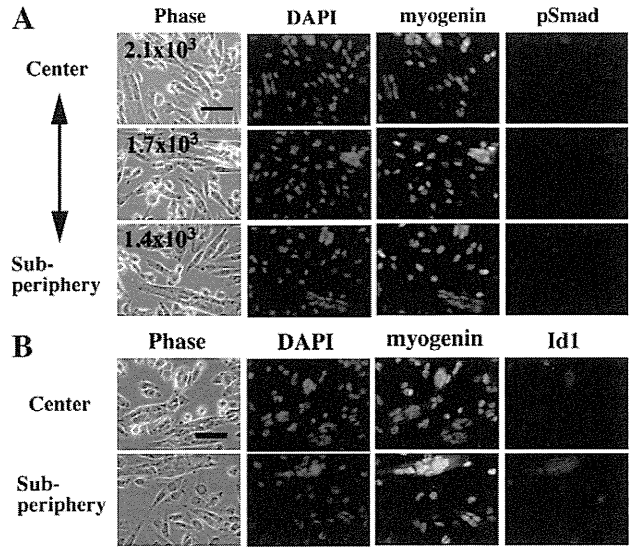


Fig. 5 – Synergistic induction of robust myogenesis by high cell density and serum reduction. (A and B) Ric10 cells (5×10^4 cells per 100 μl of spot) were cultured in micromasses in pmGM for 24 h and then further cultured in pmDM for up to 24 h. The cells were subjected to immunostaining with anti-myogenin, anti-pSmad, and anti-Id1 antibodies. Nuclei were stained with DAPI. The numbers in the phase contrast images in (A) represent cell density of the indicated field (cells per mm^2). Images in each row show the same field. Scale bars: 50 μm.

regulated even in the peripheral region of the micromass in pmDM (Fig. 5B). It should be noted that the cell density in the peripheral region of the micromass ($4–6\times10^2$ cells per mm^2) was still much higher than that of the low cell density cultures shown in Figs. 2 and 3 (less than 100 cells per mm^2). The results indicate that high cell density and serum reduction synergistically induced robust myogenic differentiation, possibly through quenching the Smad signaling pathway.

Dorsomorphin enhances myogenic differentiation in a cell density-dependent fashion under growth condition

To determine whether the Smad signaling pathway is involved in the regulation of myogenic differentiation induced in the high cell density culture, Ric10 cells were cultured in micromass under the growth condition and treated with dorsomorphin for 24 h after seeding. Myotube formation was markedly enhanced by dorsomorphin in a dose-dependent fashion (Fig. 6A). Expressions of MyHC and myogenin were also enhanced by dorsomorphin in a similar manner (Fig. 6B). Myotubes were robustly formed in both dorsomorphin-treated and untreated cultures when the cells were cultured for the prolonged period (48–60 h). The result indicates that dorsomorphin induced myogenic differentiation precociously.

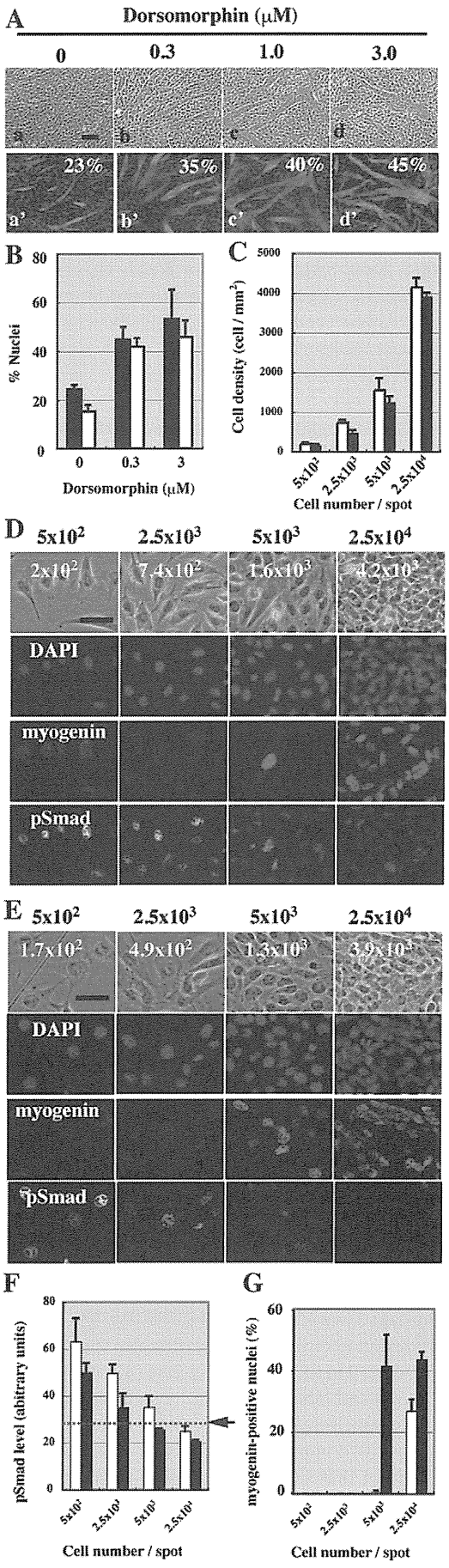
In the next series of experiments, various numbers of Ric10 cells were cultured in micromass to understand whether cell density affects the enhancement of myogenesis by dorsomorphin. The highest concentration of cells in the central region of the cell mass increased along with the number of plated cells (Fig. 6C). Phosphorylation of Smad1/5/8 was suppressed in high cell density cultures (Fig. 6D). Dorsomorphin also inhibited phosphorylation of Smad1/5/8 and markedly enhanced myogenic differentiation in high cell density culture whereas its effects were obscure in relatively lower cell density culture (Fig. 6E). Dorsomorphin continuously but limitedly decreased the level of phosphorylated Smad1/5/8 per nucleus (Fig. 6F). Dorsomorphin and high cell density synergistically down-regulated phosphorylation of Smad1/5/8. When the concentration of phosphorylated Smad1/5/8 fell below the putative threshold that is represented by an arrow in Fig. 6F, expression of myogenin was induced (Fig. 6G). Dorsomorphin-treatment also resulted in down-regulation of Id1 and up-regulation of MyoD (Supplementary Fig. S3). These results imply that down-regulation of the Smad signaling pathway induces myogenic differentiation even in pmGM under the high cell density culture condition. Similar results were obtained from another mouse myogenic cell line, COM3, that was re-cloned from C2C12 cells (Supplementary Figs. S4–6) [23].

BMP antagonist noggin triggers myogenic terminal differentiation in a cell density-dependent fashion under growth condition

To determine whether precocious cell density-dependent, serum concentration-independent myogenic differentiation is suppressed by ligand BMP, Ric10 cells cultured in micromass were exposed to a BMP antagonist, noggin. The expression of MyHC and myogenin were induced in the center of cell mass exclusively (Fig. 7A). Recombinant noggin (5 mg/ml) enhanced myotube formation and the expression of MyHC and myogenin in the region with the relatively lower cell density (Fig. 7B). However, noggin failed to induce MyHC expression in Ric10 cells in the peripheral region where cell density was lower than 1000 cells per mm^2 (Fig. 7C). Consistent with the results in myogenesis assay, noggin decreased the level of phosphorylated Smad1/5/8 (Fig. 7D). The results suggest that ligand-dependent activation of the Smad signaling pathway contributes to the suppression of precocious differentiation of myogenic cells.

Smad signaling pathway is transiently activated in myogenic progenitor cells during muscle regeneration in vivo

To examine the involvement of the Smad signaling pathway in postnatal muscle regeneration, the phosphorylation of Smad1/5/8 was determined in regenerating muscle. Muscle regeneration was induced by injection of BPV into the gastrocnemius muscle of rats. During the early phase of muscle regeneration, myogenic progenitor cells derived from muscle satellite cells express MyoD but not myogenin in BPV-injected rat muscle. Then, the cells are induced to express both MyoD and myogenin, and they give rise to myofibers [38] (Umeda and Hashimoto, unpublished observation). We detected phosphorylated Smad1/5/8 in more than 50% of nuclei of MyoD-expressing myogenic cells in regenerating muscle on days 3 and 4 after BPV injection (Fig. 8Aa–d). A fraction of the myogenin-positive myogenic cells also showed phosphorylated Smad1/5/8 in their nuclei (Fig. 8Ae–h), but the percentage of phosphorylated Smad1/5/8-positive cells in the myogenin-positive mononucleated cells was less than that in the number of MyoD-positive mononucleated cells (39% vs. 65%). The results indicate that the Smad signaling pathway is first activated and then inactivated in myogenic progenitor cells during postnatal muscle regeneration. Therefore, the Smad signaling pathway may be involved in the regulation of growth and



differentiation of postnatal myogenic cells in vivo as well as in vitro.

