tion (previously called type II lissencephaly) of intermediate severity [14,27]. The MEB gene was mapped to 1p32-p34 by linkage analysis and homozygosity mapping [28]. Thirteen independent disease-causing mutations of the POMGnT1 gene have been reported in twelve patients with MEB [25,29]. Taniguchi *et al.* [29] recently reported a slight correlation between the location of the mutation on the POMGnT1 gene and clinical severity in the brain. Patients with mutations near the 5′ terminus of the POMGnT1 coding region show relatively severe brain symptoms such as hydrocephalus, while patients with mutations near the 3′ terminus have milder phenotypes. A selective deficiency [30] and hypoglycosylation [31] of α-dystroglycan has been reported in MEB patients suggesting that α-dystroglycan is a potential target of POMGnT1 and that altered glycosylation of α-dystroglycan may play a critical role in the etiology of MEB and other forms of CMD.

Yoshida *et al.* [25] established that their MEB patients had abnormalities in the POMGnT1 gene by DNA analysis. We now report an appreciably more rapid and inexpensive diagnostic test for MEB based on POMGnT1 enzyme assays using commercially available reagents and ~20 to 30 mg wet weight of muscle biopsy tissue.

2. Materials and methods

2.1. Materials

UDP-{^{3}H}GlcNAc (New England Nuclear) was diluted with nonradioactive UDP-GlcNAc (Sigma) to a specific activity of 34 dpm/pmole (GnT1 assays) or 600 dpm/pmole (POMGnT1 assays). Manα1,6(Manα1,3)Manβ1-O-octyl and Manα1-O-benzyl were purchased from Toronto Research Chemicals, Toronto, Canada. Sep-Pak C18 reverse phase cartridges were obtained from Waters.

2.2. Muscle biopsies

We obtained frozen muscle biopsies from four MEB patients who presented either at birth or after the first few months of life with developmental delay, mental retardation and muscle weakness. All patients showed elevated serum creatine kinase activity and brain malformations (neuronal migrational abnormalities, dilated ventricles, hypoplastic corpus callosum, brain hypomyelination, hypoplastic pons and cerebellum) and a variable degree of eye abnormalities (microphthalmia, buphthalmos, anterior chamber defects, optic nerve atrophy, retinal dysplasia and myopia). Skeletal muscle biopsies from all patients showed evidence of dystrophic changes. Immunohistochemical staining of the tissues with a mouse monoclonal anti-α-dystroglycan antibody prepared with a rabbit skeletal muscle membrane preparation as antigen [32] (clone VIA4-1, Upstate Biotechnology) showed deficient α-dystroglycan.

Patient D [25,30] is of Turkish origin with a homozygous G1743A transversion in exon 19 (Ser550Asn). Patient A [25,30] is a compound heterozygote of French origin with a C1572G transversion in exon 17 (Pro493Arg) in one allele and a single base pair deletion 1970delG in exon 21 in the other allele (frameshift at Val626 and a premature termination codon at codon 633). Patient B, a 7-yr-old boy (unpublished), is a compound heterozygote with an IVS17 + 1G>A mutation in one allele which causes a splicing error, read-through of intronic sequences resulting in the introduction of a premature termination codon, and skipping of upstream exon 17 resulting in a deletion of 42 amino acids (Leu472 to His513); the mutation in the other allele has not as yet been determined. Patient C, a 2-yr-old girl (unpublished), is a compound heterozygote with a G1908A transversion in exon 21 (Arg605His) in one allele and a single base pair insertion in exon 11 in the other allele (1106insT causing a frameshift and premature termination at codon 338). Age-matched control muscle biopsy samples were obtained from the Tissue Bank at the Hospital for Sick Children. We also obtained a frozen control muscle biopsy sample that had been shipped together with the sample from MEB patient A. Muscle biopsies from a 5-yr old boy with Becker and a 3-yr old boy with Duchenne muscular dystrophy were also analyzed.

2.3. Assay of POMGnT1 enzyme activity

The frozen muscle biopsy sample (10–60 mg wet weight) was gently homogenized with about 20 strokes of a hand-held glass Potter-Elvehjem tissue homogenizer at 4°C in 0.1 to 0.2 mL homogenizing buffer (1.8% Triton X-100, 0.2 mol/L NaCl in phosphate-buffered saline) containing 1/4 tablet of protease inhibitor cocktail (Boehringer Mannheim). The homogenate was kept on ice for 1 h, centrifuged at 4°C for 10 min at 3000 rpm with a microcentrifuge, and the supernatant was used for enzyme assays. The assay incubations contained, in a total volume of 0.020 mL, 0.017 mL muscle biopsy extract, 1 mM UDP-{^{3}H}GlcNAc (34 or 600 dpm/pmole for GnT1 or POMGnT1 respectively), 10 mM $MnCl_2$, 0.2% bovine serum albumin, 75 mM MES buffer (pH 6.5 or 6.0 for GnT1 or POMGnT1 respectively), 5 mM AMP, 0.2 mol/L GlcNAc, 0.5% Triton X-100, and either 1.25 mM Manα1,6(Manα1,3)Manβ1-O-octyl (GnT1 [33]) or 62.5 mM Manα1-O-benzyl (POMGnT1 [24]). The activity of POMGnT1 with 1.25 mM Manα1,6(Manα1,3) Manβ1-O-octyl is negligible and GnT1 does not act on Manα1-O-benzyl [24]. Incubations were carried out at 37°C for 2 h. SepPak C18 cartridges were used to determine the amount of radioactive product as previously described [24,33,34]. Controls were routinely carried out in the absence of acceptor and the value obtained was subtracted. All assays were carried out in duplicate or triplicate (as indicated in Table 1) and results are reported as averages of these determinations.

Table 1
GnT1 and POMGnT1 assays on muscle biopsy extracts

Muscle Sample	GnT1 pmoles/h/mg (SD, n)	POMGnT1 pmoles/h/mg (SD, n)	Ratio of POMGnT1 to GnT1
Controls			
1	22 (5.0, 2)	7.4 (0.8, 2)	0.34
2	15 (0.8, 2)	10 (0.8, 2)	0.67
3	33 (0.5, 2)	12 (0.8, 3)	0.36
4	13 (0.2, 2)	11 (<0.1, 2)	0.85
5	20 (0.2, 2)	10 (1.1, 3)	0.50
6		8.0 (0.2, 2)	
Mean ± SD	20.6 ± 7.0	9.73 ± 1.60	
Becker	33 (0.6, 2)	9.1 (0.1, 3)	0.28
Duchenne	45 (1.9, 2)	8.2 (0.3, 3)	0.18
MEB patients			
A	41 (0.4, 2)	0.8 (0.4, 3)	0.02
B	42 (0.4, 2)	0.7 (0.3, 3)	0.02
C	29 (0.1, 2)	0.5 (0.3, 3)	0.02
Mean ± SD	37.3 ± 5.9	0.67 ± 0.12	
D	5 (0.1, 2)	<0.1 (<0.1, 2)	

Enzyme assays are averages of duplicate or triplicate determinations; SD = Standard Deviation; n = number of samples.

