considered ethical to use placebo treatment for a long-term study and the open-label design is consistent with routine clinical practice.

Sensitivity to GH therapy varies considerably, particularly in the elderly (1). It has been demonstrated that use of individualized dosing regimens reduces variation in response for most endpoints, particularly between genders (9). However, this method based on serum IGF-I concentrations has not been investigated in Japanese GHD patients. In the present study such a dosing regimen allowed for more moderate dose increases compared with the fixed-dose titration method used in the initial study (4). Data from a similar study in Caucasians also showed a lower dose when using individual dose adjustment according to IGF-I compared with a fixed-dose titration (10). The final individualized dose in that study was 0.54 mg/day, which was slightly higher than the approximate daily doses in the present study, which were 0.42 mg/day for the PL/GH group and 0.48 mg/day for the GH/GH group; this is in line with what may be expected because of the higher body weight of Caucasians compared with Japanese, and would argue for dosing based on body weight in order not to overdose those of smaller, leaner body proportions.

Consistent with other studies (11), the younger CO patients were receiving a higher mean dose of GH than the older AO patients in both groups at the end of the study. Whereas this difference in dose requirements between onsets has been suggested in Caucasian patients (12), this is the first demonstration of the difference in optimal doses by onsets in Japanese GHD patients. There was a wide range of doses even within these two groups; thus, individualized treatment based on IGF-I levels indicates that optimal dosage may differ in patients with different baseline characteristics, supporting this approach to GH treatment. Despite this difference in dose, the changes in LBM and fat mass were statistically significant in both AO and CO patients in both groups. Current recommendations suggest that IGF-I and IGFBP-3 are measured as a part of the management of patients receiving GH treatment (6). High levels of IGF-I can mediate adverse drug reactions such as arthralgia. In the present study, using the dose-adjustment method, changes in IGF-I and IGFBP-3 levels were parallel and within normal limits.

The double-blind study on Japanese adult GHD patients (4) confirmed the efficacy of GH therapy over a period of 24 weeks, in agreement with results from clinical trials over 3–6 months in Caucasian subjects (13). In another trial of Japanese adult GHD patients, mean body fat was reduced after 1 month of GH therapy and this was maintained for a further 8 months (14). In the present longer-term open-label study, Japanese adult hypopituitary patients with GHD received individualized GH therapy for 48 weeks. These patients had a significant increase in LBM and decrease in fat mass. Although there was a dose reduction in the GH/GH group between the double-blind and open-label studies, they maintained the improvements in these parameters over this treatment period.

The decrease in fat mass was in accord with a significant improvement in total cholesterol and LDL-cholesterol in those patients with abnormally high levels at baseline in this individualized-dose study, and is consistent with results from other studies (15–17). These high levels were above the Japanese validation for the Framingham threshold for cardiovascular risk and hence individualized GH therapy contributed to a reduction in these risk factors (18). The higher cholesterol values observed after 48 weeks compared with 24 weeks of treatment in both groups has been observed in other clinical trials (14). GH has complex effects on lipid metabolism (19) and it may be that cholesterol levels varied during GH treatment while attaining a new equilibrium state. In Japanese children with GHD, who have been treated with GH, mean total cholesterol and total/high-density-lipoprotein-cholesterol ratio were decreased markedly in both sexes but statistical significance was detected only in boys (20). The longest-term data in Japanese paediatric male GHD patients showed that 3 years of GH replacement caused a significant decrease in LDL-cholesterol (21).

Adverse events were predominantly mild and the most frequently reported were either common events (nasopharyngitis, pyrexia) or known to be associated with GH pharmacological action (arthralgia, headache). In the early stages of GH-replacement therapy, retention of water may occur, leading to oedema, arthralgia or carpal tunnel syndrome (22). In the present study, consistent with other studies (12), GH was administered at a low dose until week 8 in the open-label study to minimize this problem. In the 48-week open-label study the incidence of oedema was lower in both the GH/GH and PL/GH groups compared with the GH/GH group in the double-blind study. This lower incidence may reflect the decreased dose consequent on the individual dosing regimen. There were only three serious adverse events during the IGF-I-controlled regimen, suggesting a safer profile of this GH modality. As might be anticipated, from the inhibitory effects of GH on insulin sensitivity, there was a slight increase in mean serum HbA1c levels in the PL/GH group. This observation is consistent with other studies on Caucasian patients (23). It has been reported that the interaction of GH with other hormones during replacement therapy accelerates the conversion of $T_4$ to $T_3$, at least in the early stages of treatment (24). However, routine monitoring of thyroid function during the present study did not indicate any changes in these parameters.

In summary, the efficacy of individualized GH-replacement therapy in adult Japanese patients has been

demonstrated. This treatment regimen conferred advantages in terms of level of dosage and safety and in treating patients with different baseline characteristics. It is recommended that the dose-adjustment method of GH administration, based on IGF-I levels, is adopted for future treatment of adult Japanese GHD patients.

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# Transforming growth factor-β enhances connective tissue growth factor expression in L6 rat skeletal myotubes

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**Abstract**

Transforming growth factor (TGF)-β plays an important role in fibrosis of various organs and tissues. TGF-β1 stimulates fibroblastic cells to form extracellular matrix (ECM), and regulates all critical events in wound healing. Connective tissue growth factor (CTGF), a TGF-β-inducible molecule, has recently been reported to promote fibroblast proliferation, migration, adhesion and extracellular matrix formation, both in vivo and in vitro. In this study, we demonstrated that TGF-β1 enhances CTGF mRNA and protein levels in L6 rat skeletal muscle myotubes. TGF-β might, therefore, play a role in fibrosis of skeletal muscle by stimulating CTGF expression in the muscle tissue itself.


*Keywords:* TGF-β; CTGF; Myotube; Fibrosis

## 1. Introduction

Transforming growth factor (TGF)-β plays an important role in fibrosis of organs such as the liver, kidney, skin and lung. TGF-β1 stimulates fibroblastic cells to proliferate, migrate and form extracellular matrix (ECM) during the critical events of wound healing [1]. TGF-β stimulates fibroblastic cells to promote production of ECM and suppresses synthesis of matrix metalloproteinases in order to induce fibrosis of organs, and is known to play a crucial role in fibrosis of the liver [2]. Following liver injury resulting from a toxin or viral infection, hepatic stellate cells promote production of TGF-β, which causes overproduction of ECM.

In skeletal muscle, TGF-β1 was reported to be elevated in Duchenne/Becker muscular dystrophy [3,4], myotonic dystrophy [5], congenital muscular dystrophy [6], and inflammatory myopathies [7,8]. TGF-β1 thus appears to be involved in the pathogenesis of fibrosis in chronic muscular disorders.

In 1991, Bradham et al. first used the term 'Connective tissue growth factor (CTGF)' to describe a novel growth factor secreted by human endothelial cells in culture [9]. CTGF is a member of the ctgf/cyr61/nov (CCN) gene family and is expressed in various human tissues and organs, including the kidney, heart, placenta, lung, liver and pancreas. CTGF acts to promote fibroblast proliferation, migration, adhesion, and ECM formation [10,11]. CTGF is mainly induced by TGF-β in cells and tissues, although thrombin [12], VEGF [13] and dexamethasone [14] have also been known to stimulate CTGF induction. In vitro, TGF-β promotes CTGF expression in fibroblastic cells, endothelial cells, epithelial cells, vascular smooth muscle cells, and neuronal cells [10,11]. In skeletal muscle cells, however, TGF-β induction of CTGF expression has not been widely studied. In this study, we therefore examined whether TGF-β is able to enhance CTGF expression in skeletal muscle using the L6 rat skeletal myotubes.

## 2. Materials and methods

### 2.1. Materials

L6 rat skeletal myoblasts were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

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Recombinant human TGF-β1 was purchased from Sigma (St Louis, MO, USA). Anti-human CTGF-terminal peptide antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other materials and chemicals were obtained from commercial sources.

### 2.2. Cell culture

L6 rat skeletal muscle myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Subconfluent cells were harvested with trypsin/ethylenediaminetetraacetic acid (EDTA) and were seeded into 100-mm dishes ($1.8 \times 10^4$ cells/cm$^2$). Differentiation of L6 myoblasts into myotubes was achieved by growing the cells to confluence and changing the serum supplementation from 10% FBS to 2% (differentiation medium). Myogenic differentiation to myotubes was confirmed both morphologically and biochemically, as described previously [15,16]. After 72 h of exposure to differentiation medium, cells were cultured in serum-free DMEM containing different concentrations of TGF-β1 (0, 0.1, 1.0, 5.0, or 10.0 ng/ml). The effect of 5.0 ng/ml TGF-β was further studied at 0, 3, 6, 12, 24, or 48 h.

### 2.3. Northern blot analysis

Total RNA was isolated with an RNA isolation kit (NucleoSpin RNA II; Machery-Nagel, Düren, Germany). Total RNA (10 μg/lane) was subjected to electrophoresis on 1% agarose gels containing formaldehyde and was then transferred to Nylon hybridization transfer membrane. The membrane was fixed with UV-light (FUNA-UV-LINKER; Funakoshi, Tokyo, Japan). Membranes were hybridized with cDNA probes using the Gene Images AlkPhosDirect labeling and detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). A 0.23-kb cDNA fragment contained within the open reading frame of CTGF was used as a probe. Membranes were finally visualized using the CDP-Star detection reagent for chemiluminescent detection of alkaline phosphatase (Amersham). Membranes were then exposed to Hyperfilm ECL (Amersham) for 1 h at room temperature.

