의 학 박 사 학 위 논 문

COMP-Ang1 accelerates regeneration of ischemia-damaged skeletal muscle through dual regulation

:up-regulation of myogenic factor synthesis via N-cadherin signaling in muscle cells and recruitment of Tie2-expressing monocytes/macrophages releasing myogenic factors

COMP-Ang1의 허혈손상을 받은 다리에서 근육재생을 촉진하는 두 가지 기전에 관한 연구

2013년 2월

서 울 대 학 교 대 학 원

의 학 과 분 자 유 전 체 의 학 전 공

윤 석 원

의학박사 학위논문

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이 논문을 의학박사 학위논문으로 제출함

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COMP-Ang1 accelerates regeneration of ischemia-damaged skeletal muscle through dual regulation

:up-regulation of myogenic factor synthesis via N-cadherin signaling in muscle cells and recruitment of Tie2-expressing monocytes/macrophages releasing myogenic factors

by
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Abstract

Angiopoietin-1 (Ang1), one of the major angiogenic factors, is known to modulate vascular stability, endothelial cells differentiation and survival. However, the direct effect of Ang1 on myoblasts differentiation and the regulatory mechanism remain unclear.

In this study, I analyzed the regulatory mechanism of COMP-Ang1 on myoblasts differentiation and found N-cadherin as its receptor required for skeletal muscle regeneration. Tie2 expressing macrophages (TEM), infiltrated by COMP-Ang1, were also found in ischemic muscle for myogenesis acceleration.

The ischemic stress in hindlimb stimulated the deterioration of muscle quality and induced the infiltration of inflammatory cells. Interestingly, COMP-Ang1 contributed not only to increase of limb salvage but also significantly to generate regenerating muscle. COMP-Ang1 also increased myotube formation *in vitro*. Especially, COMP-Ang1 enhanced the expression of myogenin, which is one of important myogenic transcription factors. The mechanism of myogenin synthesis by COMP-Ang1 was regulated by p38MAPK and Ca^{2+} ion. Interestingly, COMP-Ang1 bound to myoblast surface through N-cadherin but not by M-cadherin and Integrin β 1. COMP-Ang1 increased the

p38 MAPK activation through directly binding to N-cadherin and augmented the interaction between N-cadherin and p120-catenin. Moreover, myoblasts that had down-regulated N-cadherin expression by siRNA could not develop myotube despite of existence of COMP-Ang1 *in vivo*.

On the other hand, COMP-Ang1 enhanced the infiltration of macrophages into ischemic muscle compared to control group. Especially, COMP-Ang1 increased the infiltration of TEM ($F4/80^{+}Tie2^{+}CD31^{-}$) which expresses IGF1, a known myogenic factor, compared with inflammatory macrophage ($F4/80^{+}Tie2^{-}CD31^{-}$, InfM). TEM isolated from ischemic muscle significantly enhanced the myogenesis of cocultured myoblasts more than both non-treated and InfM groups.

Therefore, these results suggest that COMP-Ang1 is a myogenic factor, which acts directly through N-cadherin-p120-catenin-p38MAPK-myogenin axis in myoblasts, and indirectly stimulates TEM recruitment into ischemic muscle to augment acceleration of myogenesis process.

Keywords: Myogenesis, COMP-Ang1, N-cadherin, myoblasts, Tie2 expressing myogenic macrophage

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Ang1 (angiopoietin-1)

COMP (the short coiled-coil domain of cartilage oligomeric matrix protein)

COMP-Ang1, cAng1 (COMP-angiopoietin-1)

Myog (myogenic factor 4, Myogenin)

MyoD (myogenic differentiation 1)

MyHC (myosin heavy chain)

N-cadherin (neural cadherin, CDH2)

DTSSP (3,3'-dithiobis[sulfosuccinimidylpropionate])

EGTA (ethylene glycol tetraacetic acid)

GFP (green fluorescence protein)

GFP-MB (GFP expressing myoblast)

F4/80 (pan-macrophage marker)

Tie2 (tyrosine kinase endothelial receptor 2)

CD31 (platelet endothelial cell adhesion molecule, PECAM1)

IGF1 (insulin-like growth factor 1)

PAX7 (paired box protein-7)

TEM (Tie2 expressing myogenic macrophage)

InfM (inflammatory macrophage)

ECs (endothelial cells)

EPCs (endothelial progenitor cells)

Introduction

Aging, muscle dystrophy (1, 2), obesity (3), and ischemia (4, 5) induce muscle degeneration. Therefore, the regeneration of muscle stem cells is the most important process for myogenesis in disease muscle.

Several researches have approached to enhance progression of myogenesis with myogenic stimulating factors such as IGF1, FGF, Wnt, cell-extracellular matrix, and cell-to-cell junction (6).

More specifically, cell-to-cell junction via N-cadherin activation is one of the known myogenic enhancing processes (7). The five repeated cadherin domains in N-cadherin extracellular domain bind to several calcium ions and induce stabilization of the cadherin-cadherin junction (8). N-cadherin activation initiates myogenesis through its interaction with p120-catein, and p38MAPK activation (9).

On the other hand, the anti-inflammatory macrophages promote myogenesis in inflammatory muscles induced by notexin (10). The anti-inflammatory macrophages are converted from inflammatory macrophages and releases myogenic-related cytokines such as IL-10 (10).

In embryogenesis, the general function of Angiopoietin-1 (Ang1) is to maintain vessel constructs during vessel differentiation (11) and augment

non-leaky vessel formation through the stabilization of VE-cadherin in contrast to vascular endothelial growth factor (VEGF) (12). Ang1 is produced from pericytes, smooth muscle cells, myoblasts, and muscle stem cells and binds to Tie2 receptor, which is the endothelial receptor-tyrosine kinase receptor (13-15). COMP-Angiopoietin-1 (COMP-Ang1) is modified form of Ang1 to enhance its activity (16). Our research teams have also investigated function of COMP-Ang1 for application of vascular and heart diseases (17-20).

However, the direct effect of Ang1 on myoblasts differentiation and the regulatory mechanisms remain unclear.

In this study, I explained the mechanism of COMP-Ang1 for myogenesis through the dual regulation. First, COMP-Ang1 induced the generation of center nucleus fibers, which was known to regenerating muscle fibers (21). COMP-Ang1 directly enhanced differentiation of myoblasts to myotube through p38MAPK activation and up-regulated myogenin synthesis. Interestingly, the signaling mechanism was initiated by direct binding of COMP-Ang1 and N-cadherin on myoblasts surface via Ca^{2+} ion. COMP-Ang1 also enhanced interaction between N-cadherin and p120-catenin. Moreover, N-cadherin knockdown myoblasts were not able to develop

myotubes in spite of COMP-Ang1 existence. In addition, COMP-Ang1 stimulated muscle regeneration through augmenting macrophages infiltration in ischemic damaged muscle. COMP-Ang1 recruited Tie2 expressing macrophages ($F4/80^{+}Tie2^{+}CD31^{-}$, TEM) into ischemic muscle. TEM infiltrated by COMP-Ang1 was able to induce myogenesis of myoblasts through paracrine effects such as insulin like growth factor-1 (IGF1).

Therefore, these findings suggest that COMP-Ang1 contributes to myogenesis in damaged muscle through direct modulation of COMP-Ang1/N-cadherin/120-catein/p38MAPK/myogenin axis in myoblasts and by recruiting TEM into ischemic muscle for new muscle regeneration.

Materials and Methods

Cell cultures

The mouse C2C12 myoblasts, which were kindly provided from professor Jong-Wan Park, Seoul national university hospital, were cultured with DMEM (Gibco) contained 10% FBS (Lonza) and antibiotics (Gibco). To develop myoblasts, cultured media were changed to differentiation media (DMEM contained 2% FBS, DM) every each other day.

HEK293A cells (Q.Bio) and HEK293T cells (ATCC) were cultured with DMEM contained 10% FBS and antibiotics for amplification of adenovirus and lentivirus.

Isolation of TEM and InfM, and coculture with C2C12 myoblasts

To isolate macrophages from ischemic limb, the ischemic gastrocnemius muscle was enzymatically digested with 0.1% collagenase type I (Gibco) in DMEM for 45min at 37℃. Single fibers and non-digested muscle mass were separated by gravity with cold PBS and supernatant was carefully transferred to new tube. The supernatant was meshed with 40μm strainer (Falcon). These cells were sorted between Tie2 expressing macrophages (TEM) and

inflammatory macrophages (InfM) by BD FACS aria III. The sorted cells ($5X10^4$ cells) were cultured with C2C12 myoblasts ($5X10^4$ cells) in myoblast differentiation medium with 0.4μm insert (Falcon) for myogenesis evaluation.

siRNA treatment for knockdown of specific target gene

To suppress N-cadherin, M-cadherin, and Integrin β 1, siRNA compound (40nM, Santacruz) was incubated with Metafecten pro (Biomax) in PBS for 15 min at room temperature. These mixtures were treated to flouting C2C12 myoblasts ($1X10^5$ cells/ml) and seeded on 35mm dish. The knockdown effect of siRNA was confirmed by western blot after 48 hours.

Chemical inhibition on myoblast

To inhibit p38 MAPK signaling, 10 nM of SB203580 (A.G. Scientific) chemical inhibitor was used and 2mM ethylene glycol tetaacetic acid (EGTA, Sigma) for calcium ion chelating. To confirm myogenin expression, the chemical inhibitor was removed after 4 hours because the chemical inhibitor could be harmful to myoblast survival during overnight.

Western blot and Immunoprecipitation

Western blot: Cells were washed twice with cold PBS and harvested with RIPA buffer (50mM Tris (pH8.0), 150mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, and 10mM β-glycerophosphate with 0.1M NaF, 1mM orthovanadate, and protease inhibitor cocktail (Roche)). Protein concentration was determined by BCA protein assay kit (Pierce). Western blot was performed with the following antibodies: anti-α-tubulin (Oncogene), anti-Myogenin, anti-MyoD, anti-N-cadherin, anti-M-cadherin, anti-Integrin β 1, anti-phospho-p38MAPK (Thr180/Tyr182), anti-total p38MAPK, anti-phospho-ERK (Thr202/Tyr204), p120-catenin (Santacruz), anti-total AKT (BD translation), anti-phospho-AKT (Ser473), anti-total ERK, anti-phospho-c-Jun (Ser63), anti-total c-Jun (Cell signaling), and anti-skeletal myosin heavy chain (MyHC, Sigma).

Immunoprecipitation: Immunoprecipitation was performed with immunocruz (Santacruz). Immunocruz beads were incubated with anti-N-cadherin antibody for overnight at 4℃. Total protein (1mg) and the beads mixture were incubated for 90 min with gentle rotation at 4℃, and then the beads were washed several times with lysis buffer. To evaluate interaction between hN-cadherin-Fc (R&D) and COMP-Ang1, hN-cadherin-Fc (1μg/ml) was incubated with immunocruz beads in PBS (contained with Ca^{2+} and Mg^{2+} ions)

for overnight at 4℃, and then COMP-Ang1 (200ng/ml) was incubated with or without EGTA for 30 min. The complexes were performed to protein-protein cross link by DTSSP for 1 hour at 4℃. The prepared proteins were separated on SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (0.45μm, Millipore). The membrane was blocked with 3% BSA and incubated with the indicated antibodies overnight at 4℃. Signal was detected with ECL or ECL-plus (GE healthcare).

Real time polymerase chain reaction (realtime PCR)

Total RNA (1~2ug) was isolated by Trisure (Bioline) and cDNA was generated with reverse transcription kit (TAKARA). Realtime PCR was performed with Sybergreen (Roche) by ABI 7500 (AB scientific co.). The primer sequences were described to table 1. [Myogenin, N-cadherin, M-cadherin, E-cadherin, VE-cadherin, Cadherin 11, Integrin β 1, Integrin β 2, Integrin β 3, Integrin β 4, angiopoietin-1, angiopoietin-2, Tie2, TNF-α, IGF1, and 18S RNA].

Table 1. The mouse primer sequence for realtime PCR.

Primer name		Sequence	Size
18S RNA	Forward	5'-GTAACCCGTTGAACCCCATT-3'	151bp
	Reverse	5'-CCATCCAATCGGTAGTAGCG-3'	
Myogenin	Forward	5'-GAGCGCGATCTCCGCTACAGAGG-3'	380bp
	Reverse	5'-CTGGCTTGTGGCAGCCCAGG-3'	
N-cadherin	Forward	5'-GGTGGAGGAGAAGAAGACCAG-3'	72bp
	Reverse	5'-GGCATCAGGCTCCACAGT-3'	
M-cadherin	Forward	5'-CTTGGGTGCCACGGATGA-3'	160bp
	Reverse	5'-ATGCAGGCCCTCGGAGAC-3'	
E-cadherin	Forward	5'-CCCGGGACAACGTTTATTAC-3'	72bp
	Reverse	5'-GCTGGCTCAAGTCAAAGTCC-3'	
VE-cadherin	Forward	5'-TCAACGCATCTGTGCCAGAG-3'	116bp
	Reverse	5'-CACGATTTGGTACAAGACAGTG-3'	
Cadherin 11	Forward	5'-CACAGGATGGTGTGGTGAAG-3'	170bp
	Reverse	5'-AGGCTCATCGGCATCTTCTA-3'	
Integrin β1	Forward	5'-CTGATTGGCTGGAGGAATGT-3'	173bp
	Reverse	5'-TGAGCAATTGAAGGATAATCATAG-3'	
Integrin β2	Forward	5'-AGTTCGACTACCCATCCGTG-3'	161bp
	Reverse	5'-GTTGCTGGAGTCGTCAGACA-3'	
Integrin β3	Forward	5'-CTGTTACAATATGAAGAATG-3'	177bp
	Reverse	5'-TTTTCATCACATACTGTAGC-3'	
Integrin β4	Forward	5'-TGTGTTCCAGGTGTTTGAGC-3'	507bp
	Reverse	5'-TTTCTCATCATTGCGGTTCA-3'	
Angiopoietin-1	Forward	5'-CTCGTCAGACATTCATCATCCAG-3'	138bp
	Reverse	5'-CACCTTCTTTAGTGCAAAGGCT-3'	
Angiopoietin-2	Forward	5'-TCCAAGAGCTCGGTTGCTAT-3'	114bp
	Reverse	5'-AGTTGGGGAAGGTCAGTGTG-3'	
Tie2	Forward	5'-GAGGACAGGCTATAAGGATACG-3'	281bp
	Reverse	5'-GGTGAACAGGTTTCCTCCTAT-3'	
TNF-α	Forward	5'-TTCCAGATTCTTCCCTGAGGT-3'	254bp
	Reverse	5'-TAAGCAAAAGAGGAGGCAACA-3'	
IGF1	Forward	5'-TGCTCTTCAGTTCGTGTG-3'	143bp
	Reverse	5'-ACATCTCCAGTCTCCTCAG-3'	

Adenovirus amplification

Adenovirus β-gal and COMP-Ang1 were infected to HEK293A cells for amplification. Adenovirus purification was used to CsCl (Sigma) gradient ultracentrifuge. Adenovirus COMP-Ang1 was kindly provided from Professor Gou-Young Koh, KAIST.