3. Results

Table 1 shows the results of GnT1 and POMGnT1 enzyme assays on MEB and control muscle samples. Since the amounts of MEB muscle tissue available to us were small, the protein contents were not determined. POMGnT1 activity was expressed in two ways, relative to wet weight of muscle and to GnT1 activity. We have shown that GnT1 activity can serve as an effective base-line in our previous work on Congenital Disorder of Glycosylation type IIa [35–37]. GnT1 also has the advantage over protein analysis in that it gives an indication of the quality of the muscle biopsy tissue for enzyme analysis.

The POMGnT1 activity in six samples of control muscle varied from 7.4 to 12 pmoles/h/mg (average 9.7 pmoles/h/mg). The POMGnT1 to GnT1 ratios in these samples varied from 0.34 to 0.85. The Becker and Duchenne muscle samples showed the same levels of POMGnT1 as the other controls but slightly higher levels of GnT1. MEB patients A and B also showed somewhat higher GnT1 values. Further work is required to determine whether these very small differences in GnT1 activity are statistically significant.

MEB patients A, B and C showed very similar POMGnT1 levels (0.5–0.8 pmoles/h/mg) and POMGnT1/GnT1 ratios (0.02). The differences in POMGnT1 enzyme activities between these three MEB patients and the controls range from 5 to 10 times the sums of the standard deviations and are therefore highly significant. We could not detect significant POMGnT1 activity in patient D's muscle. The GnT1 activity in the D extract was significantly lower (5 pmoles/h/mg) than the activities in all our other samples, both MEB and controls (13–45 pmoles/h/mg), suggesting that the quality of the D muscle sample was not as good as samples from the other three MEB patients. However, the D data indicate that a reliable diagnosis of MEB can be made even with partially degraded muscle samples. The data also illustrate the value of GnT1 assays as a measure of tissue quality.

The low but significant levels of POMGnT1 detected in MEB patients A, B and C (Table 1) are consistent with the suggestion by Michele *et al.* [31] based on immunologic studies "that a small amount of proper glycosylation occurs in the presence of mutated POMGnT1, due either to residual enzymatic activity or to a secondary, partially compensatory enzyme".

4. Discussion

MEB [31], Walker-Warburg syndrome (WWS, OMIM 236670) [38], Fukuyama congenital muscular dystrophy (FCMD; OMIM 253800) [31,39–41] and Congenital Muscular Dystrophy 1C (OMIM 606612; MDC1C) [42] are all CMDs with deficient α-dystroglycan in the basal lamina of skeletal muscles as demonstrated by immunohistochemical techniques using antibodies against α-dystroglycan such as VIA4-1. However, α-dystroglycan staining can be normal in muscle from MEB and FCMD patients using a polyclonal antibody which can detect hypoglycosylated full-length α-dystroglycan [31] suggesting that VIA4-1 is directed against the glycans on α-dystroglycan and that α-dystroglycan is underglycosylated in muscle from these patients. Although the exact functions of α-dystroglycan and the dystrophin glycoprotein complex (DGC) are not known, they appear to provide structural support to the sarcolemma and may play a role in signaling, cell adhesion and the regulation of the intracellular calcium concentration [15,43,44]. Several cell surface and transmembrane molecules (laminins, integrins and lectins) have been proposed as ligands for dystroglycan [1,43]. Disruption of the binding of α-dystroglycan to its ligands could lead to weakened anchorage of muscle fibers to the extracellular matrix with very early (*i.e.*, embryonic) and rapid muscle dysfunction and necrosis as a result.

MEB, WWS and FCMD are complex disorders associated with a wide spectrum of brain malformations described as the "cobblestone complex". The specific abnormalities consist of simplified or absent brain convolutions, inappropriate migration of neurons and glia into the subarachnoid space where they intermix with fibroblasts and blood vessels, cystic degeneration of white matter, hydrocephalus, and a small dysplastic brainstem and cerebellum [14,27,45]. The brain changes are least severe in FCMD, intermediate in MEB and most severe in WWS. All three syndromes also have abnormalties of the eyes that consist of microphthalmia, buphthalmos, anterior chamber defects, congenital cataracts, optic nerve atrophy, retinal dysplasia, myopia and coloboma. In contrast, MDC1C presents only with muscular dystrophy [14,46–48]. The combination of brain, eye and muscle abnormalities leads to differing degrees of motor

developmental delay, physical disability, muscle pathology, elevation of serum creatine kinase, mental retardation, and structural brain and eye defects. The most severe and often lethal phenotype is generally associated with WWS followed in decreasing severity by MEB, FCMD and MDC1C.

Mutations in the gene encoding the enzyme which attaches mannose in *O*-glycosidic linkage to the Ser/Thr residues of proteins like α-dystroglycan (Protein *O*-Mannosyltransferase 1; POMT1; Figure 1) were found in 6 of 30 unrelated WWS patients [38]. The POMT1 gene maps to 9q34. FCMD is associated with mutations in a protein called fukutin which maps to 9q31 [40,41,49]. The function of fukutin is unknown but it is predicted to be a secreted protein with an amino acid sequence suggestive of a glycosyltransferase [50,51]. The structure of a recently cloned gene encoding a protein highly homologous to fukutin, fukutin-related protein (FKRP), is also suggestive of a putative glycosyltransferase [42,47,52]. The FKRP gene has been mapped to 19q13.3 Mutations in the gene lead to either severe CMD (MDC1C) [42,52], or to a later onset and milder allelic form of CMD called limb-girdle muscular dystrophy 2I (LGMD2I; OMIM 607155) [47].

The enzymatic diagnostic test we have demonstrated in this report will allow a rapid and accurate diagnosis of MEB no matter where the mutation in the POMGnT1 gene is located. We have not as yet tested leukocytes or fibroblasts to determine whether there is sufficient POMGnT1 expression in these tissues to allow enzyme assays. We have not had access to parental muscle biopsy material. If it proves possible to use either leukocytes or fibroblasts for the POMGnT1 assay, it may be possible to carry out the assay on parental tissues and to determine heterozygosity. CMDs associated with brain malformations such as MEB, WWS and FCMD are heterogenous in clinical presentation and on radiologic examination, suggesting that the simple enzymatic test described in this paper should be used as a screening procedure for MEB in these cases. Since the mutations in WWS, FCMD and MDC1C are also in genes encoding either established or putative glycosyltransferases, it is probable that similar diagnostic tests will eventually be developed for these diseases and possibly for other CMDs.

