Expression Levels of Long Non-Coding RNAs Change in Models of Altered Muscle Activity and Muscle Mass

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Abstract: Skeletal muscle is a highly plastic organ that is necessary for homeostasis and health of the human body. The size of skeletal muscle changes in response to intrinsic and extrinsic stimuli. Although protein-coding RNAs including myostatin, NF-κB, and insulin-like growth factor-1 (IGF-1), have pivotal roles in determining the skeletal muscle mass, the role of long non-coding RNAs (lncRNAs) in the regulation of skeletal muscle mass remains to be elucidated. Here, we performed expression profiling of nine skeletal muscle differentiation-related lncRNAs (DRR, DUM1, linc-MD1, linc-YY1, LncMyod, Neat1, Myoparr, Malat1, and SRA) and three genomic imprinting-related lncRNAs (Gtl2, H19, and IG-DMR) in mouse skeletal muscle. The expression levels of these lncRNAs were examined by quantitative RT-PCR in six skeletal muscle atrophy models (denervation, casting, tail suspension, dexamethasone-administration, cancer cachexia, and fasting) and two skeletal muscle hypertrophy models (mechanical overload and deficiency of the myostatin gene). Cluster analyses of these lncRNA expression levels were successfully used to categorize the muscle atrophy models into two sub-groups. In addition, the expression of Gtl2, IG-DMR, and DUM1 was altered along with changes in the skeletal muscle size. The overview of the expression levels of lncRNAs in multiple muscle atrophy and hypertrophy models provides a novel insight into the role of lncRNAs in determining the skeletal muscle mass.

Keywords: long non-coding RNAs; disuse atrophy; muscle wasting; muscle hypertrophy; myostatin; skeletal muscle mass

1. Introduction

Skeletal muscle is an organ that plays important roles in motion, postural maintenance, and metabolic adaptation. The adult skeletal muscle exhibits high plasticity and its mass can be altered by intrinsic and extrinsic stimuli. The skeletal muscle mass is decreased not only in diseases, such as cancer cachexia, neuromuscular disorders, chronic kidney disease, heart failure, and chronic obstructive pulmonary disease, but also as a consequence of aging, immobilization, and malnutrition [1–8]. The latter type of loss in the muscle mass is better known as muscle atrophy or wasting condition, which affects the activities of daily living and leads to increased mortality from diseases [9,10]. Activated
signaling of myostatin, NF-κβ, and glucocorticoid leads to dysregulation of the ubiquitin-proteasome or autophagy-lysosome systems, inducing muscle atrophy and wasting [11–13]. In contrast, moderate exercise and good nutrition increase the skeletal muscle mass (known as muscle hypertrophy) through the activation of insulin-like growth factor-1 (IGF-1)/Akt/mTOR (mammalian target of rapamycin) and β-adrenergic pathways [12,14]. Epidemiological studies have shown that people with high skeletal muscle mass have a lower prevalence of various diseases and a longer life expectancy [15,16]. Thus, understanding the molecular mechanisms controlling skeletal muscle mass is important to extend the healthy life expectancy in humans.

Recent advances in techniques for nucleic acid detection have revealed the presence of numerous long non-coding RNAs (lncRNAs), which are defined as RNAs longer than 200 bases in length [17]. Although lncRNAs do not code for proteins, dosage compensation and genomic imprinting have been known to be controlled by lncRNAs [18–21]. It is now clear that lncRNAs work as transcriptional, epigenetic, and translational regulators; structural cores; and perform other functions through their interaction with several essential proteins [22,23]. During skeletal muscle formation, an lncRNA, SRA, is required for the terminal differentiation of myoblasts by enhancing the transcriptional activity of myoblast determination protein 1 (MyoD) [24], one of the key myogenic transcription factors. The DRR enhancer RNA, also known as MUNC, is expressed from the distal regulatory element of the Myod1 gene, which codes for MyoD, and promotes myogenic differentiation [25,26]. LncMyoD is in the immediate vicinity of the Myod1 gene and is required for terminal muscle differentiation through the translational regulation of genes involved in proliferation [27]. Other lncRNAs, such as linc-MD1, DUM1, linc-YY1, Malat1, and Neat1, also regulate myogenesis [28–33]. However, the roles of these lncRNAs in the regulation of skeletal muscle mass remain largely unknown.