### 2.4. Western blot analysis

L6 myotubes were lysed in radioimmunoprecipitation (RIPA) buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), complete protease inhibitor mixture, 1% Triton X-100, and 1 mM sodium orthovanadate. Cell lysates were centrifuged at 12,000 *g* for 20 min at 4 °C. Protein quantification was performed using BCA protein assay reagent (Pierce, Rockford, IL, USA). Equal amounts of supernatant were subjected to 10% SDS-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred to polyvinylidene difluoide. Blots were blocked with Tris-buffered saline (TBS; 20 mM Tris–Hcl [pH 7.5] and 137 mM NaCl) containing 0.1% Tween 20 and 3% dried milk powder, and were then incubated with anti-CTGF antibody at 4 °C overnight. Primary antibody was used at a 1:500 dilution. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Labs, West Grove, PA, USA), and were finally visualized using an ECL kit (Amersham).

## 3. Results

### 3.1. Northern blot analysis

Fig. 1 shows the effects of TGF-β1 on the expression of CTGF mRNA in L6 myotubes. Enhancement of CTGF mRNA was observed from 3 to 48 h after stimulation with 5 ng/ml TGF-β1, with peak expression seen at 6 h, as shown in Fig. 1(a). Expression of CTGF mRNA increased in a dose-dependent manner within a range from 0.1 to 10 ng/ml TGF-β1, as shown in Fig. 1(b). Enhancement of CTGF mRNA expression primarily occurred at 5–10 ng/ml TGF-β1.

### 3.2. Western blot analysis

Fig. 2 shows the effects of TGF-β1 on the expression of CTGF protein in L6 myotubes. The time course of CTGF protein expression induced by TGF-β1 is shown in Fig. 2(a). Stimulation with 5 ng/ml TGF-β1 increased CTGF protein levels in L6 myotubes from 3 to 48 h after treatment, with peak expression seen at 12 h, as shown in Fig. 2(a).

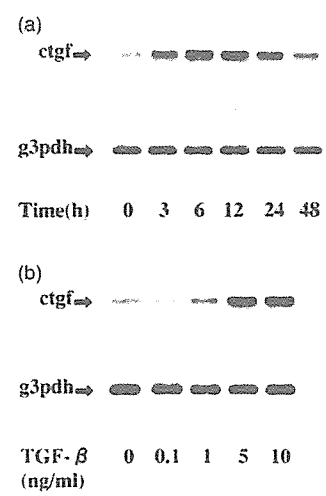


Fig. 1. (a) Time course of CTGF mRNA expression in L6 myotubes stimulated with 5 ng/ml TGF-β1. Enhancement of CTGF mRNA was observed from 3 to 48 h after stimulation. (b) Dose-dependent effects of TGF-β1 on CTGF mRNA in L6 myotubes. Enhancement of CTGF mRNA expression primarily occurred at 5–10 ng/ml TGF-β1. Equal loading was confirmed against G3PDH.

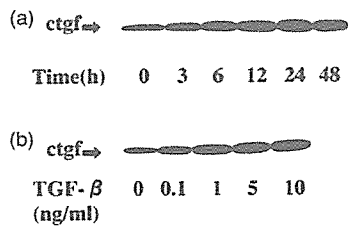


Fig. 2. (a) Time course of CTGF protein expression in L6 myotubes stimulated with 5 ng/ml TGF-β1. CTGF protein levels increased from 3 to 48 h after treatment. (b) Dose-dependent effects of TGF-β1 on CTGF protein expression in L6 myotubes.

The increase in CTGF protein was dose dependent in a range from 0.1 to 10 ng/ml TGF-β1 (Fig. 2(b)).

## 4. Discussion

Fibrosis among muscle fibers is one of the histopathological hallmarks of chronic skeletal muscle disease or injury. Abundant fibrosis is known to interfere with both regeneration of muscle fibers and recovery of contractile function. Muscle regeneration is internally regulated by various transcriptional regulators, such as MyoD and myogenin [17]. It is also regulated by numerous factors generated by different cells, including skeletal muscle cells, fibroblasts, and macrophages. Various growth factors, including platelet-derived growth factor, basic fibroblast growth factor, and TGF-β, secreted by macrophages are present within the microenvironment of regenerating muscle fibers [18,19]. Both internal regulators and external effectors are involved in the complex mechanism of skeletal muscle fibrosis.

TGF-β stimulates fibroblast proliferation and collagen synthesis and is responsible for the excessive collagen production that leads to alteration of tissue architecture and ultimately interferes with tissue regeneration. TGF-β has direct effects on skeletal muscle cells. TGF-β1 reportedly induces collagen and fibronectin synthesis in rat myoblasts [20,21]. In vitro, TGF-β1 down-regulates the expression of myogenic proteins and initiates the production of fibrosis-related proteins [22]. In vivo, TGF-β1 stimulates both the initiation of fibrotic cascades in skeletal muscle and the induction of myogenic cell differentiation into fibrotic cells [22]. TGF-β1 appears to be involved in the pathogenesis of fibrosis in muscle.

TGF-β promotes CTGF expression in fibroblastic cells, endothelial cells, epithelial cells, vascular smooth muscle cells, and neuronal cells [10,11]. TGF-β is a specific inducer of CTGF expression in both cardiac fibroblasts and cardiac myocytes [23]. In skeletal muscle, TGF-β-induced CTGF expression has not been extensively studied, although expression of CTGF reportedly increased under conditions of augmented TGF-β expression in muscular diseases [4,6, 24]. Obreo et al. recently reported that TGF-β induced an increase in CTGF expression and collagen synthesis and accumulation in L6 myoblasts [25]. In this study, we demonstrated that TGF-β1 enhanced CTGF mRNA and protein levels in L6 myotubes in dose dependent manner. This suggests that TGF-β1 is able to stimulate the induction of CTGF in skeletal muscle.

CTGF appears to play a role in the ECM remodeling that occurs in normal physiological processes, such as embryogenesis, implantation, and wound healing [11]. In pathological processes, CTGF is believed to play a key role in the fibrosis of many tissues. Overproduction of CTGF reportedly contributes to the fibrosis and scarring of major organs in fibroproliferative diseases, such as liver cirrhosis, diabetic nephropathy, lung fibrosis [26] and vascular atherosclerosis [27]. In glomerulosclerosis and tubulointerstitial fibrosis, CTGF expression was increased and CTGF levels were correlated with the progression and severity of fibrosis [28]. CTGF induced by TGF-β may promote ECM proliferation and fibrosis in skeletal muscle. This postulation would indicate that TGF-β and CTGF play a critical role in skeletal muscle fibrosis.

Suppression of TGF-β is reportedly effective in treating fibrosis in various organs [2,29]. Down-regulation of TGF-β1 in dermatomyositis patients is associated with improvements in muscle cytoarchitecture and reductions in endomysial inflammation and connective tissue proliferation [30]. In a mouse laceration model, γINF, which inhibits TGF-β1 signaling, decreased the area of fibrosis [31]. Down-regulation of TGF-β might effectively improve inflammatory myopathies by reducing fibrosis. Further studies are necessary in order to clarify the role of CTGF induction by TGF-β1 in skeletal muscle and to better understand skeletal muscle disorders and fibrosis.

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# Menin Suppresses Osteoblast Differentiation by Antagonizing the AP-1 Factor, JunD*

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**Mice null for menin, the product of the multiple endocrine neoplasia type 1 (MEN1) gene, exhibit cranial and facial hypoplasia suggesting a role for menin in bone formation. We have shown previously that menin is required for the commitment of multipotential mesenchymal stem cells into the osteoblast lineage in part by interacting with the bone morphogenetic protein (BMP)-2 signaling molecules Smad1/5, and the key osteoblast transcriptional regulator, Runx2 (Sowa H., Kaji, H., Hendy, G. N., Canaff, L., Komori, T., Sugimoto, T., and Chihara, K. (2004) *J. Biol. Chem.* 279, 40267–40275). However, menin inhibits the later differentiation of committed osteoblasts. The activator protein-1 (AP-1) transcription factor, JunD, is expressed in osteoblasts and has been shown to interact with menin in other cell types. Here, we examined the consequences of menin-JunD interaction on osteoblast differentiation in mouse osteoblastic MC3T3-E1 cells. JunD expression, assessed by immunoblot, gradually increased during osteoblast differentiation. Stable expression of JunD enhanced expression of the differentiation markers, Runx2, type 1 collagen (COL1), and osteocalcin (OCN) and alkaline phosphatase (ALP) activity and mineralization. Hence, JunD promotes osteoblast differentiation. In MC3T3-E1 cells in which menin expression was reduced by stable menin antisense DNA transfection, JunD levels were increased. When JunD and menin were co-transfected in MC3T3-E1 cells, they co-immunoprecipitated. JunD overexpression increased the transcriptional activity of an AP-1 luciferase reporter construct, and this activity was reduced by co-transfection of menin. Therefore, JunD and menin interact both physically and functionally in osteoblasts. Furthermore, menin overexpression inhibited the ALP activity induced by JunD. In conclusion, the data suggest that menin suppresses osteoblast maturation, in part, by inhibiting the differentiation actions of JunD.**

The activator protein-1 (AP-1)[1] transcription factor complex plays an important role in skeletal development and maintenance (1). AP-1 consists of dimers formed by members of the Fos, Jun, and ATF protein families. Fos proteins (c-Fos, FosB, Fra-1, Fra-2) are only able to heterodimerize with members of the Jun family, whereas the Jun proteins (c-Jun, JunB, JunD) can both homo- and heterodimerize with Fos proteins to form transcriptionally active complexes. AP-1 is implicated in cell differentiation, proliferation, apoptosis, and oncogenic transformation (2). AP-1 activity is controlled by upstream kinases and is modulated by other transcription factors. The study of genetically modified mice (and cells) has provided some insights into the biological functions of AP-1 family members (1, 3). Deletion of Fra1, c-Jun, or JunB in mice is embryonic lethal, whereas lack of c-Fos, FosB, or JunD is not. Thus, some Fos/Jun proteins are essential and these and the others may have redundant or overlapping functions.