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Csx/Nkx2-5 Is Required for Homeostasis and Survival of Cardiac Myocytes in the Adult Heart*

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***Csx/Nkx2-5*, which is essential for cardiac development of the embryo, is abundantly expressed in the adult heart. We here examined the role of *Csx/Nkx2-5* in the adult heart using two kinds of transgenic mice. Transgenic mice that overexpress a dominant negative mutant of *Csx/Nkx2-5* (DN-TG mice) showed degeneration of cardiac myocytes and impairment of cardiac function. Doxorubicin induced more marked cardiac dysfunction in DN-TG mice and less in transgenic mice that overexpress wild type *Csx/Nkx2-5* (WT-TG mice) compared with non-transgenic mice. Doxorubicin induced cardiomyocyte apoptosis, and the number of apoptotic cardiomyocytes was high in the order of DN-TG mice, non-transgenic mice, and WT-TG mice. Overexpression of the dominant negative mutant of *Csx/Nkx2-5* induced apoptosis in cultured cardiomyocytes, while expression of wild type *Csx/Nkx2-5* protected cardiomyocytes from doxorubicin-induced apoptotic death. These results suggest that *Csx/Nkx2-5* plays a critical role in maintaining highly differentiated cardiac phenotype and in protecting the heart from stresses including doxorubicin.**

The cardiac homeobox gene *Csx/Nkx2-5* starts to be expressed at embryonic day 7.5 in the heart primordia of mice (1, 2), and targeted disruption of murine *Csx/Nkx2-5* results in embryonic lethality (3). Many mutations in human *Csx/Nkx2-5* have been reported to cause a variety of congenital heart diseases and atrioventricular conduction delay (4, 5). These observations indicate that *Csx/Nkx2-5* plays a critical role in cardiac morphogenesis and contributes to diverse cardiac developmental pathways at the embryonic stage.

Knockout experiments have suggested that many genes such as *Hand1*, myocyte enhancer factor-2, atrial natriuretic peptide (*ANP*),[1] brain natriuretic peptide (*BNP*), cardiac α-actin, cardiac ankyrin repeat protein (*CARP*), N-*myc*, and *MSX2* are genetically located downstream of *Csx/Nkx2-5* at the embryonic stage (2, 6–8). Although *Csx/Nkx2-5* continues to be expressed in the adult heart (1, 2), the function of *Csx/Nkx2-5* in the later stage of development is unknown because of embryonic lethality of null mutant mice (3, 8). In our recently generated transgenic mice that overexpress human *Csx/Nkx2-5* (WT-TG mice), mRNA levels of many cardiac genes such as *ANP*, *BNP*, *CARP*, and sarcoplasmic reticulum calcium ATPase 2 (*SERCA2*) genes were up-regulated (9). These results suggest that *Csx/Nkx2-5* regulates expression of cardiac-specific genes also in the adult heart. However, because there was no difference in phenotype between WT-TG and non-transgenic (non-TG) mice (9), the significance of these gene up-regulations remains unknown. *Csx/Nkx2-5* itself is also differentially regulated by different stimuli. In response to hypertrophic stimuli such as isoproterenol, phenylephrine, and pressure overload, *Csx/Nkx2-5* is up-regulated (10, 11), whereas it is down-regulated by treatment with doxorubicin (DOX) (12). These results suggest that *Csx/Nkx2-5* has certain roles in the adult heart. In this study, we generated transgenic mice that overexpress a dominant negative mutant of human *Csx/Nkx2-5* (DN-TG mice) under the control of α-myosin heavy chain (*α-MHC*) promoter and examined the role of *Csx/Nkx2-5* in the adult heart.

EXPERIMENTAL PROCEDURES

Animal Models—Human *Csx/Nkx2-5 LP* mutant (*LP* mutant) cDNA created by substituting a proline for a highly conserved leucine in the homeodomain of human *Csx/Nkx2-5* (13) was subcloned into the *α-MHC* promoter-containing expression vector (14). The linearized DNA was injected into pronuclei of eggs from BDF1 mice, and the eggs were transferred into the oviducts of pseudopregnant ICR mice. The transgene was identified by PCR with transgene-specific primers and by Southern blot analysis. Three independent lines of DN-TG mice were obtained, and they showed the same results. We used 12-week-old heterozygous mice for analysis. A single dose of 20 mg/kg DOX was injected intraperitoneally. Mice were sacrificed 24 h after DOX injection. Protocols were approved by the Institutional Animal Care and Use

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[1] The abbreviations used are: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CARP, cardiac ankyrin repeat protein; WT, wild type; DN, dominant negative; TG, transgenic; SERCA2, sarcoplasmic reticulum calcium ATPase 2; DOX, doxorubicin; α-MHC, α-myosin heavy chain; LV, left ventricular; %FS, percent fractional shortening; TUNEL, terminal dUTP nick-end labeling; EMSA, electrophoretic mobility shift assay; TTF-1, thyroid transcriptional factor-1; EM, electron microscopic.

Committee of the University of Chiba.

Cell Culture, DNA Transfection, and Reporter Gene Assay—CL6 cells were cultured as described previously (15). To induce differentiation, 1% dimethyl sulfoxide (Me_2SO) was added to the growth medium (differentiation medium). The plasmid containing human wild type (WT) *Csx/Nkx2-5* or *LP* mutant was transfected into CL6 cells by the lipofection method (Tfx reagents, Promega) as described previously (15). Stable transformants were selected with 300 μg of neomycin/ml, and six independent cell lines were isolated. We transfected WT *Csx/Nkx2-5* and/or *LP* mutant into COS-7 cells and cardiomyocytes of neonatal rats by the standard calcium phosphate method. The luciferase activity of the 300-bp *ANP* promoter containing the reporter gene was measured 48 h after transfection with a Berthold Lumat LB9501 luminometer as described previously (16).

Physiological Analysis—Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). Cardiac function was evaluated with echocardiography (Image Point HX, Hewlett Packard) using a 10-MHz transducer as described previously (9). Left ventricular (LV) dimension, wall thickness, and percent fractional shortening (%FS) were obtained from M-mode images of the left ventricle. The quantitative measurements represent consensus estimates by two different investigators (H. Toko and E. Takimoto), and interobserver variability was less than 10%. Arterial blood pressure and heart rate were measured by tail cuff method.

