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Progranulin regulates zebrafish muscle growth and regeneration through maintaining the pool of myogenic progenitor cells

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Myogenic progenitor cell (MPC) is responsible for postembryonic muscle growth and regeneration. Progranulin (PGRN) is a pluripotent growth factor that is correlated with neuromuscular disease, which is characterised by denervation, leading to muscle atrophy with an abnormal quantity and functional ability of MPC. However, the role of PGRN in MPC biology has yet to be elucidated. Here, we show that knockdown of zebrafish progranulin A (GrnA) resulted in a reduced number of MPC and impaired muscle growth. The decreased number of Pax7-positive MPCs could be restored by the ectopic expression of GrnA or MET. We further confirmed the requirement of GrnA in MPC activation during muscle regeneration by knockdown and transgenic line with muscle-specific overexpression of GrnA. In conclusion, we demonstrate a critical role for PGRN in the maintenance of MPC and suggest that muscle atrophy under PGRN loss may begin with MPC during postembryonic myogenesis.

Vertebrate myogenesis is tightly regulated by intrinsic signals, growth factors and transcription factors, all of which contribute to a series of morphogenetic events[1]. After the initial embryonic muscle pattern is established, the adult Myogenic progenitor cell (MPC), also known as satellite cell, become responsible for postembryonic muscle growth and regeneration[2]. MPCs are mononucleated cells that are located between the basal lamina and sarcolemma of mature muscle fibres. The pool of MPCs, which can be identified by the expression of the transcription factor Pax7, is primarily maintained in an inactive state in mature muscle; when muscle regeneration or adaptive growth is needed, the MPCs rapidly proliferate and differentiate into fusion-competent myoblasts. These myoblasts are characterised by the expression of muscle regulatory factors (MRFs) such as MyoD, Myf5 and myogenin[3]. MET, the receptor for hepatocyte growth factor (HGF), is expressed on the cell surface of MPCs and has been proposed to play a role in regulating proliferation and activation from a quiescent state of muscle progenitors[4]. HGF-MET signalling promotes cell proliferation and prevents myogenic differentiation in cultured satellite cells[5]. However, the *in vivo* regulatory mechanisms involved in the maintenance of MPC quantity and function are less well understood.

Progranulin (PGRN), also known as epithelin/granulin precursor, acrogranin, or proepithelin, is a pluripotent secreted growth factor that contributes to early embryogenesis, the wound healing response and tumorigenesis[6]. The mutation and dysregulation of PGRN has also been found to correlate with human neuromuscular diseases such as amyotrophic lateral sclerosis and spinal muscular atrophy, in which denervation produces muscle atrophy with an abnormal quantity and functional ability of MPCs[7,8]. Four PGRN genes have been identified in the zebrafish genome; however, only the *grnA* gene exhibits a syntenic conservation of chromosomal localisation and is the true orthologue of human PGRN[9]. According to *in situ* hybridisation analysis, *grnA* is expressed in the myosepta and somite boundary during late- and post-embryonic myogenesis[8], suggesting that this gene may contribute to postembryonic myogenesis. PGRN has been suggested to regulate progenitor cells in caudal fin,

heart muscle, retina, and liver models[10,11]. However, the regulatory role of PGRN in MPC biology during postembryonic myogenesis has yet to be fully elucidated.

In addition to its rapid development, easy visualisation and genetic tractability, the occurrence of postembryonic myogenesis (similar to that of amniotes) in the zebrafish *(Danio rerio)* make this species an ideal model for studying the functional role of PGRN in myogenesis and modelling human disease[12]. Using the zebrafish model, we addressed the regulatory role and genetic requirements of PGRN in postembryonic myogenesis. The knockdown of GrnA expression by antisense morpholinos (MO) resulted in impaired postembryonic muscle growth. Gene expression profile and immunohistochemistry analysis provided further evidence that impaired muscle growth results from a decreased number of MPCs. This reduced MPC number in *grnA* morphants could be restored by the administration of *met* mRNA. Furthermore, we established a transgenic line with muscle-specific expression of the *grnA* gene using the Tol2 transposon system; this transgenic line displayed an increase in MPCs under cardiotoxin-induced muscle injury and an enhancement of postnatal muscle growth through hypertrophy. In conclusion, we demonstrate a critical role for PGRN in the maintenance of MPCs and suggest that muscle atrophy under PGRN loss may begin with MPCs during postembryonic myogenesis. It may provide new insights for the development of future muscular disease therapeutics.

Results

Growth hormone induces *grnA* expression in postembryonic zebrafish muscle. The growth hormone/insulin-like growth factor 1 (GH/IGF1) axis is known for its critical role in muscle growth[13]. In a previous study, we reported that hepatic *pgrn* expression could be induced by GH administration in the teleost[14]. To determine the GH responsiveness of PGRN in zebrafish muscle, we sampled the muscle tissue after intraperitoneal injections of GH into 3-month-old adult zebrafish. The expression of *igf1* and *grnA* mRNA following GH administration was examined by semi-quantitative RT-PCR and quantitative RT-PCR. Compared to the PBS-injection control, muscle *igf1* and *grnA* expression were significantly increased at 12 hours after the GH injection (Supplemental Fig. S1). These results indicate that *grnA* is a GH-responsive gene in postembryonic muscle tissue.