GFP positive myoblast generation by lentivirus

GFP expressing myoblasts were generated by lentivirus, which encode GFP. pLL3.7 (Addgene) encoded GFP expression with CMV promoter. For generating lentivirus, plasmid complexes which contained pLL3.7, pLp1, pLp2, and pLp3 (Invitrogen) were incubated with polyethylenimine (PEI, Polyscience) for 15min at room temperature and then theses mixture was added into 293T cells. After 2 days, the supernatant containing lentivirus was added in C2C12 myoblasts for 2 days. GFP positive myoblasts were sorted by FACS aria III and selected through single cells culture methods.

Non-permeable protein-protein cross-linking by DTSSP

Myoblasts were suspended by trypsinization and cultured with COMP-Ang1 in DM for 1hour at 4℃. The cells were treated with 2mM 3,3'-dithiobis

[sulfosuccinimi-dylpropionate] (DTSSP, Pierce) and incubated for 2 hours on ice for protein-protein cross-linking. DTSSP activity was stopped by 20mM Tris buffer (pH7.4) (22, 23).

Fluorescence-Activated Cell Sorter (FACS) analysis

To evaluate adhesion capacity of COMP-Ang1 on C2C12 myoblasts surface, the cells were stained with anti-Flag (Sigma) because COMP-Ang1 was a fusion protein with Flag on its N-terminal. To assess the infiltrated cells in ischemic muscle, the isolated cells from ischemic muscle were incubated with anti-IGF1 (Santacruze), anti-F4/80-APC, anti-Tie2-PE, and anti-CD31-FITC (eBioscience). In cytosolic FACS, the harvested cells were fixed and permeabilized by chilled 90% MeOH at -20℃. The COMP-Ang1 binding ability and the infiltrated cells into ischemic muscle were analyzed by BD FACS Canto II and sorted by BD FACS Aria III.

Animal experiments

All animal experiments were performed under approval from Institutional Animal Care and Use Committee (IACUC) of Seoul National University Hospital. Male C57/BL6 mice were anesthetized with a 1:1 mixture of

Rompun (10mg/ml) and Zoletil (30 mg/kg) by intraperitoneal injection. Ischemic condition was generated by removing a unilateral femoral artery as previously described (19). Mice were intramuscularly injected with adenovirus COMP-Ang1 ($1x10^8$pfu, COMP-Ang1 group) and with adenovirus β-gal (control group) after the sugary.

To evaluate recovery foot force, ischemic foot and non-ischemic foot were applied with ink. The mouse was walked on paper through tunnel. The foot print results were accessed to score (0 to 4) of recovering foot.

For calculating the muscle volume on ischemic limb, the non-ischemic limb and ischemic limb volume were measured with venire caliper. The ischemic muscle volume was representative of ischemic limb/non-ischemic limb ratio.

To evaluate the effect of COMP-Ang1 on muscle recovery, the ischemic gastrocnemius muscles were harvested dependent on time (1, 3, and 7 days) and made frozen or paraffin block.

To assess transplanted cells efficiency for myogenesis, GFP-MB ($1X10^6$cells) were injected with PBS into gastrocnemius muscle. After 3 days, the transplanted mouse was injected with adenovirus β-gal or COMP-Ang1 ($1X10^8$pfu). The transplanted muscle was harvested after 7 days. The harvested muscle was fixed by 10% neutral formaldehyde for 24 hours to

generate paraffin block. The fixed tissue was processed to dehydration and embedded with paraffin. For generating optimal cutting temperature (OCT) block, the tissue was soaked with OCT compound (Sakura) for 10min in the embedding mold and the mixed compound was solid on aluminum foil in liquid nitrogen vapors during freezing media harden.

Hematoxylin and eosin (H&E) and Masson's trichrome (MT) staining

6-8μm sliced paraffin slides were performed to deparaffination following general step. The slides were stained with eosin (Sigma) at 10 min and washed with D.W. Slides were incubated with hematoxylin (Sigma) for 3min and washed with D.W. For MT stain, the deparaffined slides were processed with MT kit (Sigma). The slides were observed with microscopy (Olympus BX50).

Immunohistochemistry staining

The antigens were retrieved by retrieval solution (DAKO) and then slides were incubated with anti-myogenin (1:200), and anti-GFP (1:200, Santacruz). Immunohistochemistry was performed with HRP ready to use kit (Vector) and

chromatin was used to DAB (brown, Vector) and NovaRED (red, Vector). Nuclear stain was used to hematoxylin. The slides were observed with microscopy (Olympus BX50).

Immunofluorescent staining

C2C12 myoblasts were cultured on 8 well 15μ-Slide Ibidi (Ibi Treat). These cells were fixed by 4% PFA (Paraformaldehyde, WAKO) at room temperature for 10 min and blocked with 1% BSA (Gibco) contained 0.05% Triton X-100 (Sigma) at room temperature for 1hour. The fixed cells were incubated with anti-skeletal myosin heavy chain (MyHC, 1:100), anti-N-cadherin (1:100) and anti-p120-catenin (1:100) during overnight at 4℃ and incubated anti-mouse-Alexa 555, anti-rabbit-Alexa488 (1:1000, Molecular probe) for 45min at room temperature.

6~12μm sliced OCT slides were used to confirm infiltrating macrophages in ischemic muscle. The slides were washed with PBS and treated to permeabilization with 1% BSA contained 0.1% Triton X-100 for 30 min. Anti-F4/80 (1:50) were incubated in antibody dilution solution (Zymed) during overnight at 4℃ and incubated with anti-rat-Alexa 555 (1:1000, Molecular probe) for 45 min at room temperature. Nuclear was stained by

DAPI (1:2000, Sigma). The fluorescent images were obtained with confocal microscope (Carl Zeiss LSM710).

Statistical analysis

Quantification of band intensity was analyzed using TINA 2.0 (RayTest). The results expressed as means ± standard error of the mean (SEM). The differences between the groups were compared by the Mann-Whitney *U-test*, the unpaired *t-test* or one-way analysis of variance (ANOVA), followed by post hoc analysis with the Bonferroni test. P values ≤ 0.05 were considered statistically significant.

Results

COMP-Ang1 accelerated muscle regeneration in ischemic limb

I previously reported that COMP-Ang1 induced angiogenesis through stromal derived factor-1 (SDF-1) synthesis in hypoxic endothelial cells and recruitment of bone marrow derived progenitor cells (24). In this study, I investigated another function of COMP-Ang1 related to muscle regeneration. C57/BL6 mouse was made to ischemic condition through removing unilateral femoral artery in left limb and infected with adenovirus (adv-β-gal and adv-COMP-Ang1) (Fig. 1A). When a lateral femoral artery of limb was removed, the ischemic foot could not stamp on paper at 1 day. However, the ischemic foot was recovered in a time dependent manner (Fig. 1B). Next, I examined foot step score of the rescued foot (Fig. 1C). Interestingly, COMP-Ang1-treated mice had increased foot score point compared to control mice (Fig. 1D). Moreover, COMP-Ang1 increased gastrocnemius muscle volume compared with control (Fig. 2A-B). In muscle histology, muscle fibers were converted to round shape at ischemic 1 day. At ischemic 3 days, muscle fibers had increased damaged muscle fibers and, especially, several infiltrating cells were also observed beside ischemic

muscle fibers. At 7 days, the ischemic muscle in control group had reduced central nuclear fibers which are known to regenerate muscle fibers and was exacerbated by generation of fibrosis and adipocyte-like cells. However, COMP-Ang1 dramatically increased central nuclear fibers and reduced fibrosis (Fig. 2C).

These *in vivo* findings suggested that COMP-Ang1 might induce muscle recovery through muscle regeneration.

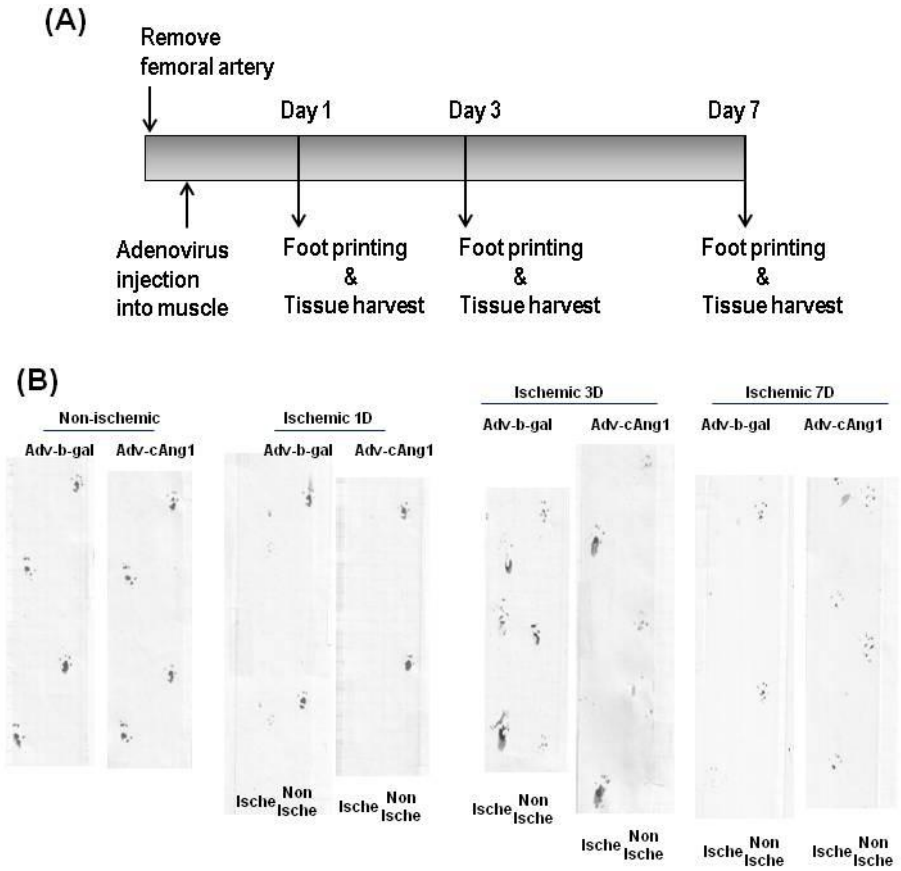

(A)
Remove
femoral artery
Day 1
Day 3
Day 7
Adenovirus
injection
into muscle
Foot printing
&
Tissue harvest
Foot printing
&
Tissue harvest
Foot printing
&
Tissue harvest
(B)
Non-ischemic
Adv-b-gal
Adv-cAng1
Ischemic 1D
Adv-b-gal
Adv-cAng1
Ischemic 3D
Adv-b-gal
Adv-cAng1
Ischemic 7D
Adv-b-gal
Adv-cAng1
Ische
Non Ische
(C)
Foot step score
0
1
2
3
4
(Score)
Foot print
(D)
Foot step score
COMP-Ang1
Beta-gal
Foot setp score
1D
3D
7D
*

Figure 1. COMP-Ang1 induced foot score of ischemic limb

(A) The schematic timeline of ischemic hindlimb model. (B) The photography of foot printing of ischemic (left foot print) and non-ischemic limb (right foot print). The followed footsteps indicate recovery of ischemic limb in a time dependent manner. (C) The foot recovery scores. Non printing foot indicates to "0" score, only touched printing foot indicates "1" score, the recovered toe's printing indicates "2" score, the slightly recovered toe and sole printing indicates "3" score, and the fully recovered foot printing indicates "4" score. (D) The foot step scores graph of each group with time dependent manner. The recovery capacity of COMP-Ang1 mouse was significantly increased compared to control mouse. The significance score between the groups were statically compared by the Mann-Whitney *U-test* (*$p<0.05$) and the unpaired *t-test* (*$p<0.05$).