Histological Analysis—Four-μm-thick paraffin sections were stained with hematoxylin-eosin and van Gieson. For the detection of apoptotic cells, TUNEL and immunohistochemical analysis to detect active caspase-3, which is one of the critical enzymes to induce apoptosis, were performed with an *in situ* apoptosis detection kit (Takara Syuzo) and with anti-active caspase-3 polyclonal antibody (Promega), respectively, according to the suppliers' instructions. For electron microscopic analysis, the specimens were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. All of the samples were coded and scored in a blind fashion as described previously (17). We examined five hearts of each group, and 10 pictures were randomly taken from each heart. Samples were coded and scored independently by two different investigators (H. Toko and M. Sakamoto) using a scale of 0–4 (score 0, normal; score 1, early degenerative alterations in some cells, *i.e.* loss of parallel orientation, swelling of mitochondria, and cell vesiculation; score 2, advanced degenerative changes, *i.e.* intracytoplasmic inclusions, loss of myofilaments, separation of intercalated discs, and nuclear modifications; score 3, myofibrillar atrophy and loss of contractile elements; score 4, myofiber degeneration accompanied by myolysis.).

Northern Blot Analysis—Total RNA was extracted by the acid guanidine method (RNAzol B, TEL-TEST), and Northern blot analysis was performed as described previously (9).

Western Blot Analysis—The plasmids expressing Myc-tagged WT *Csx/Nkx2-5* and *Csx/Nkx2-5 LP*, hemagglutinin-tagged WT *Csx/Nkx2-5*, and *GATA-4* cDNAs were transiently transfected into COS-7 cells, and 48 h after transfection, whole cell extracts were prepared for immunoprecipitation-Western blot analysis as described previously (14). Western blot analysis was also performed with anti-Bcl-2 and anti-Fas/ligand monoclonal antibodies (Transduction Laboratories) as described previously (14). Hybridizing bands were visualized using an ECL detecting kit (Amersham Biosciences).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed using double-stranded oligonucleotides corresponding to the thyroid transcriptional factor-1 (TTF-1) binding sequence as described previously (14).

Immunofluorescent Cytochemistry—The plasmids expressing Myc-tagged WT *Csx/Nkx2-5* and *Csx/Nkx2-5 LP* cDNAs were transfected into the cultured cardiomyocytes of neonatal rats plated on a cover glass. The cells transfected with *LP* mutant and WT *Csx/Nkx2-5* were marked by anti-Myc monoclonal antibody and an anti-mouse IgG conjugated to rhodamine. To detect apoptotic cells, 50 μl of TUNEL reaction mixture containing both terminal deoxynucleotidyltransferase and fluorescein isothiocyanate-conjugated dUTP was added to each sample.

Statistical Analysis—Data are shown as mean ± S.E. Multiple group comparison was performed by one-way analysis of variance followed by the Bonferroni procedure for comparison of means. Two-tailed Student's *t* test was used to compare transgenic with non-transgenic specimens under identical conditions. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Dominant Negative Mutant of Csx/Nkx2-5—Human *LP* mutant was created by substituting a proline residue for a leucine residue in the homeodomain as described previously (13). Grows *et al.* (13) have reported that overexpression of *Xenopus LP* mutant inhibits heart development, suggesting that the *LP* mutant has dominant inhibitory function. In this study, we further examined the dominant inhibitory function of *LP* mutant using the *ANP* promoter. When co-transfected with *ANP* promoter containing the luciferase reporter gene into COS-7 cells, the *LP* mutant did not activate the *ANP* promoter. However, the *LP* mutant suppressed WT *Csx/Nkx2-5*-induced activation of the *ANP* promoter in a dose-dependent manner (Fig. 1*A*). The *LP* mutant also suppressed the synergistic activation of the *ANP* promoter induced by *Csx/Nkx2-5* and *GATA-4* (Fig. 1*A*). EMSA revealed that WT *Csx/Nkx2-5*, but not the *LP* mutant, bound to TTF-1 binding sequence, and the TTF-1 binding of WT *Csx/Nkx2-5* was mildly but significantly inhibited by the *LP* mutant (Fig. 1*B*). These results suggest that a part of the dominant negative effects of the *LP* mutant is to inhibit the ability of the WT *Csx/Nkx2-5* to bind DNA. Our and other groups have demonstrated that *Csx/Nkx2-5* may interact with *Csx/Nkx2-5* itself and *GATA-4* (16, 18). Immunoprecipitation assay indicated that the *LP* mutant interacted with WT *Csx/Nkx2-5* and with *GATA-4* (Fig. 1*C*). These results suggest that the *LP* mutant suppresses *Csx/Nkx2-5*-induced activation of the *ANP* promoter possibly by sequestering associated protein.

CL6-LP Cell Line—The CL6 cell line, derived from P19 cells, is a useful *in vitro* model to study cardiomyocyte differentiation because CL6 cells differentiate into beating cardiomyocytes with high efficiency by treatment with 1% Me_2SO (15). To further examine the functions of the *LP* mutant, we isolated three permanent CL6 cell lines that overexpress WT *Csx/Nkx2-5* (CL6-WT), *LP* mutant (CL6-LP), and the empty vector (CL6-(−)). When cultured in growth medium, all of these cells grew well, and there was no difference in growth rate. When treated with 1% Me_2SO, ~80% of CL6-(−) cells were differentiated into beating cardiomyocytes (positive for MF20) (Fig. 1*D*), and the spontaneous beating was first observed on day 10 after the initiation of the Me_2SO treatment. In contrast, more than 95% of CL6-WT cells were differentiated into beating cardiac myocytes, and the spontaneous beating was first observed on day 8–9, 1 or 2 days earlier than CL6-(−) (Fig. 1*D*). In contrast, CL6-LP cells did not differentiate into beating cardiomyocytes until day 12, and less than 10% of CL6-LP cells were differentiated into MF20-positive beating cardiomyocytes on day 16 (Fig. 1*D*). These results suggest that overexpression of *Csx/Nkx2-5* promotes cardiomyocyte differentiation of CL6 cells and that overexpression of the *LP* mutant inhibits the cardiomyocyte differentiation.

Physiological Characteristics of DN-TG Mice—We obtained three independent lines of transgenic mice that overexpressed human *Csx/Nkx2-5 LP* mutant under the control of *α-MHC* promoter (Fig. 2*A*). The transgene was abundantly expressed in the adult heart, and mRNA levels of *LP* mutant were much higher (>10-fold) than those of endogenous *Csx/Nkx2-5* (Fig. 2*B*). Because the antibody against Csx/Nkx2-5 is not available at present, we estimated the abundance of Csx/Nkx2-5 proteins using EMSA. The band shift was observed when the extracts from the heart of WT-TG mice, but not of DN-TG or non-TG mice, were used (Fig. 2*C*). These results suggest that exogenous Csx/Nkx2-5 proteins are much higher than endogenous Csx/Nkx2-5.