We previously identified an lncRNA, Myoparr, from the promoter region of the myogenin gene, which codes for a transcriptional factor that is indispensable for skeletal muscle differentiation [34]. Myoparr interacts with a transcriptional co-activator, DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (Ddx17), and is essential both for the specification of myoblasts into the differentiation lineage and for the myoblast cell cycle withdrawal. Intriguingly, Myoparr is also involved in the induction of muscle atrophy caused by surgical denervation in adult skeletal muscle in mice [34,35]. These findings suggest that lncRNAs involved in muscle differentiation might also be involved in the regulation of skeletal muscle mass. In addition, we previously showed that muscle hypertrophy induced by myostatin deficiency increased the expression levels of genomic imprinting-related lncRNAs, Gtl2 (also known as Meg3) and IG-DMR [36]. Gtl2 promotes bovine myoblast differentiation by acting as an miR-135 sponge [37]. H19, another genomic imprinting-related lncRNA, also regulates myogenic differentiation [38–40], and knockout mice of H19 show muscle hypertrophy and hyperplasia [41]. Thus, in the current study, to reveal the roles of lncRNAs in the regulation of skeletal muscle mass, we performed expression profiling of nine lncRNAs (DRR, DUM1, linc-MD1, linc-YY1, LncMyod, Neat1, Myoparr, Malat1, and SRA), whose roles in skeletal muscle differentiation were well established [24–33], and three genomic imprinting-related lncRNAs (Gtl2, H19, and IG-DMR) in six muscle atrophy and two muscle hypertrophy models in mice. By using the expression data sets of these lncRNAs, we could classify the muscle atrophy conditions into two sub-groups. Our comprehensive expression data of 12 lncRNAs provide an important insight into the roles of these lncRNAs in the regulation of skeletal muscle mass.

2. Results

2.1. Changes in the Expression of Skeletal Muscle Differentiation-Related lncRNAs during Muscle Atrophy

We examined the expression of nine skeletal muscle differentiation-related lncRNAs (Myoparr, linc-MD1, LncMyod, DRR, DUM1, linc-YY1, Malat1, Neat1, and SRA) in six muscle atrophy models in mice. The first three models, including denervation, casting, and tail suspension, are the disuse atrophy group. Sciatic nerve transection significantly decreased the tibialis anterior (TA) muscle weight by
18.9% compared to that in the control side that was sham operated after 7 days of surgical denervation (Table 1). A significant decrease of 9.9% in the muscle weight was also observed after 7 days of casting (Table 1). The disuse of skeletal muscle for 3 days in the tail suspension model significantly decreased the TA muscle weight by 10.9% (Table 1). The next three models are the systemically muscle wasting group. Glucocorticoids are strong inducers of systemic muscle wasting [42]. Continuous administration of a high dose of glucocorticoid (dexamethasone, Dex) for 7 days significantly decreased the weight of the TA muscles by 9.8% (Table 1). Cancer cachexia also systemically induces the loss of skeletal muscle mass and this symptom is prominent in cancer patients [43]. Tumor-bearing mice with cachexia-inducible colon-26 adenocarcinoma (C26) cells have been used as cancer cachexia models [44]. C26-transplanted mice showed a significant decrease of 22.4% in the skeletal muscle mass as compared to the phosphate-buffered saline (PBS)-administrated control mice (Table 1). In addition, by 2 days of fasting, a 14.4% decrease in the weight of TA muscles was observed compared to that for control mice fed normally (Table 1).

| Condition                   | Muscle Weight of Control Group (mg) | Muscle Weight of Treated Group (mg) | p Value | Strain   | Muscle |
|-----------------------------|------------------------------------|------------------------------------|---------|----------|--------|
| Denervation                 | 44.0 ± 1.0                         | 35.7 ± 3.1                         | < 0.05  | C57BL/6J | TA     |
| Casting                     | 47.0 ± 1.0                         | 42.3 ± 0.6                         | < 0.01  | C57BL/6J | TA     |
| Tail suspension             | 49.0 ± 1.0                         | 43.7 ± 1.5                         | < 0.01  | C57BL/6J | TA     |
| Glucocorticoid administration| 44.0 ± 1.0                         | 39.7 ± 1.2                         | < 0.01  | C57BL/6J | TA     |
| Cancer cachexia             | 44.7 ± 1.5                         | 34.7 ± 0.6                         | < 0.001 | CD2F1    | TA     |
| Fasting                     | 41.7 ± 1.2                         | 35.7 ± 1.5                         | < 0.01  | C57BL/6J | TA     |
| Myostatin deficiency        | 43.3 ± 4.9                         | 78.7 ± 2.3                         | < 0.001 | C57BL/6J | TA     |
| Mechanical overload         | 21.2 ± 0.9                         | 39.3 ± 2.7                         | < 0.001 | C57BL/6J | Plantaris |

Data are presented as means ± SD. n = 3 per group. TA as abbreviation means tibialis anterior muscle.