Overexpression of c-Fos in transgenic and chimeric mice specifically affects bone, cartilage, and hematopoietic cell development, and the mice develop osteosarcomas (4). Homozygous c-Fos knock-out mice are growth-retarded, lack osteoclasts, and develop osteopetrosis with deficient bone remodeling (5). Thus, c-Fos is an essential regulator of macrophage/osteoclast lineage determination and bone remodeling (6). Overexpression of either Fra-1 or ΔFosB increases bone formation causing osteosclerosis in transgenic mice (7, 8). ΔFosB, a naturally occurring alternatively spliced product lacking the COOH-terminal part of FosB and hence a known transcriptional domain, nonetheless does influence transcriptional activity possibly by heterodimerizing with other AP-1 proteins such as JunD (8). Studies of engineered mice with altered Fra-2 expression indicates that this AP-1 factor plays important roles in both osteoblast and osteoclast differentiation. Bone volume is markedly reduced and both number and sizes of osteoclasts are dramatically increased in Fra-2 knock-out mice (9). Loss of JunB in mice results in reduced bone formation and severe bone turnover osteopenia mainly due to a cell-autonomous osteoblast and osteoclast differentiation defect (10). The role that JunD might play in bone biology is less clear. It is known that while the various AP-1 family members are differentially expressed in osteoblasts *in vitro*, Fra-2 and JunD predominate in differentiating osteoblasts (11). Homozygous JunD$^{-/-}$ mice show reduced postnatal growth, although

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[1] The abbreviations used are: AP-1, activator protein-1; MEN1, multiple endocrine neoplasia type 1; BMP, bone morphogenetic protein; TGF, transforming growth factor; FBS, fetal bovine serum; RT, reverse transcription; COL1, type 1 collagen; OCN, osteocalcin; ALP, alkaline phosphatase.

the nature of any altered bone phenotype has not yet been examined (12), and JunD$^{-/-}$ males exhibit defects in reproduction although no defects in fertility have been observed in JunD$^{-/-}$ females. Therefore, the evidence overall points to redundant functions for members of the Fos/Jun family including JunD during development.

Menin is the product of the multiple endocrine neoplasia type 1 (MEN1) gene, which when inactivated is responsible for an autosomal dominant cancer syndrome characterized by tumors of the parathyroid, endocrine pancreas, and anterior pituitary (13). The 610-amino acid protein has carboxyl-terminal nuclear localization sequences and has been demonstrated to be predominantly nuclear (14, 15). The physiological functions of menin are unclear but may be related to transcriptional regulation (16, 17), cell cycle control (15), and interactions with a variety of proteins including transcription factors have been demonstrated (16–20). Menin is widely expressed from an early developmental stage and found in both nonendocrine and endocrine tissues (21).

Recently, Crabtree *et al.* (22) reported that whereas mice heterozygous for menin inactivation exhibit a phenotype similar to that of the human MEN1 disorder, and develop endocrine tumors later in life, homozygous menin inactivation was embryonic lethal, and some fetuses had clear defects in cranial and facial development. This suggested that menin might play a role in osteoblast formation and differentiation. We showed previously that menin is required for the commitment of multipotential mesenchymal stem cells to the osteoblast lineage (23). This occurred, in part, by the roles played by menin in facilitating BMP signaling via Smads and the transcriptional activity of the key osteoblast regulator, Runx2 (23, 24). Thus, menin physically and functionally interacted with Smads1/5 and Runx2 in mesenchymal stem cells. In committed osteoblasts these interactions were, for the most part, lost and menin inhibited later osteoblastic differentiation. This seemed to occur, in part, by the interaction of menin with the TGF-β/Smad3 pathway (24). However, it is likely that other mechanisms are involved in the role of menin in retarding differentiation of mature osteoblasts.

JunD has been identified as an interacting partner of menin, and this interaction leads to a repression of JunD-activated transcription (16, 25). Among several AP-1 transcription factors tested, only JunD directly binds to menin. The physiological significance of menin-JunD interaction, in any tissue, is not known. In the present study, we have examined the role of JunD in osteoblast differentiation by use of mouse MC3T3-E1 cells. Moreover, we demonstrated that JunD and menin interact in these osteoblast cells and explored the consequences of this interaction with respect to the suppressive effect of menin on the further differentiation of committed osteoblasts.

## EXPERIMENTAL PROCEDURES

*Materials*—Human recombinant TGF-β was from Sigma. Anti-JunD antibody was from Sigma and Type I collagen antibody was from Calbiochem LSL Co., Ltd. (Tokyo, Japan). The menin rabbit polyclonal antibody was generated as described previously (15). All chemicals used were of analytical grade.

*Cell Culture*—MC3T3-E1 cells were cultured in α-minimal essential medium (containing 50 μg/ml ascorbic acid) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). The medium was changed twice a week.

*Construction of Expression Plasmids and Stable Transfection*—The human sense menin cDNA expression vector was constructed as described previously (17). For the antisense menin construct, human menin DNA was cloned in an antisense orientation into the EcoRI site of pcDNA3.1(+). An XbaI-HindIII fragment bearing the mouse JunD DNA was cloned into pcDNA3.1(−) to create the sense JunD cDNA construct. Mouse JunD DNA, antisense menin DNA (AS), or empty vector (V) (each 3 μg) were transfected into MC3T3-E1 cells with Lipofectamine (Invitrogen). Six hours after transfection, the cells were fed with fresh α-minimal essential medium containing 10% FBS. After 48 h, cells were passaged, and clones were selected in α-minimal essential medium supplemented with G418 (0.3 mg/ml) (Invitrogen) and 10% FBS. Reduced expression of menin by AS-DNA was detected with immunoblot analysis, using the polyclonal anti-menin antibody. To rule out the possibility of clonal variation, we characterized at least three independent clones for each transfection. V-transfected cells were used as the control.

*Luciferase Assay*—Cells were seeded at a density of $2 \times 10^5$/6-well plate. Twenty-four hours later, cells were transfected with 3 μg of the reporter plasmid (p3TP-Lux) and the pCH110 plasmid expressing β-galactosidase (1 μg) using Lipofectamine. Fifteen hours later, the medium was changed to a 4% FBS-containing medium, and the cells were incubated for an additional 9 h. Thereafter, the cells were cultured for 24 h in the presence or absence of 5 ng/ml TGF-β in medium containing 0.2% FBS. Cells were lysed, and the luciferase activity measured and normalized to the relative α-galactosidase activity, as described (17).

*Protein Extraction, Co-immunoprecipitation, and Western Blot Analysis*—Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mixture, 1% Triton X-100, and 1 mM sodium orthovanadate. Cell lysates were centrifuged at 12,000 × *g* for 20 min at 4 °C, and the supernatants were stored at −80 °C. Protein quantitation was performed with BCA protein assay reagent (Pierce). Proteins were denatured in SDS sample buffer and separated on 10% polyacrylamide-SDS gels and then transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride. Blots were blocked with TBS (20 mM Tris-HCl (pH 7.5) and 137 mM NaCl) plus 0.1% Tween 20 containing 3% dried milk powder. The antigen-antibody complexes were visualized using the appropriate secondary antibodies (Sigma) and the enhanced chemiluminescence detection system, as recommended by the manufacturer (Amersham Biosciences). For all experiments, 20 μg of protein were applied to each lane.

For co-immunoprecipitation experiments, cells were lysed with a buffer containing 1% Triton X-100, 1% deoxycholate, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1.5 mM $MgCl_2$, 2 mM EGTA, plus protease inhibitor mixture for 30 min at 4 °C, and insoluble materials were separated by centrifugation at 4 °C for 30 min at 14,000 × *g*. An aliquot of the supernatant (1 mg of protein) was clarified and incubated with anti-JunD antibody on a rocking platform at 4 °C overnight. The immune complexes were collected with protein G Plus/protein A-agarose beads (Calbiochem) for 30 min at 4 °C. The beads were washed three times with the lysis buffer, resuspended in 2× sample buffer, and boiled for 5 min. Immunoprecipitated proteins were then analyzed by SDS-PAGE and subjected to Western blot analysis, as described above.