Discussion

Smad signaling pathway determines cell fates of myogenic cells

In the present study, we provide evidence for a novel role of the BMP–ALK–Smad axis in the switch between growth and differentiation of myogenic cells during postnatal muscle growth and repair. Autonomous cell expression of BMP receptors and downstream Smad proteins in postnatal myogenic cells implies a physiological function of the BMP–ALK–Smad axis during postnatal muscle differentiation. Actually, growing undifferentiated myogenic cells show significant activation of the Smad signaling pathway without administration of any exogenous BMPs. Four independent methods of blocking the BMP–ALK–Smad axis demonstrated that this physiological Smad signaling suppresses precocious differentiation of myogenic cells and keeps their undifferentiated state (Fig. 8B): inhibition of BMP function by noggin, inhibition of ALK2 activation by dominant negative ALK2, inhibition of the interaction between Smad1/5/8 and Smad 4 or ALK2 by an inhibitory Smad, and inhibition of phosphorylation of

Fig. 6 – Induction of precocious myogenic differentiation by dorsomorphin. (A and B) Ric10 cells (5×10^4 cells per 100-μl spot) were cultured in micromass in pmGM for 24 h and then further cultured in pmGM with (Ab–d) or without (Aa) dorsomorphin for up to 24 h. Each medium contained 0.03% DMSO. The cells were subjected to immunostaining with anti-MyHC alone (A) or anti-myogenin and anti-MyHC antibodies (B). Nuclei were stained with DAPI. Images of central regions of micromasses with the highest cell density were obtained by phase contrast (Aa–d) and epifluorescent microscopy (Aa′–d′). The percentages of nuclei in MyHC-positive cells in the total number of nuclei are shown in the lower panels (Aa′–d′). The percentages of nuclei in myogenin- and MyHC-positive cells in the total number of nuclei were calculated, and averages and standard deviations of three independent cultures are shown as solid and open bars, respectively in (B). (C–G) The indicated numbers of Ric10 cells per 50-μl spot were cultured in micromass in pmGM for 24 h and then further cultured in pmGM with 3 μM dorsomorphin (E and solid bars in C, F and G) or without (D and open bars in C, F and G) for up to 24 h. The cells were subjected to immunostaining with anti-myogenin and anti-pSmad antibodies. Nuclei were stained with DAPI. Images of the central regions of micromasses with the highest cell density were obtained by phase contrast (top panels) and epifluorescent microscopy (lower panels) in (D and E). Cell density (cells per mm^2) at the end of culture was calculated and is shown as averages and standard deviations of three independent cultures (C), or represented as numbers in the top panels (D and E). The relative concentration of pSmad in nuclei (F) and the percentages of myogenin-positive nuclei in the total number of nuclei (G) were calculated, and averages and standard deviations of three independent cultures are shown. Scale bars: 10 μm.

Smad1/5/8 by a protein kinase inhibitor dorsomorphin. The correlation between the levels of phosphorylated nuclear Smad1/5/8 and myogenin indicates a threshold of the Smad signal intensity that is sufficient to maintain myogenic cells in an undifferentiated state (Fig. 6F). When the level of Smad signaling is below the threshold, myogenic progenitor cells begin to undergo terminal myogenic differentiation (Fig. 8B).

Our previous study suggests that the exposure to high concentrations of BMP2 causes extraordinary activation of the Smad signaling pathway resulting in induction of osteogenesis in myogenic cells [9]. Hyper-activated Smad1/5/8 irreversibly prevents myogenesis, whereas spontaneously activated Smad1/5/8 suppresses precocious myogenic differentiation reversibly without exposure to exogenous BMPs. Then, hyperactivation of the Smad signaling pathway results in expression of Smad target genes that are not induced during myogenesis but are required for osteogenesis (Fig. 8B).

Recently, we found that high concentrations of BMPs induce ectopic osteogenesis of Ric10 in a cell density-dependent fashion (Supplementary Fig. S7). The results suggest a continuum in the effect of BMPs between the inhibition of myogenic differentiation and transdifferentiation into an osteogenic cell fate. In addition, low concentrations of BMP2 induced osteogenesis in Ric10 cells at low cell density (Supplementary Figs. S7B and C). Taken together with the Supplementary results, the magnitude of Smad signaling might play a critical role in generation of different fates from myogenic progenitor cells (Fig. 8B). In addition, we have found that the exogenous BMP-induced osteogenesis is facilitated by a co-signal (Yanagisawa and Hashimoto, unpublished). Therefore, cellular context and co-signals may determine whether a given BMP stimulus induces which cell fates. From this point of view, it is very interesting that the migrating Ric10 cells at the margin of a cell mass were refractory when exposed to high concentrations of BMP2 (Supplementary Figs. S7D and E).

Quenching of Smad signaling is rate-limiting for myogenic differentiation

The present study indicates that quenching of the Smad signaling pathway triggers myogenic differentiation under the high cell density culture condition. Serum reduction also lowered the phosphorylation level of the Smad signaling pathway. However, high cell density was more potent for inactivating the Smad signaling pathway than low serum concentration in the medium. The present study shows that the Smad signaling pathway is also rate-limiting for myogenic differentiation induced by serum reduction. However, the enhancement of myogenic differentiation by dorsomorphin was quite limited under the serum-reduced, low cell density culture condition. Thus, it is likely that distinct

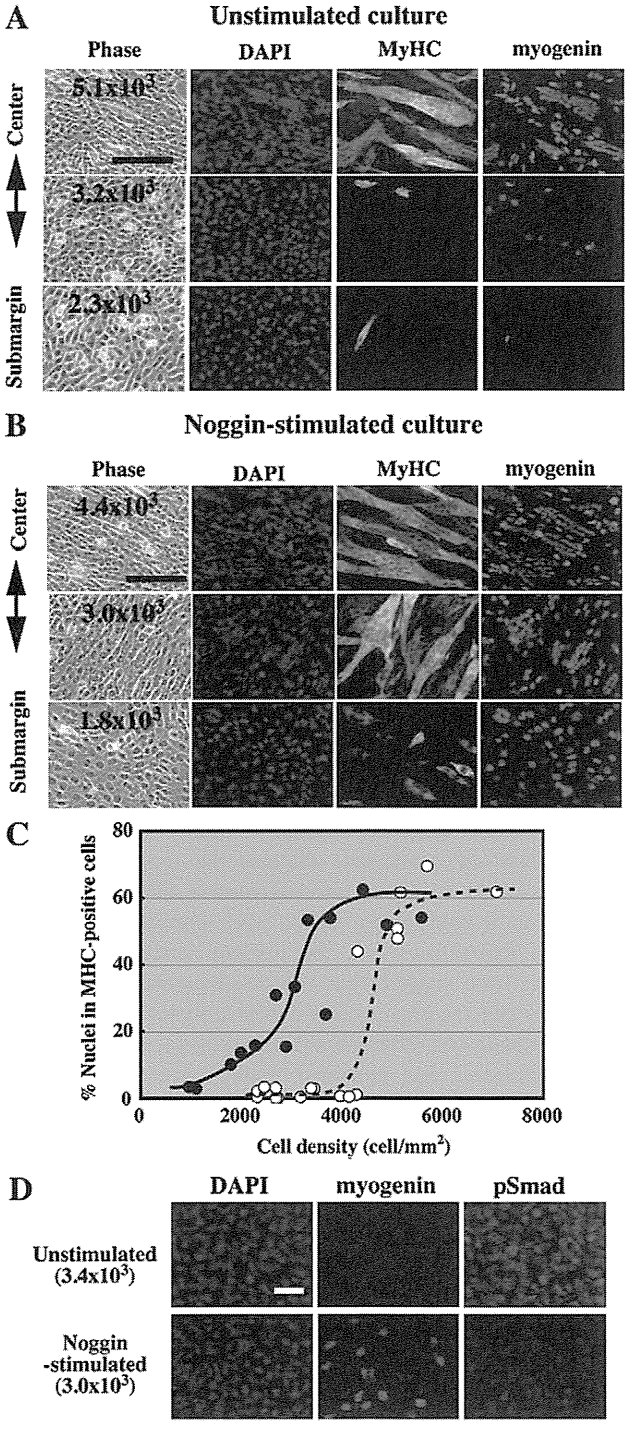


Fig. 7 – Induction of precocious myogenic differentiation by BMP antagonist noggin. (A, B and C) Ric10 cells (5×10^4 cells per 100-μl spot) were cultured in micromasses in pmGM for 24 h and then further cultured in pmGM with (B) or without (A) noggin (5 mg/ml) for up to 24 h. The cells were subjected to immunostaining with anti-myogenin and anti-MyHC antibodies. Nuclei were stained with DAPI. Images of various regions were obtained by phase contrast and epifluorescent microscopy. Numbers in the left-hand panels in A and B represent cell density (cell per mm^2). (C) Cell density and the percentages of nuclei in MyHC-positive cells in the total number of nuclei were calculated in cultures stimulated with (solid circles) or without (open circles) noggin. (D) Ric10 cells (5×10^4 cells per 100-μl spot) were cultured in micromasses in pmGM for 24 h and then further cultured in pmGM with (lower panels) or without (upper panels) noggin (5 mg/ml) for up to 24 h. The cells were subjected to immunostaining with anti-myogenin and anti-phosphorylated Smad1/5/8 antibodies. Nuclei were stained with DAPI. Images were obtained by epifluorescent microscopy. The numbers in parentheses at the left of panels represent cell density (cell per mm^2) at the end of culture. Scale bars: 50 μm.