We previously showed that surgical denervation increases the expression level of Myoparr in skeletal muscles [34]. Thus, we first examined Myoparr expression in adult skeletal muscles after surgical denervation. In accordance with a previous report [34], Myoparr expression was significantly increased by denervation (Figure 1A). However, the expression level of Myoparr was not changed significantly in other muscle atrophy models (Figure 1A). Intriguingly, we found that surgical denervation significantly increased the expression level of linc-MD1 in comparison to the expression in the muscle on the control side (Figure 1B). These results are consistent with our previous findings that Myoparr activates the expression levels of miR-133b and miR-206, which are located on the linc-MD1 locus during myogenic differentiation [34]. The expression level of linc-MD1 was also significantly increased after the casting and tail suspension treatments but did not change significantly either by the Dex treatment, cancer cachexia, or in the fasting mice (Figure 1B). In addition to linc-MD1, the denervation, casting, and tail suspension treatments significantly increased the expression level of LncMyod (Figure 1C). On the other hand, the expression level of LncMyod was observed to decrease in the fasting mice (Figure 1C). Although not significant in all muscle atrophy models, we observed that the expression levels of DRR and DUM1 tended to be decreased in all models (Figure 1D and 1E). The expression levels of linc-YY1, Malat1, Neat1, and SRA showed significant changes in each model, but they did not show consistent changes across the different muscle atrophy models (Supplementary Figure S1A–D).
Figure 1. Changes in the expression of skeletal muscle differentiation-related lncRNAs in six muscle atrophy conditions. (A–E) Box-and-whisker plots showing the results of quantitative RT-PCR (qRT-PCR) for *Myoparr* (A), *linc-MD1* (B), *LncMyod* (C), *DRR* (D), and *DUM* (E) expression in multiple muscle atrophy models. Red lines indicate the median values. Lower and upper box limits are 25th and 75th percentiles, respectively. Whiskers indicate the maximum and minimum values. Sham; tibialis anterior (TA) muscles of sham-operated C57BL/6J mice. Den; denervated TA muscles of C57BL/6J mice. Cast; casting-operated TA muscles of C57BL/6J mice. TS; TA muscles of tail suspension-operated C57BL/6J mice. Saline; TA muscles of saline-injected C57BL/6J mice. Dex; TA muscles of dexamethasone-injected C57BL/6J mice. PBS; TA muscles of control phosphate-buffered saline (PBS)-injected CD2F1 mice. C26; TA muscles of C26 tumor-bearing CD2F1 mice. Control; TA muscles of C57BL/6J mice provided with water and food ad libitum. Fast; TA muscles of fasting C57BL/6J mice. qRT-PCR data were normalized to *Rpl26* expression and shown as relative expression. *n* = 3 per group. *p < 0.05. **p < 0.01. ***p < 0.001.

2.2. Changes in the Expression of Genomic Imprinting-Related lncRNAs during Muscle Atrophy

We next examined the expression of three genomic imprinting-related lncRNAs (*H19*, *Gtl2*, and *IG-DMR*) in six muscle atrophy models. Intriguingly, we found increased *H19* expression after 7 days of surgical denervation (Figure 2A). As in the case of *linc-MD1*, this result is consistent with *Myoparr* activating *H19* expression during myogenic differentiation [34]. Fasting also significantly altered the expression level of *H19*, but its expression was not changed significantly in the other
four muscle atrophy models (Figure 2A). We observed significant decreases in Gtl2 expression by the denervation, tail suspension, and cancer cachexia and in the fasting mice (Figure 2B). The IG-DMR expression was also significantly decreased by the denervation and cancer cachexia and in the fasting mice, but the expression level of IG-DMR was observed to significantly increase by the tail suspension treatment (Figure 2C).

**Figure 2.** Changes in the expression of genomic imprinting-related lncRNAs in six muscle atrophy conditions. (A–C) Box-and-whisker plots showing the results of quantitative RT-PCR (qRT-PCR) for H19 (A), Gtl2 (B) and IG-DMR (C) expression in multiple muscle atrophy models. Sham; tibialis anterior (TA) muscles of sham-operated C57BL/6J mice. Den; denervated TA muscles of C57BL/6J mice. Cast; casting-operated TA muscles of C57BL/6J mice. TS; TA muscles of tail suspension-operated C57BL/6J mice. Saline; TA muscles of saline-injected C57BL/6J mice. Dex; TA muscles of dexamethasone-injected C57BL/6J mice. PBS; TA muscles of control phosphate-buffered saline (PBS)-injected CD2F1 mice. C26; TA muscles of C26 tumor-bearing CD2F1 mice. Control; TA muscles of C57BL/6J mice provided with water and food ad libitum. Fast; TA muscles of fasting C57BL/6J mice. qRT-PCR data were normalized to Rpl26 expression and shown as relative expression. \( n = 3 \) per group. * \( p < 0.05 \). ** \( p < 0.01 \). *** \( p < 0.001 \).