*RNA Extraction and Northern Blot Analysis*—RNA was prepared from cells with TRIzol reagent (Invitrogen). Northern blot analysis was performed, as described previously (26). In brief, 20-μg aliquots of total RNA were denatured, electrophoresed on 1% agarose gels containing 2% formaldehyde, and then transferred to nitrocellulose membranes and fixed with UV light. Membranes were hybridized to a $^{32}P$-labeled DNA probe overnight at 42 °C. The hybridization probes were type I collagen (COL1) (a gift from Dr. T. Kimura, Osaka University, Osaka, Japan) and mouse OCN. After hybridization, the filters were washed twice with saline citrate containing SDS and exposed to x-ray film. All values were normalized for RNA loading by probing blots with human β-actin cDNA (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

*Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*—Reverse transcription of 5 μg of cultured cell total RNA was carried out for 50 min at 42 °C and then 15 min at 70 °C, using the SuperScript™ first strand synthesis system for RT-PCR (Invitrogen), which contained RT buffer, oligo$(dT)_{12-18}$, 5× first strand solution, 10 mM dNTP, 0.1 M dithiothreitol, SuperScript II (RT-enzyme), and RNase H (RNase inhibitor). PCR using primers to unique sequences in each cDNA was carried out in a volume of 10 μl of reaction mixture for PCR (as supplied by TaKaRa, Otsu, Japan), supplemented with 2.5 units of TaKaRa TaqTM, 1.5 mM concentration each dNTP (TaKaRa), and PCR buffer (10×), which contained 100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM $MgCl_2$. 25 ng of each primer and 1 ml of template (from a 50 ml RT reaction) were used. Thermal cycling conditions were: 1) initial denaturation at 96 °C for 2 min; 2) cycling for cDNA-specific number of cycles (96 °C for 1 min, cDNA-specific annealing temperature for 2 min, and 72 °C for 2 min); and 3) final extension at 72 °C for 5 min. Primer sequences, annealing temperature, and cycle numbers were as follows: Runx2, 5′-CAGGAAGACTGCAAGAAGGCTCTGG-3′ and 5′-ACACG-

GTGTCACTGCGCTGAAGA-3′ (62 °C; 25 cycles); glyceraldehyde-3-phosphate dehydrogenase, 5′-ATCCCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTGATG-3′ (47 °C; 24 cycles). For semiquantitative RT-PCR, the number of cycles was chosen so that amplification remained well within the linear range. Equal volumes from each PCR were analyzed by 6% nondenaturing polyacrylamide gel electrophoresis, and ethidium bromide-stained PCR products were evaluated by densitometry (NIH Image J, version 1.08i, public domain program). Marker gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase expression in each sample.

*ALP Activity Assay and ALP Staining*—ALP activity was assayed as described previously (27). In brief, the assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol (Sigma), 1 mM $MgCl_2$, 8 mM *p*-nitrophenyl phosphate disodium, and cell homogenates. After a 3-min incubation at 37 °C, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with *p*-nitrophenol (Sigma). Each value was normalized to the protein concentration. ALP staining was performed by a standard protocol. In brief, cultured cells were rinsed in phosphate-buffered saline, fixed in 100% methanol, rinsed with phosphate-buffered saline, and then overlaid with 1.5 ml of 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Invitrogen) plus 0.3 mg/ml nitro blue tetrazolium chloride (Invitrogen) in 0.1 M Tris-HCl (pH 9.5), 0.01 N NaOH, 0.05 M $MgCl_2$, followed by incubation at room temperature for 2 h in the dark.

*Mineralization Assay*—Mineralization of MC3T3-E1 cells was determined in 6- and 12-well plates using von Kossa staining and Alizarin red staining, respectively. The cells were fixed with 95% ethanol and stained with $AgNO_3$ by the von Kossa method. At the same time, the 12-well plates were fixed with ice-cold 70% ethanol and stained with Alizarin red (Sigma). For quantitation, cells stained with Alizarin red were destained with ethylpyridinium chloride (Wako Pure Chemical Industries, Ltd.), and then the extracted stain was transferred to a 96-well plate, and the absorbance at 562 nm was measured using a microplate reader, as described previously (26).

*Statistics*—Data are expressed as means ± S.E. Statistical analysis was performed using an unpaired *t* test or analysis of variance.

## RESULTS

*JunD Expression and Osteoblast Differentiation*—To examine the role of JunD in the later differentiation of osteoblastic cells, we employed MC3T3-E1 cells. We have previously shown that in MC3T3-E1 cells ALP activity increased with time of culture (up to 21 days). The levels of COL1 and OPN expression were highest at days 7 and 14 of culture, respectively, whereas the expression of OCN mRNA, a terminal differentiation marker, was highest at day 21 of culture (see Fig. 9, *B* and *C*, of Ref. 23). We confirmed these changes in the present study (data not shown) and demonstrated that endogenous JunD expression, detected by immunoblot, increased throughout the culture period to reach its highest level in the terminally differentiated 21-day culture (Fig. 1, *A* and *B*). These findings suggest that JunD plays an important role in well differentiated osteoblasts.

*Generation of MC3T3-E1 Clones Stably Overexpressing JunD*—To investigate the role of JunD in osteoblasts we first generated a set of MC3T3-E1 clones overexpressing JunD. Twenty-four clones of cells transfected with either JunD or empty vector were picked after 3 weeks of selection with G418. The clones were screened by immunoblot with anti-JunD antibody. Several clones of the JunD transfected cells showed elevated JunD expression relative to empty vector transfected cells (Fig. 1*C*; clones #3, #9, and #12). These clones were used for further studies.

*JunD Promotes Osteoblast Differentiation*—To examine the effects of JunD overexpression on osteoblast differentiation we analyzed osteoblast-specific gene expression. The transcription factor, Runx2, is a master regulator of osteoblast differentiation. The expression of Runx2 was clearly increased in the JunD-stably transfected clones at day 7 of culture (Fig. 2*A*). At this time, levels of both COL1 and OCN mRNA were higher in JunD-transfected clones than in empty vector clones (Fig. 2*B*). This was reflected in higher COL1 protein levels in JunD-transfected clones, compared with the control clones (Fig. 2*C*). ALP plays an important role in osteoblast function and, like COL1, is an osteoblast differentiation marker. ALP staining and activity were markedly increased in the JunD-overexpressing MC3T3-E1 clones relative to empty vector-transfected controls (Fig. 3, *A* and *B*). Taken together, these findings strongly suggest that JunD promotes osteoblast differentiation.

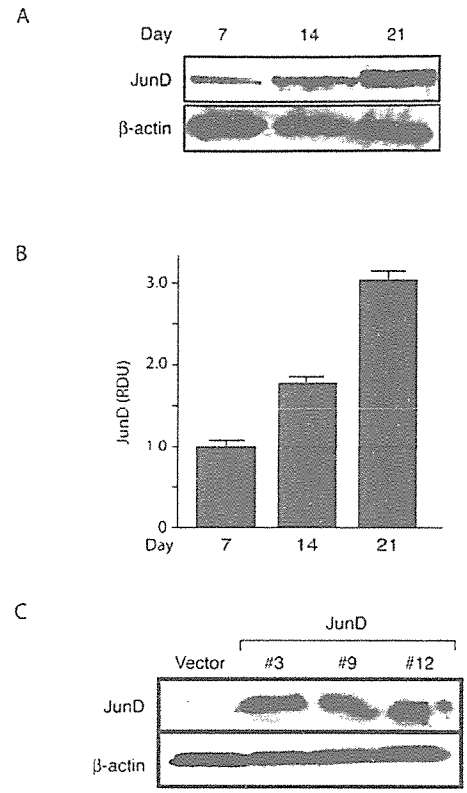


FIG. 1. **Endogenous JunD expression in MC3T3-E1 cells.** *A* shows the endogenous expression of JunD in MC3T3-E1 cells. MC3T3-E1 cells were grown until the indicated times, total protein was extracted, and Western blot analysis was performed with anti-JunD antibody as described under "Experimental Procedures." *B* shows the quantitation of the analysis in *A*. The immunoblot signals were scanned and quantitated with NIH Image analyzer. Each JunD value was normalized to the relative level of β-actin. *C* shows the stable overexpression of JunD in MC3T3-E1 cells. After JunD-transfected (clones #3, #9, and #12) and vector-transfected MC3T3-E1 cells were cultured for 7 days, total protein was extracted, and Western blot analysis was performed as described under "Experimental Procedures."

*JunD Promotes Mineralization*—Extracellular matrix mineralization is the most important phenomenon in bone formation. Accelerated mineralization occurred in the JunD-overexpressing MC3T3-E1 clones as assessed by both the von Kossa and Alizarin red methods (Fig. 4*A*). A quantitative analysis of the Alizarin red result is shown in Fig. 4*B*. The data indicate that JunD overexpression enhances osteoblast mineralization.