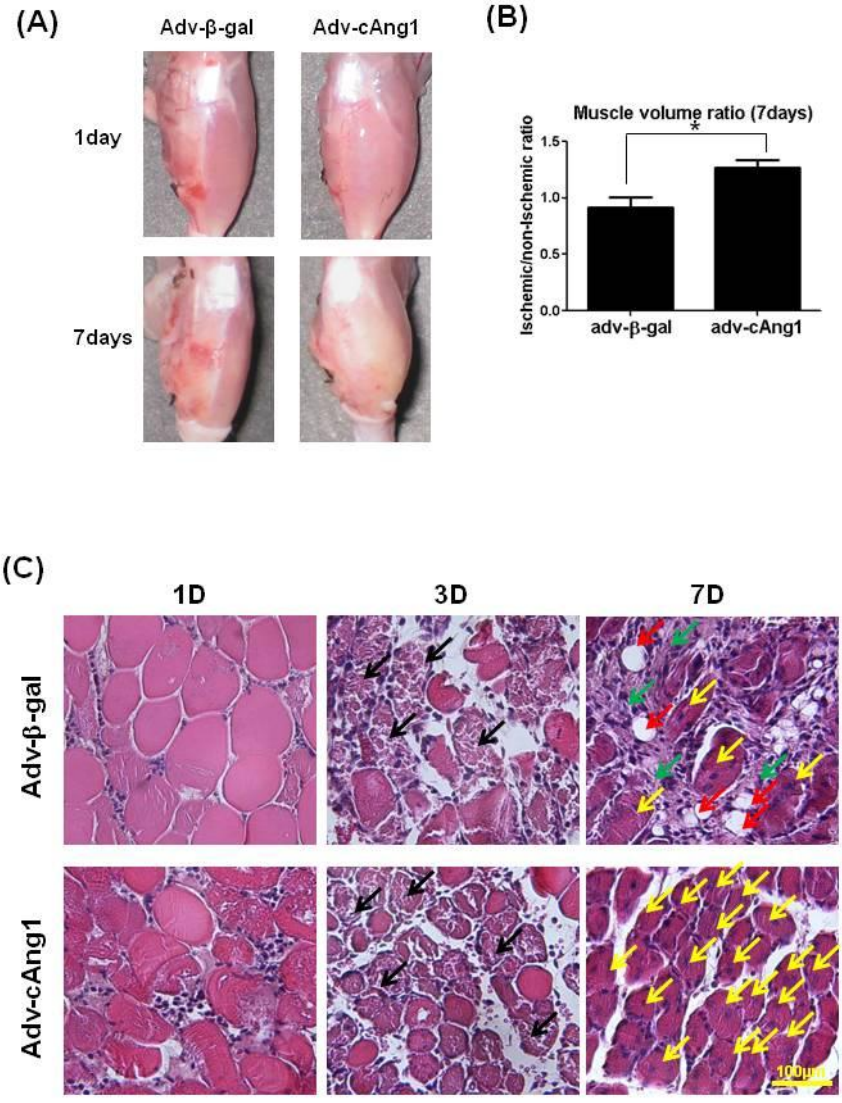

(A)
Adv-β-gal
Adv-cAng1
1day
7days
(B)
Muscle volume ratio (7days)
*
Ischemic/non-Ischemic ratio
1.5
1.0
0.5
0.0
adv-β-gal
adv-cAng1
(C)
1D
3D
7D
Adv-β-gal
Adv-cAng1
100μm

Figure 2. COMP-Ang1 repaired ischemic damaged muscle through new muscle generation

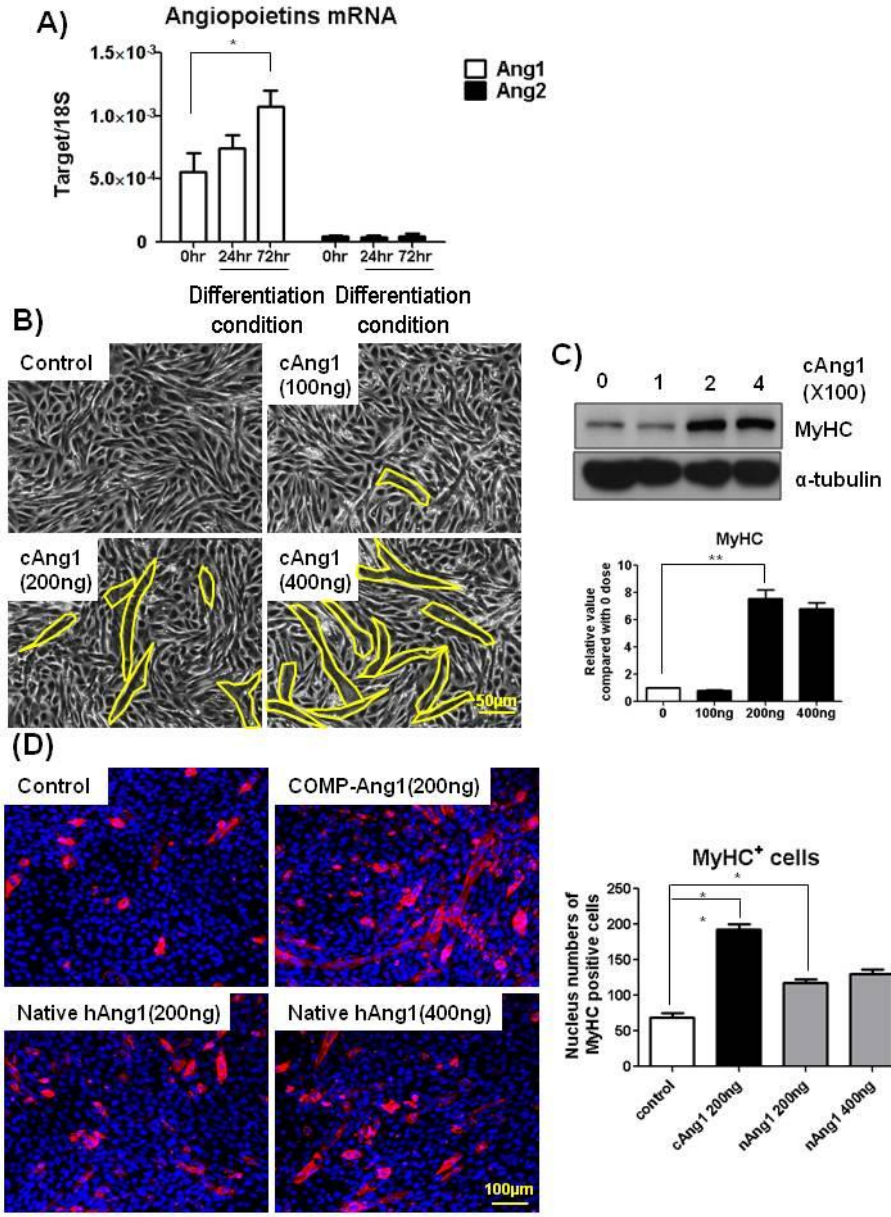
(A) The growth finding of ischemic muscle that was injected with adenovirus. (B) The ischemic/non-ischemic ratio of gastrocnemius muscle volume was measured between control and COMP-Ang1 groups. . Adv-COMP-Ang1 injected groups had slightly increased muscle volume compared with adv-β-gal injected groups at 7 days (*p <0.05). (C) Histology of ischemic damaged muscle. The muscle fibers were initially transformed to round shape at 1 day, and destructed at 3 days (dark arrows). At 7 days, Adv-β-gal injected muscle was detected regenerating muscle fiber (yellow arrows) but increased fibrosis area (green arrows) and adipocytes like cells (red arrows). However, Adv-COMP-Ang1 injected muscle significantly increased regenerating muscle fibers and rarely detected fibrosis area. Scale bar =100μm. Magnification X200.

COMP-Ang1 directly induced differentiation of C2C12 myoblasts to myotube

I studied whether angiopoietins (Ang1 and Ang2) expressions were changed during myogenesis in C2C12 myoblasts. Interestingly, mRNA expression of Ang1 was consistently increased but Ang2 expression did not (Fig. 3A). This result suggested that Ang1 might affect myogenesis. Therefore, I investigated whether COMP-Ang1 contributed to myogenesis. COMP-Ang1 enhanced conversion of myoblasts into myotubes with 200ng/ml and 400ng/ml dose at 3 days (Fig. 3B). Skeletal myosin heavy chain (MyHC), which indicates myotube marker, was also increased by COMP-Ang1 (Fig. 3C). Next, recombinant COMP-Ang1 and native Ang1 protein were treated on myoblasts to confirm whether native Ang1 was similar to COMP-Ang1. Both recombinant COMP-Ang1 and native Ang1 significantly induced generation of MyHC expressing myotube more than non-treated group at 7 days. Interestingly, COMP-Ang1 strongly enhanced myotube generation compared with native Ang1 (Fig. 3D). Moreover, adv-β-gal or adv-COMP-Ang1 was infected to C2C12 myoblasts to evaluating *in vitro* myogenesis. To investigate maturation ratio of myotubes, anti-MyHC was also stained in DM at day 3 and 7. Adv-COMP-Ang1 significantly increased synthesis of MyHC

expressing myotubes compared with adv-β-gal (Fig. 3E).

These results indicated that native Ang1 and COMP-Ang1 could promote generation of myotubes during myogenesis.


A)
Angiopoietins mRNA
Target/18S
1.5×10-3
1.0×10-3
5.0×10-4
0
Ang1
Ang2
0hr 24hr 72hr
0hr 24hr 72hr
B)
Differentiation condition
Differentiation condition
Control
cAng1 (100ng)
cAng1 (200ng)
cAng1 (400ng)
50μm
C)
0 1 2 4
cAng1 (X100)
MyHC
α-tubulin
MyHC
Relative value compared with 0 dose
0 100ng 200ng 400ng
(D)
Control
COMP-Ang1(200ng)
Native hAng1(200ng)
Native hAng1(400ng)
100μm
MyHC+ cells
Nucleus numbers of MyHC positive cells
control
cAng1 200ng
nAng1 200ng
nAng1 400ng

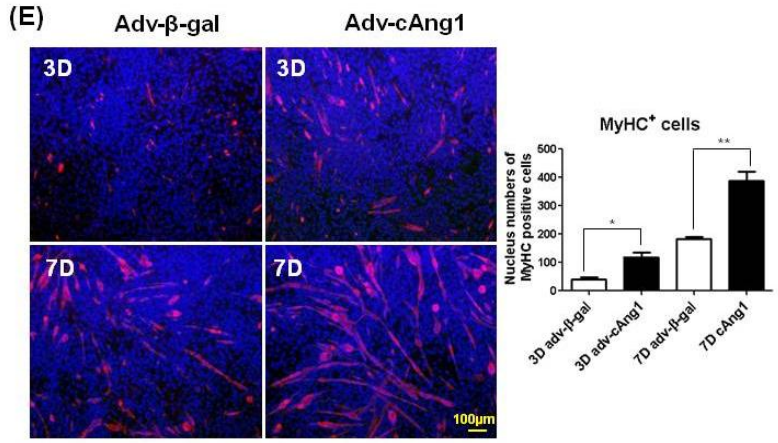


Figure 3. Both native Ang1 and COMP-Ang1 induced myogenesis.

(A) mRNA expression levels of Ang1 and Ang2 during myogenesis. The mRNA level of Ang1 was higher than Ang2 in myoblasts. Ang1 expression was increased during myogenesis but Ang2 expression did not. Ang1 and Ang2 were evaluated with relative value of 18S RNA. (B) Myotube generation was enhanced by COMP-Ang1. The fused cells (yellow line) were increased by COMP-Ang1. Scale bar = 50μm. Magnification X200. (C) Skeletal myosin heavy chain (MyHC) expression of myoblasts was increased by COMP-Ang1. COMP-Ang1 (200 and 400 ng/ml) enhanced MyHC expression in myoblast differentiation at 3 days. (D) To confirm COMP-Ang1 and native Ang1 effect for generation of myotubes, each myoblast was changed with DM and treated with COMP-Ang1 (200ng/ml) or native Ang1 (200 and 400ng/ml). Red and blue indicate MyHC and nuclear. MyHC positive nuclear numbers were counted and assessed. Scale bar = 100μm. Magnification X150. (E) Adenovirus (β-gal and COMP-Ang1) was infected to myoblasts and these cells were changed to DM. Adv-COMP-Ang1 enhanced MyHC expression at 3 and 7 days. Red and blue indicate MyHC and nuclear. MyHC positive nuclear numbers were counted and evaluated. Scale bar = 100μm. Magnification X100. (*$p<0.05$, **$P<0.01$)

COMP-Ang1 increased myogenin expression during muscle regeneration

Next, I investigated expression of the myogenic transcription factors such as MyoD and myogenin that were essentially required for myogenesis (25). The levels of MyoD expression were similar both groups, but myogenin expression was increased in COMP-Ang1 than in control at DM 1 day (Fig. 4A). The myogenin induced by COMP-Ang1 was also analyzed in ischemic limb. In normal limb, the myogenin expressing cells were rarely detected. After ischemic condition, adv-COMP-Ang1 groups had dramatically induced myogenin positive cells on fibers compared to adv-β-gal group at 1 day. However, the myogenin expressing cells were similar in both groups at 3 days (Fig. 4B). Myogenin expressing fiber score was evaluated by numbers of myogenin positive cells on single fiber (Fig. 4C). Although the total myofibers were similar in both groups, the ratio of the single myogenin positive fibers was increased in COMP-Ang1 compared with control at 1 and 3 days. However, the ratios of two or more myogenin positive fibers were similar in both groups at 3 days (Fig. 4D). Therefore, these results indicated that COMP-Ang1 could rapidly increase myogenin expression of myoblasts and satellite cells on muscle fibers in early time.