The DN-TG mice were apparently healthy and fertile, and there were no significant differences in body weight, heart weight, and blood pressure among DN-TG mice, non-TG mice,

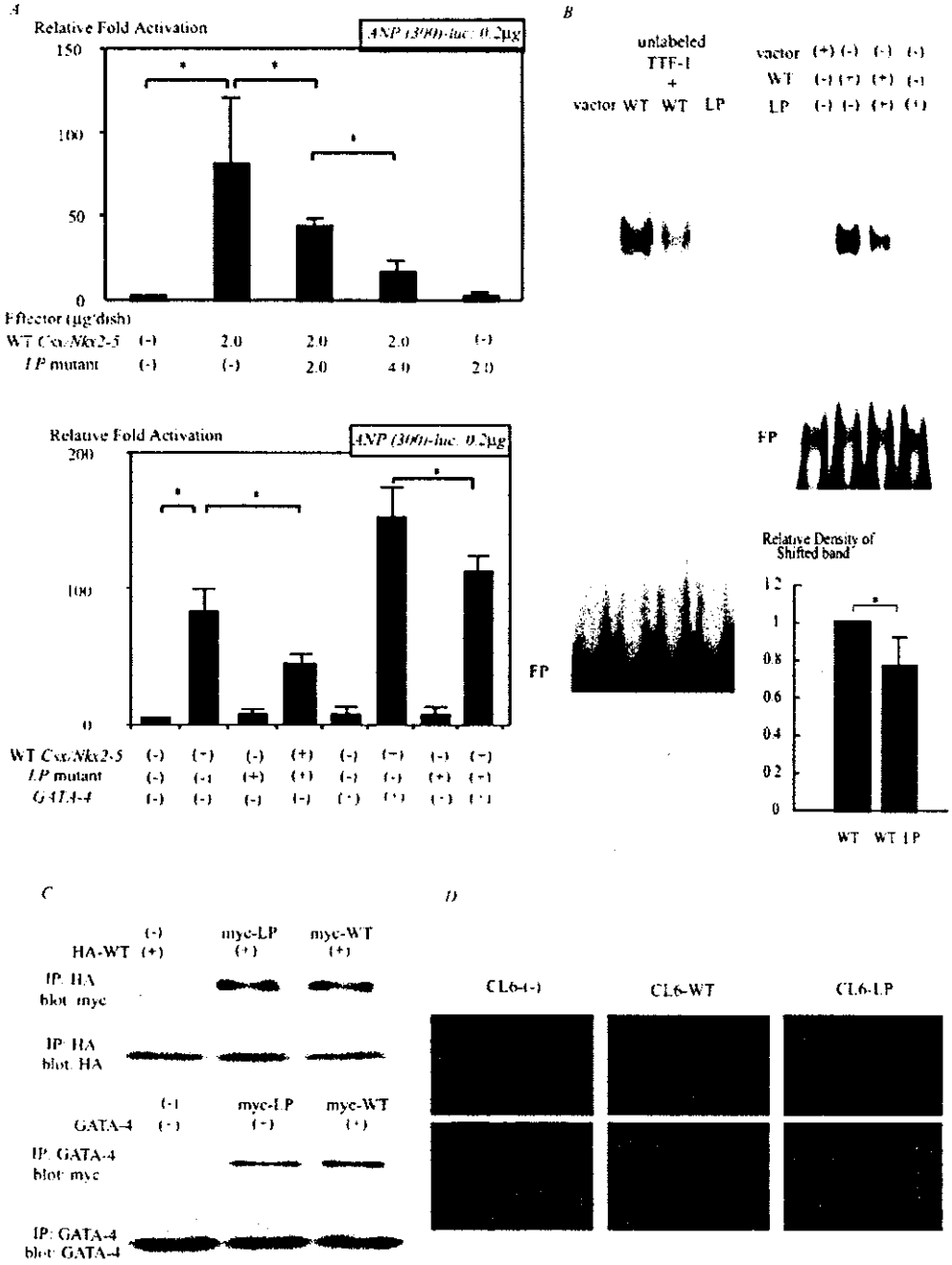

FIG. 1. **Dominant negative mutant of *Csx/Nkx2-5*.** *A*, transcriptional activity of *Csx/Nkx2-5 LP* mutant. COS-7 cells were transfected with 0.2 μg of the 300-bp *ANP* promoter containing the luciferase reporter plasmid (*ANP (300)-luc*) and various amounts of the *LP* mutant, WT *Csx/Nkx2-5*, and/or *GATA-4*. The luciferase activity was normalized to the β-galactosidase activity for each sample. The activity was presented as fold relative to the activity of the *ANP* promoter alone (=1). Values are the mean ± S.E. of data from three independent experiments performed in triplicate. *, $p < 0.05$. *B*, DNA binding activity of *Csx/Nkx2-5 LP* mutant. The DNA binding activity of *Csx/Nkx2-5* proteins was examined by EMSA using TTF-1 binding sequences. The cDNAs of WT *Csx/Nkx2-5* and the *LP* mutant were transfected into COS-7 cells, and nuclear extracts were prepared after 48 h. A ^{32}P-labeled oligonucleotide probe corresponding to the TTF-1 binding sequences was incubated with the nuclear extracts and subjected to electrophoresis on a 5% polyacrylamide gel. The binding affinity of the WT *Csx/Nkx2-5* protein was reduced by the presence of unlabeled TTF-1. The TTF-1 binding sequences bound strongly to WT *Csx/Nkx2-5* but not to the *LP* mutant (*left panel*). To elucidate whether the *LP* mutant inhibited the DNA binding activity of WT *Csx/Nkx2-5*, the cDNAs of WT *Csx/Nkx2-5* and the *LP* mutant were co-transfected into COS-7 cells, and EMSA was performed using nuclear extracts. The DNA binding of WT Csx/Nkx2-5 was mildly but significantly reduced by co-transfection of the *LP* mutant (*right panel*). *FP* indicates free probes. *, $p < 0.01$. *C*, association of *Csx/Nkx2-5 LP* mutant with WT *Csx/Nkx2-5* and *GATA-4*. The plasmids expressing Myc-tagged WT *Csx/Nkx2-5* (*myc-WT*) and *LP* mutant (*myc-LP*), hemagglutinin-tagged WT *Csx/Nkx2-5* (*HA-WT*), and *GATA-4* cDNAs were transfected into COS-7 cells. GATA-4 was immunoprecipitated (*IP*) with anti-GATA-4 antibody, and the immune complex was subjected to SDS-PAGE and immunoblotted (*blot*) with anti-Myc antibody (*top*) or with anti-GATA-4 antibody (*bottom*). (−), empty vector. *D*, CL6 cell lines. We isolated three permanent CL6 cell lines that overexpress WT *Csx/Nkx2-5* (*CL6-WT*), *LP* mutant (*CL6-LP*), and empty vector (*CL6-(−)*). Cardiomyocyte differentiation from these CL6 cells was examined using anti-sarcomeric myosin heavy chain (MF20) (*top*). Hoechst33342 DNA staining showed that there were equal numbers of cells (*bottom*).