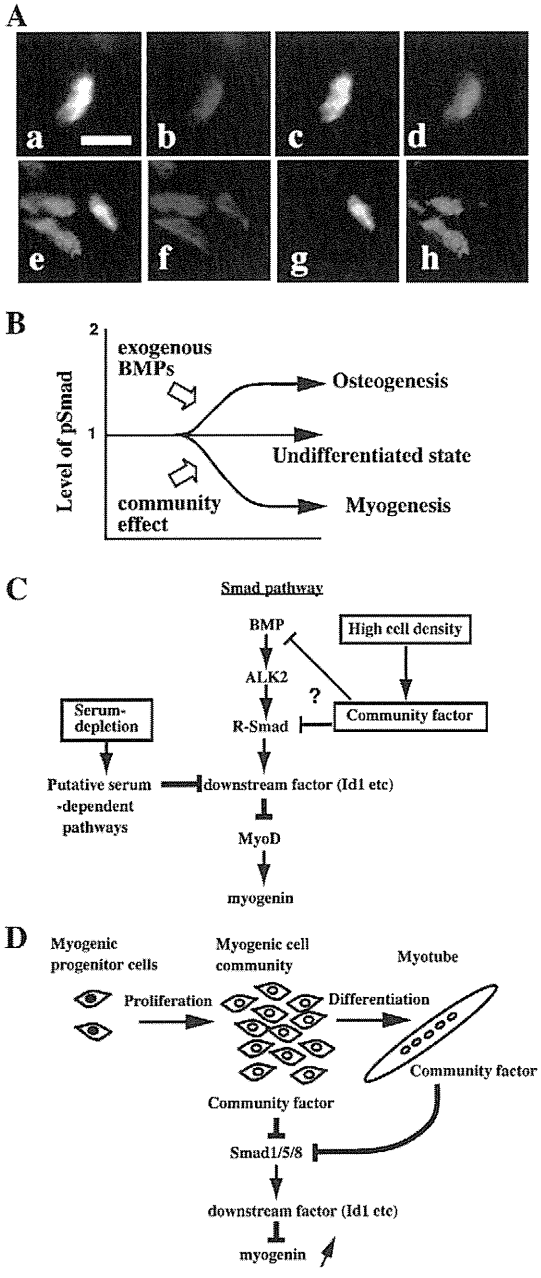


Fig. 8 – Role of Smad signaling pathway in switch between growth and differentiation of postnatal myogenic cells. (A) BPV was injected into the gastrocnemius muscle of rats. Cryosections were prepared from the muscles removed on day 3 (a–d) or 4 (e–h) after BPV injection, and stained with anti-MyoD (c), anti-myogenin (g), anti-phosphorylated Smad1/5/8 (pSmad) (d and h) and DAPI (b and f), respectively. (b, c, and d and f, g, and h) were merged in (a) and (e), respectively. Scale bar: 10 μm. (B) Crucial role of phosphorylated Smad1/5/8 (pSmad) in generation of different fates from myogenic progenitor cells. (C) Hypothetical role of down-regulation of Smad signaling pathway during myogenic differentiation induced in vitro. High cell density and serum reduction synergistically induce myogenic differentiation although they down-regulate downstream factors including Id1 through distinct pathways. (D) Community effect on myogenesis during postnatal muscle growth and repair. See detailed explanation of B, C and D in the "Discussion".

signaling pathways also play a role in the rate-determining step for myogenic differentiation under the serum-reduced, low cell density culture condition. The basal levels of the Smad signaling may induce expression of multiple target genes in undifferentiated, growing myogenic cells. *Id1* is a well-known downstream target gene of the Smad signaling pathway (Fig. 8C). *Id1* encodes an inhibitor protein of the MyoD family and is down-regulated during myogenic differentiation induced by serum reduction [37,39,40]. Previous studies have shown several factors that down-regulate Id1 protein independently of the effects of the BMP–ALK–Smad axis on serum reduction: interferon-inducible protein p204 and micro RNA miR-206 repress Id1 protein and promote myogenic differentiation [39,40]. In addition, lowering the level of p204 inhibits myogenic differentiation in serum-reduced culture [40]. In contrast, quenching of the Smad signaling pathway alone seems critical and rate-limiting for myogenic differentiation and induces myogenic differentiation under the high cell density culture condition even in the high serum-containing culture. Therefore, the two myogenic differentiation-inducing conditions, high cell density and serum reduction, may induce myogenic differentiation in different ways (Fig. 8C): suppression of the Smad signaling pathway alone is rate-limiting for myogenesis or both the Smad signaling pathway and the other signaling pathways play a role in the rate-determining step for myogenesis. It is conceivable that the Smad signaling pathway plays a role in the rate-limiting step for postnatal myogenesis in vivo. From this point of view, it is noteworthy that the Smad signaling pathway is actually down-regulated during BPV-induced muscle regeneration.

Community effect triggers terminal differentiation of postnatal myogenic cells

The present study suggests that muscle satellite cell-dependent myogenesis in postnatal mice depends on a "community effect," which means the expression of a differentiation potential when a certain cell density is exceeded [41], and also provides evidence that quenching the Smad signaling pathway in postnatal myogenic cells is required for the community effect. Skeletal muscle formation in amphibian embryos provides a paradigm of the community effect [42]. Dissociation of muscle progenitor cells reduces their differentiation, whereas the reaggregated cells differentiate [43]. In mouse embryos, muscle differentiation also depends on a community effect [44]. Previous studies on embryonic myogenesis and the present study on postnatal myogenesis both suggest that the developmental timing of a community effect is important as a critical switching mechanism between growth and differentiation of myogenic progenitor cells during embryonic and postnatal muscle growth, repair, and differentiation in mice (Fig. 8D).

Both signals from adjacent tissues and a community effect are necessary for the formation of skeletal muscle in embryos [45]. Fibroblast growth factors (FGFs) are candidates for community factors in *Xenopus* embryos [41]. During postnatal muscle growth and repair in mice and humans, muscle satellite cells and their descendant progenitor cells express and release a number of trophic factors that are candidates for community factors, including growth factors and cytokines such as BMPs, myostatin, FGFs, hepatocyte growth factor, insulin-like growth factors, interleukin-6, leukemia inhibitory factor, and tumor necrotic

factor α [21,46–52]. In embryos, BMP4 is released from the adjacent neural tube and lateral plate mesoderm, and inhibits MyoD and Myf5 gene expression [53,54]. However, the role of BMP signaling in a community effect during skeletal muscle formation in embryos remains to be determined. In contrast, the present study shows that a community effect quenches the Smad signaling pathway in postnatal myogenic cells. Thus, BMP antagonists are possible candidates for community factors. The list of BMP antagonists includes noggin, chordin, gremlin, follistatin, Cerberus, sclerostin, and their related and family proteins [55]. Careful description of the spatiotemporal expression patterns of these antagonists during myogenesis induced by high cell density may provide a hypothetical and mechanistic view of the community effect. However, the activity of the BMP–ALK–Smad axis could be also modulated by Notch signaling [56] or Src tyrosine kinase [57]. Therefore, we should observe whether a simple community factor story can explain a community effect on the terminal differentiation of postnatal myogenic cells.

The origin of ligands that stimulate the Smad signaling pathway in growing myogenic progenitor cells remains puzzling. We cannot exclude the possibility that the FBS in the medium contains an adequate amount of BMP to support the activation of Smad1/5/8, but putative BMP derived from FBS is unlikely to contribute to the activation of Smad1/5/8 in growing myogenic cells because serum reduction does not significantly affect the level of phosphorylated Smad1/5/8 except in higher density culture. In addition, the Smad signaling pathway is inactivated exclusively in the central region of a micromass, even in pmGM supplemented with 20% FBS. Therefore, BMP4 produced by myogenic progenitor cells themselves [21] is a possible candidate for ligands that stimulate their own Smad signaling pathway.

Community effect guarantees myogenic cell fusion following expression of muscle-specific genes

Skeletal muscle terminal differentiation of muscle satellite cells is composed of a highly ordered series of steps that includes activation of quiescent satellite cells, proliferation of descendent progenitor cells, expression of muscle-specific genes, and cell fusion to give rise to syncytia. Cell fusion is the last step of terminal muscle differentiation and is a multi-cellular event, whereas the other steps are uni-cellular responses. To differentiate into myotubes, a differentiating myogenic cell requires direct contact with its fusion partner cell. If a single myogenic progenitor cell is cultured without contact with other cells under the serum-reduced condition, it will undergo the myogenic differentiation process up to the expression of muscle-specific genes but be unable to form myotubes. Therefore, neighboring myogenic cells, including progenitor cells and myofibers, are required for terminal differentiation of myogenic progenitor cells. The community effect induces myogenic differentiation when a certain cell density is exceeded and guarantees myogenic cell contact relevant for syncytium formation. The community effect also provides a probable explanation of how non-synchronized and local myogenic differentiation is induced in culture when myogenic cells are distributed unevenly throughout a culture dish. In addition, high-level expression of noggin in myotubes (Hashimoto, unpublished observation) raises the possibility of differentiated cell-induced differentiation: terminally differentiated cells enhance the community effect and induce differentiation of neighboring undifferentiated myogenic cells (Fig. 8D).

