for this unsustainable effect may be the single dose infection. In order to obtain persistent fiber type remodelling effects, multiple injections or composite material could be applied.

Remarkably, our study revealed that Wnt7a could induce new myofiber formation, as evidenced by the significantly elevated level of emb-MyHC expression. Developmental myosins, such as emb-MyHCs, are typically expressed transiently during embryonic and fetal development, but disappear shortly after birth [33]. Nevertheless, emb-MyHCs can reexpress in regenerating muscles and can be used to identify the newly-formed myofiber. In addition to the established effects of Wnt7a to induce satellite cell expansion and muscle fiber hypertrophy [20-22], our work demonstrated that Wnt7a could also promote muscle fiber hyperplasia. The increase in myofiber number, as well as diameter, exert a synergistic effect to boost 00 muscle regeneration.

Use of biotherapy to enhance the OO muscle regeneration could have a profound effect on cleft lip patient treatment. In current clinical practice, the lip deficiency is frequently corrected by flap transfer or fat graft. The two surgical modes, however, have their drawbacks. Aside from donor site morbidity, the disadvantage shared by both techniques is that flap surgeries always involve multiple surgical stages. which might result in long-term consequences in anxiety and depression [34]. In fat grafting surgeries, the efficacy is reduced by fat absorption and fibrosis [35-37]. Instead of relying on ectopic tissue replenishment, the OO muscle regeneration itself could serve as an effective treatment, addressing the problem of muscle deficiency. Moreover, the enhancement of myofiber regeneration could compete for the excessive deposition of fibrotic tissue [38, 39], which further helps restore a more natural and vivid lip contour [40]. If successfully applied in clinical practice, the biotherapy will largely benefit cleft lip patients.

The study has several limitations. First, the effect of Wnt7a on the OO muscle regeneration was tested on an experimental animal, so the interpretation of these results could be limited. Second, only a single dose of Wnt7a was applied, thus the pro-regeneration effect may not be lasting. Further studies to explore the modalities of administration can aid in maximizing Wnt7a effects.

In conclusion, the OO muscle can exhibit vigorous regeneration after administration of the limb muscle growth factor Wnt7a. The ilnvestigation into the regeneration capacity of the orofacial muscles provides a biological foundation for developing bio-therapies to combat orofacial muscle deficiency, not only in congenital orofacial deformity, but also in the facial plastic surgery where lip augmentation is demanded.

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Disclosure of conflict of interest

None.

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