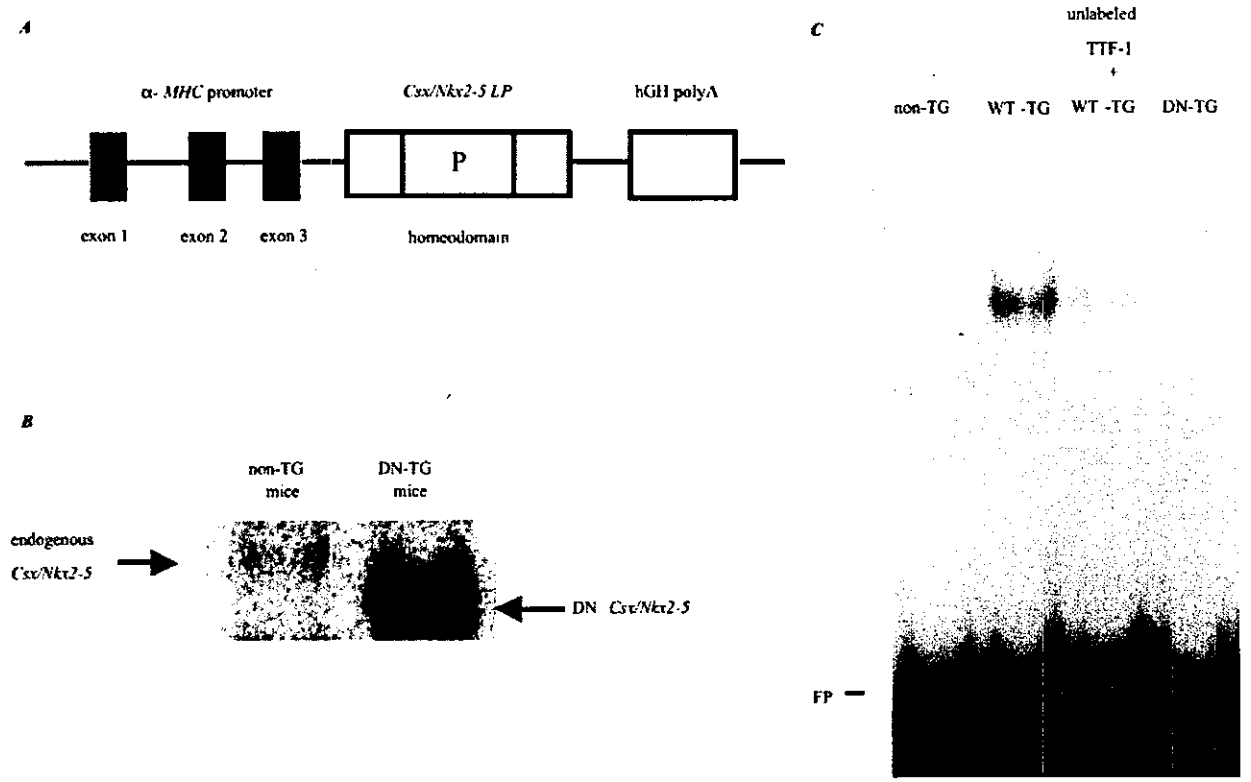

FIG. 2. **DN *Csx/Nkx2-5* transgenic mice.** *A*, schematic representation of the DN *Csx/Nkx2-5* transgene. *Csx/Nkx2-5 LP* was subcloned between the murine *α-MHC* promoter and human growth hormone (*hGH*) poly(A). The *LP* mutant was created by substituting proline (*P*) for leucine in the homeodomain. *B*, expression of endogenous *Csx/Nkx2-5* gene and DN *Csx/Nkx2-5* transgene. Northern blot analysis revealed that DN *Csx/Nkx2-5* was more abundantly (>10-fold) expressed than endogenous *Csx/Nkx2-5*. *C*, DNA binding activity *in vivo*. Nuclear extracts were prepared from hearts of WT-TG, DN-TG, and non-TG mice, and EMSA was performed using the TTF-1 binding sequence as a DNA probe.

and WT-TG mice (data not shown). In echocardiograms, however, an increase in LV end-systolic dimension and a decrease in %FS was observed in DN-TG mice but not in non-TG or WT-TG mice (Fig. 3).

Histological Analysis—Although there was no significant difference in macroscopic morphology among the three groups, light microscopic analysis revealed that interstitial fibrosis was increased in DN-TG mice compared with non-TG mice and WT-TG mice (Fig. 4*A*) (percent fibrosis: non-TG mice, 0.75 ± 0.12%; WT-TG mice, 0.42 ± 0.59%; DN-TG mice, 6.71 ± 1.33%; $p < 0.01$, non-TG mice *versus* DN-TG mice; $p < 0.05$, WT-TG mice *versus* DN-TG mice). In electromicroscopic analysis, a loss of cardiac myofilaments and an increase in the number of mitochondria were observed in the heart of DN-TG mice but not in the hearts of non-TG mice or WT-TG mice (Fig. 4*B*). The electron microscopic (EM) scores were higher in DN-TG mice (0.875 ± 0.149) than in non-TG mice (0.062 ± 0.062) and WT-TG mice (0 ± 0) (Fig. 4*C*). These results suggest that inhibition of *Csx/Nkx2-5* function induces degenerative changes of the adult heart.

Effects of DOX on Cardiac Function and Histology—To highlight the effect of loss of *Csx/Nkx2-5* on the adult heart, we injected a cardiotoxic agent, DOX, into these mice. After DOX injection, an enlargement of the LV dimension and a decrease of %FS were observed in non-TG mice and DN-TG mice. Depression of %FS was more prominent in DN-TG mice than in non-TG mice. In WT-TG mice, LV dimension and cardiac function were not significantly changed even after administration of DOX (Fig. 3). Furthermore, although there were no histological changes in all groups of mice after DOX treatment in light microscopic analysis (data not shown), electromicroscopic analysis revealed that DOX induced cytoplasmic vacuolization and myofibrillar loss of cardiomyocytes in both non-TG mice and DN-TG mice, and these ultrastructural changes were more prominent in DN-TG mice than in non-TG mice. In contrast, these ultrastructural changes were barely detectable in the ventricle of WT-TG mice (Fig. 4*B*). DOX induced an increase of the EM score in non-TG mice (0.489 ± 0.139) and DN-TG mice (1.270 ± 0.104) but not in WT-TG mice (0.116 ± 0.044), and EM scores were increased more in DN-TG mice than in non-TG mice (Fig. 4*C*). These results suggest that *Csx/Nkx2-5* protects the heart from DOX-induced impairment of myocardium.

