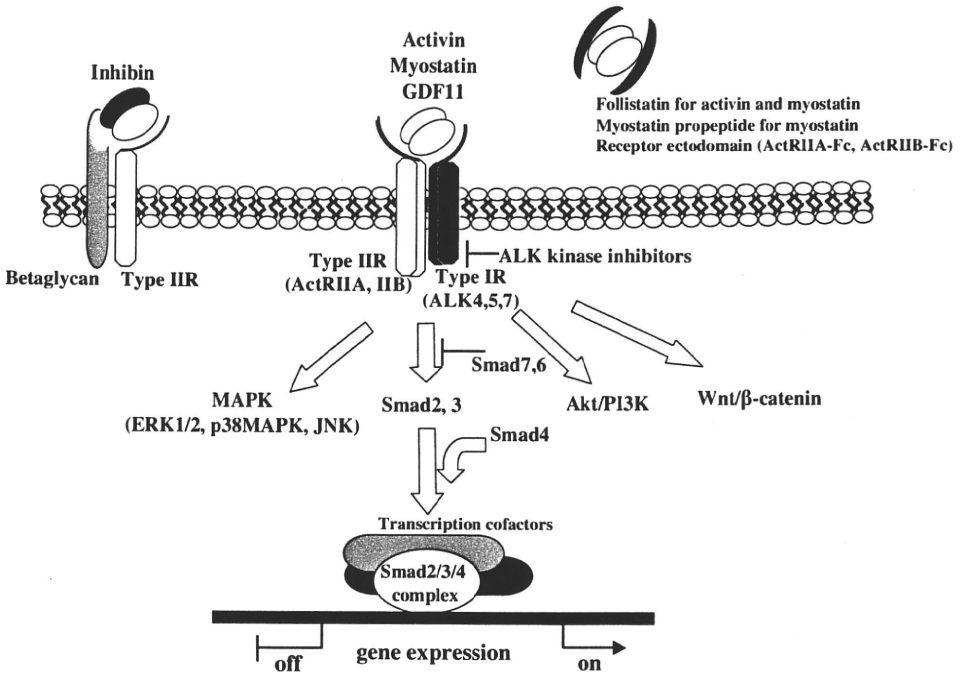

Figure 1

Signal transduction through activin receptors. Activin, myostatin and GDF11 signal through type II and type I serine/threonine kinase receptors. Type IIR is the principal ligand binding receptors, and ligand/typeIIR complexes recruit and associate with type IR. Type IR is phosphorylated and activated by type IIR kinase. Smad2 and 3, activin/TGF-β specific Smads, are phosphorylated by activated type IR. In the nucleus, Smad2/3/4 complexes regulate gene expression with additional transcriptional cofactors. Smad-independent pathway such as MAPK is also activated downstream of activin receptors. Inhibin acts antagonistic to activin by forming high affinity complexes with ActRII and betaglycan. Follistatin, myostatin propeptide and receptor ectodomain inhibit the activities of activin and related factors in the extracellular space to prevent ligand/receptor interaction. Chemical type IR kinase inhibitors act in the cell to disrupt receptor/intracellular signaling.

known as synaptic scaffolding protein, S-SCAM [18]. A recent study showed that activin induces long-lasting NMDA receptor activation by ARIP1 in hippocampal neurons [19]. ARIP2 is a small protein that has one PDZ domain [20]. Several ARIP2 splicing isoforms exist, and, depending on the isoform, ARIP2 either augments or inhibits activin signaling [21]. Gene trapping analysis identified the RasGAP-binding protein Dok-1, which acts downstream of receptor tyrosine kinases as an essential adapter molecule for activin-induced apoptotic signaling in B cells. Dok-1 interacts simultaneously with activin receptors and Smads. Stimulation by activin induces association of Dok-1 and Smad3 [22].

Posttranslational modification of the activin/TGF-β receptor is an additional important mechanism for the regulation of receptor activation. The ubiquitin-proteasome pathway tightly regulates TGF-β family signaling. HECT-type E3 ubiquitin ligases, Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2 have been implicated in Smad degradation. Smurf1 and Smurf2 bind to TGF-β family receptors via the inhibitory Smads, Smad6 and Smad7, to induce their ubiquitin-dependent degradation [23]. In addition, TGF-β type I receptor is sumoylated in response to ligand stimulation. Posttranslational receptor sumoylation, the covalent attachment of a small ubiquitin-like modifier (SUMO) is required for the kinase activities of both the TGF-β type I and type II receptors, and enhances receptor function by facilitating the recruitment and phosphorylation of Smad3 [24].

Regulation of activin signaling through Smads

Smad signaling in the cytoplasm and the nucleus is under tight control. Smads consist of an NH_2-terminal MH1 and a COOH-terminal MH2 domain. The L45 loop of type I receptors directly interacts with the MH2 domain of receptor-regulated Smad (R-Smad), and determines Smad specificity [2]. Type I receptors phosphorylate Smads at their COOH-terminal two serine residues. Smad2 and 3, R-Smads for activin and TGF-β undergo constant shuttling

between the cytoplasm and nucleus, and the activation of R-Smads triggers nuclear accumulation [2]. PPM1A may act as a Smad COOH-terminal phosphatase [25]. Linker regions between MH1 and MH2 domains of Smads are phosphorylated by mitogen-activated protein kinase (MAPK). This phosphorylation enhances the binding of ubiquitin ligase to Smad, resulting in polyubiquitination and degradation [26].

Smads have intrinsic DNA-binding activity [2]. However, to fully activate target genes, Smad physically associates with a diverse set of DNA-binding cofactors such as CBP/p300, TGIF, c-Ski and Evi-1 [11]. This characteristic determines the cell type-specific transcription and complexity of activin/TGF-β signaling. A number of transcription factors including forkhead proteins, bHLH family, AP1 family, homeodomain protein family and nuclear receptors act as Smad-interacting transcription factors [2]. Once activated, Smad complexes recruit additional transcriptional activators or repressors to regulate target genes (Figure 1).

Negative feedback regulation by the inhibitory Smads, Smad6 and Smad7 is an important shutoff system for signaling by the TGF-β family including activins [2,11].

Smad-independent activin signaling and receptor crosstalk

In addition to the canonical Smad pathway, activin signaling through activin receptors regulates other intracellular pathways. p38 MAPK, ERK1/2 and JNK are activated by activin in a cell type-specific manner [27,28]. For example, activin synergizes with basic fibroblast growth factor to activate tyrosine hydroxylase expression via the ERK1/2 pathway [27]. Activin negatively regulates the pituitary transcription factor Pit-1 through p38 MAPK-dependent and Smad-independent pathways [28]. Independently of Smad4, ActRIB/Smad2 acts as a co-activator of the canonical Wnt signaling pathway. Upon activation, Smad2 physically interacts with Tcf4, β-catenin and the co-activator p300 to enhance transcriptional activity of β-catenin/Tcf4 through the histone acetyltransferase activity of p300 [29]. Transactivation by Smad2 is independent of the Smad binding element. Furthermore, recent characterization revealed that TGF-β stimulates phosphorylation of BMP-specific Smad1 independently of BMP receptors [30-32]. Smad-independent activin signaling and receptor crosstalk increase the complexity of activin/TGF-β signaling.