Concluding remarks

We have shown a novel physiological role of the Smad signaling pathway in a switch between growth and differentiation of postnatal myogenic cells. Further studies identifying factors that quench the Smad signaling pathway will provide mechanistic insight into a community effect on postnatal myogenesis.

Supplementary materials related to this article can be found online at doi:10.1016/j.yexcr.2010.10.011.

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Phospholipid synthetic defect and mitophagy in muscle disease

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Mitophagy, selective autophagy of mitochondria, has been extensively demonstrated in cultured cell models but has never been described in skeletal muscle in the context of muscle disease. We recently reported the first example of human muscle disease where mitophagy plays a role in the peculiar muscle pathology. This disease is caused by loss-of-function mutations in the *CHKB* gene encoding choline kinase β. "Patients" and rostrocaudal muscular dystrophy (*rmd*) mice, spontaneous *Chkb* mutants, develop congenital muscular dystrophy with a peculiar mitochondrial abnormality—mitochondria are markedly enlarged at the periphery of muscle fibers and absent from the center. Choline kinase is the first enzymatic step in a biosynthetic pathway for phosphatidylcholine, the most abundant phospholipid in eukaryotes. Our discovery demonstrates that a phosphatydilcholine biosynthetic defect leads to mitochondrial dysfunction and increased mitophagy.

Keywords: choline kinase beta, phosphatidylcholine, mitochondria, mitophagy, congenital muscular dystrophy

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Congenital muscular dystrophy with mitochondrial structural abnormalities (CMDmt) (OMIM:602541) is an autosomal recessive disorder. "Patients" have various biallelic mutations—nonsense, missense or splice-site—in the *CHKB* gene. This is the only human disease caused by a phospholipid de novo synthetic enzyme defect. *rmd* mice have a 1.6-kb deletion in the *Chkb* gene, an ortholog of *CHKB*. Choline kinase activity in muscle is undetectable and the phosphatidylcholine level is decreased in muscles from both "patients" and *rmd* mice and also in isolated mitochondria from *rmd* mice (**Fig. 1**).

Interestingly, both CMDmt "patients" and *rmd* mice show distinct mitochondrial morphological abnormalities in muscle fibers: Mitochondria are nearly absent in the center, while they are enlarged at the periphery. This morphological change seems specific to this disease; we tested 15 "patients" with this distinct muscle pathology and identified mutations in *CHKB* gene in all of them. We hypothesize that altered phospholipid composition in muscle mitochondrial membrane may lead to mitochondrial structural and functional abnormalities.

In *rmd* mice, muscles are involved with the rostrocaudal gradient of severity; hindlimbs are more severely affected while forelimbs are relatively spared, and mitochondria are more sparse in hindlimbs. Isolated mitochondria from skeletal muscle of *rmd* mice show impaired respiratory chain enzyme activities, decreased ATP synthesis, and increased superoxide production. Not surprisingly, these mitochondrial bioenergetic dysfunctions are more severe in hindlimbs than in forelimbs, presumably reflecting the different degree of severity in muscle damage.

Mitochondrial bioenergetic abnormalities are also seen in mitochondrial myopathies; genetic diseases of the mitochondrial respiratory chain caused by a variety of defects in mtDNA or nuclear DNA. However, the muscle pathology of mitochondrial myopathies is different from that of CMDmt. In mitochondrial myopathies, mitochondria are commonly increased both in number and size in muscle fibers, showing a ragged red appearance upon modified Gomori trichrome staining. In contrast, in CMDmt, mitochondria are sparse in the center of muscle fibers, while they are enlarged at

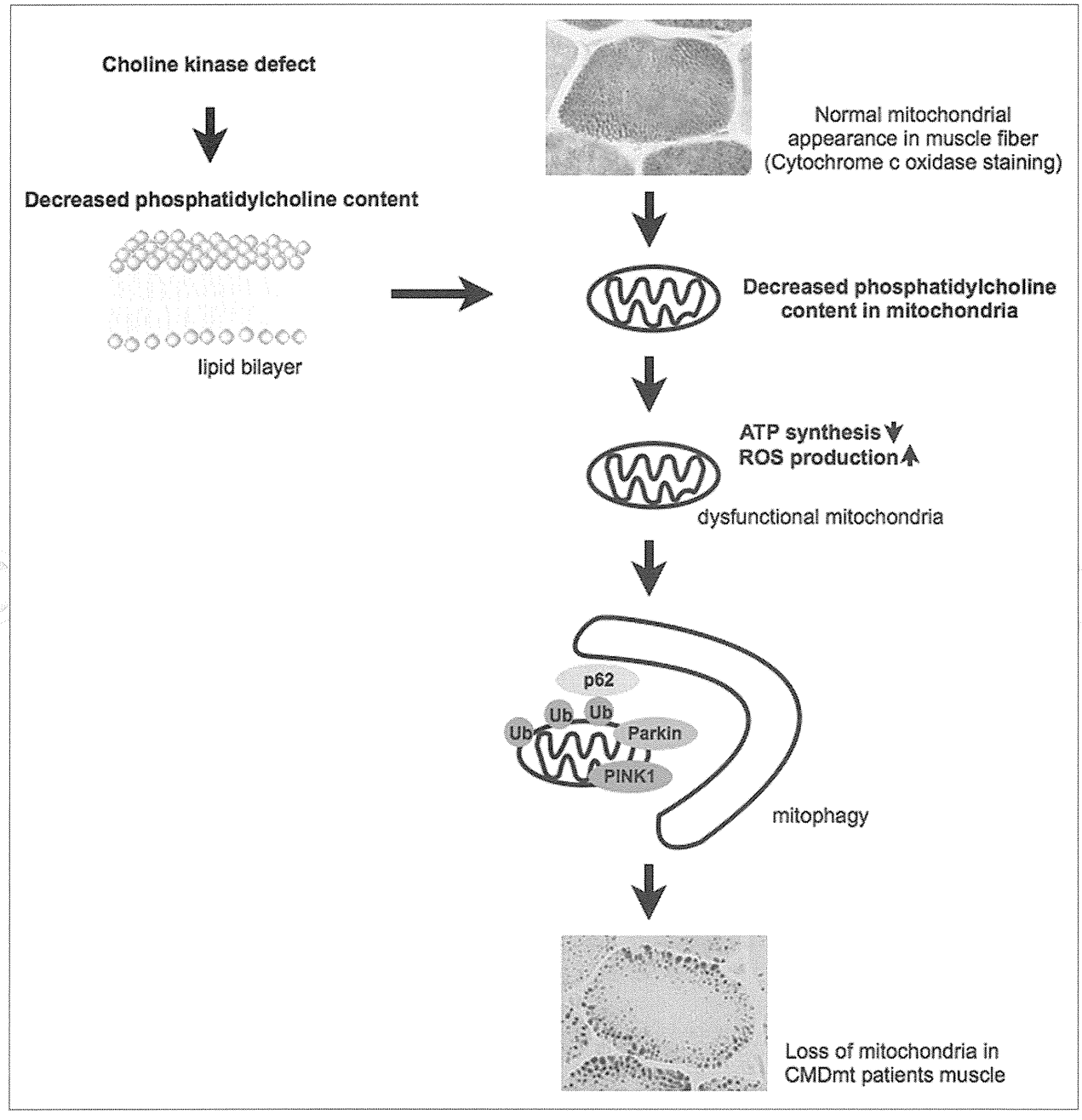

Figure 1. In normal muscle fiber, mitochondria distribute uniformly. Choline kinase defect causes a decreased phosphatidylcholine level in the mitochondrial membrane, leading to mitochondrial dysfunction. These mitochondria are eliminated by mitophagy, probably resulting in sparse mitochondria in muscle fiber.

the periphery. This mitochondrial decrease in number with concomitant increase in size can be confirmed by quantitative analysis in *rmd* mice. Mitochondrial DNA copy number is also decreased in *rmd* muscle in an age-dependent manner, most likely due to decreased mitochondrial number, because the pathological change of sparse mitochondria is also progressive with age.

In this disease, mitochondria are eliminated by mitophagy because (1) mitochondria are observed in autophagosomes by EM; (2) mitochondria are polyubiquitinated, and p62 and LC3 are recruited to mitochondria based on immunohistochemistry; and (3) PINK1 and Parkin in isolated mitochondria are increased based on western blotting, suggesting that they are recruited to mitochondria to promote mitophagy. Our data suggest that the decreased number of mitochondria is most likely due to increased mitochondrial clearance in skeletal muscle.

This discovery raises a number of important questions. Is it mitochondrial bioenergetic dysfunction that induces mitophagy in *rmd*? What will happen if mitophagy is blocked in *rmd* muscle? Does mitophagy have a protective role, or

promote muscle degeneration? In other words, is muscular dystrophy in *rmd* mice due to impaired mitochondria or excessive clearance of mitochondria?