*Menin-JunD Interaction and Osteoblast Differentiation*—Menin is known to directly interact with JunD and inhibit its transcription although this has not been investigated in osteoblasts. We have shown that menin promotes the commitment of multipotential mesenchymal stem cells to the osteoblast lineage, but inhibits their later differentiation (23). In that study, menin inactivation achieved by menin antisense DNA stimulated osteoblast differentiation and mineralization in MC3T3-E1 cells. Therefore, the effects of JunD overexpression observed in the present study are analogous to those seen with menin inactivation in our previous study (23). We hypothesized

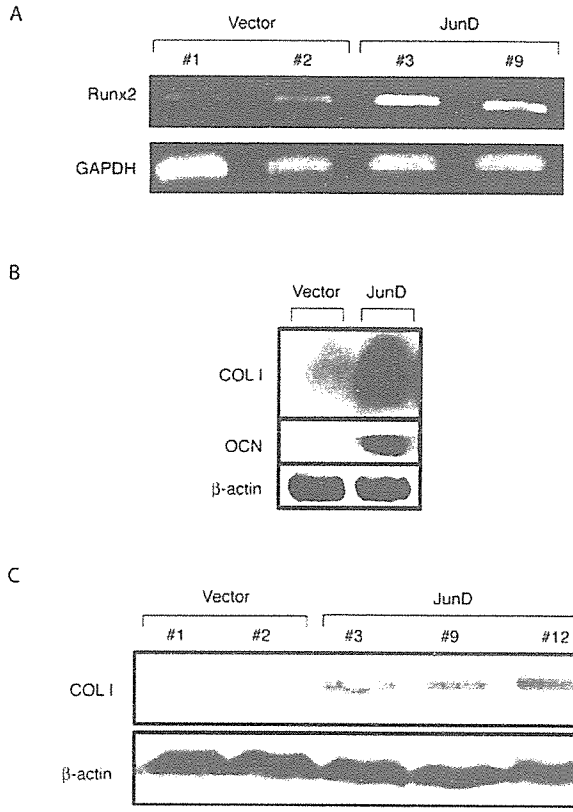


FIG. 2. **Effects of JunD on the expression of Runx2, bone matrix proteins, and ALP in MC3T3-E1 cells.** *A*, effect of JunD on Runx2 expression in MC3T3-E1 cells. After cells were grown for 7 days, total RNA was extracted, and semi-quantitative RT-PCR was performed as described under "Experimental Procedures." *B*, vector-transfected or JunD-transfected MC3T3-E1 cells were grown for 7 days, total RNA was extracted, and Northern blot analysis was performed for COL1, OCN, and α-actin mRNAs as described under "Experimental Procedures." *C*, vector-transfected (clones #1 and #2) and JunD-transfected (clones #3, #9, and #12) MC3T3-E1 cells were cultured for 7 days, total protein was extracted, and Western blot analysis was performed with anti-COL1 and β-actin antibodies as described under "Experimental Procedures."

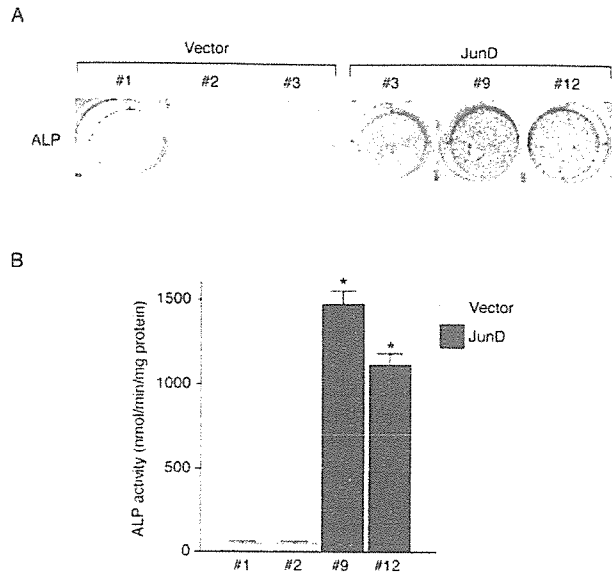


FIG. 3. **Effects of JunD on ALP in MC3T3-E1 cells.** Cells were grown for 7 days and ALP staining was performed (*A*) or ALP activity was measured (*B*) as described under "Experimental Procedures." Each value is the mean ± S.E. of four determination. *, $p < 0.01$, compared with the vector-alone-transfected group.

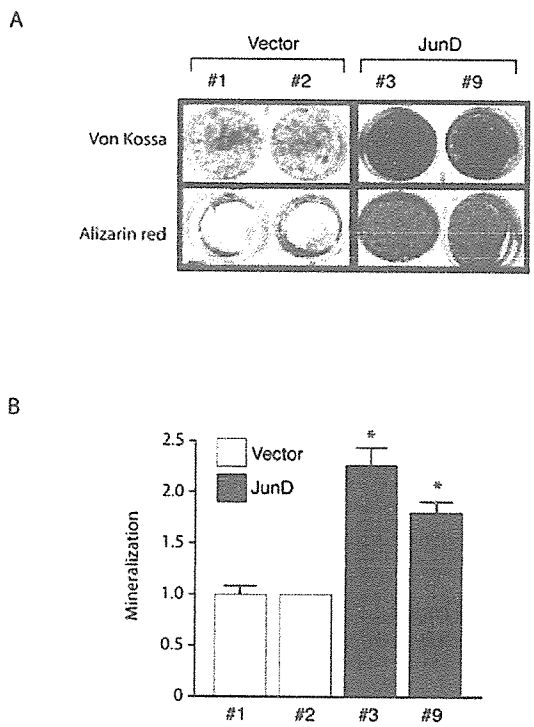


FIG. 4. **Effects of JunD on mineralization in MC3T3-E1 cells.** Confluent cells were cultured in medium with 10 mM α-glycerophosphate for 14 days. *A*, then cells were stained by the von Kossa method or with Alizarin red as described under "Experimental Procedures." *B*, cell layers stained with Alizarin red were destained, and the mineralization was quantitated as described under "Experimental Procedures." *, $p < 0.01$, compared with the vector-alone-transfected group.

therefore that menin might suppress osteoblast differentiation by antagonizing the actions of JunD. To test our hypothesis, we first examined the effects of menin inactivation on endogenous JunD expression in MC3T3-E1 cells. Second, we examined the physical and functional interactions between JunD and menin.

We generated menin-inactivated MC3T3-E1 cell clones (AS-MC) by stable expression of menin antisense DNA (23). JunD expression, assessed by immunoblot, was higher in the menin-inactivated cells compared with empty vector-transfected cells (Fig. 5*A*). These results indicate that menin inactivation stimulates JunD expression in osteoblasts.

When JunD and menin were co-transfected into MC3T3-E1 cells, JunD co-immunoprecipitated with menin (Fig. 5*B*). This suggests that JunD physically interacts with menin in osteoblasts compatible with findings in non-bone cells (16).

The promoter-reporter construct, 3TP-lux, is driven by increased AP-1 activity. The luciferase activity of the reporter was increased 4-fold in MC3T3-E1 cells in which the JunD construct had been transfected relative to the vector-alone control (Fig. 6*A*). However, co-transfection of a menin sense construct, while having no affect on basal luciferase activity, led to complete abrogation of the JunD-stimulated increase (Fig. 6*A*). Overall, these results suggest that JunD interacts both physically and functionally with menin in osteoblasts.

Finally, we investigated whether menin affects JunD-induced differentiation in osteoblasts. Co-transfection of menin significantly inhibited the ALP activity induced by JunD transfection in MC3T3-E1 cells (Fig. 6*B*). The data indicate that

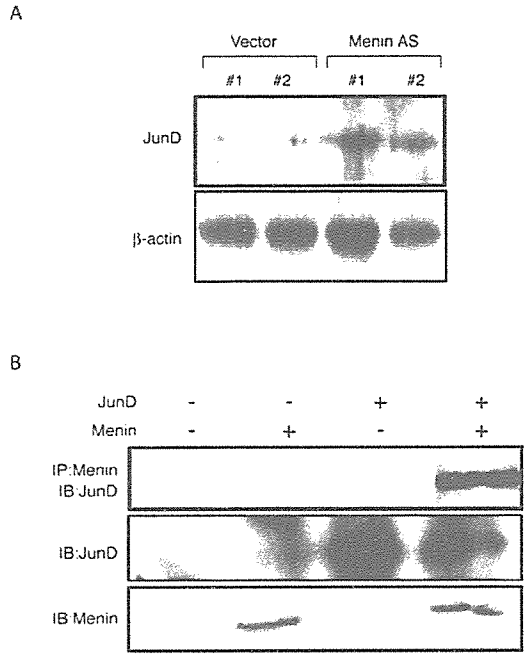


FIG. 5. **Effect of menin inactivation on expression of JunD and physical interaction of menin and JunD in MC3T3-E1 cells.** *A*, after cells were grown for 7 days, total protein was extracted, and Western blot analysis was performed as described under "Experimental Procedures." *B*, menin and/or JunD were transfected into MC3T3-E1 cells. Cell extracts were immunoprecipitated (*IP*) with anti-JunD antibody, followed by immunoblotting (*IB*) with anti-menin antibody, as described under "Experimental Procedures." Whole cell extracts were immunoblotted with anti-JunD and anti-menin antibodies.

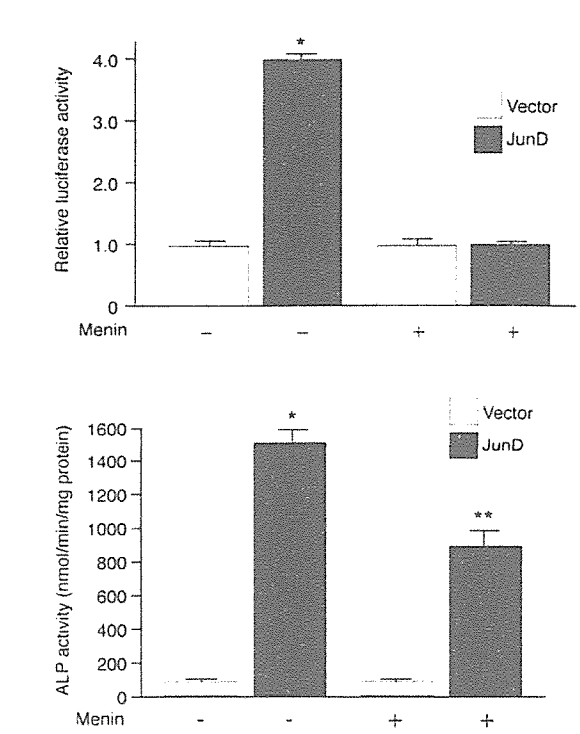


FIG. 6. **Functional interaction of menin and JunD in MC3T3-E1 cells.** *A*, the AP-1 responsive 3TP-lux promoter-reporter construct was transfected into MC3T3-E1 cells with empty vector, JunD, and/or menin expression plasmids, and relative luciferase activity was measured as described under "Experimental Procedures." Each value is the mean ± S.E. of four determination. *, $p < 0.01$, compared with the vector alone-transfected group. *B*, effect of menin on JunD-stimulated ALP activity in MC3T3-E1 cells. Menin was transfected into empty vector or JunD stably transfected MC3T3-E1 cells, and after 48 h, ALP activity was measured as described under "Experimental Procedures." Each value is the mean ± S.E. of four determination. *, $p < 0.01$, compared with corresponding empty vector stably transfected group. **, $p < 0.01$, compared with the corresponding non-menin-transfected group.

menin inhibits osteoblast differentiation by suppressing JunD-induced transcriptional activity and JunD expression.