Ligand binding proteins

Extracellular activin-binding proteins control activin signaling [1]. Follistatin (FST) is a prototype of activin-binding proteins. FST is a cysteine-rich single chain glycoprotein that does not possess sequence similarity to the TGF-β family [33]. Structural analysis of FST with activin showed that two FST molecules encircle activin, and neutralize the ligand by burying one-third of its residues and both type II and type I receptor binding sites [34-36] (Figure 1). FST not only binds and inhibits activins, but also binds and neutralizes the actions of myostatin and GDF11 [1,37]. Mice with a disrupted follistatin gene have musculoskeletal and cutaneous abnormalities, reflecting the abnormal signaling of activins, myostatin and GDF11 [38]. The follistatin-related gene, FLRG, is a follistatin domain-containing protein structurally similar to FST [39,40]. Whereas FST has three follistatin domains, FLRG has only two. Like FST, FLRG binds and neutralizes activins, myostatin and GDF11 [37,39]. Proteomics analyses indicate that FLRG associates with myostatin in sera [37]. Although functionally redundant, expression and transcriptional regulation of FST and FLRG are different [39-41]. FLRG gene deleted mice show dysregulated glucose metabolism and fat homeostasis [42](see below).

Biological activities and roles of activin signaling as a target of therapeutic interventions

After the purification and identification of activins as regulators of follicle-stimulating hormone secretion from the anterior pituitary, important roles of activins in the hypothalamus-pituitary-gonadal axis have been described [1]. However, activin activity is not limited to reproductive tissues. Activins and related factors have pleiotropic actions in extragonadal tissues. In this section, we focus on selective actions of activins and related growth factors from a therapeutic point of view.

Activins and their regulators in metabolic disorders

Activin signaling is required for proper development of the endocrine and exocrine pancreas, and dysregulation of the activin signaling pathway contributes to the genesis of metabolic diseases. In human embryonic stem cells, activin B mediates the induction of homeoprotein Pdx1, a key regulator of endocrine pancreas development [43]. ActRIIA mutant mice show hypoplasia of the pancreas and develop diabetes [44]. ActRIIB and Smad2 activity use the same signaling pathway to regulate pancreas islet formation [45]. ALK7, a type I receptor for activin B, activin AB and nodal, is expressed abundantly in pancreatic β cells and adipose tissues, and regulates insulin biosynthesis and secretion [46-48]. Recent characterization revealed that ALK7 transmits signals of GDF3, another TGF-β family member [49,50]. GDF3, ALK7 and co-receptor Cripto are all expressed in adipose tissues, and Gdf3(-/-) null mice and ALK7(-/-) null mice showed reduced fat accumulation and resistance to diet-induced obesity [49,50].

The expression of activin receptors, myostatin and their binding protein FLRG can be modulated in adipose tissue and skeletal muscle by chronic obesity. In subcutaneous and visceral fats, myostatin and ActRIIB mRNA levels in

ob/ob mice are 50- to 100-fold higher than that in wild-type mice [51]. By contrast, FLRG mRNA levels are increased in subcutaneous fat, but decreased in visceral fat of ob/ob mice compared to wild-type mice [51]. In humans, myostatin was shown to increase in skeletal muscle and plasma of obese and insulin resistant women [52].

FLRG gene disrupted mice showed an increased pancreatic islet number and size, β cell hyperplasia, decreased visceral fat mass, improved glucose tolerance, and enhanced insulin sensitivity. This phenotype is caused through increased signaling by activin or myostatin in a tissue-specific manner [42].

Myostatin and activin in muscular diseases

Myostatin, the skeletal muscle specific member of the TGF-β family, restricts muscle growth and determines skeletal muscle mass [5]. Myostatin signals through activin type I receptors (Alk4 and 5) and type II receptors [5]. Mice with a targeted deletion of the myostatin gene have a 25–30% increased muscle mass resulting from hypertrophy and hyperplasia [53]. Double muscling phenotypes upon inactivation of the myostatin gene have been observed in cattle, sheep, race dogs, fish and even in humans [54-59]. Myostatin is regarded as a good drug target since therapeutics that stimulate skeletal muscle growth may be useful for muscle-wasting conditions such as muscular dystrophy, sarcopenia and cachexia. Whereas activins and TGF-β function in almost every cell type, myostatin specifically affects skeletal muscle growth. Thus, targeting myostatin is a rational therapeutic strategy to increase skeletal muscle mass. Several myostatin inhibitors such as monoclonal antibodies and myostatin propeptide, as well as FST and its derivatives are promising candidates for the treatment of muscle wasting disorders [60-67] (Table S2; Additional file 2). Skeletal muscle fibrosis is also ameliorated by myostatin inhibition [68]. The effectiveness of myostatin inhibition has been studied using various muscular dystrophy animal models. Monoclonal antibody-mediated myostatin blockade results in an increase of muscle mass and absolute muscle strength in *mdx* mice, an animal model of Duchenne-type muscular dystrophy [60]. Muscles in *mdx* mice with myostatin inhibition showed less fibrosis, reduced fatty remodeling and an improved regeneration process [61]. Myostatin circulates in the serum in a latent form complexed with multiple binding proteins. NH_2-terminal myostatin propeptide is a major myostatin-binding protein and non-covalently associates with myostatin [5,37]. Myostatin propeptide, stabilized by fusion to IgG-Fc, has been shown to be effective in ameliorating dystrophic pathophysiology [62]. Muscle atrophy caused in mutant caveolin-3 transgenic mice, a model of limb-girdle muscular dystrophy (LGMD) 1C, was reduced dramatically by crossing these mice with myostatin propeptide transgenic mice [63]. In calpain 3-deficient LGMD2A model mice, both muscle mass and muscle force were recovered upon gene therapy using myostatin propeptide [64]. Myostatin blockage at an early stage in a model of δ-sarcoglycan-deficient muscular dystrophy was effective in reducing muscle loss and fibrosis, and in improving regeneration [65]. It is of note that the elimination of myostatin did not suppress the phenotype of a laminin-α2-deficient mice, but increased postnatal lethality due to fat loss [69]. Soluble forms of an extracellular domain of ActRIIB fused with IgG-Fc may block myostatin effectively *in vivo*, and have strong muscle mass increasing activities [70]. In addition to myostatin, activin and GDF11 are recognized by soluble forms of ActRIIB [71]. FST and FST-derived myostatin inhibitors are also effective for increasing muscle mass and ameliorating muscular dystrophy [66,67]. It is worth noting that neurogenic muscle atrophy caused by amyotrophic lateral sclerosis and spinal muscular atrophy may be ameliorated by myostatin inhibition either by myostatin antibody or follistatin [72,73].

The expression of activin, myostatin, TGF-β, activin receptors, and FST in cardiac muscle is also deregulated in pathological conditions such as cardiac failure and cardiomyopathy [74,75]. However, in contrast to the observations in skeletal muscle, myostatin does not counteract cardiac hypertrophy or fibrosis [75].