Another important question is: How does membrane lipid compositional change affect mitochondrial function in vivo? Native PAGE analysis of *rmd* mitochondria shows that respiratory enzyme activity is decreased, but the respiratory chain protein complexes are properly formed. This finding supports the evidence that proper membrane lipid composition, and thereby lipid-protein interaction, is crucial for the activities of respiratory chain enzymes. Further study is necessary to elucidate the regulation mechanism of respiratory chain enzyme activities by membrane lipid.

CMDmt, due to the choline kinase β defect, should be a good model to investigate the regulation of mitochondrial number by mitophagy and lipid-protein interaction in mitochondrial membrane.

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ORIGINAL ARTICLE

Effects of enzyme replacement therapy on five patients with advanced late-onset glycogen storage disease type II: a 2-year follow-up study

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Abstract We examined the efficacy of 2-year enzyme replacement therapy (ERT) using recombinant human α-glucosidase (GAA; Myozyme®) in five long-term ventilator-dependent adults and aged patients with advanced, late-onset glycogen storage disease type II (GSDII, also known as Pompe disease). Although all patients had advanced respiratory failure and were ventilator-dependent for more than 6 years, four showed obvious improvements in muscle strength, pulmonary function, and activities of daily living after ERT. Improvement in each parameter was more prominent in the first year than in the second year. Values in the second year were still significantly better than those at study entry and indicate stabilization in the clinical status of all patients. These results suggest that ERT continues to be effective in the second year of treatment even in patients suffering from advanced late-onset GSDII disease with severe respiratory failure.

Introduction

Glycogen storage disease type II (GSDII), or Pompe disease, is an autosomal recessive lysosomal glycogen storage disease

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resulting from a deficiency in α-glucosidase (GAA) activity (OMIM #232300). The different clinical phenotypes of GSDII include classic infantile-onset; non-classic infantile-onset; childhood, juvenile, and adult forms of GSDII; and late-onset GSDII. However, GSDII presents as a broad spectrum with varying degrees of severity and rates of progression. The classic infantile-onset form is characterized by hypertrophic cardiomyopathy and generalized muscle weakness, which appear in the first few months of life (Hirshhorn and Reuser 2001; Engel et al. 2004). Late-onset GSDII is characterized by progressive skeletal muscle weakness and loss of respiratory function.

Enzyme replacement therapy (ERT) using recombinant human GAA (rhGAA) derived from transfected Chinese hamster ovary cells resulted in marked improvement in the survival rate of 18 patients with infantile-onset GSDII (Kishnani et al. 2008). Nicolino and colleagues also reported that rhGAA reduced the risk of death and invasive ventilation by 79 and 58%, respectively, in infants and children with advanced Pompe disease (Nicolino et al. 2009). The use of ERT with Myozyme® (α-glucosidase) was approved by the U.S. Food and Drug Administration (FDA) in 2006 and by the Japan Ministry of Health, Labor and Welfare (MHLW) in 2007.

Previous studies confirmed the efficacy of ERT in late-onset GSDII patients with acute respiratory failure or relatively mild respiratory dysfunction (Winkel et al. 2004; Pascual-Pascual et al. 2006; Merk et al. 2007, 2009; Case et al. 2008; Yamamoto et al. 2008; Rossi et al. 2007; van Capelle et al. 2008; Strothotte et al. 2010; van der Ploeg et al. 2010). On the other hand, ERT efficacy in advanced patients seemed to be lower than that in milder patients (Orlikowski et al. 2011). It is not clear whether ERT is continuously effective in ventilator-dependent patients with advanced disease and long-term respiratory failure. Because ERT is relatively expensive, it is important to determine whether continuous administration is effective, or whether therapy is only effective for a short duration. In the present study, we evaluated the efficacy of ERT in five patients with advanced late-onset GSDII for 2 years and analyzed factors related to its efficacy.

Patients and methods

Patients

Patients with late-onset Pompe disease diagnosed based on both muscle biopsies and fibroblast/muscle residual GAA activity, and who had undergone ERT at the National Center Hospital (National Center of Neurology and Psychiatry), were included in this study. Written informed consent was obtained before enrollment. The study protocol was approved by the National Center Hospital Ethics Committee. Patients 4 and 5 have been reported previously (Sasaki et al. 1992; Yamazaki et al. 1992). Table 1 lists the characteristics of all five patients (two men and three women).

Genomic DNA was extracted from blood or muscle biopsy samples according to standard protocols. All exons and flanking intronic regions of GAA were amplified and sequenced using an automated 3100 DNA sequencer (Applied Biosystems, Foster, CA). Primer sequences are available upon request. All patients had previously reported mutations (Tsujino et al. 2000; Tsunoda et al. 1996; Lam et al. 2003; Pipo et al. 2003; Hermans et al. 2004). The average (SD) age at ERT initiation was 47 (13.6) years (range 32–66 years), and the average duration of disease was 26 (4.5) years (range 20–31 years). The average duration of mechanical ventilatory support before ERT was 8.0 (1.9) years (range 6–11 years). Patients 1, 2, 4, and 5 had been treated with noninvasive ventilation (NIV), and patient 3 had been treated with invasive ventilation. All patients were wheelchair-bound for a mean of 7.0 (5.1) years (range 2–14 years). Only patient 4 was able stand for a few minutes or walk a few steps with assistance. Others were completely wheelchair-bound.

Methods

ERT (Myozyme®) was administered at 20 mg/kg body weight biweekly at a dose of 1 mg/kg/h for the first 30 min, 3 mg/kg/h for the second 30 min, and then increased to 5 mg/kg/h, and finally 7 mg/kg/h every 30 min. Patients were carefully monitored for infusion-related reactions during and after ERT administration. Clinical condition was assessed every 6 months, including physical examination, manual muscle test (MMT), ECG, Holter ECG, ultrasound cardiography (UCG), and pulmonary function tests [% vital capacity (%VC), % force vital capacity (% FVC), forced expiratory volume in the first second (FEV1.0), peak expiratory flow rate (PEF), peak cough flow (PCF; Bach 2004)], and lean body mass (Discovery Bone Densitometer, Hologic, Bedford, MA). Muscle strength, including grip power (Dynamometer®, TTM, Japan, for patient 1; Grip Strength Dynamometer®, Takei, Japan, for patients 2–5) and pinch power (PinchTrack™, Jtech, Japan), was assessed every 2 weeks. The Barthel index and gross motor function measure manual (GMFM) were assessed every 6 months from the second year (Hosoda and Yanagisawa 2000; Kondo and Fukuda 2000). Occlusal force in the right and left first molar was measured using the Occlusal Force Meter GM10® (Nagano Keiki, Japan) every 6 months. In this test, which was repeated three times, patients were asked to bite on a block as hard as possible. All patients rested for more than 2 h before each muscle strength test. Normal values for grip power

Table 1 Baseline patient characteristics and conditions

Patient no.	1	2	3	4	5
Sex	Male	Male	Female	Female	Female
Age at inclusion (years)	66	55	44	38	32
Age at onset (years)	35	35	25	8	7
Observation period (weeks)	104	104	104	104	104
Symptom at onset (weakness)	Lower extremities	Lower extremities	Lower extremities	Neck	Lower extremities
Ventilator since (age in years)	58	49	36	32	21
Duration of ventilator use (years)	8	7	8	6	11
Wheelchair-bound	Complete	Complete	Complete	Complete	Partial
Ventilator use (h/day)	24	10 (at night)	24	22	10 (at night)
Tracheotomy (age in years)	None	48	36	None	None
Wheelchair since (age in years)	51	48	36	36	29
Genotype	c.1585–1586TC > GT(p.S529V) homozygote	c.546 G > T(p.T182T) homozygote	c.307 T > C(p.C103R)/ c.546 G > A(p.T182T)	c.1309 C > T(p.R437C)/ c. 1857 C > G(p.S619R)	c.546 G > T(p.T182T)/ c.1798 C > T(p.R600C)
Enzyme activity[a]	1.2 (M)	0.6 (M)	1.88 (M)	0.46 (F)	3.8 (M)
Complications	Diabetes mellitus	Atrial fibrillation	Interstitial pneumonia pneumothorax	Pneumothorax subcutaneous/ mediastinal emphysema	—
Pathology	Myopathic changes	Myopathic changes	Myopathic changes	Myopathic changes	Myopathic changes
AcP- and PAS-positive vacuoles	Few	Scattered	Scattered	Stained for acid phosphatase	Many

[a] *(M)* Muscle (nmols 4MU/mg/h) (14.6±4.4), *(F)* fibroblast (mmol/pg protein) (161±32.4)

and occlusal force were provided by the manufacturer, and three healthy volunteers were tested as controls for pinch power [see Table in Electronic Supplementary Material (ESM)]. Blood cell counts and blood chemistry tests were conducted regularly. We interviewed patients and their families about activities of daily living (ADL). IgG antibodies to rhGAA were measured regularly by enzyme-linked immunosorbent assay (ELISA) (Kishnani et al. 2006).