## DISCUSSION

In the present study, we show that JunD plays an important role in bone formation and stimulates differentiation and mineralization of osteblasts. JunD enhanced the expression of the key transcriptional regulator Runx2 and that of the differentiation markers, COL1 and OCN, and ALP activity and mineralization in mouse osteoblast MC3T3-E1 cells.

Unlike the majority of the Fos/Jun family members, JunD is antimitogenic. The expression of JunD is regulated in a cell cycle-dependent manner in some cells (5, 15, 28) and is generally constitutive being relatively refractory to growth factor or hormone stimulation (29). The full range of physiological roles played by JunD is not clear. Mice with disruption of the JunD gene develop post-weaning growth retardation and male mice have impaired spermatogenesis and reproductive defects (12). Recent studies indicate that JunD mediates survival signaling by the c-Jun $NH_2$-terminal kinase (JNK) signal transduction pathway (30) and protects against chronic kidney disease by regulating paracrine mitogens (31). It has been proposed that JunD is part of a network controlling proliferation and preventing pathological progression in renal disease, for example (31). These recent findings suggest that JunD is involved in important physiological processes in several organs, although the loss of JunD may be compensated for by other factors in JunD knock-out mice (12).

With respect to bone, several JunD-related actions have been noted. The expression of JunD in osteoblasts can be modulated by external stimuli, either mechanical strain (32) or basic fibroblast growth factor (33). In the latter case, JunD is one of several AP-1 factors that may regulate collagenase-3 gene transcription in response to the growth factor (33). Overexpression of JunD and Fra2 represses Runx2-induced collagenase-3 gene promoter activity in differentiated osteoblasts suggesting that Runx2/AP-1 interaction regulates this matrix metalloprotease important for skeletal development and normal and pathological remodeling of bone (34). Moreover, expression of osteocalcin, the most abundant osteoblast-specific non-collagenous protein, is up-regulated by overexpression of JunD and Fra2 in rat osteoblasts (11). A recent study suggested that diminished AP-1 activity, especially JunD, and the resultant decline in the expression of the osteogenic cytokine, interleukin-11 (IL-11), by bone marrow stromal cells plays a role in the impaired bone formation of a strain of senescence-accelerated mice, an animal model of senile osteoporosis (35). However, there have been no reports as yet on the effects on JunD itself on osteoblast differentiation and mineralization.

The protein product of the multiple endocrine neoplasia type 1 gene, menin, is being intensively investigated with respect to identifying those properties responsible for its action as a tumor suppressor (18). However, it is also evident that menin has other physiological roles, for example, in modulating production of hormones such as parathyroid hormone (36), prolactin (37, 38), and insulin (39). With respect to functions of menin in bone, we recently showed that menin inactivation specifically inhibits the commitment of pluripotent mesenchymal stem cells to the osteoblast lineage (23). In the mesenchymal stem

cells, not yet committed to the osteoblast lineage, menin interacts with the crucial mediators of BMP-2 signaling, Smad1 and Smad5, as well as the key osteoblast transcriptional regulator, Runx2, to promote osteoblast commitment (23, 24). In committed osteoblasts, these functional interactions are lost and menin inhibits the later osteoblast differentiation. Although this occurs, in part, by the interaction of menin with the TGF-β/Smad3 pathway (24), it appears likely that other mechanisms are also involved in the ability of menin to retard the differentiation of committed osteoblasts. This we have investigated in the present study.

Previous studies in nonosteoblast cells showed that menin interacts directly with JunD and inhibits JunD-activated gene transcription (16, 25). Only the full-length isoform of JunD binds to menin and is considered to be its functional target (40). Menin represses JunD-mediated transcriptional activity by association with an mSin3A-histone deacetylase complex (41). The inhibition of the activity of one anti-mitogenic protein (JunD) by another, namely menin, appears paradoxical (15). However, it has recently been suggested that the presence of menin is important for the growth suppressor activity of JunD and in the absence of menin JunD switches from a growth suppressor to a growth promoter (42). However, the broader significance of menin/JunD interaction unrelated to tumor suppressor function is not known. We are demonstrating in our study, for the first time, the importance of menin/JunD interaction to osteoblast biology. Even though a particular protein-protein interaction has been shown for one type of cell, this may not hold as one moves to other cell types. Therefore, it is important to explore the mechanism for each cell type examined.

In MC3T3-E1 cells as osteoblast differentiation progresses menin is highly expressed at days 7 and 14 days of culture but is decreased at day 21 (23). As demonstrated in the present study, the inverse pattern is seen with respect to JunD with a modest level of expression occurring at day 7 but then increasing through day 14 to day 21. To examine the role of JunD in osteoblasts we generated clones of MC3T3-E1 cells stably overexpressing JunD. These cells demonstrated enhanced expression of the differentiation markers, Runx2, COL1, and OCN and ALP activity and mineralization. This emphasized the important role played by JunD in promoting osteoblast differentiation. The observed phenotype was strikingly similar to that seen in our previous study in which inactivation of menin in MC3T3-E1 cells, achieved by stable transfection of antisense menin cDNA, enhanced differentiation marker expression and ALP activity and mineralization (23). This raised the hypothesis that menin might suppress osteoblast maturation by antagonizing the differentiating actions of JunD. As noted above, there is a normal reciprocal relationship between the expression levels of menin and JunD with the latter predominating in the most differentiated osteoblasts. This reciprocity of expression was reinforced in the present study with the demonstration that MC3T3-E1 clones stably expressing antisense menin cDNA had increased levels of JunD.

We then demonstrated that menin and JunD physically and functionally interact in osteoblasts. Menin suppressed JunD-induced transcriptional activity and inhibited ALP activity in the MC3T3-E1 cells. Therefore, menin suppresses osteoblast maturation, in part, by inhibiting the bone anabolic actions of JunD. This is the first evidence of menin-JunD interaction having important consequences distinct from anti-tumor effects. Interaction of menin and JunD may have a similar role in tissues other than bone.

Given our previous findings linking menin and the actions of TGF-β superfamily members to the commitment and differentiation of osteoblasts (23, 24), the known involvement of JunD in TGF-β action in cells (other than osteoblasts) (43–47), and the present demonstration of critical menin-JunD interactions in osteoblasts it will be important to study these mechanisms further. The potential cross-talk between these pathways is currently being examined in our laboratories.

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# Gene expression profile in the heart of spontaneous dwarf rat: In vivo effects of growth hormone

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**Abstract**

Excess and deficit of growth hormone (GH) both affect cardiac architecture as well as its function. To date, experimental and clinical studies have reported that GH has an inotropic effect on animal and human heart, however, it remains controversial whether GH is applicable to the treatment for the patients with chronic heart failure. Also, the mechanism by which GH exerts these biological effects on the heart is not well understood. In this study, we attempted to specify the genes regulated by GH in the heart of spontaneous dwarf rat using a microarray analysis. We found that soluble forms of guanylate cyclase, cofilin1, and thymosin β4 mRNA were up-regulated in the heart by GH treatment. On the other hand, acyl-CoA synthetase, aldosterone receptor, myosin regulatory light chain, troponin T, laminA, and β-actin mRNA were down-regulated. These results suggest GH regulates essential molecules that regulate structural, contractile, remodeling, and regenerative functions. Collectively, our data indicate a new integrative understanding for the biological effects of GH on cardiac function.


*Keywords:* Expression profile; Growth hormone; Spontaneous dwarf rat; Microarray analysis; Heart

Accumulating evidence indicates that an excess of growth hormone (GH) gives a significant effect on cardiac function [1–3]. Impaired cardiovascular function has been demonstrated to potentially reduce life expectancy of patients with acromegaly [1]. Hypertension, diabetes mellitus, cardiac hypertrophy, and hyperlipidemia are common in the patients with acromegaly, and they have an increased risk of cardiac failure [4], which is partially reversed after normalization of GH and IGF-I levels by octreotide treatment [5]. On the other hand, long-standing GH deficiency is also known to cause abnormalities in cardiac performance and structure, which increase the risk of cardiovascular mortality [6], and GH replacement therapy appears to correct such abnormalities [7–10]. Furthermore, both animal and human studies revealed that GH administration improved cardiac function of heart failure secondary to idiopathic and ischemic cardiomyopathy in both experimental [11–14]) and clinical trials [15–18] despite some adverse effects of GH such as water and mineral retention.