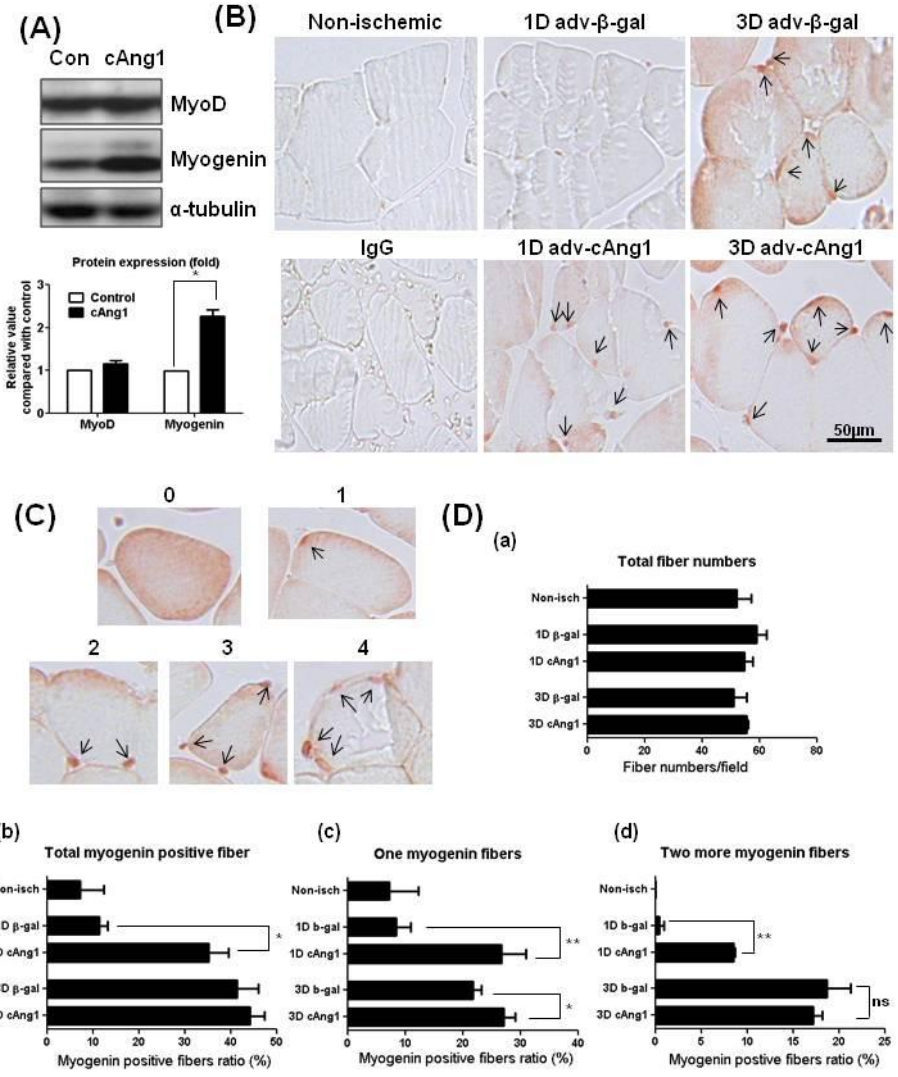

(A)
Con cAng1
MyoD
Myogenin
α-tubulin
Protein expression (fold)
Control
cAng1
Relative value compared with control
MyoD
Myogenin
(B)
Non-ischemic
1D adv-β-gal
3D adv-β-gal
IgG
1D adv-cAng1
3D adv-cAng1
50μm
(C)
0
1
2
3
4
(D)
(a)
Total fiber numbers
Non-isch
1D β-gal
1D cAng1
3D β-gal
3D cAng1
Fiber numbers/field
(b)
Total myogenin positive fiber
Myogenin positive fibers ratio (%)
(c)
One myogenin fibers
1D b-gal
3D b-gal
Myogenin positive fibers ratio (%)
(d)
Two more myogenin fibers
ns
Myogenin postive fibers ratio (%)

Figure 4. COMP-Ang1 enhanced myogenin expression of myoblasts.

(A) Myogenic factors were analyzed by immunoblot. Myoblasts were changed to DM for 24 hours and analyzed expression of MyoD and Myogenin. COMP-Ang1 group increased the myogenin expression more than control but not MyoD. (B) Immunohistochemistry for myogenin in muscle. The activating satellite cells on single fibers were stained with myogenin (brown). Scale bar = 50 μm. Magnification X400. (C) The fiber score was determined by numbers of the myogenin expression on single fiber. (D) The evaluation of the myogenin expression pattern. Total fiber numbers in field were similar both groups (a). Total myogenin expressing fiber ratio (myogenin positive fibers/total fibers) was evaluated by relative value for total fibers (b). Single myogenin expressing fiber ratio (c). Two more myogenin expressing fiber ratio (d). Adv-COMP-Ang1 induced numbers of single myogenin positive fibers at 1 and 3 days compared with adv-β-gal. (*$p<0.05$, **$P<0.01$)

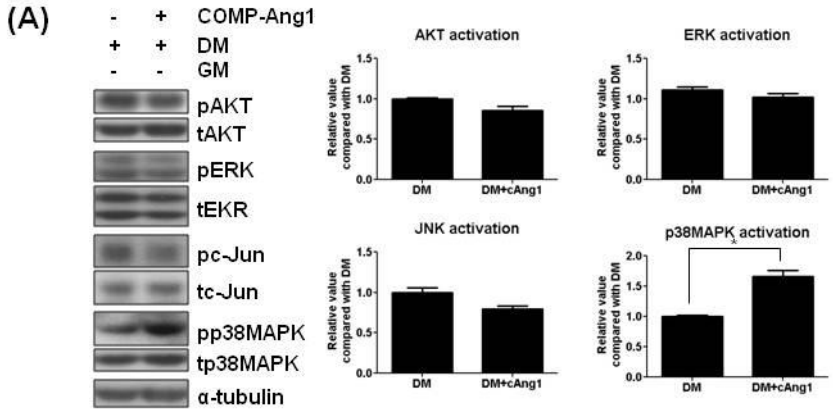
COMP-Ang1 induced expression of myogenin through p38MAPK activation dependent on Ca^{2+} ion

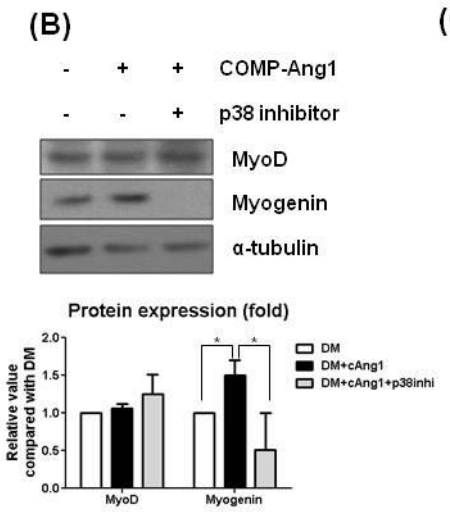
To investigate what kind of signal factors were activated by COMP-Ang1 during myogenesis, C2C12 myoblasts were treated with COMP-Ang1 in DM for 3 hours. Interestingly, COMP-Ang1 induced p38MAPK activation but not AKT, ERK, and JNK, which was indicated by c-Jun phosphorylation (Fig. 5A). Next, to examine relationship between myogenin expression and p38MAPK activation, the cells were treated with p38MAPK chemical inhibitor. Despite of COMP-Ang1 treatment, the myogenin expression was completely suppressed by p38MAPK inhibitor (Fig. 5B). These results suggested that the p38MAPK activation by COMP-Ang1 enhances myogenin expression in myogenesis.

On the other hand, I wondered whether Tie2 expression, which is a receptor of Ang1 in endothelial cells but not expressed in myoblasts (26), influences myogenesis. Interestingly, human Tie2 (hTie2) plasmid did not induce phospho-p38MAPK but induced phospho-AKT and phospho-Tie2 at 3 hours. After 24 hours, myogenin expression was also not induced by hTie2 plasmid, despite of COMP-Ang1 treatment (Fig. 5C). This result indicated that Tie2 expression could suppress myogenic effect or hinder activation of

unknown regulatory factor, which bound to COMP-Ang1, on myoblast surface for myogenesis.

Therefore, I investigated what kind of surface molecules could bind with COMP-Ang1 in myoblast. Both native Ang1 and COMP-Ang1 have fibrinogen like domain at C-terminal. The fibrinogen like domain includes Ca^{2+} ion binding sites such as fibrinogen (Fig. 6A). Thus, I investigated whether binding of COMP-Ang1 to myoblast surface is dependent on Ca^{2+} ion. After C2C12 myoblasts were detached by trypsin, the suspended cells were incubated with COMP-Ang1 for 1 hour. These cells were fixed by DTSSP, non-permeable protein-protein cross linker, and analyzed by FACS (Fig. 6B). Interestingly, COMP-Ang1 did not bind on C2C12 myoblast surface without DTSSP because COMP-Ang1 and Ca^{2+} ion might be removed by washing step. In contrast, binding of COMP-Ang1 to myoblast surface was increased by DTSSP but reduced by EGTA, which was Ca^{2+} ion chelating chemical, in the presence of DTSSP treatment (Fig. 6C). Moreover, the p38MAPK activation and myogenin expression by COMP-Ang1 were also suppressed by EGTA (Fig. 6D). Thus, these results described that COMP-Ang1 could bind to myoblast surface and activate p38MAPK and myogenin synthesis for myogenesis by Ca^{2+} ion.


(A)
- + COMP-Ang1
+ + DM
- - GM
pAKT
tAKT
pERK
tEKR
pc-Jun
tc-Jun
pp38MAPK
tp38MAPK
α-tubulin
AKT activation
ERK activation
JNK activation
p38MAPK activation
Relative value compared with DM
DM
DM+cAng1


(B)
- + + COMP-Ang1
- - + p38 inhibitor
MyoD
Myogenin
α-tubulin
Protein expression (fold)
Relative value compared with DM
DM
DM+cAng1
DM+cAng1+p38inhi
MyoD
Myogenin

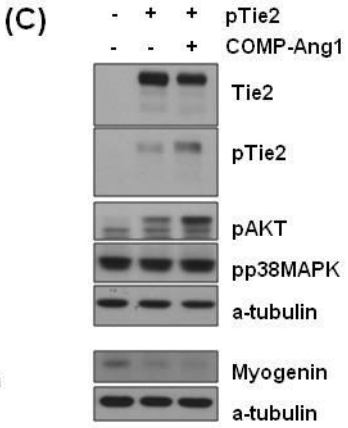

(C)
- + + pTie2
- - + COMP-Ang1
Tie2
pTie2
pAKT
pp38MAPK
a-tubulin
Myogenin
a-tubulin

Figure 5. COMP-Ang1 induced myogenin expression via p38MAPK activation.

(A) Immunoblotting for signal molecules by COMP-Ang1 (200ng/ml) in DM at 3hours. p38MAPK activation was significantly induced by COMP-Ang1 but not AKT, ERK, and JNK. Quantitative graph from three independent experiments express AKT, ERK, JNK, and p38MAPK activation levels upon normalization by total form. (B) Myoblasts were treated with p38 inhibitor for p38MAPK signaling evaluation. COMP-Ang1 increased synthesis of myogenin at DM 24 hours but reduced myogenin synthesis by p38MAPK inhibitor. MyoD expression was not responded by COMP-Ang1 and p38MAPK inhibitor. (C) Competitive experiment with phTie2. The phTie2 transfected myoblasts were increased to activation of Tie2 and AKT by COMP-Ang1 but not responded p38MAPK. Myogenin expression was also reduced by phTie2 at 24 hours. (*$p<0.05$, **$P<0.01$)

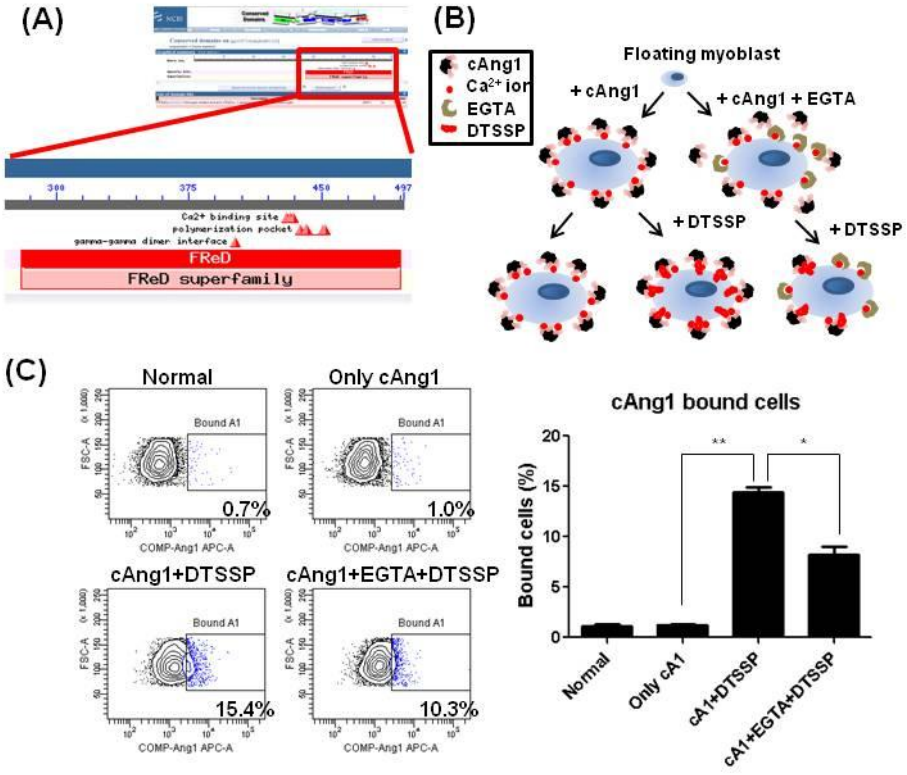

(A)
300
375
450
497
Ca2+ binding site
polymerization pocket
gamma-gamma dimer interface
FReD
FReD superfamily
(B)
cAng1
Ca2+ ion
EGTA
DTSSP
Floating myoblast
+ cAng1
+ cAng1 + EGTA
+ DTSSP
+ DTSSP
(C)
Normal
Only cAng1
cAng1+DTSSP
cAng1+EGTA+DTSSP
Bound A1
0.7%
1.0%
15.4%
10.3%
FSC-A
COMP-Ang1 APC-A
cAng1 bound cells
Bound cells (%)
Normal
Only cA1
cA1+DTSSP
cA1+EGTA+DTSSP
(D)
COMP-Ang1
EGTA
p-p38MAPK
α-tubulin
Myogenin
α-tubulin
p38MAPK activation
Relative value compared with DM
DM
DM+cAng1
DM+cAng1+EGTA
Myogenin expression

Figure 6. COMP-Ang1 bound on myoblast surface dependent on Ca^{2+} ion.