Induction of Apoptosis by DOX—Apoptosis of cardiac myocytes has been reported to be a cause of cardiac dysfunction (19). We thus examined whether DOX induced apoptotic cell death in the hearts of these mice using TUNEL analysis and anti-active caspase-3 antibody. TUNEL- and active caspase-3-positive cardiomyocytes were barely detectable in the heart of all groups of mice without DOX treatment. DOX increased the number of TUNEL- and active caspase-3-positive cells in non-TG mice and DN-TG mice but not in WT-TG mice, and positive cells were more abundant in DN-TG mice than in non-TG mice (Fig. 5*A*). These results suggest that *Csx/Nkx2-5* inhibits DOX-induced cardiomyocyte apoptosis.

To further clarify the protective role of *Csx/Nkx2-5*, we transfected the cDNAs of WT *Csx/Nkx2-5* and *LP* mutant into the cultured cardiomyocytes and examined cell death after DOX treatment for 24 h. ~70% of *LP* mutant-transfected cells were TUNEL-positive, while only ~10% of WT *Csx/Nkx2-5*-transfected cells were TUNEL-positive (Fig. 5*B*). Furthermore, when differentiated CL6 cell lines were exposed to DOX for 24 h, the number of surviving cells was much lower in CL6-LP than in CL6-(−). In contrast, the number of surviving cells of CL6-WT was more than that of CL6-(−) (Fig. 5*C*). These results suggest

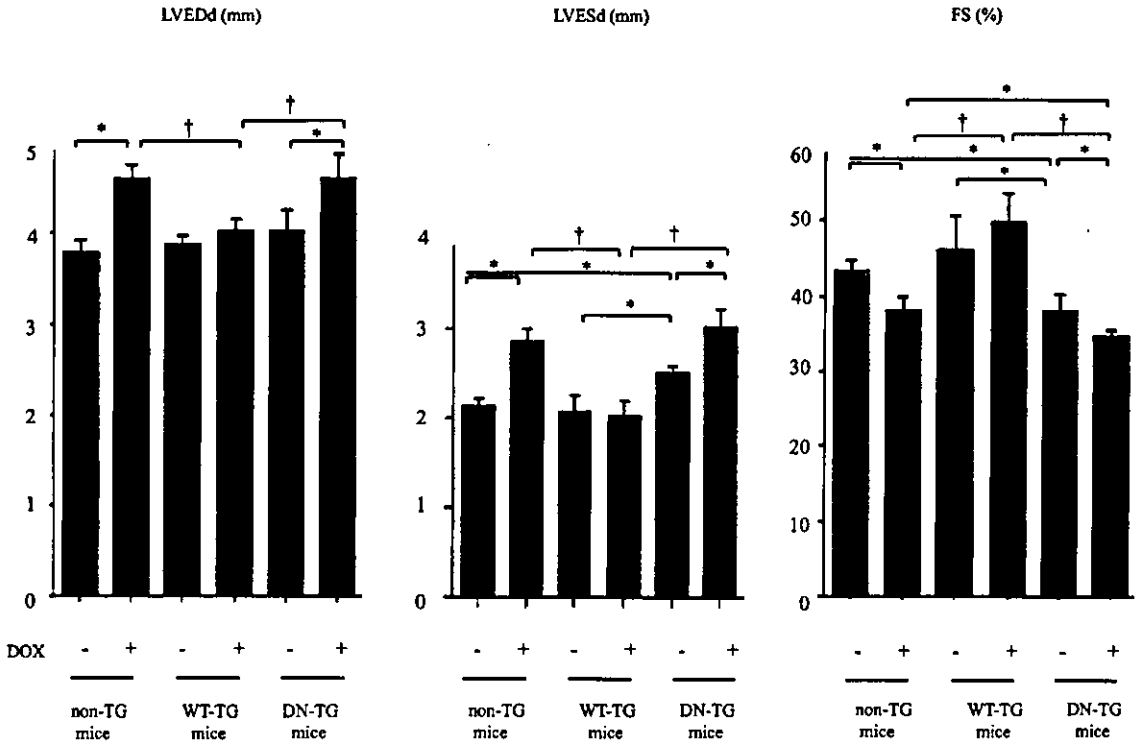

FIG. 3. **Cardiac function before and after DOX injection.** At base line, there was no significant difference between non-TG and WT-TG mice, but in DN-TG mice left ventricular end-systolic dimension (*LVESd*) was increased and %FS was reduced as compared with non-TG and WT-TG mice. In non-TG mice, LV internal dimensions were increased, and %FS was decreased after DOX treatment. Enlargement of LV dimension and depression of %FS were more prominent in DN-TG mice than in non-TG mice. In WT-TG mice, LV dimension and cardiac function were not significantly changed even after DOX treatment. *LVEDd*, left ventricular end-diastolic dimension. *, $p < 0.01$; †, $p < 0.05$.

that *Csx/Nkx2-5* also inhibits DOX-induced cardiomyocyte apoptosis *in vitro*.

Cardiac Gene Expression—We examined expression of cardiac genes such as *ANP*, *BNP*, *CARP*, and *SERCA2*. As we reported previously, all of these genes were up-regulated in WT-TG mice (9). There was no significant difference in mRNA levels of these genes between the DN-TG and non-TG mice (Fig. 6*A*). We next examined mRNA levels of these genes after DOX injection. mRNA levels were all down-regulated by DOX injection in all of these mice (Fig. 6*A*). It is noteworthy that the suppressed mRNA levels of all these genes in WT-TG mice were comparable to or still higher than basal mRNA levels of these genes in non-TG mice; however, mRNA levels in DN-TG mice were lower than those in non-TG mice after DOX injection (Fig. 6*A*). These results suggest that DOX specifically inhibits the transcription of cardiac genes and that *Csx/Nkx2-5* prevents DOX-induced suppression of gene expression.

Expression of Death-related Proteins—To get insights into the mechanism by which *Csx/Nkx2-5* protects cardiomyocytes from DOX, some death-related proteins were examined after DOX injection using Western blot analysis. There was no significant difference in the basal protein levels of an anti-apoptotic protein, Bcl-2, among non-TG, WT-TG, and DN-TG mice. Following DOX injection, Bcl-2 protein levels were increased most markedly in DN-TG mice and moderately in non-TG mice but not changed in WT-TG mice (Fig. 6*B*). There was no significant difference in protein levels of Fas/ligand in all of these mice before or after DOX injection (Fig. 6*B*).