Roles of activin and BMP signaling in osteoporosis and bone formation

Although both BMP and activin regulate bone formation, their modes of action are distinct. BMPs are potent inducers of osteoblast differentiation. Activins are expressed abundantly in bone tissues, and regulate bone formation by controlling both osteoblast and osteoclast functions. Different from the activity of BMP, activins enhance the receptor activator of NF-κB ligand (RANKL)-mediated osteoclast differentiation, and act as commitment factors for osteoclastogenesis [76]. Both antiresorptive and anabolic drugs are useful for the treatment of osteoporosis [77]. Bisphosphonates, selective estrogen-receptor modulators and estrogen are currently available antiresorptive drugs, whereas recombinant human parathyroid hormone is an anabolic drug. Intriguingly, the extracellular domain of ActRIIA stabilized by fusion to IgG-Fc increases bone mass and strength by activin inhibition, and is a novel promising agent for osteoporosis in early human trials [77,78] (Table S2; Additional file 2).

As mentioned above, the extracellular domain of ActRIIB fused to IgG-Fc increases muscle mass. Thus, two activin type II receptor decoys have different clinical uses. Consistent with the activity of activin in bone formation, inhibin A, an activin antagonist, works as an endocrine stimulator of bone mass *in vivo* by increasing osteoblast-

ogenesis [79]. Inhibin antagonizes activin by forming a complex of ActRIIs and betaglycan [2,4](Figure 1).

Fibrodysplasia ossificans progressive (FOP), a genetic disorder of progressive heterotypic ossification, is caused by missense mutations in ACVR1A (ALK2), a BMP type I receptor, which increase BMP signaling [80]. A recurrent activating mutation in the juxtamembrane GS domain of ACVR1A was reported in sporadic and familial cases of classic FOP [80]. Thus, the activin and BMP pathway are therapeutic targets for the treatment of low bone mass.

Roles of activins and related growth factors in cancer

Inhibition of cancer cell growth is one of the activities of activins in the early phase of cancer development. Facilitating activin signaling either by Cripto silencing or FLRG silencing inhibits human breast cancer cell growth [81,82](Table S2; Additional file 2). Mutations in several genes involved in the activin signaling pathway have been characterized in cancers. Two 8-bp polyadenine tracts of the ACVR2 gene were targets for frameshift mutations in gastrointestinal cancers with microsatellite instability [83]. Somatic ACVR1B gene mutations have been found in pancreatic carcinoma [84] and Smad2 and Smad4 are mutated in colorectal and pancreatic carcinomas [85]. Thus, dysregulation of activin receptors and activin/TGF-β Smads is directly involved in carcinogenesis.

Interestingly, inhibin-deficient mice develop gonadal sex cord-stromal tumors [86]. They develop adrenal cortical tumors when gonadectomized. Therefore, inhibins act as secreted tumor suppressors in gonads and adrenal glands. Supraphysiological levels of activins in inhibin-deficient mice are responsible for the development of tumors. Overproduction of activins was observed in a cachexia-like wasting syndrome that includes hepatocellular necrosis and metastasis [86-88]. Thus, the actions of activin in tumor development are highly context-dependent.

Myofibroblasts present in tumor stroma facilitate tumor development and invasion [2]. TGF-β and activin stimulate the differentiation of myofibroblasts from mesenchymal progenitors, suggesting the facilitation of invasive properties of cancers.

Regarding metastasis, inhibition of activin and/or TGF-β suppresses experimental metastasis to multiple organs including lung, liver and bone [89,90](Table S2; Additional file 2). Chemical inhibitors for type I receptor kinases for activin/TGF-β (ALK4, 5 and 7) are promising cancer therapies [89,91]. They may offer an option for preventing tumor angiogenesis, the motility of cancer cells, fibrosis and metastasis [92].

TGF-β and TGF-β type I receptor are upregulated at the tumor-bone interface and modulate RANKL-dependent osteolysis, and TGF-β inhibition reduces mammary tumor-induced osteolysis [93]. Since activin works as a cofactor for RANKL, similar to TGF-β, activin may modulate osteoclastogenesis in the tumor-bone interaction.

TGF-β produced by cancer cells has immunosuppressive effects, resulting in the evasion of cancers from destruction by the immune system. A novel TGF-β kinase inhibitor reverses this effect, inhibits cell growth and enhances the immunogenicity of cancer cells [94]. Whether activins also act as regulators in immunosuppression in cancers has not yet been determined.

Activities of activins in the brain

Activins and activin receptors are expressed highly in the central nervous system and have crucial roles in neuronal development [95,96]. However, compared with classical neurotrophic factors, our knowledge about the functions of activins in the brain is limited. Importantly, the expression of inhibin βA mRNA, which encodes activin A, is induced by excitatory synaptic input [97,98]. It is induced in granule cell neurons of the hippocampus by high-frequency synaptic stimuli that produce long term potentiation (LTP). This induction is NMDA receptor-dependent [97,98]. Activin increases the number of synaptic contacts by modulating actin dynamics in the spine of the neurons, which may be responsible for the establishment of LTP [99]. This modulation is mediated by the classical MAP kinase cascades via Erk1/2 [99]. Similarly, inhibin βA mRNA is transiently induced in dentate gyrus neurons through NMDA receptor activation after unilateral mechanical brain injury by saline injection [100]. Inhibin βA mRNA is also induced during amygdala kindling, and accurately marks excitatory neurons with synaptic alterations from seizures [101].

Accumulating evidence indicates that activin also has neurotrophic and neuroprotective effects on selective neurons [102]. Treatment with recombinant activin following ischemic injury rescues neurons from damage [103]. Overexcited neurons are protected by the neurotrophic effect of basic fibroblast growth factor, which depends on the induction of activin A [104] (Table S2; Additional file 2). It is also of note that activin and fibroblast growth factor act in synergy in dopaminergic neurons [27].

Neuronal-specific transgenic approaches using the αCaMKII promoter revealed further functions of activins [105,106]. Hippocampal neurons in αCaMKII promoter-driven dominant negative ActRIB transgenic mice were more vulnerable to kainate injection [105]. These mice also showed a reduced NMDA current with an impaired LTP. Reciprocally, activin potentiates NMDA receptor-mediated signaling by forming complexes with activin receptors, NMDA receptors and Fyn on postsynaptic scaffolding proteins [19]. Interestingly, activins tune pre- and

postsynaptic GABAergic transmission affecting anxiety [107]. αCaMKII promoter-driven activin and FST transgenic mice are affected in their anxiety-related behavior by modulation of their postnatal neurogenesis in the subgranular zone of the dentate gyrus in the hippocampus [106]. Infusion of activin into the dentate gyrus of the hippocampus produces an antidepressant-like effect in the forced swim test. Conversely, antidepressants such as fluoxetine and desipramine increase Smad2 phosphorylation [108]. These data suggest that the activin signaling pathway may be a novel target for neuroprotection and psychopharmacological therapy.