(A) The structure analysis of fibrinogen like domain in Ang1. The fibrinogen like domain of COMP-Ang1 and Ang1 has Ca^{2+} ion binding site, polymerization pocket, and gamma gamma dimer interface. (B) The diagram of surface binding experiment. Detached myoblasts were incubated with COMP-Ang1 and protein-protein cross-linked by DTSSP. Ca^{2+} ion was blocked by EGTA. COMP-Ang1 binding capacities were evaluated by FACS. (C) The analysis of COMP-Ang1 binding capacity on myoblast. The COMP-Ang1 binding capacity was accessed by anti-Flag. In myoblast without DTSSP, COMP-Ang1 was not bound on myoblast, but increasingly bound on myoblast by DTSSP. The binding capacity of COMP-Ang1 was reduced by EGTA. The graph indicates COMP-Ang1 binding cells (%). (D) Immunoblot for COMP-Ang1 signaling related Ca^{2+} ion. The myoblasts were treated with COMP-Ang1 with or without EGTA. COMP-Ang1 induced p38MAPK activation compared to non-treated group but EGTA suppressed p38MAPK activation by COMP-Ang1 at 3 hours. Although COMP-Ang1 existed, EGTA inhibited myogenin expression at 24 hours. The graph of p38MAPK activation and myogenin expression were evaluated with densitometry of three different experiments. (*$p<0.05$, **$P<0.01$)

COMP-Ang1 directly bound N-cadherin and induced N-cadherin downstream signaling

Next, I investigated what kinds of surface molecules were activated by Ca^{2+} ion and COMP-Ang1. Cadherin and integrin families were well known to possess high number Ca^{2+} ion binding domains and activated by Ca^{2+} ion complexes (27-31). Therefore, I evaluated the expression levels of cadherin and integrin β families in C2C12 myoblast (Fig. 7A). Among cadherin families, N-cadherin and M-cadherin were dominantly expressed, and integrin β 1 among integrin β families was highly expressed in myoblasts. The specific target molecules (N-cadherin, M-cadherin, and integrin β 1) were suppressed by siRNA (Fig. 7B) and then COMP-Ang1 binding capacity on cell surface was examined. In the both M-cadherin and Integrin β 1 knockdown cells, the binding capacities of COMP-Ang1 were similar to COMP-Ang1 treated cells. However, in N-cadherin knockdown cells, COMP-Ang1 binding capacity was significantly reduced (Fig. 7C). The p38MAPK activation by COMP-Ang1 was also similar to control in M-cadherin and integrin β 1 knockdown myoblasts, but reduced in N-cadherin knockdown myoblast despite of COMP-Ang1 treatment (Fig. 7D). Additionally, I also wondered whether COMP-Ang1 directly bound N-cadherin with *in vitro*

binding assay. hN-cadherin-Fc was weakly bound to COMP-Ang1 without DTSSP but the binding was greatly increased in the presence of DTSSP. As expected, this binding was reduced by EGTA treatment (Fig. 7E).

These results suggested that COMP-Ang1 could directly bind to C2C12 myoblast surface and induce p38MAPK activation through N-cadherin.

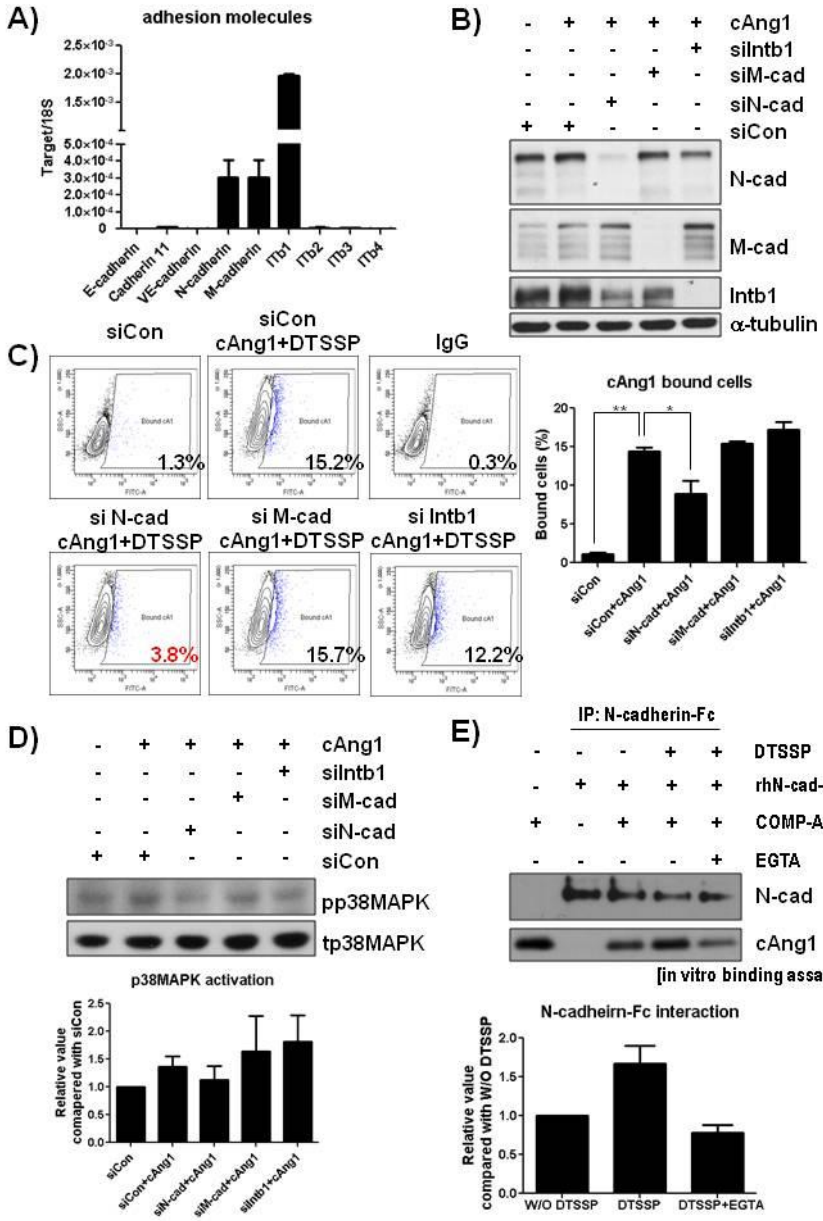

A)
adhesion molecules
Target/18S
E-cadherin
Cadherin 11
VE-cadherin
N-cadherin
M-cadherin
ITb1
ITb2
ITb3
ITb4
B)
cAng1
siIntb1
siM-cad
siN-cad
siCon
N-cad
M-cad
Intb1
α-tubulin
C)
siCon
siCon cAng1+DTSSP
IgG
1.3%
15.2%
0.3%
si N-cad cAng1+DTSSP
si M-cad cAng1+DTSSP
si Intb1 cAng1+DTSSP
3.8%
15.7%
12.2%
cAng1 bound cells
Bound cells (%)
siCon
siCon+cAng1
siN-cad+cAng1
siM-cad+cAng1
siIntb1+cAng1
D)
cAng1
siIntb1
siM-cad
siN-cad
siCon
pp38MAPK
tp38MAPK
p38MAPK activation
Relative value comapered with siCon
E)
IP: N-cadherin-Fc
DTSSP
rhN-cad-Fc
COMP-Ang1
EGTA
N-cad
cAng1
[in vitro binding assay]
N-cadheirn-Fc interaction
Relative value compared with W/O DTSSP
W/O DTSSP
DTSSP
DTSSP+EGTA

Figure 7. COMP-Ang1 increased p38MAPK activation through N-cadherin binding.

(A) mRNA levels of cadherin and integrin β families in myoblasts. N-cadherin and M-cadherin were dominantly expressed among cadherin family. The integrin β 1 was largely expressed in myoblasts. (B) Immunoblot to confirm siRNA effect. Each target molecule suppressed by specific siRNA was confirmed. (C) COMP-Ang1 binding assay in siRNA knockdown myoblasts. These cells transfected with siRNA (N-cadherin, M-cadherin, and Integrin β 1) were performed to adhesion assay with DTSSP. The graph indicates COMP-Ang1 binding cells (%). (D) Immunoblot for confirming p38MAPK activation by COMP-Ang1 with siRNA transfection. The activation of p38MAPK in M-cadherin and integrin β 1 knockdown myoblast was similar to COMP-Ang1 treated group, but the p38MAPK phosphorylation increased by COMP-Ang1 was reduced by N-cadherin knockdown. (E) N-cadherin and COMP-Ang1 *in vitro* binding assay. Anti-human N-cadherin-Fc was incubated with COMP-Ang1 whether EGTA treated or not. The interaction between N-cadherin and COMP-Ang1 was confirmed by western blotting using anti-N-cadherin and anti-Flag. COMP-Ang1 binding to hN-cadherin-Fc was increased in DTSSP but reduced by EGTA. (*$p<0.05$, **$P<0.01$)

COMP-Ang1 promoted interaction between N-cadherin and p120-catenin

I investigated whether COMP-Ang1 induces N-cadherin activation in myoblasts. N-cadherin activation was evaluated by interaction between N-cadherin and p120-catenin and this interaction is usually observed in cell junction (32). When myoblasts were stimulated by DM, N-cadherin was accumulated on cell junction point and p120-catenin also interacted with N-cadherin in both groups (Fig. 8Aa). However, at non-junctioning site, N-cadherin and p120-catenin interaction in control cells was disrupted, whereas COMP-Ang1 stimulated the interaction (Fig. 8Ab). I also checked that the N-cadherin-p120-catenin interaction by COMP-Ang1 was present in a single cell level (Fig. 8B). Next, to confirm whether COMP-Ang1 enhanced the interaction between N-cadherin and p120-catenin, the binding was analyzed by immunoprecipitation in a sparse culture condition. The N-cadherin-p120-catein interaction was weak in control, but dramatically induced by COMP-Ang1 (Fig. 8C).

These results indicated that COMP-Ang1 could directly induce interaction of N-cadherin and p120-catenin even without cell junction.

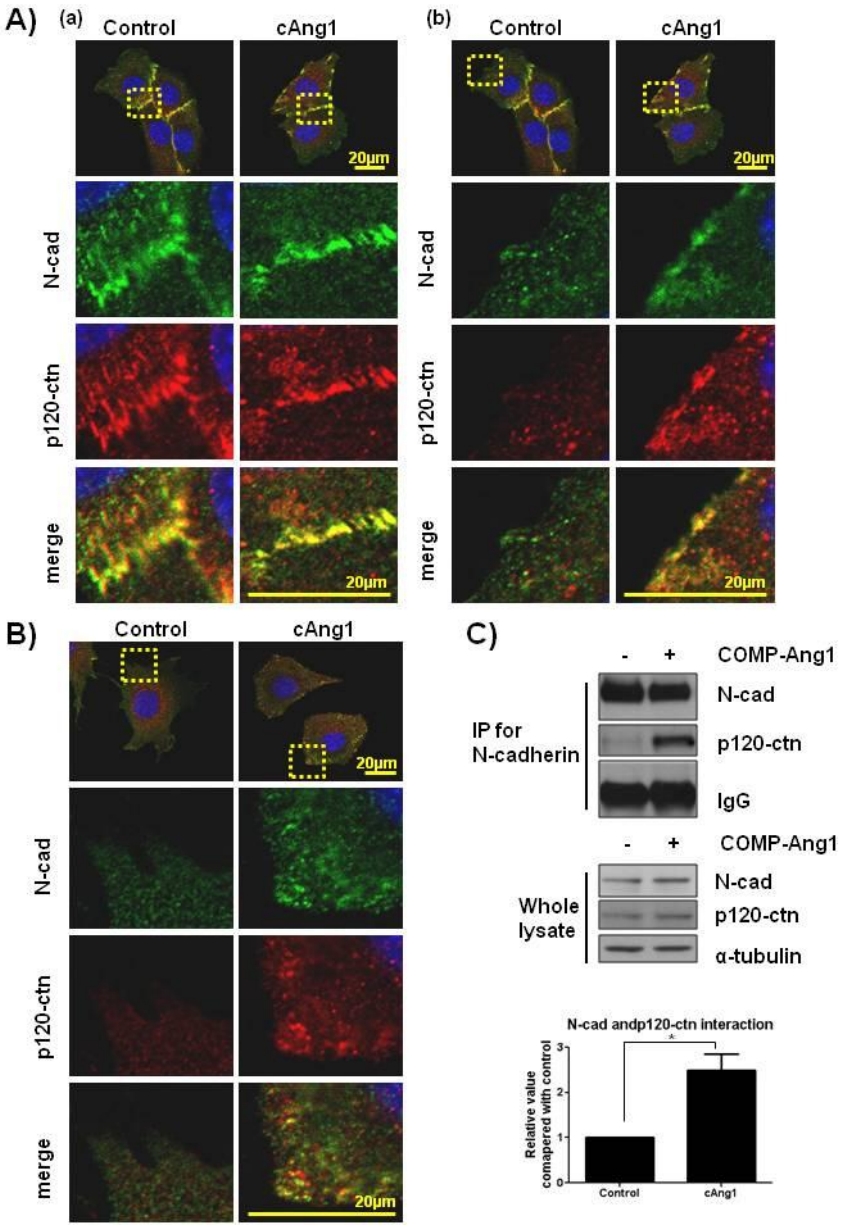

A)
(a)
Control
cAng1
20μm
N-cad
p120-ctn
merge
(b)
Control
cAng1
20μm
N-cad
p120-ctn
merge
20μm
B)
Control
cAng1
20μm
N-cad
p120-ctn
merge
20μm
C)
- +
COMP-Ang1
IP for
N-cadherin
N-cad
p120-ctn
IgG
- +
COMP-Ang1
Whole
lysate
N-cad
p120-ctn
α-tubulin
N-cad andp120-ctn interaction
*
Relative value
comapered with control
3
2
1
0
Control
cAng1

Figure 8. COMP-Ang1 promoted N-cadherin activation through enhancing interaction of N-cadherin and p120-catenin.