The knockdown of GrnA impairs postembryonic muscle growth. To determine the genetic requirement for *grnA* during embryonic myogenesis, we injected validated antisense morpholinos (MOs)[10] to suppress GrnA expression. MOs (0.25 ng) were injected into wild-type zebrafish or Tg(*mlc2*:EGFP) zebrafish expressing EGFP under the control of the myosin light chain 2 promoter (*mlc2*). We obtained a comparable myogenic differentiation 1 (*myod1*) and myogenic factor 5 (*myf5*) expression pattern via whole-mount *in situ* hybridisation (WISH) in 5 to 9 somite period *grnA* morphants (Fig. 1a-d, n = 30). At the end of somitogenesis, enhanced myogenin (*myog*) expression (Fig. 1f; 75%, n = 24; i.e., 18 embryos with enhanced *myog* expression out of 24 *grnA* morphants) and suppressed *pax7* expression were observed in the trunk myotome of 24-hpf *grnA* morphants (Fig. 1h; 78.6%, n = 28), suggesting that GrnA is not required for the formation of the initial muscle pattern but is required for the terminal differentiation that occurs during late embryonic myogenesis. To further identify the role of GrnA in postembryonic muscle growth, HE staining and a computer-assisted image analysis system were used to examine the cross-sections of myofibres adjacent to the cloaca in 1-, 3- and 6-dpf fishes. MO administration decreased the average myofibre cross-sectional area (CSA) compared to controls in 1-, 3- and 6-dpf larva (n = 3; Fig. 2b, d, f and Table 1; based on the myofibres in the upper-left quarter of the myotome). Hypotrophic myofibres were also observed in *grnA* morphants, which exhibited diminished EGFP expression, compared with the mock-injected control Tg(*mlc2*:EGFP) zebrafish larva, which exhibited intact EGFP expression in the muscle fibres at 6 dpf (Fig. 2h, 80%, n = 10). In contrast to the average CSA, the average myofibre number within the upper-left quarter of the trunk myotome (2.5×10^3 μm^2) was increased in the 3- and 6-dpf *grnA* morphants (n = 3; Table 1). These findings demonstrate that impaired postnatal muscle growth under GrnA deficiency is mainly the result of decreasing myofibril size.

The gene expression profile of trunk muscles in *grnA* morphants. To explore the molecular regulation of GrnA during trunk muscle growth, the gene expression profile of trunk muscle was assessed using a zebrafish oligonucleotide microarray with control and *grnA* MO-injected embryos at 16, 24, 48 and 72 hpf (Fig. 3a). The Ingenuity Pathway Analysis (IPA) program was used to group the differentially expressed genes into transcription factors (Supplemental Table S1) and canonical pathways (Supplemental Table 2), including "EIF2 Signaling," "Regulation of eIF4 and p70^{S6K} Signaling" and "Protein Ubiquitination Pathway", which are related to muscle protein synthesis and breakdown. The known PGRN-regulated pathways "FAK Signaling" and "TNFR1 Signaling," were also included in the IPA analysis. Among the

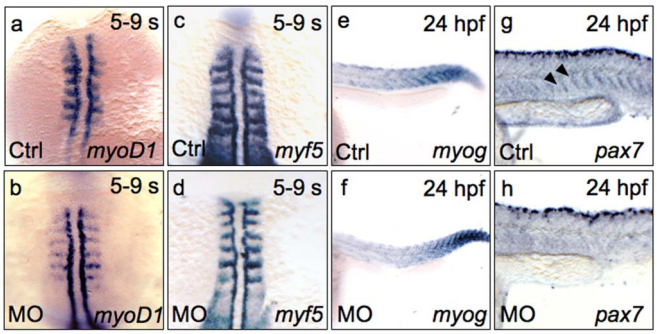

Figure 1 | Early embryonic myogenesis is similar in *grnA* morphants. The expression patterns of *myod1* (a, b), *myf5* (c, d), *myog* (e, f) and *pax7* (g, h) were examined by WISH in the control and the *grnA* morphants. At the 5-9-somite stage, the *myod1* and *myf5* expression patterns were similar in the control and the *grnA* morphants. At 24 hpf, the expression of myogenin was enhanced. However, the expression of *pax7* (arrowhead) was reduced in the *grnA* morphants. (a-d) dorsal views, anterior up; (e-h) lateral views, anterior left.

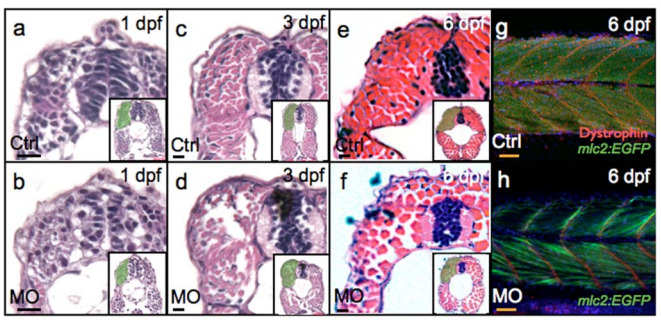

Figure 2 | The morphology of trunk myofibres in the control and the *grnA* morphants fish. Control (a, c, e) and *grnA* morphants (b, d, f) were compared by examining myofibre cross-sections adjacent to the cloaca after haematoxylin and eosin staining at 1 dpf, 3 dpf and 6 dpf. The morphology of the muscle fibres was also examined by examining *mlc2* promoter-driven EGFP expression and anti-dystrophin-stained myosepta at 6 dpf (g, h). DAPI was used for nuclear staining. (j-l) Lateral views, anterior left. Scale bars, 50 μm.

Table 1 | Cellular morphometric properties of trunk myotome in control, *grnA* morphant and Tg(*mlc2*:grnA) lines

	Group	Fiber area (μm²)	Number of myofibers#
1 dpf	Control	7.6 ± 0.3	64.3 ± 3.8
	MO	6.2 ± 0.2 **	92.7 ± 5.0 **
	Tg(*mlc2*:grnA)	7.2 ± 0.3	69.3 ± 5.1
3 dpf	Control	9.3 ± 0.3	104.7 ± 12.7
	MO	6.7 ± 0.1 **	151.3 ± 9.5 **
	Tg(*mlc2*:grnA)	26.4 ± 0.5 **	42.7 ± 3.2 **
6 dpf	Control	24.7 ± 3.2	47.3 ± 6.7
	MO	15.1 ± 4.4 **	70.7 ± 14.8 **
	Tg(*mlc2*:grnA)	40.9 ± 2.1 **	28.3 ± 3.5 **

Values are given as means together with standard deviation. #, number of myofibers within 2.5×10^3 μm². *p*, unpaired *t*-test analysis that compared with wild type group. N = 3. *, $p < 0.05$; **, $p < 0.01$.

differentially expressed genes, the MRFs and muscle-growth-related genes were further validated by quantitative RT-PCR. The expression of *myod1*, *myf5*, *mrf4* and *myog*, all of which are members of the myogenic regulatory factor family, were initially suppressed during the mid-somitogenesis stage (i.e., 16 hpf) but were enhanced during postembryonic myogenesis in the *grnA* morphants. By contrast, the critical genes for the maintenance and activation of MPCs, including *pax7*, *pax3* and *met,* were significantly inhibited in GrnA deficiency (Fig. 3b). The key mediators of skeletal muscle atrophy, ubiquitin-ligase MuRF1 (*murf1*) and Atrogin-1 (*fbxo32*), were up-regulated in GrnA deficiency; however, the knockdown of GrnA down-regulated *mstn* expression slightly (Fig. 3b). In summary, the gene expression profile illustrates the molecular signalling involved in GrnA-regulated postembryonic muscle growth and suggests a role for GrnA in the maintenance of MPCs and the suppression of myogenic differentiation.