Annual changes in quantitative parameters (pulmonary function tests, grip power, pinch power, and occlusal force) were calculated for the first and second years by subtracting old data from new data. Changes were analyzed with the Mann-Whitney *U* test. Statistical analyses were performed with SPSS for Macintosh (version 18, SPSS, Chicago, IL).

Results

Case presentation

Patient 1 suffered from limb muscle atrophy at age 35. He could not climb stairs and visited us at age 44. Muscle biopsy and acid maltase activity revealed Pompe disease. He lost ambulation at age 51. He experienced dyspnea, and %VC was 22.4 at age 58. Nocturnal NIV was initiated; he required continuous NIV from age 63 and was able to remove the NIV mask for <1 min before ERT. ERT was initiated at age 66. After 6 months of ERT, the patient was able to stop NIV for 9 min, allowing for a much easier transfer of the patient from car to wheelchair by the caregiver. This also provided the caregiver more than 5 min for shaving and/or cleaning the patient's face, compared to the 1-min limit before ERT.

Patient 2 had difficulty climbing stairs from age 36. He experienced dyspnea in the supine position at age 47 and visited a physician due to morning headache and severe dyspnea. He presented with pneumonia and CO_2 narcosis; nocturnal oxygen therapy was initiated after recovery. A muscle biopsy led to the diagnosis of Pompe disease. The patient lost ambulation during hospitalization. He visited us at age 50 and nocturnal NIV was initiated. The patient had difficulty lying down in the supine position without NIV before ERT. After ERT was initiated at age 55, he was able to lie down for 10 min at 24 weeks of ERT and for 60 min at 48 weeks without respiratory support. He was also less fatigued in the afternoons and able to drive alone for 2 h after 40 weeks.

Patient 3 noticed gait disturbance at age 22, visited a neurologist at age 26, and was diagnosed with limb-girdle

muscular dystrophy. At age 36, she complained of morning headache and drowsiness; she was intubated and tracheostomy was performed due to CO_2 narcosis and pneumonia. The patient lost ambulation during hospitalization and had recurrent pneumothorax and pneumonia. She visited us at age 39 and was diagnosed with Pompe disease by muscle biopsy and GAA activity. Recurrent pneumonia due to *Pseudomonas aeruginosa* required hospitalization with intravenous antibiotics once every 2 months before ERT. After ERT was initiated at age 44, she developed a mild fever of <38°C twice at 12 and 36 weeks after ERT, and recovered without antibiotics. She was able to open a plastic bottle unaided after 24 weeks of treatment, a task that could not be completed for 8 years prior to treatment. She was able to easily move from bed to wheelchair after 44 weeks. She also noticed less fatigue during meals, was able to pull up both legs unaided after 2 years of ERT, and could put on socks while sitting in the wheelchair.

Patient 4 had proximal weakness at age 15. She was referred to a neurologist and found to have high creatine kinase levels (1,256 U/L) and mild respiratory dysfunction (%VC: 77) at age 21. She was diagnosed with late-onset Pompe disease by muscle biopsy and fibroblast acid maltase activity. At age 32, she experienced dyspnea and initiated NIV during the night. At age 35, her %VC decreased to 18.9 and she required NIV all day. She began to use a wheelchair due to exertional dyspnea. At age 36, she presented with a right-sided pneumothorax, and %VC decreased to 15.8. She was able to turn off NIV only for 5 min to take a bath and could not comb her hair by herself before ERT. At 24 weeks after ERT initiation, pinch power increased from 48.4 N to 55.2 N, and she was able to stand with less effort. At 64 weeks of treatment, she was able to switch off NIV for 15 min while taking a bath and combing her hair. However, she experienced severe dyspnea and recurrent pneumothorax after 64 weeks of ERT and became fully dependent on NIV thereafter. She developed pneumothorax and emphysema at 80 weeks of ERT again and was completely bedridden and required cuirass ventilation in addition to NIV. She was also treated with parenteral hyperalimentation, including standard calorie and protein, for approximately 1 month due to inability to eat caused by dyspnea. After recovery from severe emphysema, she remained bedridden and consequently lost ambulation. Occlusal force was also lower after parenteral hyperalimentation.

Patient 5 could not stand without hand support and visited a pediatrician at age 13 and visited us and muscle biopsy and acid maltase activity. She initiated NIV at age 21 and required a wheelchair at age 29. After ERT was initiated at age 31, she found it easier to expectorate sputum through coughing than before ERT and could move her hip from floor to chair unaided after 44 weeks, which had been impossible for several years. She also noticed alleviation of lumbago, and after three doses of ERT, she was able to discontinue non-steroidal anti-inflammatory drugs (NSAIDS) used for back pain. The patient suffered from emaciation before ERT and was advised that this could not be resolved, but she gained 3 kg of body weight after ERT. At present, she can drive 2.5 h to go to the hospital every 2 weeks, which was impossible before ERT due to fatigue and back pain.

ERT-induced changes

Table 2 lists the results of clinical and laboratory tests before and after ERT. The mean duration of follow-up was 104 weeks. Grip power (Fig. 1a) and pinch power (Fig. 1b) showed gradual improvement in all patients. In patient 4, both grip and pinch powers continued to improve until 60 weeks after ERT initiation, but deteriorated thereafter. Occlusal force improved markedly in patients 1 and 3 (Fig. 1c), but deteriorated in patient 4. No changes in MMT were noted in any of the patients. GMFM improved slightly in patients with a score of >25, while it remained unchanged in those with a score of <5. After initiation of ERT, all patients, except patient 4 who had severe emphysema and pneumothorax, showed improvement in %VC (Fig. 2a), PEF (Fig. 2b), PCF (Fig. 2c), %FVC (Fig. 2d), and/or FEV1.0 (Fig.2e).

Creatine kinase (CK) levels decreased during treatment in patients 2, 4, and 5, and particularly in patient 4 (Table 2). CK levels were normal in patients 1 and 3 at the commencement of treatment and did not show marked changes during and after treatment. Body weight [44.4 (17.0) to 43.6 (16.1) kg, $p=0.93$)] and lean body mass [25.8 (7.9) to 25.8 (10.2) kg, $p=0.99$] did not change.

Changes in the first year were greater than in the second year (Table 3). Most data were not available for patient 4 at the first year evaluation because bed rest was required for pneumothorax therapy. Changes in %VC, %FVC, PEF, PCF, pinch power, and occlusal force were greater in the first year than in the second year ($p<0.05$). While %VC, %FVC, PEF, PCF, pinch power, and occlusal force significantly changed in the first year after ERT, changes in these parameters were not significant in the second year.

IgG antibody against Myozyme® was measured in patients 1, 3, 4, and 5 (see figure in ESM). All patients were IgG antibody positive at around weeks 12 to 16, but patients 4 and 5 became negative thereafter. Furthermore, IgG antibody titers increased to a peak level in patient 3, and increased in patient 1 to 25,600. The antibody titer of patient 2, measured once at 108 weeks after ERT, was negative. Only patient 3 developed a skin rash immediately after Myozyme® infusion at 12 weeks, but the rash disappeared completely after treatment with an antihistamine. Other patients did not experience any infusion-related reactions.

Table 2 Results of clinical and laboratory tests before and after ERT

		Patient 1			Patient 2			Patient 3			Patient 4			Patient 5		
		Pre	1 year	2 year	Pre	1 year	2 year	Pre	1 year	2 year	Pre	1 year	2 year	Pre	1 year	2 year
MMT	Neck flexion	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2
	Shoulder flexion	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2
	Shoulder abduction	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2
	Elbow flexion	1	1	1	3	3	4	3	3	3	4	4	4	3	4	4
	Elbow extension	1	1	1	4	4	4	4	4	4	4	4	4	3	3	3
	Wrist flexion	4	4	4	5	5	5	5	5	5	4	4	4	5	5	5
	Hip flexion	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2
	Knee flexion	1	1	1	2	2	2	2	2	2	3	3	3	2	2	2
	Knee extension	1	1	1	2	2	2	2	2	2	3	3	3	2	2	2
	Ankle flexion	1	1	1	5	5	5	2	2	2	4	4	4	5	5	5
Body weight (kg)		44	43	43	73.0	70	69	42	40	42	33	31	31	30	31	33
Lean body mass (kg)		23.9	22.6	22.6	39.8	39.8	39.8	23.0	24.4	24.4	21.1	NT	19.9	21.4	22.2	22.2
Pulmonary function	%VC	4.9	10.7	9.6	45.6	62.0	67.2	12.1	15.4	17.3	17.6	NT	9.2	13.1	19.5	21.4
	%FVC	0.0	26.8	7.7	46.3	51.2	66.1	9.3	12.5	16.1	14.2	NT	7.0	10.3	17.7	20.4
	FEV1.0	0.00	0.62	0.21	1.52	1.78	1.99	0.24	0.49	0.41	0.32	NT	0.14	0.29	0.50	0.55
	PEF (L/s)	0.38	0.93	0.50	3.72	6.40	5.49	0.46	0.63	0.70	0.58	NT	0.25	1.24	1.63	1.70
	PCF (L/s)	0.34	0.74	0.69	4.87	7.26	7.16	0.60	0.82	0.85	1.52	NT	0.86	1.19	1.96	2.17
Grip power (kg)		3.4	4.1	4.4	39.6	42.7	44.1	14.2	17.4	16.5	17.0	18.0	17.7	17.5	23.9	25.0
Pinch power (N)		14.7	21.1	15.5	81.9	96.1	98.8	23.6	42.4	42.5	48.3	56.3	53.0	44.3	48.5	47.3
Occlusal force (kgf)		6.4	15	15.9	NT	50.0	55.2	24.1	42.8	46.3	16.4	NT	8.4	NT	65.8	64.0
GMFM		NT	3	3	NT	25	31	NT	5	5	NT	56	59	NT	32	35
CK (IU/l)		47	36	50	238.0	132	10	166	132	100	621	NT	154	241	161	166
Barthel index		20	20	20	75.0	75	75	55	55	55	80	80	70	80	80	80