Role of activins in embryonic stem cells

Activin A is a potent mesoderm inducer in *Xenopus* embryos, and numerous tissues can be differentiated from *Xenopus* animal cap cells and embryonic stem cells [109]. A sophisticated strategy to differentiate mouse embryonic stem cells into insulin-producing cells or other cell types by activin has been developed [110,111]. Intriguingly, activin signaling is indispensable to maintain self-renewal and the stemness of human embryonic stem cells [111]. Activin signaling sustains the expression of pluripotency-associated genes such as nanog and inhibits BMP signaling, which promotes self-renewal in human embryonic stem cells [112].

Conclusion

Activin signaling as a target for therapeutic intervention

Although activins were first discovered as powerful factors to stimulate follicle-stimulating hormone production from the anterior pituitary, activins act on almost all cell types and have diverse roles. Furthermore, activin receptors are shared by other TGF-β family members such as myostatin, GDF11, nodal and a subset of BMPs. The TGF-β family members are key regulators of myogenesis, neurogenesis and organogenesis, left-right asymmetry and bone formation. Actions of activins through activin receptors and Smads are pleiotropic and context-dependent, and alterations in signaling through activin receptors are the cause of a variety of disorders. In this review, we focused on recently characterized aspects of activin signaling in relationship to metabolic diseases, musculoskeletal diseases, cancers and neuroprotection.

Various strategies have been designed for the inhibition of activin signaling through receptors. Soluble forms of the extracellular domains of activin receptors, FST and related ligand binding proteins, chemical kinase inhibitors for activin receptors, and siRNAs either for ligand or signaling molecules interfere with activin signaling. Intriguingly, histone deacetylase inhibitors or nitric oxide have been demonstrated to inhibit the progression of muscular dystrophy in a mouse model by transcriptional activation of FST [113,114].

In muscle wasting disorders, the inhibition of myostatin is a possible therapeutic strategy. Soluble ActRIIB-Fc, FST and its derivatives, myostatin propeptide, monoclonal myostatin antibodies and myostatin siRNA are myostatin inhibitors that have been shown to be beneficial for preventing muscle loss. Cachexia from cancers and neurogenic muscle atrophy are also targets for myostatin inhibition [72,73,115](Table S2; Additional file 2).

In cancers, activins have multiple roles such as regulation of cancer cell growth, promotion of organ-specific cancer progression and metastasis. Soluble ActRIIA-Fc is a novel promising drug for osteoporosis, cancer-related bone loss and cachexia [77,78,88]. Activin also has neuroprotective functions, and the augmentation of activins may have favorable protective effects on neurons (Table S2; Additional file 2).

Although targeting activin and related factors may become part of future therapies, given the complexity of their action, some side-effects of such therapies are certainly possible. The dysregulation of activin may affect functions of gonads and adipose tissues [4,42]. It is also possible that activation or targeting activin/TGF-β may in some contexts cause uncontrollable tumor growth or detrimental cellular apoptosis [22,86].

Once promising proteins or chemicals targeting activin signaling are discovered, methods of the drug delivery system are important issues for effective treatment. The stabilization of peptides by fusion with IgG-Fc or other stable proteins is a strategy for targeting activin signaling. Delivery of genes by adeno-associated viral vectors is also potentially promising [64,116]. Finally, nanoparticles such as liposomes and atellocollagen are efficient delivery vehicles for siRNA and proteins [117], and may be useful in delivering agents that target activin signaling.

In summary, therapeutic interventions targeted to signaling through activin receptors may provide novel strategies for the development of effective treatments against a variety of diseases.

Abbreviations

TGF-β: transforming growth factor-β; GDF11: growth and differentiation factor 11; ACVR2 or ActRIIA: activin type II receptor; ACVR2B or ActRIIB: activin type IIB receptor; BMP: bone morphogenetic protein; ALK: activin receptor-like kinase; ACVR1B or ActRIB: activin type IB receptor; ACVR1C: activin type IC receptor; BAMBI: BMP and activin membrane-bound inhibitor; PDZ: PSD-95/Discs-large/ZO-1; ARIP: activin receptor interacting protein; NMDA: N-methyl-D-aspartate; MAPK: mitogen-activated protein kinase; FST: follistatin; FLRG: follistatin-related gene; LGMD: limb-girdle muscular dystrophy; RANKL:

receptor activator of NF-κB ligand; FOP: fibrodysplasia ossificans progressive; ACVR1A: activin type IA receptor; LTP: long term potentiation; αCAMKII: α calmodulin kinase II.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MN participated in the analysis of MSTN/activin signaling and muscle diseases. KH participated in the analysis of growth factor signaling and the interaction of growth factors. AU participated in the analysis of skeletal muscle differentiation. YS participated in therapy for muscular dystrophy. HA and KI participated in the functions of activins in the central nervous system. KT conceived of the study, and participated in its coordination. All authors approved the manuscript.

Additional material

Additional file 1

Table S1. Ligand/receptor combination for activin and related factors. *The table provided represents the ligand/receptor combination for activins, inhibins, myostatin, GDF11 and nodal.*

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Additional file 2

Table S2. Activin signaling as a target for therapeutic interventions. *The table provided represents activin signaling as a target for therapeutic interventions and lists the disease, therapeutic strategy, methods and references.*

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Table 1. Ligand/receptor combination for activin and related factors

Ligand	Type II Receptor	Type I Receptor	Coreceptor	Smad
Activin A	ActRIIA, IIB	ALK4, (7)	-	Smad 2,3 with Smad4
Activin B	ActRIIA, IIB	ALK7, (4)	-	
Activin AB	ActRIIA, IIB	ALK4, 7	-	
Inhibin A, B	ActRIIA, IIB	-	Betaglycan	
Myostatin	ActRIIB, (IIA)	ALK5, (4)	-	Smad 2,3 with Smad4
GDF11	ActRIIB, IIA	ALK4, 5	-	
Nodal	ActRIIB, IIA	ALK4, 7	Cripto	Smad 2,3 with Smad4

Note: weak interaction of ActRIIA and ALK4 with myostatin.
weak interaction of ALK7 with activin A.
weak interaction of ALK4 with activin B.