2.3. **Cluster Analysis of Changes in the Expression of lncRNAs in Muscle Atrophy Models**

We classified the muscle atrophy models using the expression profiling of 12 lncRNAs. The value of the log2 fold change in the expression levels of lncRNAs in each model was converted to a z-score and cluster analysis was performed. The result is shown as a heatmap sorted by hierarchical clustering trees. We could classify the six muscle atrophy models into two sub-groups; one was the disuse atrophy group containing denervation, tail suspension, and casting treatments. The other was the systemically wasting group containing dexamethasone administration, C26-induced cachexia, and fasting (Figure 3). A subset of lncRNAs showed similar expression profiles in each group. Intriguingly, highly upregulated expression levels of LncMyod and linc-MD1 were observed in the disuse group but not in the systemically wasting group.
2.4. Changes in the Expression Levels of Skeletal Muscle Differentiation-Related IncRNAs in Skeletal Muscle Hypertrophy Conditions

Altered lncRNA expression levels in muscle atrophy models prompted us to examine the expression profiling of nine skeletal muscle differentiation-related lncRNAs (Myoparr, linc-MD1, LncMyod, DRR, DUM1, linc-YY1, Malat1, Neat1, and SRA) in the skeletal muscle hypertrophy condition. Because of the difficulty in inducing muscle hypertrophy in mice by resistance training, we used both genetic hypertrophy and mechanical overload (MOV)-induced chronic hypertrophy models for the analysis. As a genetic hypertrophy model, we used myostatin knockout mice [45]. Loss of the myostatin gene in mice resulted in a significant increase of 81.5% in the TA muscle mass compared to that in the wild-type mice (Table 1). Mechanical overload of the plantaris muscle by synergistic ablation was achieved by surgically removing the soleus and gastrocnemius muscles. Although not completely identical, plantaris muscle has a similar fiber-type composition with that of TA muscle in mice [46]. To examine the effect of chronic muscle hypertrophy as well as myostatin knockout mice, we collected muscles 6 weeks after surgery in the MOV model. By 6 weeks of synergistic ablation, an 85.7% increase in the weight of plantaris muscles was observed compared to that in sham-operated mice (Table 1).

Although the expression level of Myoparr was not changed significantly by the myostatin deficiency, the MOV treatment significantly increased the expression level of Myoparr (Figure 4A). The expression level of linc-MD1 was significantly decreased and increased by the myostatin deficiency and the MOV treatment, respectively (Figure 4B). A significant increase in LncMyod expression was only observed by the MOV treatment (Figure 4C). The expression level of DRR was highly altered by both the myostatin deficiency and the MOV treatment, but the direction of the changes was the opposite in both models (Figure 4D). The expression level of DUM1 was significantly increased only by the MOV treatment (Figure 4E). Although the administration of recombinant myostatin decreases the expression of Malat1, both in vitro and in vivo [47], no significant increase or decrease in the expression of other skeletal
muscle differentiation-related lncRNAs, including Malat1, was found in myostatin knockout mice (Supplementary Figure S2A–D). The MOV treatment also affected the expression levels of linc-YY1 and SRA (Supplementary Figure S2A,D).

Figure 4. Changes in the expression levels of skeletal muscle differentiation-related lncRNAs in skeletal muscle hypertrophy conditions. (A–E) Box-and-whisker plots showing the results of quantitative RT-PCR (qRT-PCR) for Myoparr (A), linc-MD1 (B), LncMyod (C), DRR (D), and DUM1 (E) expression in two muscle hypertrophy conditions. Wild; tibialis anterior (TA) muscles of control C57BL/6J mice. MSTN KO; TA muscles of C57BL/6J-background myostatin knockout mice. Sham; plantaris muscles of sham-operated C57BL/6J mice. MOV; plantaris muscles of mechanical overload (MOV)-operated C57BL/6J mice. qRT-PCR data were normalized to Rpl26 expression and shown as relative expression. n = 3 per group. * p < 0.05. ** p < 0.01. *** p < 0.001.

2.5. Changes in the Expression Levels of Genomic Imprinting-Related lncRNAs in Skeletal Muscle Hypertrophy Conditions

We next examined the expression of three genomic imprinting-related lncRNAs (H19, Gtl2, and IG-DMR) in the muscle hypertrophy condition. The expression level of H19 was significantly increased by the MOV treatment but not by the myostatin deficiency (Figure 5A). In accordance with the results of our previous study [36], myostatin deficiency significantly increased the expression levels of Gtl2 and IG-DMR (Figure 5B,C). As well as in the case of myostatin deficiency, significant increases in the expression levels of Gtl2 and IG-DMR were also observed after the MOV treatment (Figure 5B,C).
Figure 5. Changes in the expression levels of genomic imprinting-related lncRNAs in skeletal muscle hypertrophy conditions. (A–C) Box-and-whisker plots showing the results of quantitative RT-PCR (qRT-PCR) for H19 (A), Gtl2 (B), and IG-DMR (C) expression in two muscle hypertrophy conditions. Wild; TA muscles of control C57BL/6J mice. MSTN KO; TA muscles of C57BL/6J-background myostatin knockout mouse. Sham; plantaris muscles of sham-operated C57BL/6J mice. MOV; plantaris muscles of mechanical overload (MOV)-operated C57BL/6J mice. qRT-PCR data were normalized to Rpl26 expression and shown as relative expression. \( n = 3 \) per group. * \( p < 0.05 \). ** \( p < 0.01 \). *** \( p < 0.001 \).