%VC Percent vital capacity, *%FVC* percent force vital capacity, *FEV1.0* forced expiratory volume in the first second, *PEF* peak expiratory flow, *PCF* peak cough flow, *GMFM* gross motor function measure, *CK* creatine kinase, *NT* not tested

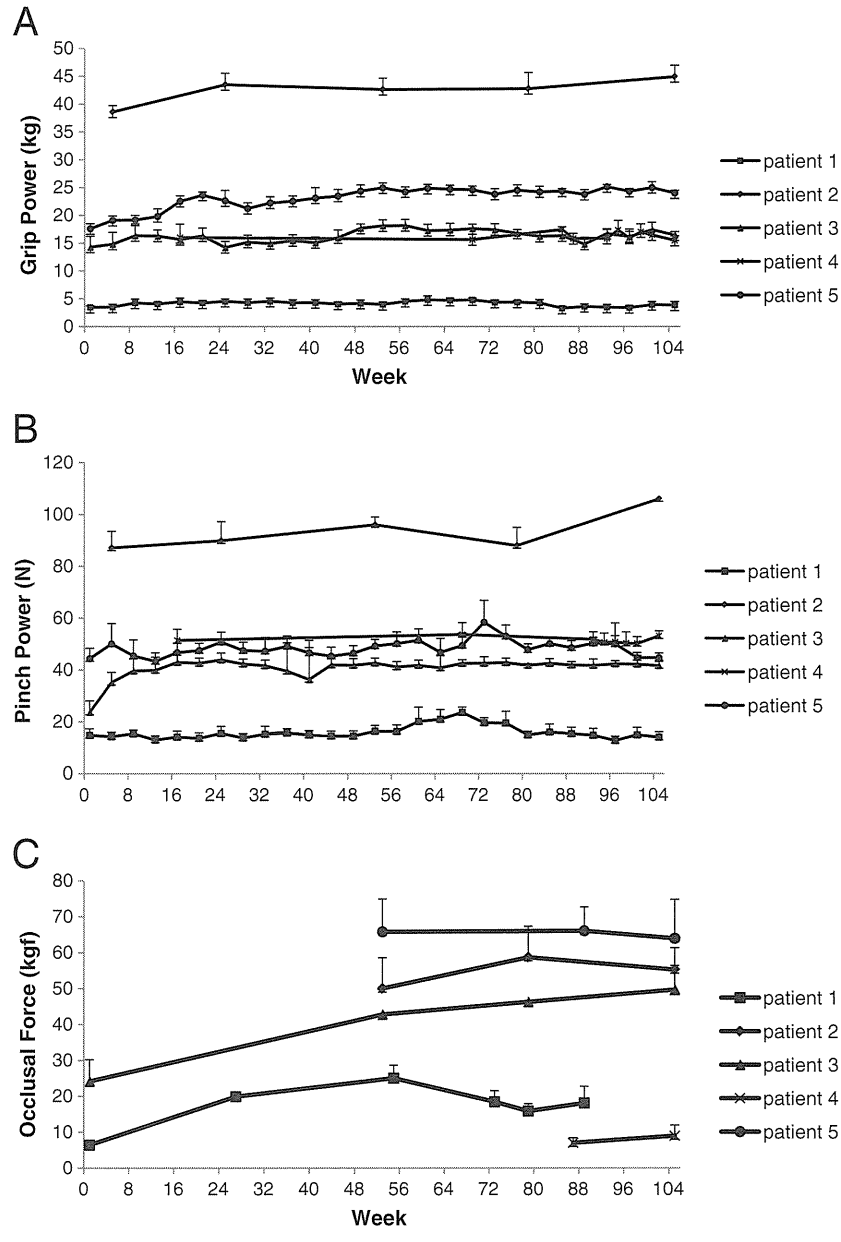


Fig. 1 Effects of ERT on grip power (**a**), pinch power (**b**), and occlusal force (**c**). Each data point represents the average of three bilateral measurements. ERT improved all of these parameters in four of five patients (with the exception of patient 4). Data are presented as mean ± SEM

Discussion

ERT is often difficult to initiate in the early stages of subclinical GSDII or in early-stage GSDII because the disease is difficult to diagnose due to heterogeneity in clinical presentation and overlapping symptoms with other neuromuscular diseases. Accordingly, it is important to gain an understanding of ERT efficacy in patients with advanced GSDII. Our study demonstrated that ERT is effective for 2 years without severe complications in adult patients who have advanced GSDII and are dependent on ventilator and wheelchair support. During the 2 years of ERT, all patients showed some improvements in muscle and pulmonary function and ADL.

All parameters improved during the first year of treatment. While the results of various tests in the second year were lower than those recorded at the end of the first year, they were still better than before ERT initiation. Although the rate of improvement differed widely among patients, our results indicate that ERT is more effective in the first year and it maintains its efficacy for 2 years. At present, there is no explanation for the better outcome in the first year compared to the second year. Taking into consideration the muscle pathology associated with GSDII, intracellular accumulation of large amounts of glycogen may cause displacement, replacement, or compression of normal cellular organelles. Thus, ERT may normalize cell function by reducing such accumulation in surviving