Table 2. Activin signaling as a target for therapeutic interventions

Disease	**Therapeutic strategy**	**Methods**	**Ref**
Muscular dystrophy Muscle atrophy	Increase of muscle mass by myostatin inhibition	Monoclonal MSTN Ab MSTN propeptide Soluble ActRIIB-Fc Follistatin and its derivatives HDAC inh MSTN siRNA	[60, 65, 72] [62, 63, 64] [63, 70] [66, 67, 73, 116] [113] [117]
Osteoporosis	Increase of bone mass by activin inhibition	Soluble ActRIIA-Fc Inhibin A	[78] [79]
Cancer tumor growth	Suppression by activin activation	Cripto silencing FLRG silencing	[81] [82]
cachexia	Activin inhibition Myostatin inhibition	Soluble ActRIIA-Fc Follistatin, MSTN propeptide	[88] [115]
metastasis	Activin inhibition	Follistatin	[90]
angiogenesis and motility	Suppression by TGF-β/activin inhibition	ALK4, 5, 7 kinase inhibitors	[89, 91, 92, 94]
Neuron damage Depression	Recombinant Activin A application	Activin A	[102, 103, 104, 108]

Abbreviations: MSTN, myostatin; HDAC inh, histone deacetylase inhibitor

Wnt4による筋分化促進作用と今後の創傷治癒への展望

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Myostatin（MSTN）は骨格筋形成の過程で一過性に出現し，筋芽細胞の増殖と分化を負に調節し，その機能欠失変異によって過剰な筋肉が形成される。近年，このMSTNがWnt4を抑制し，それにより筋芽細胞の増殖と分化を負に調節している可能性が示唆された。今回，Wnt4をニワトリ胚の肢芽に過剰発現させ，筋分化，特に速筋に対しWnt4が促進的に作用することを確認した。また，C2C12を用いた*in vitro*でのWnt4過剰発現でも同様の結果を得た。このことはMSTNの機能欠失による表現形と同様であり，Wnt4が，MSTNの下流シグナルである可能性が強く示唆される結果となった。
（皮膚の科学，増*11*：*21*－*24*，*2009*）

キーワード：*Wnt4*，*MSTN*，*TGF*-β，ニワトリ胚，筋分化

はじめに

骨格筋形成過程に関与するmyostatin（MSTN）は筋前駆細胞や筋芽細胞の増殖と分化を負に調節する因子であり，筋肉形成に対して抑制的な作用を持つ。MSTNはⅡ型activin受容体であるActRII，およびⅠ型受容体としてALK4，ALK5，ALK7を介してシグナルを細胞内へ伝えており，このMSTNが欠損している動物は過剰な筋肉を持つことが知られている[1]。

近年，MSTNのノックアウトによりWnt4の発現上昇とその結合タンパク質であるsFrp 1，2の発現抑制が起こることが報告された[2]。MSTNはこの経路を負に調節することで筋分化を抑制している可能性が高いと考えられる。Wnt familyによる筋分化への作用は現在盛んに研究されており，Wnt5aやWnt11による筋分化の促進作用などが知られている[3]。しかし，Wnt4による筋分化への作用はあまり知られていない。今回，Wnt4の筋分化に対する作用を*in vivo*，*in vitro*にて評価し，MSTNとの相互関係を検討した。

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実験材料と方法

遺伝子発現系はニワトリASLVのサブグループAに由来するRCASベクター，および発現ベクターpcDNA3.2DESTを用いた。

1．ニワトリ胚への遺伝子過剰発現

ニワトリ胚の発生ステージはHumburger and Hamilton[4]に従った。Wnt4をRCASベクターに組換えてウイルスを調製した。これをステージ15～17のニワトリ胚の予定肢芽領域へmicroinjectorにて注入し，右側のみで過剰発現を行った。ステージ28でwholemount in situ hybridization，ステージ30でsection in situ hybridizationを行いWnt4の発現を確認し，その後，ニワトリ胚をステージ37前後で固定して，5μmの連続切片とし，下腿最大径の部位にてウイルスが感染していない左側の肢芽を対照として筋肉形成をHematoxylin-eosin（HE）染色，Myosin heavy chain（MyHC）免疫染色で評価した。

2．筋芽細胞に対する効果

マウス筋芽細胞株C2C12を用い，Wnt4，およびコントロール群としてEGFPをトランスフェクトして発現し，3日間培養の後細胞を固定し，筋分化に与える効果をMyHC免疫染色にて評価した。C2C12へのDNAの導入はLipofectamine 2000（Invitrogen）を使用した。C2C12は10％ウシ胎児血清を含むD-MEMで継代維持し，トランスフェクション後は2％ウマ血清を含む培地に換えて筋分化を誘導した。

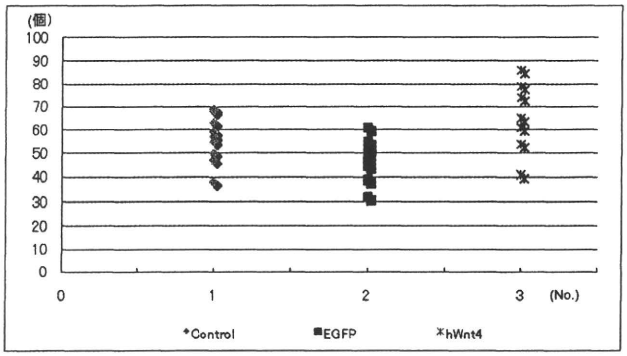

Fig. 1 The number of nuclei/field with fast myosin heavy chain expressing cells in cultures
Effect of overexpression of Wnt4 on fiber-type differentiation in cultured C2C12 myoblasts.

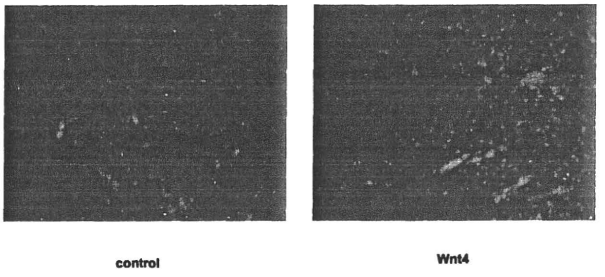

Fig. 2 Immunofluorescent staining against fast myosin heavy chain
Fast myofiber myosin heavy chain is shown in red. Cell nuclei were counterstained with DAPI

3．MSTNを添加することによるWnt4の作用への影響

Wnt4，EGFP，をlenti virusベクターを用いてC2C12（10％ FBS D-MEM）にインフェクションを行い，1日後に2％HS D-MEMに培地を交換し，MSTNを添加した。5日後にMyHC免疫染色を行った。

結　果

Wholemount in situ hybridization，及びsection in situ hybridizationにて，ステージ28ではWnt4の右側肢芽全体での発現が確認され，ステージ30では衛星細胞のマーカーであるPax7の発現も対照の左側肢芽に比べて上昇を認めた。MyoDの発現も同様に処理側で発現上昇が確認された。また，ステージ37の切片において，Wnt4を過剰発現した右側肢芽では対照である左側に比べてHE染色にて有意な筋肥大が見られた。MyHC免疫染色では，遅筋型MyHCによる領域が減少し，速筋型MyHCによる領域が有意に拡大していた。

C2C12を用いた実験においても同様に，Wnt4を発現した群では，EGFP発現群に比べ全体に筋分化が促進しており，特に速筋型MyHCに対し，著明な促進効果を認めた。

MSTNを添加した実験においては，MSTN添加による筋分化の抑制が，Wnt4発現群においてはMSTNもWnt4も加えなかったコントロール群を超えて筋分化が促進する傾向がみられた（**Fig. 1**）。また，この実験においても特に速筋型MyHCに対し，著明な促進効果を認めた（**Fig. 2**）。