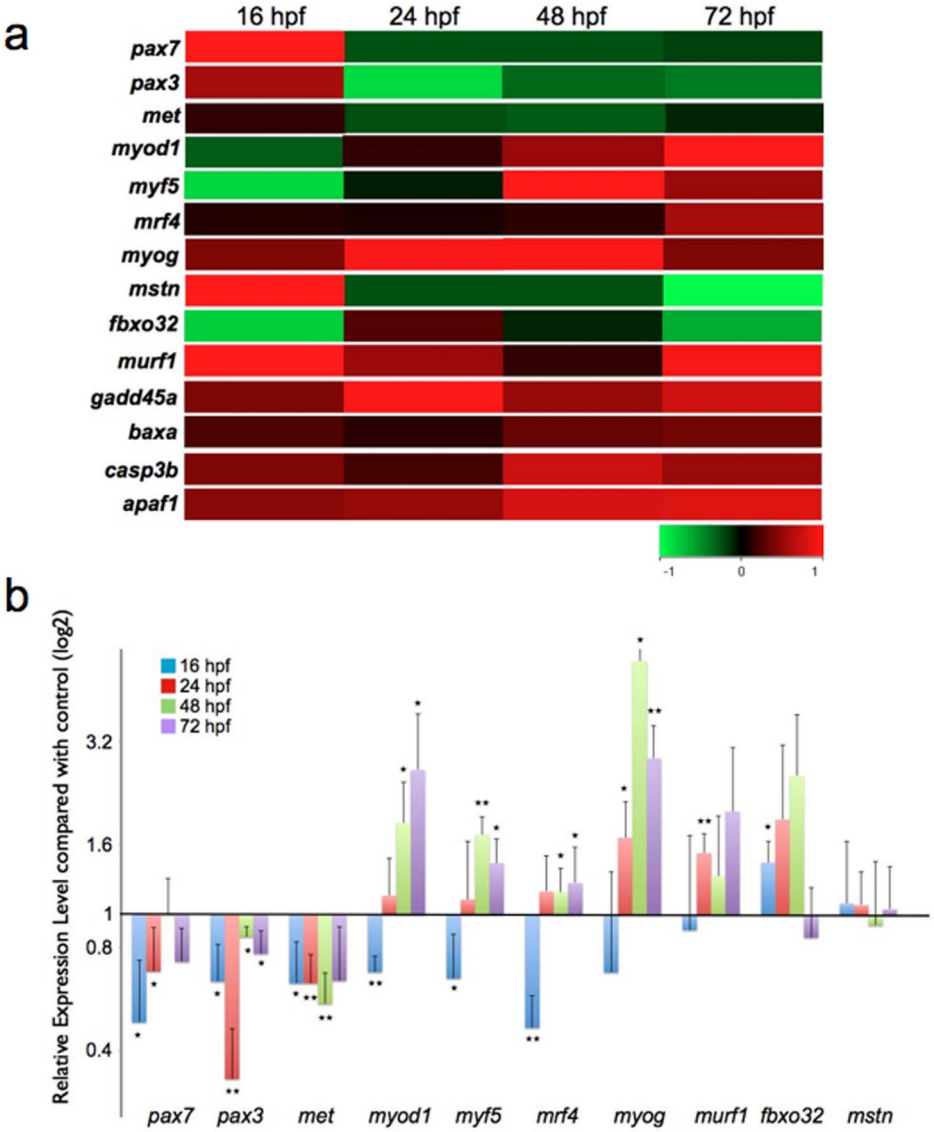

Figure 3 | The microarray gene expression analysis of trunk muscle in GrnA deficiency. Trunk muscle tissues of control and *grnA* morphants were sampled at various time points, and total RNA extracts were analysed using microarray analysis. (a) A heat map reveals the gene expression values at 16, 24, 48 and 72 hpf. Expression levels (log2) above 0 represent up-regulation, whereas those below 0 represent down-regulation. (b) The transcriptional expression levels of *pax7*, *pax3*, *met*, *myod1*, *myf5*, mrf4, *myog*, *murf1*, *fbxo32* and *mstn* were validated by qRT-PCR at several time points in the control and *grnA* morphants. The relative gene expression was normalised to *ef1a* expression and compared with the control treatment. The error bars indicate the standard deviation. *, $P < 0.05$; **, $P < 0.01$, t-test.

The knockdown of GrnA reduces the quantity of Pax7-positive MPCs. To examine the role of GrnA in the maintenance of MPCs, we used a monoclonal antibody that was previously shown to recognise Pax7 in zebrafish[15]. The Pax7 transcription factor, a key marker of muscle progenitors in all vertebrates, can be used as a label to detect the layer of external dermomyotome cells on the surface of the zebrafish somite[15]. Most of the Pax7-positive ($Pax7^+$) cells were rounded and located near the somite surface, corresponding to MPCs, which could be clearly identified in the 24-hpf embryos. In addition, some intensively labelled $Pax7^+$ cells in the dorsal superficial somite were xanthophores (Fig. 4a). The quantity of MPCs was determined by counting the number of $Pax7^+$ cells per somite, excluding the intensively stained xanthophores. Compared to the wild-type (Fig. 4a, 30.7 ± 1.5, n = 3; a′, high magnification of boxed region) and the 5 base-pair-mismatch (*5 mm*) control morphants (Fig. 4c, 28.3 ± 4.7, n = 3), the MO knockdown of GrnA reduced the number of $Pax7^+$ cells within a somite in the 24-hpf *grnA* morphants (Fig. 4b, 10.3 ± 2.5, n = 3). Moreover, the number of $Pax7^+$ cells was affected to a lesser extent by the knockdown of the *grnA* orthologue *grnB* (Fig. 4d, 22.3 ± 3.8, n = 3).