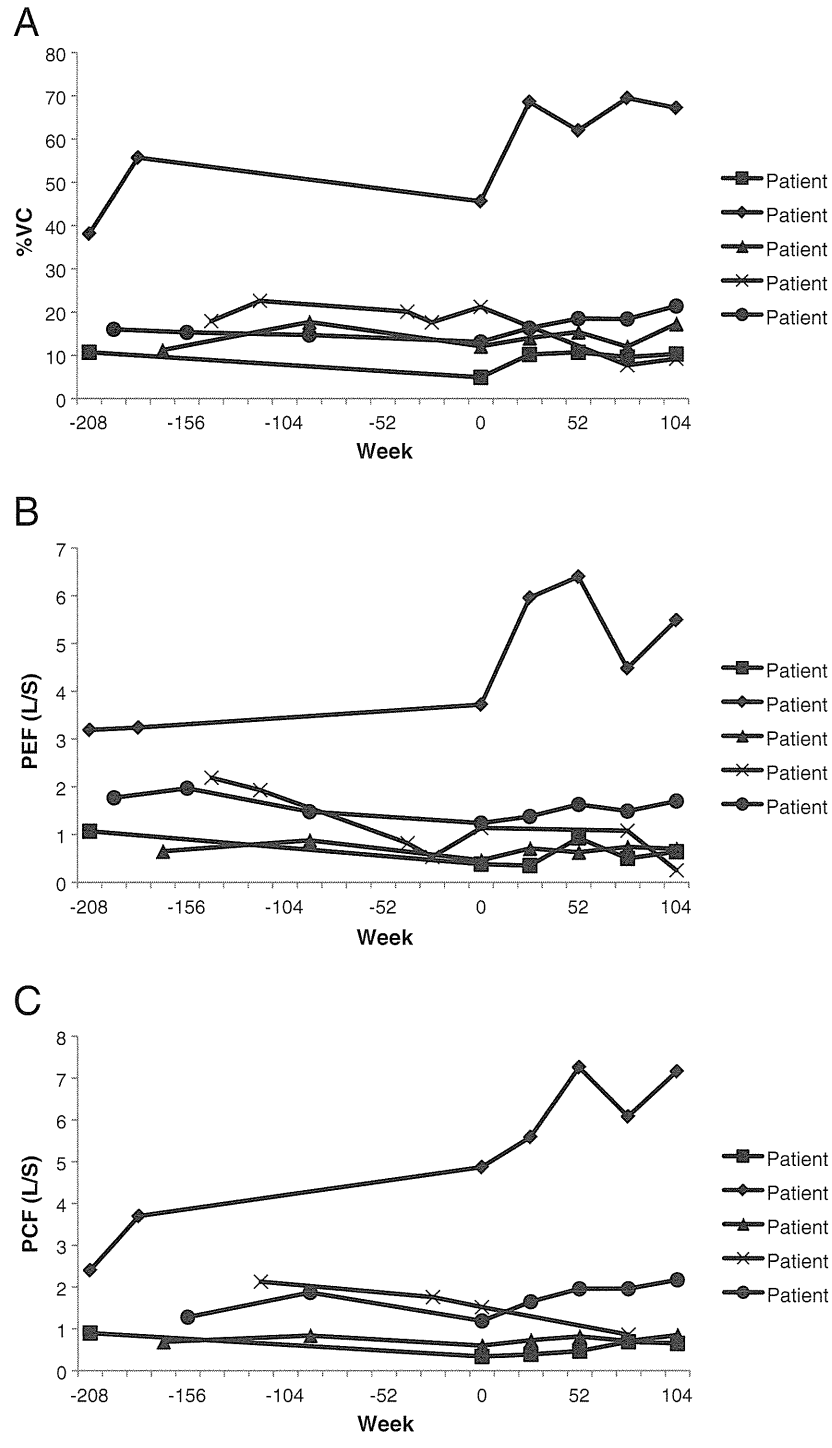
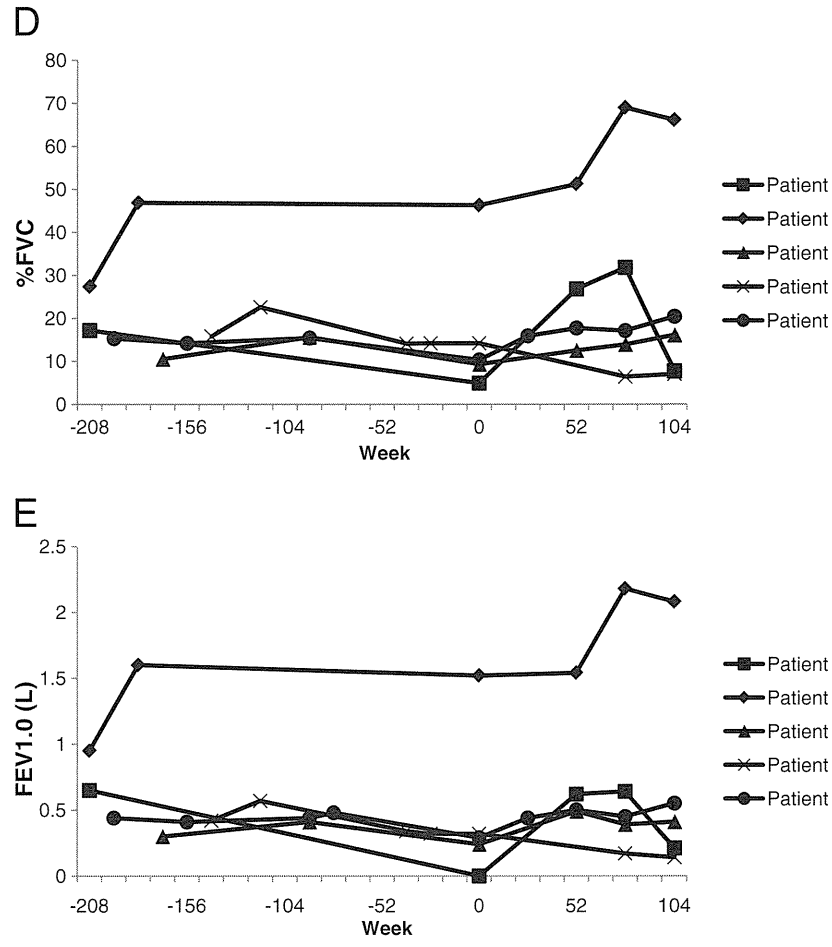


Fig. 2a–d Effects of ERT on respiratory function. Percent vital capacity (**a**), peak expiratory flow (**b**), peak cough flow (**c**), percent force vital capacity (**d**), and forced expiratory volume in the first second (**e**). Note the low values of all parameters prior to ERT and their improvement after ERT. The improvement is more pronounced in patients with spared baseline functions

myotubes, followed by a gradual regeneration of myofibers. The observed effects of ERT may represent its acute effect on intracellular glycogen accumulation.

Younger or milder cases, including those presented in a randomized controlled study of ERT, showed a greater improvement over advanced cases (Winkel et al. 2004; Pascual-Pascual et al. 2006; van der Ploeg et al. 2010). Van der Ploeg and colleagues reported on ambulant patients whose %VC was greater than 30 (van der Ploeg et al. 2010). In this clinical study, ERT elicited significant improvements in walking distance and stabilization of pulmonary function. On the other hand, efficacy of ERT in patients with advanced GSDII seemed to be milder or partial. A case report of a 67-year-old wheelchair-bound woman described alleviation of muscle symptoms following ERT, although pulmonary function tests showed no improve-

Fig. 2a–d (continued)



ment, suggesting cases with no respiratory recovery (Merk et al. 2007). Furthermore, one open-label observational study of ERT in 44 late-onset GSDII patients showed that both motor function tests and CK levels improved, and pulmonary function stabilized (Strothotte et al. 2010). Orlikowski et al. reported a 52-week follow-up of five patients (Orlikowski et al. 2011) with respiratory dysfunction as severe as in our patients, and respiratory and motor functions in all patients improved somewhat. Our data further these findings by suggesting that the improvements continue through the second year of ERT and that ERT is beneficial even for patients with advanced-stage Pompe disease.

Only patient 4 failed to show a clear recovery at the end of the follow-up period. However, grip and pinch powers increased in this patient at 60 weeks of ERT. Immobility and suspension of oral feeding resulted in reduction of muscle power, particularly in the masseter muscles. Pneumothorax also influenced the improvement in pulmonary function. Thus, we speculate that the small improvement was offset by the negative influence of pneumothorax. Because patients in similar condition at the beginning of the study responded to treatment (patients 3 and 5), one can rule out any effects of age, body weight, lean body mass, and lung dysfunction on the prognosis. Variability in the response to treatment may reflect individual differences in disease severity at treatment initiation and rate of disease progression.

The benefits conferred by ERT may not be adequate when considering ERT costs, as none of the patients exhibited an improvement in Barthel index; however, observation before ERT indicated gradual deterioration before the therapeutic intervention was initiated (Table 2). In one study, dramatic changes did not occur at the advanced stage, although certain benefits were evident (Orlikowski et al. 2011). However, we speculate that patient conditions will deteriorate if ERT is terminated after the first year, a period showing the greatest improvements. Serial pulmonary function tests indicated that the respiratory function of our patients will sequentially deteriorate (Fig. 2).

Based on our assumption that therapeutic effects of ERT cannot be measured by MMT or morbidity function in 6-min walk tests, we attempted to measure muscle power in relatively spared functions. Occlusal force is known to decrease in parallel with disease progression in Duchenne muscular dystrophy (DMD) (Ueki et al. 2007). Occlusal,

Table 3 Annual changes in parameters

	%VC						%FVC						FEV (L)						PEF (L)					
Years	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2
Patient 1	5.8	−1.1	4.7				21.9	−19.1	2.8				0.6	−0.4	0.21				0.55	−0.43	0.1			
Patient 2	16.4	5.2	21.6				4.9	14.9	19.8				0.3	0.2	0.47				2.68	−0.91	1.8			
Patient 3	3.3	1.9	5.2	0.043	0.773		3.2	3.6	6.8	0.248	1.000		0.3	−0.1	0.17	0.200	0.306		0.17	0.07	0.24	0.020	0.468	
Patient 4[a]	Not tested					0.057	Not tested					0.248	Not tested					0.043	Not tested					0.430
Patient 5	5.4	2.9	8.3				7.4	2.7	10.1				0.21	0.05	0.26				0.39	0.07	0.46			

PCF (L)							Grip power (kg)						Pinch power (N)						Occlusal force (kgf)					
1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2		1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2
0.4	−0.05	0.35					0.7	0.3	1.0				6.4	−5.6	0.8				8.6	0.9	9.5			
2.39	−0.1	2.29					3.1	1.4	4.5				14.2	2.7	16.9				50	5.2	55.2			
0.22	0.03	0.25	0.028	0.885	0.020		3.2	−0.9	2.3	0.083	0.905	0.142	18.8	0.1	18.9	0.009	0.69	0.016	18.7	3.5	22.2	0.021	0.886	0.021
Not tested							Not tested		0.7				8	−3.3	4.7				Not tested					
0.77	0.21	0.98					6.4	1.1	7.5				4.2	−1.2	3				65.8	−1.8	64			

%VC Percent vital capacity, *%FVC* percent force vital capacity, *FEV1.0* forced expiratory volume in the first second, *PEF* peak expiratory flow, *PCF* peak cough flow

[a] Patient 4 could not be evaluated at 1 year after ERT initiation due to severe pneumothorax

grip, and pinch powers were relatively spared in all patients, except patient 1. Four of five patients could write, use utensils, fasten a button, or bite foods as efficiently as healthy people, although their data revealed some decrements compared to normal controls. Cranial muscle involvement is thought to be rare, but we found that occlusal force was mildly reduced in patients with advanced Pompe disease. This suggests that occlusal force is a sensitive parameter for assessing the response to ERT.

Conclusions

The present study showed that ERT improved respiratory function and muscle power for 2 years even in adult patients with advanced GSDII. Improved muscle strength resulted in better ADL and quality of life during the long follow-up period. Taking our results into consideration, we recommend the initiation of ERT in GSDII patients, irrespective of age and disease severity.

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