考　察

近年，筋萎縮，筋肥大のメカニズムに関し，これを負に調節する因子としてMyostatinが発見された。MyostatinはTGF-βスーパーファミリーに属する分子であり，骨格筋形成の過程で一過性に出現し，筋芽細胞の増殖と分化を負に調整し，その機能欠失変異によって過剰な筋肉が形成される．この作用をブロックすることにより筋ジストロフィーの治療や，癌，AIDS等によるcachexiaの改善，高齢者の筋力低下の改善を得ることができると考えられている。Myostatinを抑制するため，Dominant-Negative Activin receptorやMyostatinに直接結合して機能を阻害するFollistatin，抗Myostatin抗体や，propeptide等，様々な方法が研究されている[5, 6]。

今回，Wnt4の過剰発現にて筋肉の過形成を認めた。その作用は筋線維の中でも速筋型に対してより顕著であった。この効果はC2C12を用いた実験でも同様の結果であり，この結果はこれまでに報告されているMSTN機能欠失による表現型と類似している。MSTNのノックアウトで発現が上昇するWnt4が，MSTNの下流シグナルである可能性を強く示唆させる結果となった。また，Wnt4とMSTNをともに添加した実験の結果より，Wnt4は，MSTNの抑制と同様，もしくはよりダイレクトに筋分化，増殖に作用すると考えられる。

Wntは，現在哺乳動物において約20種類同定されており，多彩な生理作用を有する分泌性タンパク質である。胚発生に伴う形態形成において，様々な局面で時間的，空間的に発現し，動物の胚発生を広く制御しており[7]，また，出生後の細胞に対しても分化，増殖を制御し，恒常性維持に重要な役割を担っていることが知られている[8]。

細胞レベルにおいては，細胞の運命や，極性を決定している。

そのシグナル伝達系には主となるCanonical経路と呼ばれるβ-catenin経路，Non-canonical経路と呼ばれるPCP経路（JNK経路），Wnt/Ca^{2+}経路の3経路が知られており，また，反対にβ-catenin経路に抑制的に働く経路にWntが関与しているものもある[9]。このようにWntのシグナル伝達は複雑なネットワークを構成している[10]。

さらに，近年ではWntシグナルが幹細胞の自己複製に重要な役割を果たすことが報告されており[11]，再生医療への応用も模索され始めている。

また，Wntのシグナル伝達経路の中での異常が関わる疾患は，糖尿病や骨粗鬆症，悪性腫瘍など数多くのものが報告されている[12]。

また，上皮幹細胞の制御機構にWntとTGFβが強く関わっていることが示唆されており，Wntの抑制とTGFβの活性化により，幹細胞の静止期を維持しているのではないかと推測されている[13]。多くの細胞でWntシグナルが細胞増殖を誘導し，TGFβシグナルが細胞増殖を抑制することが報告されており，上皮幹細胞においても同様の働きをしていると思われる。

創傷治癒においてWnt4，Wnt5a，Wnt11が系時的に発現しており，また，創傷でのWnt5a，βcateninの過剰発現にて皮膚附属器の形成が得られることが知られている[14]。また，マウスの創傷においてWntを過剰発現すると再生する毛嚢の数が増加し，Wntシグナル伝達を阻害すると毛嚢形成が阻害されるとの報告もあり[15]，Wntは皮膚の再生のPromote signalである可能性があり，Wntシグナルにて毛嚢新生，皮膚付属器新生を操作できる可能性が示唆される。今後，上皮幹細胞の分化制御機構のさらなる解析により，創傷治癒，美容医療等においてWntファミリーが新たな治療となる可能性があると考える。

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Effect of Wnt4 on Myogenic Differentiation and the Potential Usefulness for Wound Healing

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Key words : *chick embryo, MSTN, Wnt4, TGF-β, myogenesis*

Myostatin (MSTN) is transiently expressed in the developing skeletal muscle, and negatively regulates muscle growth. A loss of function mutation of the MSTN gene is known to result in excess muscle formation with elevated expression of Wnt4. To examine direct effect of Wnt4 on skeletal muscle formation, Wnt4 cDNA was misexpressed in the presumptive limb field of chick embryos using retrovirus vector. Significant increase in muscle mass was observed in the Wnt4-treated limb compared to the control. The area for fast-type myosin heavy chain-expressing cells showed a significant increase, suggesting the possible involvement of Wnt4 during fast-type muscle formation after MSTN knockout.

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Expression Pattern of *WWP1* in Muscular Dystrophic and Normal Chickens

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The WW domain containing E3 ubiquitin protein ligase 1 (WWP1) is classified into one of ubiquitin ligases which play an important role in ubiquitin-proteasome pathway. Previously, we identified the *WWP1* gene as a candidate gene of chicken muscular dystrophy by linkage analysis and sequence comparison. However, the mechanism causing pathological changes and underlaying gene function remains elucidated. In the present study, we analyzed the *WWP1* gene expression in various muscles and tissues of normal chickens, and compared with those from muscular dystrophic chickens. Two mRNA isoforms were detected in all tissues examined and revealed almost equal expression level. The *WWP1* expression of dystrophic chickens was decreased in almost all skeletal muscles including unaffected muscles. These data indicate that there might not be a causal relationship between the alteration of *WWP1* expression level and the severity of muscular dystrophy.

Key words: chicken, expression analysis, fast twitch muscle fiber, muscular dystrophy, WWP1

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Introduction

The WW domain containing E3 ubiquitin protein ligase 1 (*WWP1*) is classified into an ubiquitin ligase (E3) which plays an important role in ubiquitin-proteasome pathway (UPP) to degrade unneeded or damaged proteins (Scheffner and Staub, 2007). E3 recognizes and catalyzes ubiquitin (Ub) conjugation to specific protein substrates (Liu, 2004). Comparative genome analysis reveals few genes encoding E1, tens of E2 encoding genes and hundreds of E3 encoding genes (Semple *et al.*, 2003).

The *WWP1* gene is classified into HECT (homologous to the E6-AP carboxyl terminus)-type E3 which possesses one C2 domain, multiple WW domains and one HECT domain (Pirozzi *et al.*, 1997; Flasza *et al.*, 2002). The C 2 domain binds to the cellular membranes in a Ca^{2+}-dependent manner (Plant *et al.*, 1997) and mediates interactions with other proteins (Plant *et al.*, 2000; von Poser *et al.*, 2000; Augustine, 2001). The WW domain has two conserved tryptophan residues and binds proline-rich region (Sudol *et al.*, 1985). HECT domain, similar to E2s structurally, has a cysteine residue as an active center that transfers the activated Ub from E2 onto first itself, and then onto its substrates (Jackson *et al.*, 2000).

The muscular dystrophies are the group of inherited diseases with progressive weakness and degeneration of skeletal muscle (Partridge, 1991). It is well known that abnormalities of muscle proteins linking sarcolemma and basal lamina lead to cause muscular dystrophies (Lisi and Cohn, 2007), but there are a number of muscular dystrophies and related diseases of which causes are still unknown. We identified *WWP1* gene as a candidate responsible for the chicken muscular dystrophy by the linkage analysis (Matsumoto *et al.*, 2007) and the sequence comparison between normal and dystrophic chickens (Matsumoto *et al.*, 2008). The R441Q missense mutation was found in *WWP1* gene to cause the phenotype of muscular dystrophy.