GrnA deficiency suppresses MPC proliferation and enhances apoptosis. To examine the cell fate of the reduced number of MPCs under GrnA deficiency, an antibody against the cell proliferation marker phospho-histone H3 (PH3) was used to co-stain with Pax7 in the *grnA* morphants. Suppressed cell proliferation was observed in the 24-hpf *grnA* morphants (Fig. 4g, h; 2.1 ± 0.4, PH3 and Pax7 double-positive ($Pax7^+/PH3^+$) cells per somite in the controls versus 1.1 ± 0.2 in the *grnA* morphants; n = 3). The reduction in the number of $Pax7^+/PH3^+$ cells could be detected in the 20-hpf *grnA* morphants (Fig. 4e, f; 2.5 ± 0.9 in the controls; 1.1 ± 0.4 in the *grnA* morphants; n = 3). According to the microarray and IPA analysis of the 24-hpf *grnA* morphants, the group of differentially expressed apoptosis signalling genes included several that are known to enhance pro-apoptotic growth arrest and are DNA-damage-inducible, including alpha (*gadd45a*), bcl2-associated X protein, a (*baxa*), caspase 3, apoptosis-related cysteine protease b (*casp3b*), and apoptotic peptidase activating factor 1 (*apaf1*) (Fig. 3a). We further examined the apoptotic events of the MPCs after GrnA knockdown using the TUNEL assay. A massive apoptosis event occurred under GrnA deficiency at 20 hpf (Fig. 4j). Indeed, the number of cells co-stained with Pax7 and TUNEL increased 5.1-fold in the 20-hpf *grnA* morphants (Fig. 4i, j; 3.5 ± 0.8, $Pax7^+/TUNEL^+$ cells per somite in the controls; 17.8 ± 2.4 in the *grnA* morphants; n = 3). The apoptosis level of the MPCs was maintained in the 24-hpf *grnA* morphants (Fig. 4k, l; 3.4 ± 0.9 in the controls; 9.1 ± 0.3 in the *grnA* morphants; n = 3). In summary, the impairment of myogenic progenitors observed upon GrnA knockdown may have been caused by an increased level of apoptosis combined with the suppression of MPC proliferation.

GrnA regulates muscle progenitor cells via MET

In a previous study, we demonstrated that GrnA could regulate hepatic progenitor cell proliferation through the regulation of MET expression[10]. MET has been shown to be involved in the proliferation of myogenic progenitor cells in the limb muscle[16]. Therefore, we conducted mRNA rescue experiments to determine whether GrnA regulates myogenic progenitors through MET signalling. The WIHC results revealed a significant reduction in the quantity of $Pax7^+$ MPCs in the trunk-muscle region of the 24-hpf

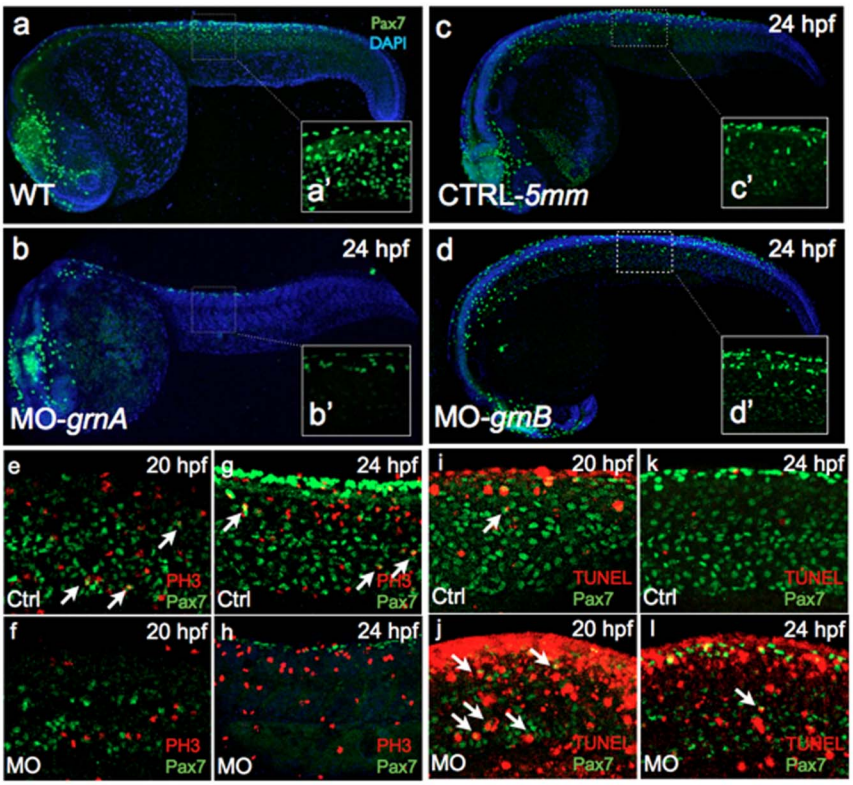

Figure 4 | MPC loss results from suppressed proliferation and enhanced apoptosis in 24-hpf *grnA* morphants. The number of Pax7-positive cells was significantly decreased in 24-hpf *grnA* morphants (b) high magnification of the boxed region compared with the non-injection wild type (a) and 5-mismatch paired control (CTRL-5 mm) morphants (c). The number of Pax7-positive cells was less affected by the knockdown of the *grnA* orthologue *grnB* (d). The mitotic status of the MPCs was determined by co-staining with anti-PH3 and anti-Pax7 (arrow). The control (e, g) and *grnA* MO-injected (f, h) embryos were examined at 20 and 24 hpf. The number of cells co-stained with Pax7 and TUNEL was increased in the 20- and 24-hpf *grnA* morphants (j, l). Lateral views, anterior left.