We finally performed a correlation analysis between the muscle weight and expression levels of lncRNAs in multiple muscle atrophy and hypertrophy models and found that the changes in skeletal muscle mass showed a positive correlation with the expression levels of Gtl2 and IG-DMR (\( R = 0.88 \) and 0.77, respectively, Figure 6A,B). Although no significant increase in DUM1 expression was observed in myostatin knockout mice, DUM1 expression also showed a positive correlation with the changes in skeletal muscle mass (\( R = 0.76 \), Figure 6C). Thus, these results indicate that the expression levels of Gtl2, IG-DMR, and DUM1 are highly correlated with the skeletal muscle mass.

Figure 6. The expression levels of Gtl2, IG-DMR, and DUM1 show a positive correlation with skeletal muscle weight. (A–C) Pearson correlation plot with a coefficient of correlation (\( R \)) and \( P \)-value (\( P \)) between skeletal muscle weight (fold-change with respect to control) and Gtl2 (A), IG-DMR (B), and DUM1 (C) expression (fold-change with respect to control) in multiple muscle atrophy and hypertrophy models. Each dot indicates the independent samples (\( n = 3 \) per group). Dots with white, blue, red, green, light blue, purple, yellow, and orange indicate the denervated tibialis anterior (TA) muscles of C57BL/6J mice (Den), the casting-operated TA muscles of C57BL/6J mice (Cast),...
the TA muscles of tail suspension-operated C57BL/6J mice (TS), the TA muscles of
dexamethasone-injected C57BL/6 mice (Dex), the TA muscles of C26 tumor-bearing CD2F1 mice (C26),
the TA muscles of fasting C57BL/6 mice (Fast), the TA muscles of C57BL/6J-background myostatin
knockout mice (KO), and the plantaris muscles of mechanical overload (MOV)-operated C57BL/6 mice
(MOV), respectively.

3. Discussion

Recent findings indicate that denervation, immobilization, cancer cachexia, chronic kidney
disease, fasting, and aging affect the expression of Petl1, IncMUMA, Atrohc, and MARI IncRNAs
in the skeletal muscles [48–51]. In addition, the expression of Myoparr and Chronos is increased
by surgical denervation and aging, respectively, and regulates skeletal muscle mass through BMP
signaling [35,52], which antagonizes muscle atrophy [53,54]. Other IncRNAs, namely AK017368,
Charme, and Inc-mg, are also involved in the regulation of skeletal muscle mass through multiple
mechanisms [35–57]. Therefore, IncRNAs have been thought to be new regulators controlling the skeletal
muscle mass. Although the pivotal roles of IncRNAs (DRR, DUM1, linc-MD1, linc-YY1, LncMyod, Neat1, Malat1, and SRA) in skeletal muscle differentiation have been reported [24–33], their involvement in
the regulation of skeletal muscle mass is largely unknown. In this study, we performed expression
profiling of nine IncRNAs, whose expression was related to skeletal muscle di-

expression; however, it remains unclear how these treatments increased the expression

mTOR signaling [35,52], which antagonizes muscle atrophy [53,54]. Other IncRNAs, namely AK017368,
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the regulation of skeletal muscle mass is largely unknown. In this study, we performed expression
profiling of nine IncRNAs, whose expression was related to skeletal muscle differentiation, and three
IncRNAs involved in genomic imprinting, using skeletal muscle atrophy and hypertrophy models.
Comprehensive analysis of the expression of IncRNAs showed no common changes in the expression
during muscle atrophy and hypertrophy situations, suggesting that the expression of these IncRNAs is
complex and is tightly regulated during various muscle atrophy and hypertrophy conditions. However,
we report the following new findings: (1) Muscle atrophy models can be divided into two sub-groups
based on the expression of IncRNAs, especially the expression levels of linc-MD1 and LncMyod; and (2)
the expression levels of Gtl2, IG-DMR, and DUM1 are highly correlated with skeletal muscle mass.
Collectively, these findings suggest that the IncRNAs involved in muscle differentiation and genomic
imprinting might also be involved in the regulation of skeletal muscle mass.