(A) Immunofluorescence for N-cadherin and p120-catenin in myoblasts. The interaction of N-cadherin and p120-catenin was similar both COMP-Ang1 and control in cell junction site (a), but the interaction was increased by COMP-Ang1 in the peripheral site, which was not existent to cell junction (b). The indication of N-cadherin and p120-catein is green and red color. Co-localized signal of N-cadherin and p120-catenin presents yellow color. Blue indicates cell nucleus. Scale bar = 20μm. Magnification X630. (B) The interaction of N-cadherin (green) and p120-catenin (red) was analyzed in single cells level. The interaction signaling (yellow) was increased by COMP-Ang1. Scale bar = 20μm. Magnification X630. (C) Immunoprecipitation for confirming interaction between N-cadherin and p120-catenin. The interaction between N-cadherin and p12-catenin was weakly bound in control but the interaction was dramatically increased by COMP-Ang1. Whole lysate indicates input protein. Quantitative graph from independent experiments express interacting p120-catein levels upon normalization by N-cadherin. (*$p<0.05$)

COMP-Ang1 accelerated myogenesis through N-cadherin

Next, I investigated whether COMP-Ang1 could undoubtedly activate myogenesis of myoblast through N-cadherin *in vivo*. The GFP expressing myoblast (GFP-MB) was generated by lentivirus. For N-cadherin knockdown, GFP-MB was transfected with N-cadherin siRNA. After, these cells were transplanted into gastrocnemius of nude mouse together with adv-β-gal or adv-COMP-Ang1 injection (Fig. 9A). After 10 days, the transplanted myoblasts developed myotubes in nude mouse gastrocnemius muscle. The transplanted GFP-MB had significantly increased generation of myotubes by COMP-Ang1. However, in the N-cadherin knockdown GFP-MB, there was rarely observed to myotube and had undergone fibrosis, even though COMP-Ang1 was treated (Fig. 9B). Interestingly, when GFP was stained to indicate GFP-MB, the myotube of GFP-MB showed more staining by anti-GFP than undifferentiated GFP-MB. The number and size of GFP positive myotubes were increased more in COMP-Ang1 treated than in non-treated groups, and rarely detected in N-cadherin knockdown GFP-MB (Fig. 9C). COMP-Ang1 enhanced myogenin expression of GFP-MB more than non-treated and N-cadherin knockdown groups (Fig. 9D). Thus, these results indicated that N-cadherin signaling was an important factor for myogenesis by COMP-Ang1.

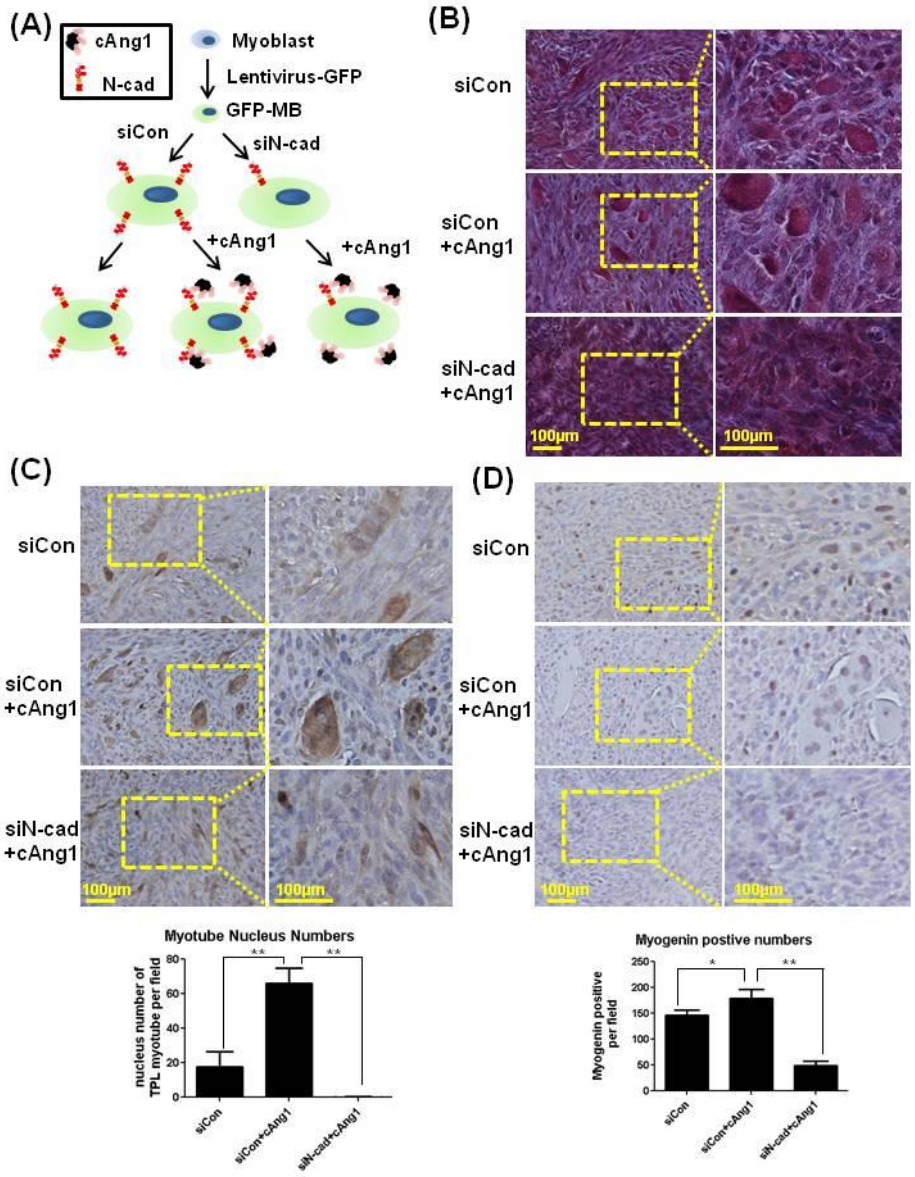

(A)
cAng1
N-cad
Myoblast
Lentivirus-GFP
GFP-MB
siCon
siN-cad
+cAng1
+cAng1
(B)
siCon
siCon
+cAng1
siN-cad
+cAng1
100μm
100μm
(C)
siCon
siCon
+cAng1
siN-cad
+cAng1
100μm
100μm
(D)
siCon
siCon
+cAng1
siN-cad
+cAng1
100μm
100μm
Myotube Nucleus Numbers
nucleus number of TPL myotube per field
80
60
40
20
0
**
**
siCon
siCon+cAng1
siN-cad+cAng1
Myogenin postive numbers
Myogenin positive per field
250
200
150
100
50
0
*
**
siCon
siCon+cAng1
siN-cad+cAng1

Figure 9. COMP-Ang1 directly induced myogenin expression and myotube generation of transplanted GFP-expressing myoblasts via N-cadherin existence.

(A) The diagram of GFP-MB transplantation experiment with N-cadherin knockdown myoblasts. (B) The histology of transplanted GFP-MB at 10 days after transplantation. In COMP-Ang1 treated groups, the generated myotubes were dramatically increased, whereas N-cadherin knockdown GFP-MB were not generated myotube despite of COMP-Ang1 treatment. (C) Immunohistochemistry for GFP. GFP (brown) positive cells were detected in the transplanted cells. Myotubes of GFP-MB strongly expressed GFP. Blue indicates nucleus. The graph indicates that the nuclear numbers of myotubes were counted on several fields. (D) Immunohistochemistry for myogenin. Myogenin positive cells (red) were stained in transplantated GFP-MB. Blue indicates nucleus. Myogenin expressions were increased in COMP-Ang1 treated group compared with control. In the contrast, myogenin of N-cadherin knockdown groups were reduced, although COMP-Ang1 existence. The graph indicates that the numbers of myogenin positive cells were counted on several field. Scale bar = 100μm. Magnification X200. (*$p<0.05$, **$P<0.01$)

Macrophages were infiltrated into ischemic muscle by COMP-Ang1

In the previous paper, anti-inflammtory macrophages contributed to enhancement of myogenesis (10). In this study, several infiltrating cells were observed at 3 days after limb ischemia. Therefore, I investigated whether COMP-Ang1 could affect macrophage infiltration. At ischemic day 3, F4/80 postive macrophages were detected on both groups, a pan macrophage marker positive macrophages. (Fig. 10A). The infiltration of F4/80 positive mono/macrophages was increased after ischemic condition. At ischemic day 1, F4/80 positive cells were very low but dramatically increased at ischemic day 3. Interestingly, the F4/80 positive cells were significally increased by COMP-Ang1 at ischemic day 3 (Fig. 10B).

These results suggested that COMP-Ang1 could influence macrophage infiltration into ischemic muscle.

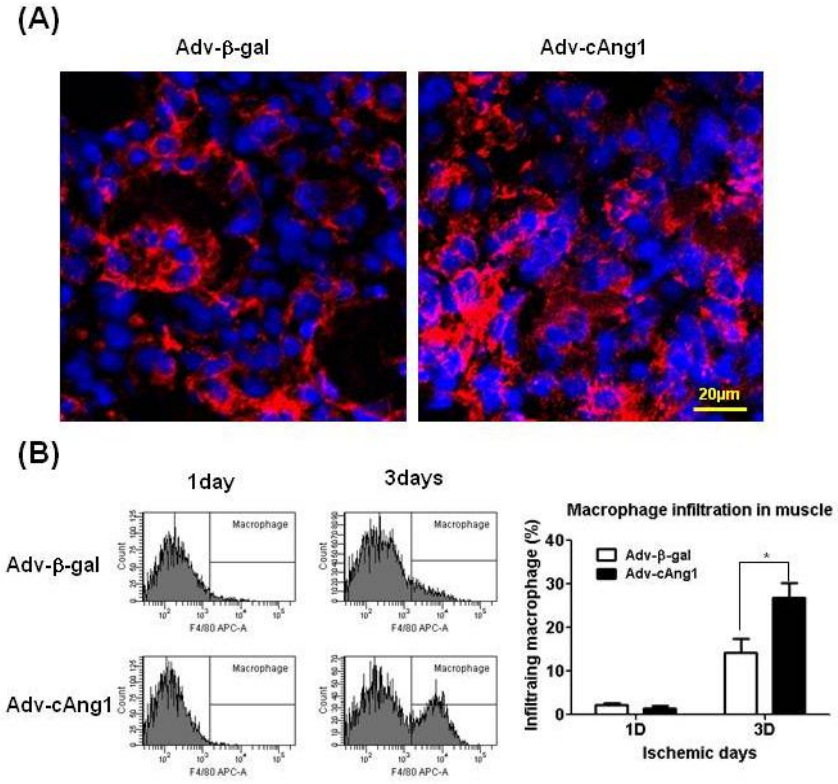


Figure 10. Infiltration of F4/80 positive mono/macrophage to ischemic muscle by COMP-Ang1.

(A) Immunofluorescence stain for F4/80 at ischemic 3 days. Red cells indicates F4/80 positive mono/macrophages and blue color indicates nucleus. Scale bar = 20μm. Magnification X400. (B) FACS analysis of F4/80 positive population in ischemic muscle. The mono/macrophage population was assessed by anti-F4/80-APC. At ischemic 3 days, F4/80 positive cells in adv-COMP-Ang1 dramatically increased infiltration compared with adv-β-gal. The graph indicates that the infiltrating mono/macrophage population percent was assessed. (*$p<0.05$)

COMP-Ang1 recruited Tie2 expressing macrophage in ischemic muscle

In previous reports, COMP-Ang1 was known as an anti-inflammtory factor (33, 34). However, in other previous reports, Tie2 expressing macrophages (TEM) were migrated to tumors stimulated by angiopoietin-2 secreted from cancer cells for tumor angiogenesis. Therefore, I investigated whether COMP-Ang1 could induce infiltation of TEM into ischemic muscle. In control muscle, TEM ($F4/80^{+}Tie2^{+}$) infiltration was increased in a time dependent manner. However, infiltration of Tie2-negative macrophages, which indicate inflammtory macrophages ($F4/80^{+}Tie2^{-}$, InfM), was similar in each groups. Interstingly TEM infiltration was significantly increased by COMP-Ang1 (Fig. 11A).

Tie2 positive cells in ischemic muscle are possibly consist of endothelial cells (ECs) (35), endothelial progenitor cells (EPCs) (18), satellite cells (15), and TEM. ECs and satellite cells did not express F4/80 but EPCs (36, 37) and TEM expressed F4/80. Therefore, I evaluated the separation between EPCs and TEM using CD31, which are expressed in ECs and EPCs but not macrophages (36-38). In ischemic day 7, the single cells in ischemic muscle were separated to three gates - ECs ($F4/80^{-}Tie2^{+}$), TEM ($F4/80^{+}Tie2^{+}$),

and InfM ($F4/80^{+}Tie2^{-}$). The ECs highly expressed CD31, but its expression was weakly detected in TEM and not in InfM (Fig. 11B).

Next, I investigated whether insulin like growth factor 1 (IGF1), one of myogenic factors, was expressed in infiltrated cells. The cells in ischemic muscle were also separated into three groups (ECs, TEM, and InfM) and IGF1 expression was analyzed the by FACS. IGF1 was not expressed in InfM and weakly expressed in ECs. However, TEM highly expressed IGF1 (Fig. 11C).

Therefore, these results indicated that TEMs, which were significantly infiltrated into ischemic muscle by COMP-Ang1, was not EPCs and might enhance the myogenesis through paracrine effect such as IGF-1 secretion.