grnA morphants (Fig. 5c) and *met* morphants (Fig. 5g, 8.7 ± 3.1, $Pax7^+$ cells per somite; n = 3). Furthermore, this reduced number of MPCs could be rescued by co-injecting *grnA* MO with 0.25 ng *grnA* mRNA (Fig. 5e; 20.7 ± 3.2; n = 3) or 0.125 ng *met* mRNA (Fig. 5d; 18.3 ± 3.2; n = 3). In addition, the *met* mRNA rescued the quantity of MPCs in a dose-dependent manner (Fig. 5b, d, f). Comparable information was obtained by WISH, which demonstrated a significant reduction in *pax7* expression in the trunk muscle region of *grnA* morphants (Supplemental Fig. S2c; 64%, n = 28). This reduced *pax7* expression could be rescued by co-injecting *grnA* MO with 0.25 ng *grnA* mRNA (Supplemental Fig. S2e; 74%, n = 27) or *met* mRNA (Supplemental Fig. S2b; 79%, n = 28). Furthermore, the injection of *met* MO led to an extensive reduction in *pax7* expression in the *met* morphants (Supplemental Fig. S2d; 80%, n = 25). However, this reduction could not be recovered by co-injection with *grnA* mRNA (Supplemental Fig. S2f; 89%, n = 19). These results suggest that the quantity of GrnA-regulated MPCs can be controlled by MET during postembryonic myogenesis.

GrnA regulates MPC activation in cardiotoxin-induced muscle injury. MPCs contribute to the regeneration of skeletal muscle under conditions of injury or disease. Rattlesnake cardiotoxin can be used to induce muscle injury in zebrafish because it depolarises cell membranes and disrupts the sarcomeric structure[17]. To study the functional role of GrnA in MPC activation during muscle regeneration, we injected rattlesnake cardiotoxin into the trunk muscle of somites around the yolk extension at 3 dpf. While scarcely any MPCs were activated in the control larvae injected with PBS (Fig. 6a), after cardiotoxin administration, a population of $Pax7^+$ MPCs could be observed at the injury site in the control wild-type 5-dpf larvae (Fig. 6b, 11.7 ± 1.5; n = 3). By contrast, in the GrnA-deficient larvae, the number of $Pax7^+$ MPCs was significantly decreased (Fig. 6c, 6.3 ± 1.5; n = 3; Fig. 6e), indicating an essential role of GrnA in MPC-mediated muscle regeneration.

The muscle-specific overexpression of GrnA enhances the function of MPCs in postembryonic muscle growth and muscle regeneration. To study the effects of a *grnA* gain-of-function during postembryonic myogenesis and to bypass the role of PGRN in early embryonic myogenesis, we used the Tol2 transposon system to establish a transgenic zebrafish Tg(*mlc2*:grnA) using the myosin light chain 2 promoter for muscle-specific expression of the *grnA* gene (Supplementary Methods)[18]. To confirm that the *grnA* gene was overexpressed in the transgenic line, qRT-PCR was used to demonstrate that the expression level of *grnA* was higher in 1-dpf F1 transgenic larvae than in control larvae (a 13.67-fold enhancement in the transgenic line compared with the control; Fig. 7a; n = 3). Similarly, the expression of the GrnA protein in Tg(*mlc2*:grnA) larvae was increased 1.62-fold compared to controls at 1 dpf (Fig. 7b, n = 3), and the mRNA expression levels of *pax7*, *met*, *myog* and *myhc* were also enhanced in the trunk region of 1-dpf Tg(*mlc2*:grnA) larvae (Fig. 7a; n = 3).

To determine the effect of *grnA* overexpression on the role of MPCs in postembryonic muscle growth, cross-sections of the myofibres adjacent to the cloaca muscle fibres of Tg(*mlc2*:grnA) larvae were examined at 1, 3 and 6 dpf. Compared to the control wild-type larvae, Tg(*mlc2*:grnA) exhibited more compact/organised muscle fibres with intensive EGFP expression at 6 dpf (Fig. 7f, 90%, n = 10). HE staining of cross-sections of Tg(*mlc2*:grnA) myofibres revealed a significantly increased CSA compared to the controls at 3 and 6 dpf (Fig. 7c, d and Table 1). By contrast, the average myofibre number within the muscle was also significantly decreased following *grnA* overexpression (Table 1). These findings indicate that GrnA enhances postembryonic muscle growth mainly through hypertrophy. Consequently, we examined the effect of *grnA* overexpression in juvenile fish 2.5 months after fertilisation. HE staining confirmed that the cross-sections of 2.5-month-old Tg(*mlc2*:grnA) zebrafish were approximately 1.35 times bigger than those of control zebrafish (Fig. 7g, h). The average myofibre areas in the Tg(*mlc2*:grnA) and control zebrafish were 345.1 ± 89.3 μm^2 and 255.7 ± 58.6 μm^2, respectively, indicating that long-term GrnA expression promotes muscle growth in the juvenile stage. Finally, to determine the effect of *grnA* overexpression on MPC activation