Our cluster analysis of comprehensive expression profiling of IncRNAs classified the six muscle
atrophy models into two sub-groups, namely the disuse-mediated atrophy and systemically wasting
atrophy sub-groups. This classification was mainly dependent on the expression of linc-MD1 and LncMyod;
the expression levels of linc-MD1 and LncMyod were largely increased in the disuse models, including
the denervation, casting, and tail suspension models, but not in the systemically wasting models,
including the dexamethasone-administration, cancer cachexia, and fasting models. During muscle
differentiation, the expression of linc-MD1 and LncMyod is controlled by the MyoD protein [27,28].
Although denervation and immobilization activate Myod1 expression [58,59], considering the fact that
immobilization did not increase the expression of DUM1, linc-YY1, Myoparr, and H19, whose expression
levels are also positively controlled by the MyoD protein during muscle differentiation [29,30,34,60],
molecular mechanisms other than those involving MyoD might regulate the expression of linc-MD1 and
LncMyod in the disuse-mediated muscle atrophy conditions. Denervation and MOV treatments also
increased Myoparr expression; however, it remains unclear how these treatments increased the expression
level of Myoparr. Therefore, future studies investigating factors whose expression changes in common
in denervation and MOV models may reveal the molecular mechanism regulating Myoparr expression in
adult skeletal muscle. Intriguingly, linc-MD1 increases the myocyte-specific enhancer factor 2C (MEF2C)
and mastermind-like protein 1 (MAML1) expression by sponging mir-133 and mir-135 during skeletal
muscle differentiation [28]. Baruffaldi et al. showed that MEF2C promotes the growth of myofibers by
activating Akt/mTOR/S6K signaling [61]. MAML1 promotes the transcriptional activity of MEF2C [62].
Therefore, the increased linc-MD1 expression would work as a compensatory mechanism, attenuating
the muscle atrophy in the disuse conditions by activating MEF2C. By contrast, despite the fact that
IGF2-mRNA-binding protein 2 (IMP2) is required to maintain the skeletal muscle mass [63], LncMyoD
blocks the function of IMP2 in muscle differentiation [27], suggesting that increased LncMyoD expression
might contribute to the cause of muscle atrophy in the disuse situations. The expression levels of
linc-MD1 and LncMyoD were also increased by MOV treatment. Therefore, in that case, linc-MD1 might contribute to inducing muscle hypertrophy and LncMyoD might work as a compensatory mechanism to attenuate muscle hypertrophy, as opposed to the disuse situations. Both linc-MD1 and LncMyoD are not expressed in intact mature muscle [27,28]. The mechanism by which these lncRNAs are expressed in individual muscle components, such as mature myofibers, satellite cells (adult skeletal muscle stem cells), or mesenchymal progenitor cells, during muscle atrophy conditions is unclear. Therefore, further studies examining the exact expression sites of linc-MD1 and LncMyoD in multiple muscle atrophy models would be useful to reveal the roles of these lncRNAs in adult skeletal muscle.

Genomic imprinting-related lncRNAs, Gtl2 and IG-DMR, are located in the Dlk1-Dio3 imprinting locus at chromosome 12qF1 in mice. A paternally inherited DNA mutation of this locus, called the callipyge mutation, results in a 30%–40% increase in the hindlimb muscle mass in sheep through activation of the Dlk1 or Rtl1 gene [64]. On the other hand, a maternally inherited mutation of this locus increases the expression of microRNAs that inhibits Dlk1 expression and counteracts muscle hypertrophy [65,66]. Gtl2 is also maternally expressed from this locus and promotes muscle differentiation by sponging miR-135 in bovine myoblasts [37]. Skeletal muscle defects were observed in mice with the maternal deletion of Gtl2 [67]. IG-DMR is a maternal lncRNA and works like an enhancer RNA that controls gene expression on the maternal chromosome at the Dlk1-Dio3 locus in embryonic stem cells [68]. However, it remains unclear whether Gtl2 and IG-DMR are involved in the regulation of adult skeletal muscle mass. Intriguingly, our data showed that the expression levels of Gtl2 and IG-DMR were increased in two muscle hypertrophy models and were decreased in multiple muscle atrophy models. We and others previously found that the expression levels of lncRNAs at this locus are gradually decreased during muscle growth and aging in mice and pigs [36,69–71]. It is of note that the level of DUM1 expression, which was correlated with the skeletal muscle mass in this study, also gradually decreased during muscle growth and aging in mice [29]. Therefore, although the function of these lncRNAs in the regulation of skeletal muscle mass remains unknown, it is likely that their expression levels are useful as a promising indicator for the skeletal muscle mass.