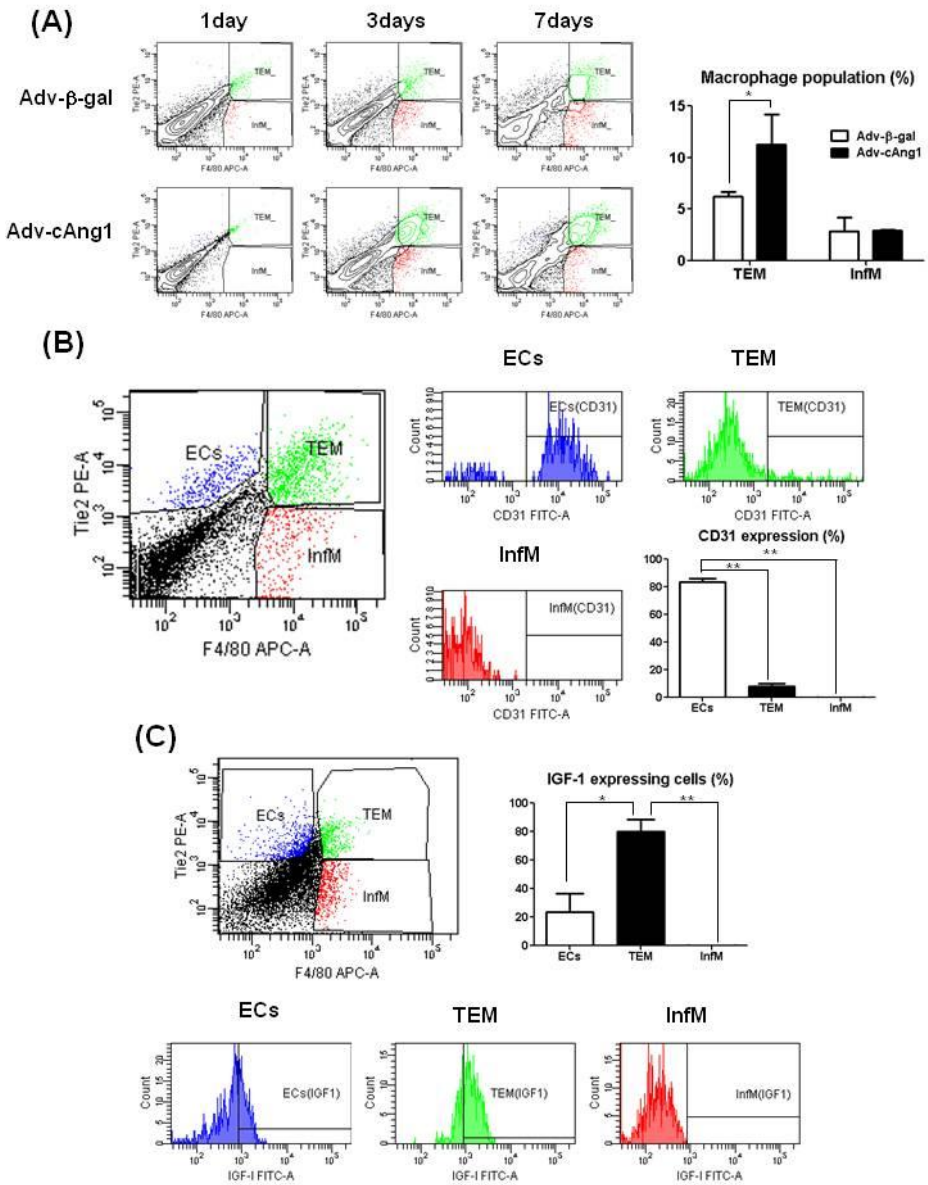

(A)
1day
3days
7days
Adv-β-gal
Adv-cAng1
TEM
InfM
F4/80 APC-A
Tie2 PE-A
Macrophage population (%)
Adv-β-gal
Adv-cAng1
TEM
InfM
(B)
ECs
TEM
InfM
Tie2 PE-A
F4/80 APC-A
ECs(CD31)
TEM(CD31)
InfM(CD31)
Count
CD31 FITC-A
CD31 expression (%)
ECs
TEM
InfM
(C)
IGF-1 expressing cells (%)
ECs(IGF1)
TEM(IGF1)
InfM(IGF1)
IGF-I FITC-A

Figure 11. TEM, not ECs and EPCs, was infiltrated by COMP-Ang1 and expressed IGF1.

(A) FACS analysis of ischemic muscle dependent on time. Anti-Tie2-PE and anti-F4/80-APC were used to evaluate cell population in ischemic muscle. $F4/80^{+}Tie2^{+}$ cells (green) indicates TEM and $F4/80^{+}Tie2^{-}$ cells (red) indicates InfM. TEM was dramatically increased in COMP-Ang1 but not InfM. The graph indicates that the ratio of TEM and InfM population at ischemic 7 days. (B) FACS analysis to separate infiltrating cells. The infiltrating cells were separated to three gates which indicate ECs ($F4/80^{-}Tie2^{+}$, blue), TEM ($F4/80^{+}Tie2^{+}$, green), and InfM ($F4/80^{+}Tie2^{-}$, red). Each gate was evaluated by CD31 positivity. ECs strongly expressed CD31 but TEM and InfM weakly expressed or did not express. The graph indicates CD31 expression in cells of three gates. (C) The analysis of IGF1 expression in three gates. The three gates were evaluated by anti-IGF1-FITC. ECs weakly expressed IGF1. TEM significantly increased IGF1 expression compared to InfM. The graph indicates IGF1 expression in cells of three gates. (*$p<0.05$, **$P<0.01$)

TEM expressed myogenic and angiogenic factor such as IGF1 and induced myogenesis

Next, I investigated whether TEM and InfM could induce myotube generation of myoblasts. These two types of macrophages were isolated by FACS sorting. The ischemic muscle at 3 days was enzymatically digested and the cells were excluded by CD31. The CD31 negative cells were segregated by F4/80 and then separated by Tie2. TEM were sorted by $F4/80^{+}Tie2^{+}CD31^{-}$ expression and InfM by $F4/80^{+}Tie2^{-}CD31^{-}$ expression (Fig. 12A). Interestingly, Tie2 and IGF1 expression of TEM was higher than InfM, but TNFα expression, one of inflammatory factors, was slightly lower than InfM in mRNA level (Fig. 12B).

Next, I examined whether TEM could induce myogenesis through the paracrine effect. TEM and InfM were cocultured with myoblasts through the insert which suppressed physical contact between myoblasts and macropahges, but allowed small molecules to pass from macrophages to myoblasts (Fig. 12C). TEM significantly induced myogenin expression in cocultured myoblasts compared with InfM and single myoblasts (Fig. 12D). Moreover, the cocultued myoblasts with TEM had increased myotube generation than in myoblasts cocultured with InfM or in myoblasts alone 5

days after cocultue (Fig. 12E).

These results suggested that TEM infiltrated by COMP-Ang1 could promote myogenin expression and myotube generation of myoblasts through paracrine effects such as IGF1 secretion.

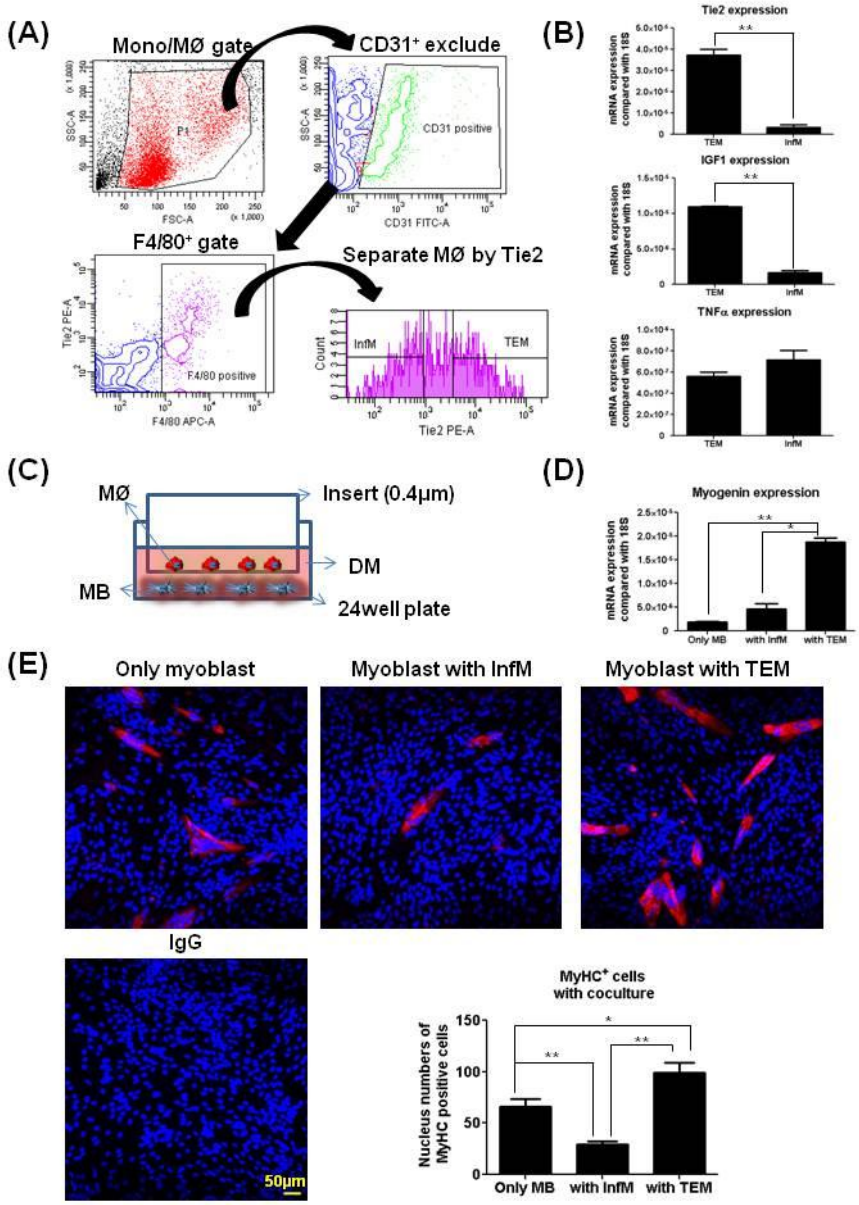

(A)
Mono/MØ gate
CD31+ exclude
P1
CD31 positive
F4/80+ gate
Separate MØ by Tie2
F4/80 positive
InfM
TEM
(B)
Tie2 expression
IGF1 expression
TNFα expression
mRNA expression compared with 18S
TEM
InfM
(C)
MØ
Insert (0.4μm)
DM
MB
24well plate
(D)
Myogenin expression
Only MB
with InfM
with TEM
(E)
Only myoblast
Myoblast with InfM
Myoblast with TEM
IgG
50μm
MyHC+ cells with coculture
Nucleus numbers of MyHC positive cells

Figure 12. TEM induced myogenesis through paracrine effects such as IGF1.

(A) TEM and InfM were isolated by FACS sorting. At 3 days, ischemic muscle was digested with 0.1% collagenase type I and cells were stained with anti-CD31-FITC, anti-Tie2-PE, and anti-F4/80-APC. The infiltrated cells were segregated by P1 gate which indicated to mono/macrophage gate. The gated cells were excluded from CD31 positive cells by inverted gating. The CD31 negative cells were separated by Tie2 and F4/80. The F4/80 gate represented macrophages into ischemic muscle. This gate was divided by Tie2 expression; Tie2 positive cells indicate TEM and the Tie2 negative cells indicate InfM. (B) Realtime PCR to analyze characters of sorted cells. Tie2 and IGF1 expression in TEM increased more than InfM. However, InfM highly expressed TNFα compared with TEM. (C) The schematic diagram of coculture condition with myoblasts and macrophages. Insert separated to contact myoblasts and macrophages but allowed to transfer small molecules with below 0.4 μm filter. (D) Myoblasts and macrophages were cocultured in DM for 1 day and then analyzed the mRNA levels of myogenin in myoblasts by realtime PCR. (E) Myotube generation and MyHC expression of cocultured myoblasts at 5 days. Only myoblast (Only MB) represents that myoblasts were cultured in differentiation medium without macrophages. Myoblasts with InfM (with InfM) and TEM (with TEM) indicate that myoblasts were cocultured with each macrophage. Myotube generation was evaluated by nuclear numbers of MyHC positive cells. Red and blue indicate MyHC and nuclear. The scale bar = 50μm. Magnification X200. (*$p<0.05$, **$P<0.01$)

Discussion

Tie2, a receptor of Ang1, is expressed on endothelial cells, hematopoietic lineages, and satellite cells (15, 35, 39). However, myoblasts does not express Tie2 (Fig. 5C) (26) and the mechanisms of Ang1 myogenesis remain unclear.

In this study, I described the several novel mechanisms of COMP-Ang1 for myogenesis. First, COMP-Ang1 accelerated muscle regeneration via increasing generation of central nucleus fibers in ischemic muscle. Second, COMP-Ang1 directly bound to N-cadherin on myoblast surface in Ca^{2+} ion dependent manner. Third, COMP-Ang1 induced myogenesis of myoblast through activation of N-cadherin-p120-catein-p38MAPK axis. Forth, p38MAPK activation by COMP-Ang1 induced synthesis of myogenin, an important myogenic factor. Fifth, COMP-Ang1 was a factor for TEM recruitment into ischemic muscle. Lastly, TEM infiltrated by COMP-Ang1 enhanced myogenesis with releasing myogenic factor containing IGF1.

COMP-Ang1 increased muscle regeneration and prevented fibrosis in ischemic muscle

Fibrosis and adipocyte like cells were increased in ischemic damaged

muscle (Fig. 2C). Stimulated by exercise or inflammation, satellite cells are reported to develop myofiber (40, 41). However, the damaged satellite cells in the scar tissue were changed to adipocyte or fibroblast, and muscle volume was also reduced (42). These events also happened in ischemic damaged muscle. When muscle was exposed to ischemic condition, inflammation was increased in damaged muscle region (5). The inflammation might increase the apoptosis of muscle fiber and stimulate satellite cells to change their fats that were differentiated to myotube, fibroblast, or adipocyte like cells. When COMP-Ang1 was treated to ischemic muscle, regenerating muscle fibers were increased in damaged muscle instead of fibrosis and adipocyte like cells generation (Fig. 2C). These results suggest that COMP-Ang1 could act as a key factor to regulate fat of satellite cells. I observed that myoblasts in hypoxic condition were induced to generate myotube such as normoxic myoblasts but, in long time culture, the generated myotube was rapidly regressed despite of COMP-Ang1 existence (data not shown). This result indicates that oxygen is a very important factor for myogenesis. Asahara, T., *et al*. showed that only Ang1 did not induce in neovasculogenesis, but combination of Ang1 and VEGF is extremely boomed neovasculogenesis (43). Additionally, I also reported COMP-Ang1 rescued ischemic muscle through

angiogenesis that mechanisms increased survival of hypoxic endothelial cells and recruitment of angiogenic progenitor cells into ischemic muscle (17, 19). Neovasculogenesis of Ang1 and COMP-Ang1 in ischemic muscle could suppress oxygen deprivation and might enhance to help myotube generation.