Spontaneous dwarf rat (SDR) harbors a mutation in the GH gene yielding undetectable levels of GH, indicating that SDR is an excellent animal model for isolated growth hormone deficiency (GHD) [19].

In this study, using a cDNA microarray technique, we have attempted to identify genes regulated by GH in the SDR heart to clarify the mechanism of GH effect on cardiac function.

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## Materials and methods

*Animals.* Five-week-old male spontaneous dwarf rats were purchased from Japan SLC (Shizuoka, Japan). Recombinant human GH (1 mg/kg), dissolved by saline was injected intra-peritoneally. Three, 24, and 72 h after GH injection, three rats for each group were sacrificed and the heart was dissected immediately. Rats with the vehicle (PBS) injection were used as the control. The isolated heart was cut, washed with PBS to remove the blood, and frozen immediately by liquid nitrogen. The left ventricular muscle was used for RNA extraction. All experiments were conducted according to the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Kobe University Graduate School of Medicine (Kobe, Japan) Animal Care Committee.

*RNA extraction.* Total RNA was extracted from each group using Trizol LS reagent (Gibco-BRL) according to the manufacturer's protocol. Trace of DNA contamination in RNA preparations was removed by DNase I (Sigma) digestion.

*Microarray technique.* Probe was synthesized using sample RNA, $^{33}$P-labeled dATP (Amersham Biosciences), 10× dNTP mix, 100 mM DTT, Power Script Reverse Transcriptase, random primer mix, and 5× Power Script reaction buffer. After purified by column chromatography, labeled cDNA was hybridized with Clontech rat 4k plastic microarray (Clontech) film overnight at 60 °C, and the film was washed by SSC containing 0.1% SDS as manufacturer's protocol. Then it was exposed to BAS2040 (FUJIFILM) for two days and each signal was detected by FLA8000 (FUJIFILM). Data were analyzed by Image Reader software (FUJIFILM) and Array Gauge software (FUJIFILM). Each signal intensity was corrected by local background. We selected the genes, which were up-regulated in SDR heart by rhGH more than twice higher than control levels. On the other hand, we also selected the genes, which were down-regulated less than a half of control levels.

*Real-time quantitative PCR.* For precise quantitative analysis of mRNA, real-time quantitative PCR was carried out on ABI Prism 7000 Sequence Detector (Applied Biosystems). Taqman probe, sense and anti-sense primers were designed by primer express software (Applied Biosystems) (Table 1). Data were collected using ABI Prism 7000 Sequence Detection System software (Applied Biosystems). cDNA was generated as described before. For each experiment, 2 μl of the reverse transcription reaction was used with PCR master mix (Applied Biosystems) for PCR as manufacturer's protocol. Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each reaction was repeated three times on a MicroAmp Optical 96-Well Reaction Plate and Optical Caps (applied biosystems). GAPDH was used as an internal standard. Threshold cycles, in which PCR products were reached the given amount, were determined to indicate the initial copies of each mRNA.

*Data analysis.* The results were shown as means ± SD. Data analysis was performed using Student's *t* test. A $p$ value less than 0.05 was considered as statistically significant.

Table 1
Nucleotide sequences of the primers and probes for quantitative real-time PCR (forward primer, **TaqMan probe**, reverse primer, respectively)

| | |
|---|---|
| Acyl-CoA synthetase | (5′-CAGACAAACCCGGAAGTCCAT-3′<br>**5′-TCGCTCTGTCACGCACTTCGACTCA-3′**<br>5′-TCTGCTCCAGGGATGTCTATGA-3′) |
| Aldosterone receptor | (5′-GCTTGAGTGGGTCAGCGTTT-3′<br>**5′-TCACCATGCAGGCAACATTACCGTG-3′**<br>5′-GGCAGGCGTCGTCTGAGA-3′) |
| Troponin T | (5′-TGTTCGACAAAGCTCTGTTCCTT-3′<br>**5′-TGCCCTTGCCCTGTGAATCCCA-3′**<br>5′-CGGGTGCCTGGCAAGA-3′) |
| Myosin regulatory light chain | (5′-GAAACGCCTTCGCTTGCTT-3′<br>**5′-TGAGGAAGCCACAGGCACCATCC-3′**<br>5′-AGCAGCTCCCTCAGGTAATCC-3′) |
| β-Actin | (5′-ACTGGTGAAGGCTGGCTTTG-3′<br>**5′-TGATGATGCTCCCAGAGCTGTCTTCC-3′**<br>5′-GGCGACCCACGATGGA-3′) |
| Cofilin1 | (5′-TGCACCCTGGCAGAGAAAC-3′<br>**5′-TGGCAGCGCCGTCATTTCCC-3′**<br>5′-TGGAGGTGGCTCACAAAGG-3′) |

Nucleotide sequences of primers and TaqMan probes design for quantitative real-time PCR.

## Results

The treatment with GH in SDR significantly altered the profile of gene expression in the heart. Three hours after GH administration, the expression of mRNA of guanylate cyclase, soluble α1 was increased by 2.29-folds. The expression of mRNA of acyl-CoA synthetase, aldosterone receptor, troponin T, and myosin regulatory light chain was decreased by 0.27-, 0.38-, 0.38-, and 0.46-folds, respectively (Table 2). After 24 h, the expression of mRNA of A-kinase-anchoring-protein, thymosin β4, olfactory protein, and cofilin 1 was increased by 2.35-, 2.35-, 2.36-, and 2.86-folds, respectively, and the expression of mRNA of β-actin and zinc finger (OCZF) protein were decreased by 0.32- and 0.41-folds, respectively (Table 2). After 72 h, the expression of mRNA of transcription factor HES-3, late gestation lung protein 2 (Lgl2), $N^G$-dimethylarginine dimethylaminohydrolase, small proline-rich protein (spr), and lamin A was decreased by 0.37-, 0.37-, 0.45-, 0.48-, and 0.49-folds, respectively (Table 2).

To assure the reliability of the results of the microarray analysis, we made a validation using quantitative real-time PCR. We successfully confirmed the changes in the expression of mRNA of acyl-CoA synthetase, aldosterone receptor, troponin T, myosin regulatory light chain, β-actin, and cofilin1 as were indicated by microarray analysis (Table 3).

Next, we analyzed the time-dependent alternations in the expression of these genes using quantitative real-time PCR (Fig. 1). GH-induced increment of guanylate cyclase mRNA reached the peak at three hours after GH administration and returned to the basal level at 24 h. In contrast, the expression of mRNA of cofilin 1 and thymosin β4 reached the peak at 24 h. The mRNA of acyl-CoA synthetase, aldosterone receptor, troponin T, myosin regulatory light chain, and lamin A decreased with troughs at three hours after rhGH administration. Besides, the nadir of β-actin expression appeared at 24 h.

## Discussion

It is well known that in acromegaly, cardiac function and structure are markedly affected and these changes influence the prognosis significantly [1]. It is also reported that adult GHD patients show impaired cardiac function and GH treatment improves its dysfunction in these patients [8–10]. GH exerts its effects either directly or

Table 2
Up- or down-regulated genes in the heart of SDR after GH treatment

| | Index | Locus link/GenBank ID | Ratio |
|---|---|---|---|
| *3 h* | | | |
| Up-regulated genes | | | |
| Guanylate cyclase, soluble, α1 | L15a4/b4 | 25201/U60835 | 2.30 |
| Down-regulated genes | | | |
| Acyl-CoA synthetase | E20a8/b8 | 113976/D85189 | 0.27 |
| Aldosterone receptor | F14c2/d2 | 25672/M36074 | 0.38 |
| Troponin T cardiac | I06a2/b2 | 24837/M26052 | 0.38 |
| Myosin regulatory light chain | Na6/b6 | 50685/X52840 | 0.46 |
| *24 h* | | | |
| Up-regulated genes | | | |
| A-kinase-anchoring-protein | P19a7/b7 | 60332/AJ002474 | 2.35 |
| Thymosin β4 | O03c4/d4 | 81814/M34043 | 2.35 |
| Olfactory protein | O23c4/d4 | /M64388 | 2.36 |
| Cofilin1 | P23c3/d3 | 29271/X62908 | 2.86 |
| Down-regulated genes | | | |
| β-Actin | L07a4/b4 | 29275/X00306 | 0.32 |
| Zinc finger (OCZF) protein | 24a8/b8 | 117107/D88450 | 0.41 |
| *72 h* | | | |
| Down-regulated genes | | | |
| Transcription factor HES-3 | E09a8/b8 | 64628/D13418 | 0.37 |
| Late gestation lung protein 2 | b5 | 116458/AF110195 | 0.37 |
| $N^G,N^G$-Dimethylarginine | G02a8/b8 | 64157/D86041 | 0.45 |
| Small proline-rich protein (spr) | F14a8/b8 | 60461/L46593 | 0.48 |
| Lamin A | L04a6/b6 | 60374/X66870 | 0.49 |

Effect of GH treatment on cardiac gene expression in SDR. Index of the Clontech rat 4k plastic microarray sheet, Locus link/GenBank ID, and the ratio (the average spot intensity of GH-treated group/the average spot intensity of control group) is shown.