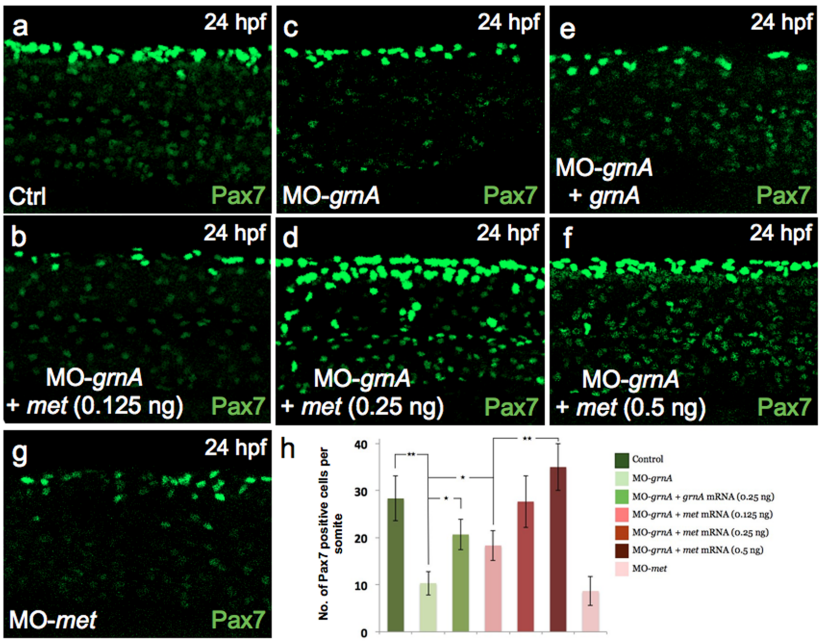

Figure 5 | GrnA regulates the quantity of MPCs via MET. At 24 hpf, the number of Pax7-positive cells per somite was determined for embryos injected with the control MO (a), the *grnA* MO (c), the *grnA* MO with *grnA* mRNA (e) or *met* mRNA (b, d, f) and the *met* MO (g) and assessed using WIHC analysis. (h) The statistical figure represents the number of Pax7-positive cells per somite under various conditions. The error bars indicate the standard deviation. *, $P < 0.05$; **, $P < 0.01$, t-test. Lateral views, anterior left.

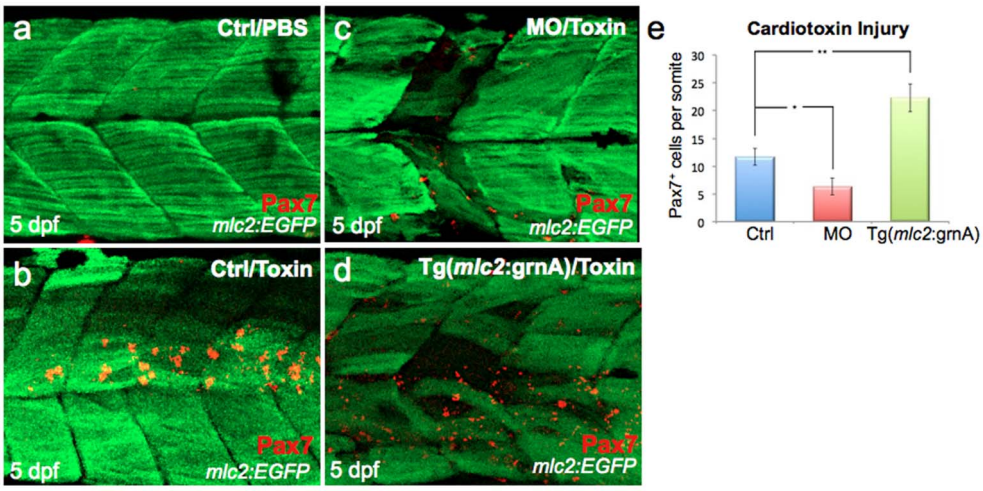

Figure 6 | GrnA promotes MPC activation in cardiotoxin-induced muscle injury. Cardiotoxin or PBS was injected within the somites of 3-dpf zebrafish. (a) The control larvae injected with PBS at 5 dpf. (b) The cardiotoxin-injected control larvae at 5 dpf. (c) The 5-dpf *grnA* morphants injected with cardiotoxin. (d) The 5-dpf Tg(*mlc2*:grnA) zebrafish injected with cardiotoxin. (e) Statistical results were calculated from three somites in three fish. The error bars indicate the standard deviation. *, $P < 0.05$; **, $P < 0.01$, t-test. Lateral views, anterior left.

in muscle regeneration, WIHC analysis was performed. This analysis revealed a 1.9-fold increase in $Pax7^+$ cells around the cardiotoxin-injured somite (Fig. 6d and 6e; 22.3 ± 2.5, $Pax7^+$ cells per somite in Tg(*mlc2*:grnA); n = 3). In conclusion, a gain of *grnA* function not only promotes postembryonic muscle growth but also enhances MPC activation under conditions of muscle injury.

Discussion

The myogenic progenitor cells located in the myotome govern post-embryonic muscle growth. In the present study, we manipulated the expression of the *grnA* gene to examine the functional role of PGRN in postembryonic myogenesis and, in particular, in MPC biology through MO knockdown and muscle-specific overexpression stud-