Additionally, COMP-Ang1 could directly increase myotube generation of satellite cells. Satellite cells in injured or exercised muscle developed myotube for repair myotube (44, 45). However, fibrosis was increased by ischemic condition. The increased fibrosis might be derived from satellite cells because Zhang, L., *et al*. showed the dysfunctional satellite cells could be changed to fibroblasts and induce fibrosis area (46). In contrast, the fibrosis region was significantly reduced by COMP-Ang1, and, especially, in this study, COMP-Ang1 directly induced myogenesis of myoblasts. This evidence suggests that COMP-Ang1 induces to develop satellite cells to muscle regeneration and prevents fibroblast differentiation. The further study is needed to evaluate the suppressed mechanism of fibroblast and adipocyte like cell generation in satellite cells by COMP-Ang1.

COMP-Ang1 induced p38 MAPK activation and bound to myoblast surface dependent on Ca^{2+} ion during myogenesis

Ang1 was known to bind integrin β 1 with Ca^{2+} ion and enhance

survival signaling such as AKT/ERK in myoblast (26). However, I confirmed that COMP-Ang1 did not activate AKT/ERK in differentiation condition (Fig. 5A). These evidences indicate that COMP-Ang1-integrin β 1 signaling in myoblast couldn't regulate myogenesis. On the other hand, COMP-Ang1 induced p38MAPK activation and the myogenin expression via p38MAPK pathway. This result describes that the upstream of p38MPAK was contributed to myogenesis by COMP-Ang1. Additionally, p38 MAPK activation and myogenin expression by COMP-Ang1 was suppressed by calcium ion chelating. But, several papers reported that myogenesis was dependent on Ca^{2+} ion such as calcium ion intake and calmodulin-dependent kinase (47, 48). Therefore, the myogenin expression reduced by chelating calcium ion cannot be completely described by COMP-Ang1 signaling. However, COMP-Ang1 bound to myoblast surface relied on Ca^{2+} ion in cell surface binding experiment. The binding effect of COMP-Ang1 could be considered for the interaction between COMP-Ang1 and Ca^{2+} ion related factor on myoblast surface. Thus, these results describe that COMP-Ang1 could activate p38MAPK pathway and bind to myoblast surface for myogenesis in a Ca^{2+} ion dependent manner.

COMP-Ang1 promoted myogenesis via direct binding N-cadherin and inducing N-cadherin-p120-catenin activation

I checked surface molecules that are related to Ca^{2+} ion signaling in myoblast. Interestingly, N-cadherin, M-cadherin, and integrin β 1 are highly expressed in myoblast. When I performed specific knockdown of each molecules with siRNA, surface binding and p38MAPK activation of COMP-Ang1 were significantly suppressed by N-cadherin knockdown only. Especially, COMP-Ang1 directly interacted with N-cadherin which relied on Ca^{2+} ion. Moreover, COMP-Ang1 promoted interaction between N-cadherin and p120-catenin in a non-junction site and single cell. These evidences suggest that function of COMP-Ang1 was similar to N-cadherin extracellular domain. More specifically, cell to cell junction enhanced N-cadherin signaling which was dependent on Ca^{2+} ion (7, 32) and N-cadherin was activated with its homophilic interaction on cell junction point and p120-catenin also interacted with C-terminal domain of N-cadherin (31). Previous studies reported that COMP-Ang1 was known to form pentameric formation and induce binding with target molecules such as Tie2 receptor (49-51). Therefore, when COMP-Ang1 was bound to N-cadherin on myoblast surface, N-cadherin might be induce clustering of N-cadherin by COMP-Ang1, and then, the N-

cadherin clusters were bound to p120-catenin on N-cadherin C-terminal region. Thus, these results suggest COMP-Ang1 could directly bind to N-cadherin dependent on Ca^{2+} ion and promote interaction between N-cadherin and p120-catenin for myogenesis.

Tie2 expressing satellite cells might have down-regulated Tie2 expression mediated by ischemic condition

Abou-Khalil, R., *et al.* showed that satellite cells expressed Tie2 receptor and remained quiescent state by Ang1/ERK pathway (15). In this study, I found that N-cadherin was one of candidates that can bind Ang1 and accelerated to myogenesis. However, when myoblasts were transfected by hTie2 plasmid, COMP-Ang1 and endogenous Ang1 induced AKT activation and suppressed myogenic signaling such as p38MAPK activation and myogenin expression (Fig. 5C). These results suggest that COMP-Ang1/Ang1-Tie2 signaling in myoblasts could suppress myogenesis through hindering intercellular signaling such as p38MAPK activation or suppressing COMP-Ang1-N-cadheirn interaction because COMP-Ang1/Ang1 affinity might be higher with Tie2 receptor than N-cadherin. In fact, quiescent satellite cells expressed Tie2 receptor but, during myogenesis, satellite cells could be

developed into myoblasts, which do not express Tie2 receptor (15). Therefore, ischemic condition may contribute satellite cells to activate myogenesis and the activated satellite cells could be developed into myoblasts. Thus, these evidences suggest that COMP-Ang1-N-cadherin signaling could induce myogenesis of myoblasts in ischemic muscle.

COMP-Ang1 enhanced myogenesis of transplanted myoblasts in vivo

I confirmed COMP-Ang1-N-cadheirn signaling with the myoblast transplantation in nude mouse gastrocnemius muscle. The transplanted myoblasts were increased to myotube generation. Interestingly, when N-cadherin expression was suppressed by siRNA, the myogenic enhancing effect of COMP-Ang1 was completely diminished and these cells were transformed to fibroblast and generated fibrosis. Therefore, N-cadherin signaling in myoblasts was important to determine the fate of myogenic cells, which decided myogenesis or fibrogenesis. On the contrary, Charlton, C.A., *et al*. reported that N-cadherin null primary myoblasts were normally transformed to myotubes (52). I also made stable cell-line of N-cadherin knockdown myoblast by lentivirus shRNA infection. The N-cadherin knockdown myoblasts by shRNA had reduced N-cadherin expression but,

unexpectedly, didn't reduce myogenin expression and myotube generation like as Charlton, C.A., *et al*. report (data not shown). Interstingly, M-cadherin expression in N-cadherin knockdown myoblasts by shRNA was increased more than control myoblasts (data not shown). This cadherin switch phenomenon could compensate for loss of N-cadherin in myoblasts because the increased M-cadherin expression was also a factor of myogenesis (53, 54).

Therefore, I decided to knockdown N-cadherin was by siRNA. Actually, N-cadherin knockdown effect of siRNA was not maintained for 10 days. However, N-cadherin knockdown myoblasts by siRNA did not induce M-cadherin expression and myotube generation, despite of COMP-Ang1 existence. These evidences suggest that N-cadherin signaling is important to progress myogenesis in the early stage and COMP-Ang1 is a key factor of N-cadherin activation during myogenesis.

COMP-Ang1 was a factor for TEM recruitment in ischemic muscle

In previous our report, I confirmed that COMP-Ang1 was required for a recruitment of bone marrow progenitor cells to ischemic muscle for enhancing angiogenesis through SDF-1 expression in hypoxic endothelial cells (19). Similarly in this study, I also observed F4/80 positive macrophages

were infiltrated into ischemic muscle. Previously, Ang1 and COMP-Ang1 were known as anti-inflammatory factor (33, 34). However, I found that COMP-Ang1 induced F4/80 positive macrophage infiltration into ischemic muscle. Therefore, I thought the infiltrated F4/80 positive macrophages by COMP-Ang1 might be different compared to commonly known inflammatory macrophages. On the other hand, TEM in cancer cells were known to enhance tumor angiogenesis through paracrine effects of cytokines and increased migration by Ang2 (37, 38, 55-57). It was reported that Tie2 affinity of Ang1 was larger than Ang2 (58). Therefore, I checked whether TEM was infiltrated into ischemic muscle by COMP-Ang1. Interestingly, infiltration of TEM into ischemic muscle was increased by COMP-Ang1, which explained why infiltration of F4/80 positive macrophages was highly stimulated by COMP-Ang1. Moreover, TEM increased IGF1 expression more than InfM, which is one of the myogenic and angiogenic factors (59, 60). When TEM was cultured with C2C12 myoblasts, myoblasts were induced to express myogenin and myotube generation by TEM was increased more than by InfM.

Thus, these results suggest the TEM infiltrated into ischemic muscle by COMP-Ang1 could secrete paracrine factor such as IGF1 and enhance myogenesis.

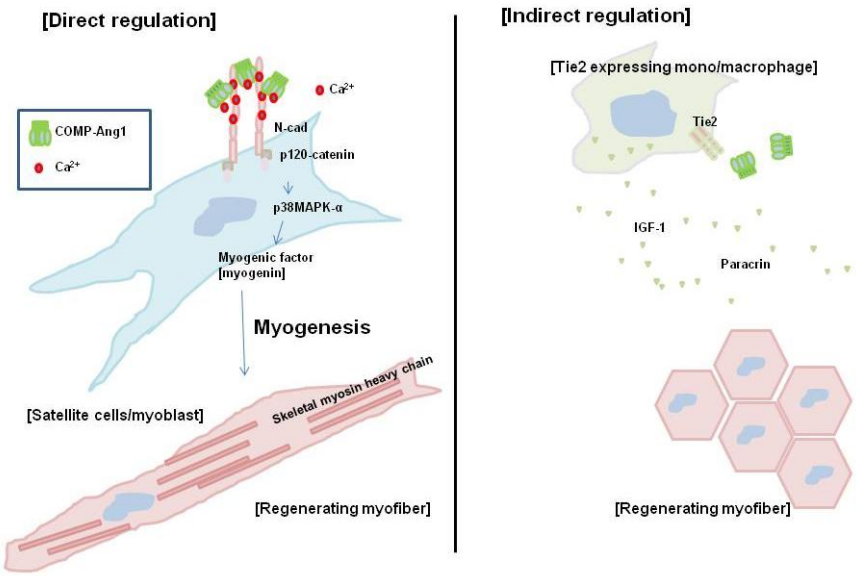


Figure 13. Dual regulation of COMP-Ang1 effects for myogenesis.

Conclusion

In this study, I propose that COMP-Ang1 is a myogenic factor, induces myogenesis via N-cadherin activation, and recruits TEM expressing myogenic factor into damaged muscle. Herein, these results could be applied to clinical approaches of ischemic muscle diseases or damaged muscle for muscle rescue. The damaged muscle was increased to muscle and vessel regression and the results progress to limb lost. However, COMP-Ang1 could promote vessel stability and regeneration of new muscle through mechanisms I propose in this study. Therefore, if COMP-Ang1 is purified to clinical grade protein for human diseases, I could propose that COMP-Ang1 can be used as therapeutic drug for the treatment of vascular and muscular diseases.

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Abstract (Korean)

COMP-Ang1의 허혈손상을 받은 다리에서 근육재생을 촉진하는 두 가지 기전에 관한 연구

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혈관 병에 의해 생성되는 저 산소 농도 는 주변조직의 apoptosis 와 necrosis 를 유발하게 된다. 허혈손상을 복구하게 위해서 신생혈관를 증가시키는 치료법을 개발하는 연구가 시행되고 있다. 혈관의 성숙과 permeabilization 을 억제시키는 작용으로 알려져 있는 angiopoietin-1 의 허혈손상을 복구에 관한 연구는 기초단계이다. 우리의 선행 연구에서 임상적 이용이 가능하도록 제작한 COMP-angiopoietin-1 (COMP-Ang1)을 통하여 허혈손상을 받은 쥐의 다리근육에서 신생혈관 증가에 관한 연구를 시행하던 중 근육재생이 되는 현상을 관찰 하였다. 아직까지

Angiopoietin-1 에 의한 근육재생에 관한 연구는 미비하다. 이에 본 연구에서는 허혈손상를 받은 쥐의 다리근육에서 COMP-Ang1 을 통한 근육재생기전을 규명하고자 하였다.

쥐의 넙적다리 동맥을 제거 함으로써 근육에 허혈상태를 만들었다. Adenovirus COMP-Ang1($1X10^9$pfu)을 근육내에 주사 하여 근육내부에서 COMP-Ang1 을 발현하도록 하였다. COMP-Ang1 은 허혈하지의 발의힘, 허혈근육의 부피와 신생 근육 섬유를 증가시켰다. 또한, COMP-Ang1 은 C2C12 myoblast 의 분화를 증가 시켰다. 그 기전은 COMP-Ang1 이 C2C12 myoblast 의 N-cadherin 과 부착하여 N-cadherin 과 p120-catenin 의 상호작용을 증가시켜 p38MAPK 를 활성화 시키고, 그로 인한 myogenin 의 발현을 증가시킴으로 최종적으로 myotube 형성을 증가 시켰다. 이와 같은 작용은 calcium chelator 인 EGTA 와 p38MAPK inhibitor 를 사용하였을 때 억제되었다. 또한, 허혈근육내의 mono/macrophage 들이 침투되는 것을 확인 하였다. 흥미롭게도, COMP-Ang1 에 의해서 Tie2-expressing macrophages ($F4/80^+Tie2^+CD31^-$, TEM)가 허혈근육으로 침투가 증가 되었고, inflammatory macrophages($F4/80^+Tie2^-CD31^-$, InfM)의 침투에는 큰 영향이 없었다. TEM 은 근육재생인자인

IGF-1 의 발현을 증가시켜 myoblast 의 myogenesis 를 증가시켰다.

본 연구를 통하여 COMP-Ang1 이 myoblast 에서 발현하는 N-cadherin 을 직접적으로 활성 시켜 myogenesis 를 증가시킴을 확인 할 수 있었고, 또한 근육 재생인자를 분비하는 TEM 을 허혈하지로의 침투을 증가시켜 간접적으로 근육재생을 증가시킴을 확인하였다. 그럼으로 COMP-Ang1 은 기존에 알려져 있던 혈관신생의 효과와 더불어 허혈손상을 받은 골 근육의 재생을 촉진시킬 수 있는 후보 물질이라고 판단된다.

주요어: 근육재생, COMP-Ang1, N-cadherin, 근육줄기세포, 하지허혈

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