Besides muscle mass, all of the models used in this study affect several muscle properties. For example, the disuse atrophy (denervation, casting, and tail suspension treatments) and the myostatin deficiency cause a slow-to-fast fiber-type shift [72,73], whereas the systemically muscle wasting (dexamethasone administration, cancer cachexia, and fasting treatments) and MOV treatment lead a fast-to-slow fiber-type shift [72,74]. Moreover, the effect of atrophy and hypertrophy models on the satellite cells is more complex. Denervation and cancer cachexia treatments activate the satellite cells [75,76]. Hindlimb suspension and fasting treatments impair satellite cell proliferation [77,78]. Immobilization decreases the number of satellite cells, whereas it increases the markers of satellite cell activation [79]. Satellite cells play little or no role in muscle hypertrophy induced by myostatin deficiency [80]. Expression levels of oxidative metabolic genes and extracellular matrix components are also affected in these models [81–83]. In addition, the contralateral sham-operated muscles, which are commonly used for the experimental controls in denervation and casting studies [84–88], might undergo hypertrophy to compensate for the immobilized muscles [89]. The proteasome system is also activated in the contralateral-innervated muscles compared with the muscles of non-operated mice [90]. It is also worthwhile to note that relatively low n numbers in each group would result in an increased false-positive change in lncRNA expression. Thus, it cannot be ruled out that the expression levels of lncRNA in our models might be influenced by the changes in these muscle properties and microenvironment.

4. Materials and Methods

4.1. Animals

The C57BL/6J and CD2F1 mice were purchased from the Japan SLC and Charles River Laboratories Japan, respectively. Mice were housed in cages with a constant temperature (24 °C) and a 12-h light:12-h dark cycle and were provided with water and food ad libitum, except during the fasting experiments.
Animal experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of Fujita Health University, Japan (#AP16055, approved on June 24th, 2016). Mechanical overload experiments were approved by the Animal Care and Use Committee of the National Cerebral and Cardiovascular Center in Japan (#16035, approved on March 28th 2016) and were conducted under institutional and national guidelines. All animal experiments were performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki and its later amendments.

4.2. Cell Culture

Cachexia-inducible colon-26 adenocarcinoma (C26) clone 20, described previously [44], was a kind gift from Dr. K. Soda, Jichi Medical University in Japan. C26 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Wako, Osaka, Japan), supplemented with 10% fetal bovine serum (HyClone, GE Healthcare, Little Chalfont, Buckinghamshire, UK), 2 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA), and PS (100 units of penicillin G per mL, 10 µg of streptomycin sulphate per mL, Thermo Fisher Scientific) in an atmosphere of 5% CO₂ at 37 °C.

4.3. Muscle Atrophy and Hypertrophy Models

For induction of muscle atrophy by denervation, a 3-mm segment of the sciatic nerve was excised from the hind leg of each 8-week-old male C57BL/6J mouse under anesthesia, as described previously [34]. Contralateral non-denervated hindlimb served as the control. Seven days after the operation, the mice were sacrificed and the TA muscles were collected, measured, and used for RNA preparation.

Left hindlimb of 8-week-old male C57BL/6J mice was immobilized in plaster casts under anesthesia, as described previously, for the induction of muscle atrophy by casting [91]. The right hindlimb was allowed to move freely and was used as the control. Mice were sacrificed seven days later, and the TA muscles were collected, measured, and used for RNA preparation.

Muscle atrophy was induced by tail suspension as follows: 8-week-old male C57BL/6J mice were suspended for 3 days by their tails, as described previously, such that the hindlimbs could not touch the floor, wall, and the lid of the cage [92]. Three days later, the mice were sacrificed. The TA muscles were then collected, measured, and used for RNA preparation.

Muscle atrophy was also induced by dexamethasone as follows: 7-week-old male C57BL/6J mice were intraperitoneally administrated 25 mg/kg/day water-soluble dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) for 7 days under anesthesia, as described previously [93,94]. Mice administered normal saline were used as controls. The mice were sacrificed, and the TA muscles were collected, measured, and used for RNA preparation after dexamethasone treatment.

Trypsinized C26 cells were washed with PBS and 1 × 10⁶ cells resuspended in 200 µL of PBS were subcutaneously injected into the dorsum of 6-week-old male CD2F1 mice under anesthesia, as described previously [44]. Equal volumes of PBS were injected into age-matched mice in the control group. The mice were sacrificed, and the TA muscles were collected, measured, and used for RNA preparation 14 days after the injection.

For the induction of muscle atrophy by fasting, 8-week-old male C57BL/6J mice were allowed free access to water but were prohibited from eating solid food for 2 days, as described previously [95]. Control mice were provided water and food ad libitum. Next, the mice were sacrificed, following which the TA muscles were collected, measured, and used for RNA preparation.

Myostatin knockout mice, described previously [45], were a kind gift from Dr. S.-J. Lee, Johns Hopkins University (Baltimore, MD, USA). Age-matched C57BL/6J mice purchased from the Japan SLC were used as controls [36]. The TA muscles were collected from 9-week-old male mice under anesthesia. After measuring the weight, the TA muscles were used for RNA preparation.

For muscle hypertrophy model induction using MOV, the soleus and gastrocnemius muscles were surgically removed from 10-week-old male C57BL/6J mice under anesthesia, as described
previously [74]. Sham-operated mice were used as controls. Six weeks after the surgery, mice were
sacrificed and then the plantaris muscles were collected, measured, and used for RNA preparation.