The WWP1s of human (Flasza *et al.*, 2002; Komuro *et al.*, 2004), mouse (Dallas *et al.*, 2006) and *C. elegans* (Huang *et al.*, 2000) were intensively studied and known

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that the *WWP1* gene is expressed ubiquitously, but strongly in liver, bone marrow, testis and skeletal muscles (Flasza *et al.*, 2002; Komuro *et al.*, 2004). In chicken, however, the *WWP1* expression has not been studied. The expression analysis of *WWP1* gene is important since it was reported that altered expression of known responsible gene could lead dystrophic phenotype (Smythe and Rando, 2006).

In this study, we analyzed the mRNA expression of *WWP1* in various skeletal muscles and other tissues of normal and dystrophic chickens by using Northern blotting and reverse transcription (RT)-PCR analysis to know the differences in the general expression pattern between them.

Materials and Methods

Chickens

A two-month-old dystrophic chicken (New Hampshire: NH-413) and an age-matched normal chicken (White Leghorn: WL-F) were used in this study. The New Hampshire (NH-413) strain is a homozygous dystrophic line introduced from University of California, Davis to Japan in 1976 (Kondo *et al.*, 1982). The disease in this strain is transmitted co-dominantly by a single gene, but the phenotype is modified by other background genes (Kikuchi *et al.*, 1981, 1987; Wilson *et al.*, 1979). The White Leghorn (WL-F) strain was established in 1970s, and maintained as closed colony in the Nippon Institute of Biological Science in Yamanashi, Japan. This study was carried out according to the guidelines of Animal Experimentation of Kobe University.

Expression analysis

For Northern blotting, mRNAs were isolated from *M. pectoralis superficialis* (PS), *M. tensor fascia lata* (TFL), *M. biceps femoris* (BF), *M. triceps surae* (TS), *M. peroneus longus* (PL), heart (H), brain (B), liver (L), kidney (K) and whole embryo (E) with PolyATtract mRNA Isolation kit (Promega, Madison, WI, USA). The 2μg of mRNAs, which were measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), were resolved by 1.2% agarose gel electrophoresis in the presence of formaldehyde and blotted on to Hybond-N+membrane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The mRNAs were visualized using digoxigenin (DIG) reagents, and kits for non-radioactive nucleic acid labeling and detection system (Roche Diagnostics, Basel, Switzerland) according to the procedure specified by the manufacturer excepting that the washing was done with 4×SCC 0.1% SDS at room temperature for 10 min, 4×SCC 0.1% SDS at 40°C for 8 min and then 2×SCC 0.1% SDS at 40°C for 8 min twice. The DIG-labeled DNA probes were prepared by PCR using DIG-dUTP using pectorals cDNA sample of a WL-F strain female as a template. The primers applied in this procedure were 5′-tccctcataaatgttgaaagcagaca-3′ (WWP1p-F), 5′-gtaataacccaaggtaatatgtaaac-3′ (WWP1 p-R) (NM_001012554), 5′-ccgtgtgccaacccccaatgt ctctg-3′ (GAPDHp-F) and 5′ -cagtttctatcagcctctcccacctc- 3′ (GAPDHp-R) (NM_204305). The PCR was done for 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec (*WWP1*) and for 35 cycles at 94°C for 30 sec, 63°C for 30 sec, 72°C for 30 sec (*GAPDH*) using TaKaRa Ex Taq® Hot Start Version (Takara Bio Inc., Tokyo, Japan). Quantitative analysis was performed with Scion Image (Scion Corporation, Frederick, MD, USA).

In order to analyze mRNA expression of *WWP1* gene in the PS, *M. anterior latissimus dorsi* (ALD) and H, RT-PCR method was applied. The concentration of cDNA derived from these muscles was calculated by NanoDrop ND-1000 (NanoDrop Technologies) and commeasurable cDNAs were used as template. The primers applied were 5′-attaggaagagccactgtagact-3′ (WWP1r-F) and 5′-tctgttgattgaggttctgctgt-3′ (WWP1r-R) (NM_001012554). The PCR was done for 35 and 40 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec using TaKaRa Ex Taq® Hot Start Version (Takara Bio Inc.).

Histology

The PS, ALD and H were snap-frozen in liquid nitrogen-cooled isopentane and sectioned in a cryostat (Leica Microsystems Japan, Tokyo, Japan). The histopathology was made by hematoxylin-eosin staining (HE) method (Kikuchi *et al.*, 1981).

Results

The mRNA expression of *WWP1* gene was detected by Northern blotting in various muscles and other tissues of normal and muscular dystrophic chickens (Fig. 1). Two bands were detected in all tissues examined, and revealed almost equally expression level in any muscles and tissues observed.

In the PS, BF, TS, PL, B and K, *WWP1* gene was strongly expressed in normal than in dystrophic chickens (Fig. 1). *GAPDH* was used as an internal control of *WWP 1* expression analysis. In TFL, L and E, similar *WWP1* expression level was observed between two phenotypes (Fig. 1).

RT-PCR analysis indicated that *WWP1* gene was expressed in slow tonic ALD, not only in PS and H of both phenotypes (Fig. 2A). Figure 2B shows histopathological changes in PS, ALD and H of normal and dystrophic chickens. The pathological findings in dystrophic PS were characterized by the degenerating fibers with many vacuoles in cytoplasm, the fatty infiltration into connective tissue, and the proliferation of nuclei within muscle fibers with large variation in sizes. However, no such lesions were observed in ALD and H from age-matched dystrophic chickens (Fig. 2B).

Discussion

Northern blotting with *WWP1* specific probe detected two bands in all tissues and muscles examined (Fig. 1). Northern blot analysis of *WWP1* expression in human tissues also exhibited two bands (Mosser *et al.*, 1998), and RT-PCR analysis showed that human *WWP1* gene had at

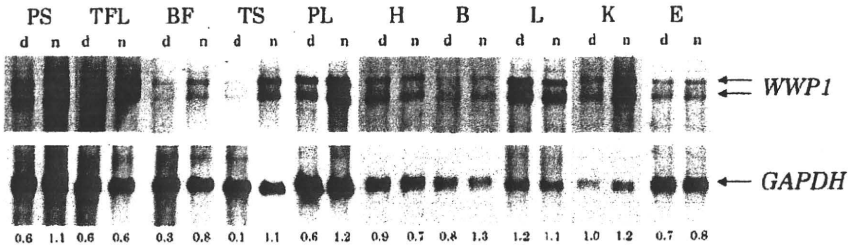

Fig. 1. **Expression of chicken *WWP1* in various tissues.**
A *WWP1* cDNA probe was used to detect *WWP1* mRNA transcripts by Northern blotting using blots containing 2μg of mRNAs from chicken muscles or various other tissues. *M. pectoralis superficialis* (PS), *M. tensor fascia lata* (TFL), *M. biceps femoris* (BF), *M. triceps surae* (TS), *M. peroneus longus* (PL), heart (H), brain (B), liver (L), kidney (K) and embryo (E) were analyzed. A doublet band is detected at variable levels in all tissues. "d" indicates mRNAs from dystrophic chickens. "n" indicates mRNAs from normal chickens. The numbers below the *GAPDH* bands represent the relative ratios of *WWP1/GAPDH*.