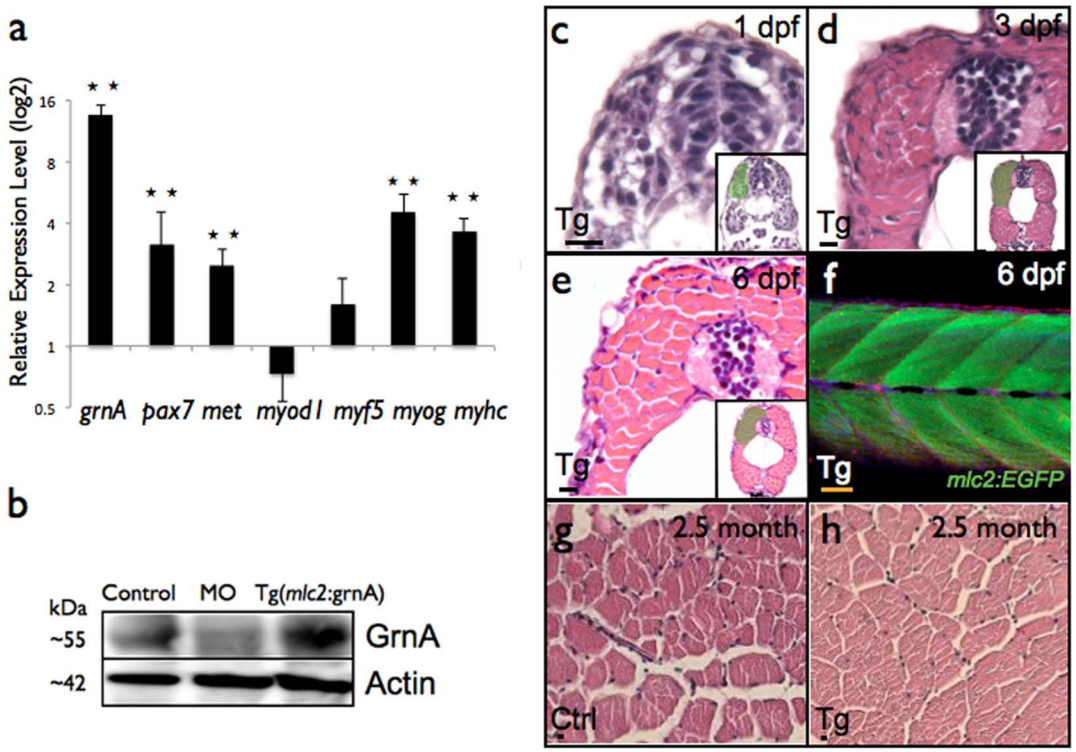

Figure 7 | Overexpression of *grnA* induces muscle hypertrophy in Tg(*mlc2*:GrnA) zebrafish. (a) The expression levels of *grnA*, *myod1*, *myf5*, *myog*, *myhc*, *met* and *pax7* in the control and Tg(*mlc2*:grnA) lines were determined at 1 dpf by qRT-PCR. The relative gene expression levels were normalised by *ef1a* expression and compared to the control larvae. Error bars indicate standard deviation. **, $P < 0.01$, t-test. (b) The protein levels of GrnA and Actin were examined by western blot analysis in the control, *grnA* morphants, and the Tg(*mlc2*:grnA) line at 1 dpf. (c-f) Myofibre cross-sections adjacent to the cloaca of Tg(*mlc2*:grnA) lines were examined after haematoxylin and eosin staining at 1 dpf (c), 3 dpf (d) and 6 dpf (e). (f) The morphology of the muscle fibres was examined by examining *mlc2* promoter-driven EGFP expression and anti-dystrophin-stained myosepta at 6 dpf. Lateral views, anterior left. The cross-sections of the fast muscle around the spinal cord in 2.5-month-old control (g) and Tg(*mlc2*:grnA) zebrafish (h). Scale bars, 50 μm.

ies. PGRN is a pleiotropic growth factor that mediates cell-cycle progression, and its regulation has been shown to have autocrine and paracrine effects, particularly during tissue impairment[19]. The expression of *grnA* has been detected in somite boundaries during late embryonic myogenesis and in the myosepta during the postembryonic stage[8]. In zebrafish embryos, the GrnA was expressed in the myotome and MPCs suggesting that GrnA may contribute to embryonic myogenesis (Supplemental Fig. S4). Postembryonic myogenesis in fish is regulated by myogenic regulatory factors that promote the proliferation and differentiation of myogenic progenitor cells. These cells are responsible for muscle growth with hyperplasia (an increase in the number of myofibres) and hypertrophy (an increase in myofibre size)[20]. The role of GH in regulating muscle growth through IGF1 signalling has been extensively studied[21]. Previously, we demonstrated that GH induces the co-expression of PGRN and IGF1 in the liver[14]. Here, we determined the ability of GrnA to respond to GH in postembryonic muscle tissues (Supplemental Fig. S1). Based on the response of GrnA to GH, we hypothesised that the differential expression of GrnA may result in physiological changes in skeletal muscle growth. Supporting this hypothesis, the knockdown of GrnA resulted in impaired muscle growth in 1-, 3- and 6-dpf *grnA* morphants; conversely, the overexpression of GrnA enhanced muscle growth through hypertrophy (Fig. 7 and Table 1). These data indicate a critical role for GrnA downstream of GH in postembryonic muscle growth. As a result, gene expression profiles revealed that protein turnover-related pathways including "EIF2 Signaling," "Regulation of eIF4 and $p70^{S6K}$ Signaling" and "Protein Ubiquitination Pathway" were differentially expressed under GrnA deficiency (Supplemental Table S2). The TNFα signalling pathway may also be involved in GrnA-mediated postembryonic muscle growth (Supplemental Table S2). TNFα binds to its receptor to activate the NFκB transcription pathway[22]. NFκB activation is then sufficient to promote cytokine-induced muscle atrophy. This process could be induced by the transcriptional up-regulation of MuRF1[23]. In a recent study, Tang et al. reported that PGRN binds TNFα receptors, blocking the interaction between TNFα and TNFR[24]. Our microarray results demonstrated a significant up-regulation of *murf1* and *fbxo32* in response to GrnA knockdown (Fig. 3b). Therefore, it is possible that GrnA may also regulate postembryonic muscle growth by blocking TNFα-induced muscle atrophy. However, the expression level of myostatin, a member of the TGF-β family that is a dominant inhibitory factor in muscle growth, is not disturbed in *grnA* morphants (Fig. 3b).

Muscle growth in fish is regulated by the primary MRFs, which are responsible for the differentiation of MPCs, myoblast fusion and subsequent formation of myotubes. Our histological analyses indicate that GrnA directly regulates the hypertrophic tendencies of myofibres and suggests that GrnA may have an effect on the expression profile of MRFs and MPC-related genes. According to microarray analysis and qRT-PCR validation, the expression of the *pax3*, *pax7* and *met* genes that govern the commitment and activation of MPCs was significantly decreased under GrnA deficiency relative to the wild-type control. This decrease was accompanied by an increased expression of MRFs under GrnA deficiency, indicating that GrnA may maintain MPC stemness and suppress the myogenic differentiation. A recent study demonstrated that PGRN suppresses myogenic differentiation and establishes a negative feedback loop with MyoD in C2C12 myoblasts[25], providing another example of the role of PGRN in the inhibition of myogenic differentiation. Based on these findings, we postulated that GrnA may regulate MPC during postembryonic myogenesis. Our data demonstrated that GrnA could regulate the quantity and mitotic status of $Pax7^+$ cells. A decrease in Pax7- and PH3-positive cells was observed, and a significant increase in apoptotic events, indicated by co-staining of Pax7 and TUNEL signals, was confirmed in 24-hpf *grnA* morphants, supporting our prediction that GrnA is required for the maintenance of MPCs during postembryonic myogenesis.