4.4. RNA Purification, Reverse Transcription Reaction, and Quantitative PCR

Total RNA was extracted from the TA or plantaris muscles and purified using the miRNeasy Mini
Kit (QIAGEN, Hilden, Germany) with DNase I (QIAGEN) treatment, according to the manufacturer’s
protocol. One microgram total RNA was subjected to the reverse transcription reaction using
SuperScript III reverse transcriptase (Thermo Fisher Scientific) or Protoscript II reverse transcriptase
(New England Biolabs, Ipswich, MA, USA) with random primers. The quantitative real-time PCR
(qRT-PCR) was conducted using SYBR Premix Ex Taq (Takara, Shiga, Japan). Data were normalized to
Rpl26 expression and shown as relative expression compared to the control samples. The raw data
of qRT-PCR are shown in Supplementary Table S1. The GenBank accession numbers of lncRNAs are
as follows: Myoparr (NR_160520), linc-MD1 (NR_131249), DUM1 (NR_028300), linc-YY1 (AK081464),
Malat1 (NR_002847), Neat1 (NR_131212), SRA (NM_025291), H19 (NR_130973), and Gtl2 (NR_027652).
The full-length sequences of DRR (MUNC) and LncMyoD were described in [26,27], respectively. Since
the full-length sequence of IG-DMR lncRNA has not identified yet, we used the previously described
primer-pairs [68] to detect IG-DMR lncRNA. The primers used are listed in Table 2.

Table 2. Primer sequences used for quantitative RT-PCR.

| Target Name | Forward | Reverse |
|-------------|---------|---------|
| Myoparr     | GTGCCCTATCGTCCATGGAG | CACGACTTCACCTGACCCC |
| linc-MD1    | GCCAGAAAACCACAGGAGG | GTGAAGCTCCTTGAGTTTGG |
| LncMyoD     | CTGAGGACACAAAGGTGCTCT | AACTGAGGCTCCACGTAAGA |
| DRR (MUNC)  | ACTAGTTGCTCAAGTGGTGTGA | TGCTGTTAGTAAATTGACTGATG |
| DUM1        | GGGATGCCAGAAGGTGCTCT | GAGCAGCTGGCTGCTTTG |
| linc-YY1    | AGCTACAGGAAAGTGGGCTTAC | AGGCACAGGCTGGCTTG |
| Malat1      | CAGGGGCAATGCGTGTGA | CGTGCCACGACGATAGAT |
| Neat1       | TTGGGACAGTGGAGCTGGTG | TCAAGTGCCACGACGAGCA |
| SRA         | TCCACCTCCCTTCAAGTGCTGT | GAGCTCAGCTACAGTGCTACA |
| H19         | CTGGGGAATGCCTGGCTG | TAGAGGCTGCTGCTCCAGG |
| Gtl2(Meg3)  | TTGGCAACTTCTCTTGGCAC | AACACACATGAGGCCACTAG |
| IG-DMR      | AGAAGCTGTTGGGATGCT | AGGCGCACCTGGATCAGT |
| Rpl26       | GTCTATGCCATCGGGAAGG | TCTGTCGATGAGATGACGTACT |

4.5. Statistical Analysis

Statistical analyses were performed using unpaired two-tailed Student’s t-tests. A value of \( p < 0.05 \)
was considered statistically significant. Data are presented as box-and-whisher plots and statistical
significance is reported in the figure legends as * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).

4.6. Cluster and Correlation Analysis

The value of the log2 fold change in the expression levels of lncRNAs was converted to a z-score
using R software with the Genefilter package (https://bioconductor.org/packages/release/bioc/html/
genefilter.html). The z-score was used for the cluster analysis of gene expression changes under muscle
atrophy. Correlation analysis was performed by R software using Pearson correlation coefficient.
A value of \( R > 0.7 \) was considered a strong correlation.

5. Conclusions

The expression profiling of 12 lncRNAs, which are related to skeletal muscle differentiation
and genomic imprinting, in multiple muscle atrophy and hypertrophy conditions performed in
this study provides a novel insight about lncRNAs in determining the skeletal muscle mass.
However, further studies including expression analysis of lncRNAs using more suitable models
or animal experiments using conditional transgenic or knockout mice of these lncRNAs are needed to mechanistically relate the changes in skeletal muscle mass to lncRNA expression.

**Supplementary Materials:** Supplementary Materials can be found at http://www.mdpi.com/1422-0067/21/5/1628/s1. Supplementary Table S1: The raw data of qRT-PCR. Figure S1: Changes in the expression of skeletal muscle differentiation-related lncRNAs in six muscle atrophy conditions. Figure S2: Changes in the expression levels of skeletal muscle differentiation-related lncRNAs in skeletal muscle hypertrophy conditions.

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