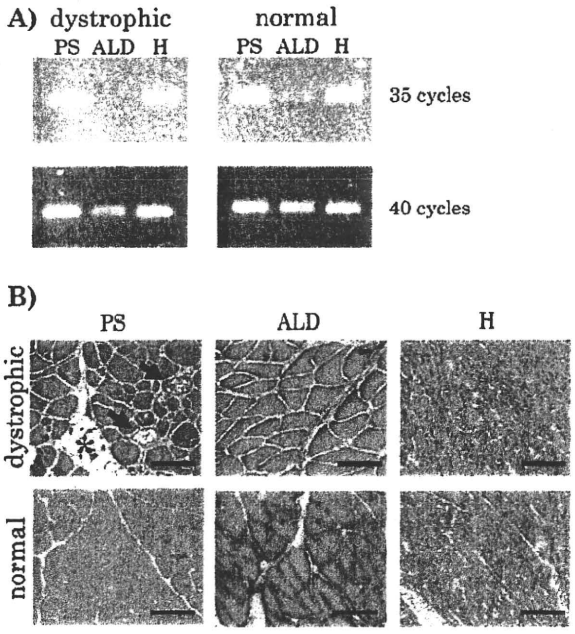

Fig. 2. **RT-PCR detection of *WWP1* gene and histological analysis for three representative muscle types.**
M. pectoralis superficialis (PS), *M. anterior latissimus dorsi* (ALD) and heart (H) expressed *WWP1* less in muscular dystrophic chicken, but only dystrophic PS was severely harmed. A) Expression of *WWP1* in PS, ALD and H was analyzed by RT-PCR method. PCR was performed for 35 or 40 cycles. B) The PS, ALD and H of dystrophic (NH-413) and normal (WL-F) chickens were analyzed with HE staining. Vacuoles (arrows) and fatty infiltration (asterisk) are observed in PS of dystrophic chickens. It is also remarkable that, in dystrophic PS, many muscle fibers have many nuclei in cytoplasm and vary widely in size. These pathological features are not observed in ALD and H of dystrophic chicken. Scale bar=120μm.

least six mRNA isoforms synthesized through the alternative splicing, two of which were strongly expressed and commonly observed in various tissues (Flasza *et al.*, 2002). The mRNA doublet bands of chicken *WWP1* by Northern blot analysis might be equivalent to two bands of human tissues, while a single band was observed by RT-PCR analysis in chicken (Fig. 2A), suggesting that the amplified region does not include alternative spliced site. Flasza *et al.* (2002) also mentioned that the relative ratio of these isoforms from human *WWP1* varied in a tissue-specific manner, but the doublet bands of chicken *WWP1* were expressed almost equally in all tissues examined.

The *WWP1* gene expression in *M. pectoralis superficialis* (PS) of dystrophic chicken was less than that of normal chicken (Fig. 1). The PS of chicken is a fast twitch muscle composed of two types of fast twitch fibers (aW and bW). TFL, BF, TS and PL muscles from wing and leg are mixed muscles co-existing fast twitch (aW and bW) with slow twitch fibers (bR) in a mosaic pattern (Ashmore and Doerr, 1971a), except that the ALD and *M. adductor magnus* are composed of slow tonic fibers (ST) innervated multiply (Ashmore *et al.*, 1978; Kikuchi *et al.*, 1986). In chicken muscular dystrophy, fast twitch fibers are initially and most severely affected, while slow twitch and slow tonic muscles persist relatively harmless throughout the life span (Ashmore and Doerr, 1971b; Barnard *et al.*, 1982). The *WWP1* expression in dystrophic BF, TS and PL showed a similar downward trend as observed in dystrophic PS (Fig. 1). These data indicate that there might not be a causal relationship between the alteration of *WWP1* expression level and the severity of muscular dystrophy, since not only affected muscles but unaffected ones exhibited the same pattern. Moreover, the alteration of *WWP1* expression level was observed in other unaffected tissues, such as B and K, which reinforces our hypothesis that the alteration of *WWP1* expression levels

does not link directly to the dystrophic phenotype (Fig. 1).

To assess the genetic influence of mutant *WWP1* upon chicken muscular dystrophy, we examined *WWP1* gene expression and histological changes in three distinct muscle types, PS as a fast twitch type, ALD as a slow tonic type, and H as a different type of muscle. RT-PCR was applied to this study since ALD was not enough quantity of mRNA for Northern blotting. The *WWP1* mRNA expression was confirmed in all muscles examined (Fig. 2 A).

Figure 2B shows HE stained sections of PS, ALD and H from normal and dystrophic chicken. The dystrophic PS was severely affected, while ALD and heart of dystrophic chicken remained relatively intact (Fig. 2B) as described in a previous study (Kikuchi *et al.*, 1981). The *WWP1* was expressed even in unaffected muscles and the downward alteration of *WWP1* expression was observed commonly in almost all dystrophic muscles examined (Figs. 1, 2). The observation suggests that the alteration of *WWP1* might not be the cause of the pathological change in chicken muscular dystrophy. Hence, the mutation identified previously (Matsumoto *et al.*, 2008) might play a crucial role in leading the onset of chicken muscular dystrophy. The detected mutation lay between WW domains, highly conserved region among tetrapods (Matsumoto *et al.*, 2008), which has been predicted as substrate binding region (Pirozzi *et al.*, 1997; Flasza *et al.*, 2002). This suggests that mutated *WWP1* could not recognize its substrates.

Many HECT-type E3s with WW domains including *WWP1* regulate membrane proteins (Chen and Matesic, 2007). Therefore, aberrant regulation of membrane protein may lead the onset of chicken muscular dystrophy. For example, *WWP1* could bind to β-dystroglycan, which is one of important muscle proteins consisting of membrane (Pirozzi *et al.*, 1997). Abnormal glycosylation of α-dystroglycan in chicken muscular dystrophy has been reported (Saito *et al.*, 2005). Furthermore, the fact that some E3s can recognize sugar chain (Yoshida *et al.*, 2002, 2003; Lederkremer and Gliskman, 2005) leads to the hypothesis that mutated *WWP1* might not be able to recognize the sugar chain of α-dystroglycan to regulate the glycosylated molecules, and that insufficiently glycosylated α-dystroglycan accumulates and causes the disease.

In the present study, we analyzed the mRNA expression of *WWP1* in various skeletal muscles and other tissues of normal and dystrophic chickens. The results suggest that *WWP1* expression level lowered in dystrophic phenotype is not directly related to the cause of disease in chicken muscular dystrophy, whereas mutated *WWP1* does not function normally to cause the onset of chicken muscular dystrophy.

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