After the quiescent MPCs receive extrinsic activation signals, such as HGF-MET signalling, they activate and rapidly undergo proliferation before entering the differentiation process for postembryonic myogenesis. The regulatory mechanism that controls MPC activation and proliferation remains elusive. The MET tyrosine kinase receptor has been shown to play a role in promoting the migration, activation and proliferation of MPCs[4]. Because we previously identified GrnA-mediated MET signalling in hepatoblast proliferation, we propose that a shared regulatory mechanism for GrnA may promote MPC proliferation via MET. Our results show that the knockdown of GrnA leads to a suppression of MET expression (Fig. 3), indicating a positive regulatory role for GrnA in *met* expression in the trunk muscle. Furthermore, the regulation of MPCs via GrnA-mediated MET signalling was verified by the co-injection of *met* mRNA with a *grnA* MO, which rescued the observed decrease in $Pax7^+$ cells expression in *grnA* morphants. By contrast, *grnA* mRNA was not able to rescue the *met* morphants (Fig. S2f). However, the details of how GrnA regulates MET expression are still unclear. One known regulator of *met* expression *in vitro* is the Pax3 transcription factor, which acts via the transactivation of its promoter[26]. Pax3 expression could be activated by β-catenin in skeletal myogenesis[27]. We have demonstrated the β-catenin as a downstream gene of PGRN-mediated MET signaling in zebrafish[10]. In the trunk muscle of *grnA* morphants, the expression of *pax3* was significantly decreased from mid-somitogenesis (Fig. 3b). In addition, our microarray date indicated the β-catenin (*ctnnb1*) expression was suppressed in the trunk region of *grnA* morphants from 16 hpf. This result infers the involvement of Pax3 in GrnA-mediated MET signalling during the postembryonic stage, although further examination is needed.

In addition to postembryonic muscle growth, MPCs contribute to the regeneration of skeletal muscle under conditions of injury or disease. Upon activation and proliferation, MPCs differentiate and fuse with *de novo* or existing MPCs, leading to muscle regeneration[28]. In our cardiotoxin-based zebrafish muscle-regeneration model, GrnA was essential for the activation of MPCs in the wound region; by contrast, the *mlc2*-driven overexpression of *grnA* increased the quantity of MPCs. These results support our prediction that GrnA is required for the activation of MPCs in muscle injury. Furthermore, the expression pattern of *mlc2*-driven EGFP that was not co-localized with the Pax7 expression (Supplementary fig. S5); it demonstrates that the GrnA is not overexpressed in the MPCs. Therefore, this result infers that GrnA could regulate MPCs function through not only autocrine but also in paracrine manner.

In conclusion, our results demonstrate that GrnA is essential for postembryonic myogenesis and that GrnA acts, at least partially, through MET signalling to maintain the quantity and functional ability of MPCs. We also present an *in vivo* model for studying both the genetic and functional factors that are involved in postembryonic myogenesis. The regulatory role of PGRN in MPC biology suggests it may be a candidate for therapeutic applications.

Methods

Fish strains. The wild-type (AB) zebrafish (*Danio rerio*) and the transgenic lines Tg(*mlc2*:EGFP) and Tg(*mlc2*:grnA) were maintained under standard conditions. The embryos were collected using natural mating and were cultured at 28.5°C in Ringer's solution. All experiments were approved by the Institutional Animal Care and Use Committee of Academia Sinica, Taiwan.

Morpholino knockdown and mRNA rescue assay. The *grnA* antisense MOs, composed of MO1 (5′- TTGAGCAGGTGGA TTTGTGAACAGC-3′) and MO2 (5′- GGAAAGTAAATGATCAGTCCGTGGA-3′), and met MO (CM2)[29](Gene Tools, USA) were administered by microinjection at the one-cell stage at the designated concentrations[10]. Zebrafish*met*mRNAswere synthesised using the mMESSAGE mMACHINE Kit (Ambion, USA) and co-injected with *grnA* MOs and met MOs (0.5 ng/embryo) at the one-cell stage for the rescue assay.

Whole mount immunohistochemistry (WIHC) and whole mount *in situ* hybridisation (WISH). Zebrafish larvae were fixed with fresh 4% paraformaldehyde, and WIHC and WISH were performed as described (Supplementary Methods).

Terminal transferase dUTP nick end-labelling (TUNEL) assay. For the TUNEL assay, embryos from the control and *grnA* morphants were fixed in 4% PFA overnight and prepared for use with the *In Situ* Cell Death Detection Kit (Roche, Germany).

Microarrays analysis. Total trunk-muscle RNA was collected and analysed as described in the Supplementary Methods. Microarray expression data were loaded into the Gene Expression Omnibus database (GEO, National Center for Biotechnology Information) under accession number GSE38441.

Quantitative RT-PCR. First-strand cDNAs were synthesised using the Superscript III first-strand synthesis system (Invitrogen, USA), and primers were designed using Primer Express 2.0 software (Applied Biosystems, USA). The qRT-PCR analysis to determine the expression levels of the muscle-growth-related genes was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, USA), as described previously[10]. The levels of *ef1a* were used to normalise the relative mRNA abundance.

Cardiotoxin-induced muscle injury. An injection of 1.5 ng cardiotoxin from the rattlesnake *Naja naja atra* (Sigma, USA) was administered to the trunk region of 3-dpf fish using a fine glass capillary needle as described previously[17].

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Author contributions

Y.-H. Li planned and performed experiments and wrote the manuscript; H.-Y. Chen performed the RT–PCR experiments and did all the animal work. Y.-W. Li performed the microinjection. S.-Y. Wu performed the regeneration assay. G.-H. Lin performed microarray hybridization. S.-Y. Hu and H.-Y. Gong established the Tg(*mlc2*:grnA) lines. C.-H. Liao and K.-Y. Chiang helped with the experiments with Tg(*mlc2*:grnA) fish. Z.-K. Chang performed the tissue section. W. Liu and C.-W. Huang contributed to interpretation of the experiments. J.-L. Wu contributed to interpretation of the experiments and completed the manuscript.

Additional information

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