Table 3
Comparison of the threshold cycle numbers of the genes in the heart of control and GH-treated SDR using quantitative real-time PCR analysis

| | Control $C_t$ | GH $C_t$ |
|---|---|---|
| Acyl-CoA synthetase (3 h) | 31.29 ± 0.59 | 33.45 ± 0.30 |
| Aldosterone receptor (3 h) | 31.80 ± 0.54 | 33.29 ± 0.23 |
| Troponin T cardiac (3 h) | 26.80 ± 0.12 | 28.93 ± 0.18 |
| Myosin regulatory light chain (3 h) | 29.78 ± 0.16 | 31.59 ± 0.27 |
| β-Actin (24 h) | 25.83 ± 0.22 | 26.81 ± 0.07 |
| Cofilin1 (24 h) | 29.38 ± 0.45 | 27.92 ± 0.52 |

Comparison of the threshold cycle numbers of the genes in the heart of control and GH-treated SDR. The numbers of the threshold cycle, in which PCR products reached the given amount, were determined to assess the initial copies of each mRNA. Data were calculated using ABI Prism 7000 Sequence Detection System software (Applied Biosystems). Threshold cycles ($C_t$) are shown as means ± the standard deviation (SD) ($n = 3$). The changes in the threshold cycles of the genes affected by GH treatment were compatible with the results of the microarray analysis.

indirectly via IGF-I. Some of these effects have been explained, however, the molecular basis of its effects is largely unknown. In the present study, we have demonstrated that GH regulates the expression of some of the key molecules that play roles in the structural, contractile, remodeling, and regenerative functions in the heart.

Accumulating evidence indicates that renin–angiotensin–aldosterone (R-A-A) system plays a critical role in cardiac function and remodeling. It is known that GH induces retention of sodium and its mechanism may involve the activation of the renin–angiotensin–aldosterone (R-A-A) system [20–23]. In this study, we demonstrated GH reduced the expression of aldosterone receptor mRNA in the heart of SDR. The decreased expression of aldosterone receptor could reduce the sensitivity to aldosterone. It is possible that GH-regulated reduction in the expression level of aldosterone receptor contributes to the impaired cardiac function in AGHD.

Acyl-CoA synthetase is a regulatory enzyme of β-oxidation in mitochondria. In terms of lipid metabolism, AGHD patients are often accompanied with hyperlipidemia and hepatic steatosis [6]. In the bovine GH-transgenic mouse, mitochondria in myocytes were swollen with dissolution of the regular cristae arrangements [11]. In this study, GH treatment decreased the expression of acyl-CoA synthetase in the SDR heart. In the heart, fatty acid is considered as the main energy source. These results suggest that GH regulates cardiac lipid oxidation pathways by affecting β-oxidation and is comparable to the fact that GH regulates β-oxidation pathway decreasing the expression level of PPARα [24].

Soluble guanylate cyclase (sGC) is a main receptor for nitric oxide (NO) in cytosol, and the binding of NO to sGC results in increasing cellular cGMP concentration [25,26]. The increase of cellular cGMP level is responsible for the decrease in intracellular calcium level. It is well known that the decrease in calcium level by NO causes

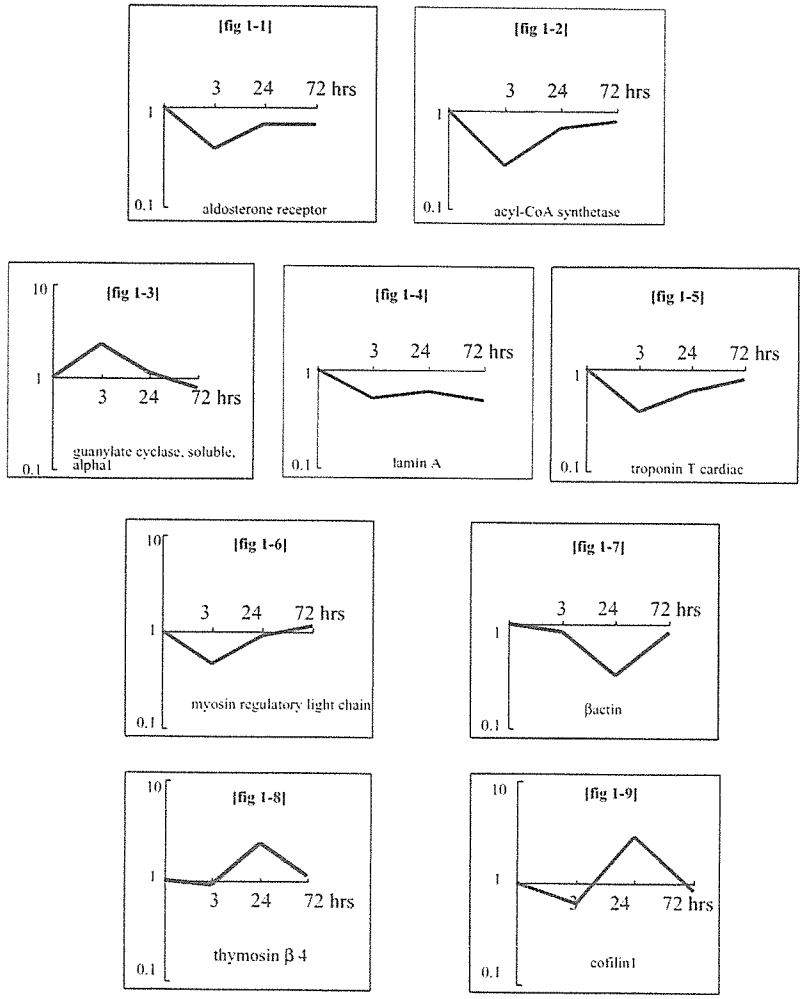


Fig. 1. Time-dependent changes of the cardiac gene expressions in the GH-treated SDR analyzed by quantitative real-time PCR. (1) A line graph indicates the expression change of aldosterone receptor. The horizontal axis represents the time after GH injection, and the vertical axis represents the expression ratio compared to control level. (2) A line graph for acyl-CoA synthetase. (3) A line graph for soluble guanylate cyclase α1. (4) A line graph for lamin A. (5) A line graph for troponin T. (6) A line graph for myosin regulatory light chain. (7) A line graph for β-actin. (8) A line graph for thymosin β4. (9) A line graph for cofilin1.

the improvement of endothelial function. On the other hand, it is also known that myocyte function is suppressed by the same mechanism [26]. Our results indicate NO receptor, sGC, was up-regulated by GH. This could cause an increased sensitivity to NO in myocardium and regulate cardiac function as well as enhancing GC activity [27,28].

Lamin A/C, the nuclear envelope protein which lines the inner nuclear membrane is encoded by the LMNA gene and its defect is responsible for the familial dilated cardiomyopathies (DCM) associated with atrioventricular block, such as autosomal dominant Emery–Dreifuss muscular dystrophy [29,30]. In this study, the expression of Lamin A was decreased by GH. Our result also shows contractile proteins, such as myosin regulatory light chain, troponin T, and β-actin, were down-regulated by GH treatment. These results suggest that GH regulates cardiac contractility via alternation of the expression level of these proteins.

Thymosin β4 is a main intracellular G-actin sequestering peptide [31,32]. It binds monomer acting in a 1:1 complex and acts as acting buffer, preventing polymerization into acting filaments but supplying a pool of acting monomers when the cell needs filaments. It is thought to be an important mediator in myocyte proliferation, migration, and differentiation. On the other hand, cofilin1 is also a small acting binding protein and belongs to a member of the cofilin1/ADF family, and it enhances acting filament turnover by increasing the rate of acting depolymerization from the pointed ends [33,34]. In contrast to thymosin β4, cofilin binds more efficiently to ADP-actin subunits in filaments and promotes filament disassembly. Recently, it is reported that thymosin β4 expression is abundant mainly in proliferative region during heart development and it promotes cardiac cell migration, survival, and cardiac repair in adult mice after myocardial infarction and improves cardiac

function [35]. In this study, we demonstrated that GH increases the expression of both genes. Given the fact that in patients with dilated or ischemic cardiomyopathy, the administration of rhGH results in significant improvement in hemodynamics and cardiac function [16–18], it is considerable that GH improves cardiac function via up-regulating the expression of thymosin β4 and cofilin.

Previous microarray analysis of GH effect indicated that myosin light chain is up-regulated in the heart of hypophysectomized rat [36]. These results are compatible to our data. On the other hand, several genes related with fatty acid metabolism such as long-chain acyl-CoA synthetase in hypophysectomized rat heart were reported to be decreased [36], however, in our study, GH treatment suppressed acyl-CoA synthetase mRNA expression in the heart of SDR. It is not clear as to the reason for this discrepancy, however, it is considerable that SDR is deficient only in GH, however, hypophysectomized rat is deficient for all pituitary hormones even some hormones are replaced. In this aspect, SDR is more appropriate to analyze specific changes depending on GH.

Our study demonstrated that the expression of sGC, acyl-CoA synthetase, aldosterone receptor, myosin regulatory light chain, and troponin T in SDR heart was changed in 3 h. Together with the fact that the GH receptor is expressed in myocardium [37], it is possible that GH directly regulates the expression of these genes. On the other hand, the expression of cofilin1, thymosin β4, and β-actin was altered in 24 h. These late-phase changes in the expression levels suggest indirect action of GH via such as IGF-I.

In conclusion, GH significantly changed gene expression levels of sGC, acyl-CoA synthetase, aldosterone receptor, troponin T, cardiac myosin regulatory light chain, thymosin β4, β-actin, and cofilin1 in SDR heart. These changes in the gene expression profiles seem to explain some of the physiological and pharmacological effects of GH in the heart. Although, further studies are required to gain an insight into the precise mechanisms of GH action, this approach shows effective to obtain a comprehensive understanding for GH action in vivo.

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