DISCUSSION

In the present study, we examined the role of *Csx/Nkx2-5* in the adult heart using two transgenic mice that express WT *Csx/Nkx2-5* and *Csx/Nkx2-5 LP* mutant. We obtained the following results: (i) DN-TG mice showed impaired cardiac function and cardiomyocyte degeneration; (ii) DOX induced impairment of cardiac function and loss of myofilaments in DN-TG and non-TG mice, and the degree was more prominent in DN-TG mice than in non-TG mice; and (iii) DOX-induced cardiomyocyte apoptosis was enhanced by overexpression of *LP* mutant and suppressed by overexpression of WT *Csx/Nkx2-5 in vivo* and *in vitro*.

It has been reported that *LP* mutant has dominant negative effects (13). Luciferase assay revealed that the *LP* mutant used in our experiments inhibited WT *Csx/Nkx2-5*-induced activation of the *ANP* promoter in a dose-dependent manner. EMSA revealed that the DNA binding of WT *Csx/Nkx2-5* was mildly but significantly inhibited by the *LP* mutant. These results suggest that a part of the dominant negative effects of the *LP* mutant is to inhibit the ability of the WT *Csx/Nkx2-5* to bind DNA. Our and other groups have reported that *Csx/Nkx2-5* and *GATA-4* display synergistic transactivation of the *ANP* promoter (16, 18). Luciferase assay revealed that the *LP* mutant inhibited the synergistic activation of *ANP* by WT *Csx/Nkx2-5* and *GATA-4*, and immunoprecipitation assay showed that the *LP* mutant interacted with WT *Csx/Nkx2-5* and *GATA-4*. Previous studies indicated that *Csx/Nkx2-5* and *GATA-4* synergistic action required the interaction of the two factors (16). Since the *LP* mutant partially inhibited the DNA binding of *Csx/Nkx2-5*, there should be other mechanisms by which the *LP* mutant inhibits the function of WT *Csx/Nkx2-5*. A possible mechanism of dominant negative effects of the *LP* mutant is consumption of *Csx/Nkx2-5*-associated proteins including *GATA-4*.

In *Xenopus*, injection of RNA of the *LP* mutant of *Csx/Nkx2-5* suppressed normal heart formation (13). In our present study, the CL6 cells that express the *LP* mutant did not well differentiate into cardiomyocytes. Following these observations and

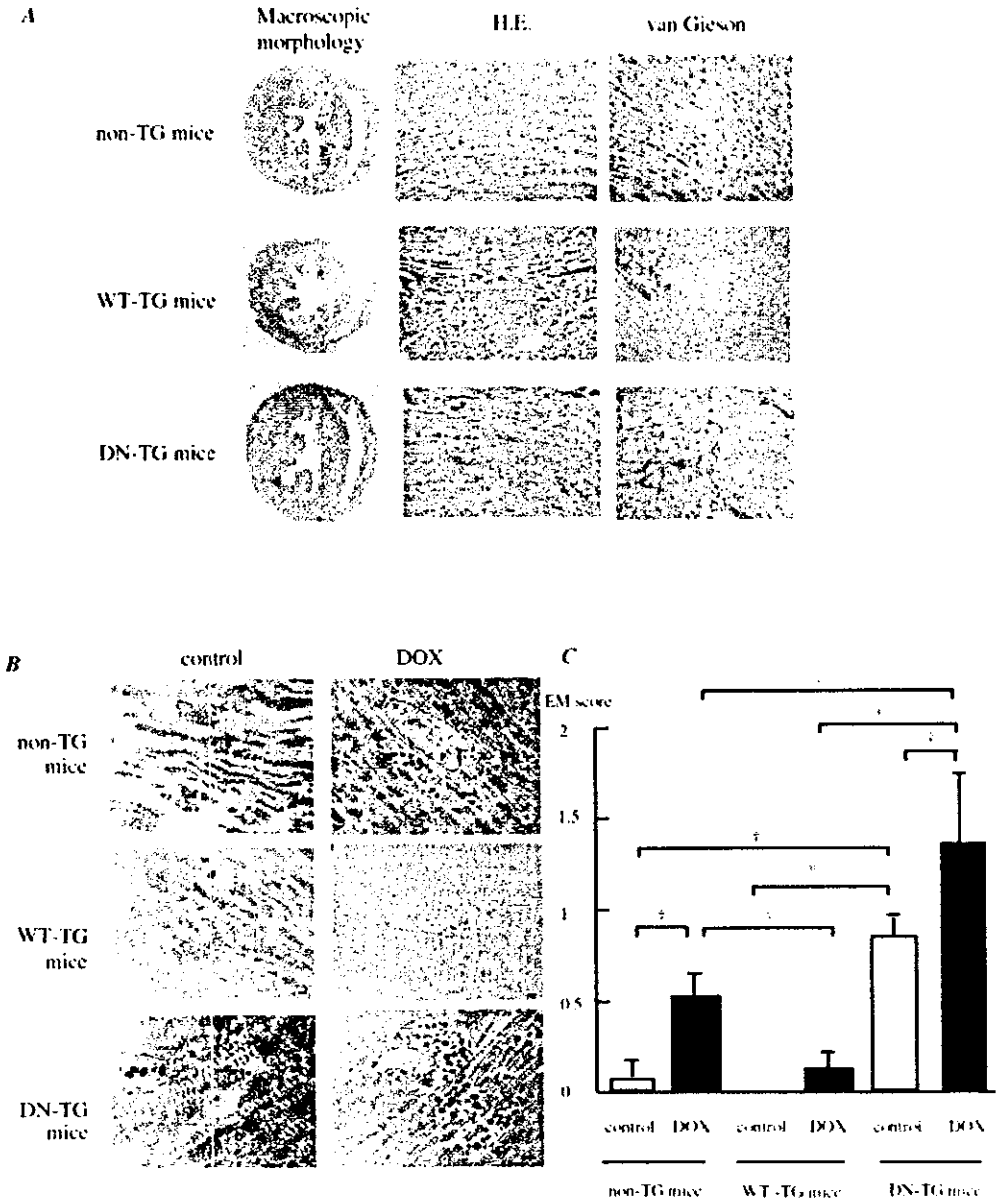

FIG. 4. **Histological analysis.** *A*, light microscopic analysis. Histological analysis was performed on hearts from non-TG (*top row*), WT-TG (*middle row*), and DN-TG (*bottom row*) 12-week-old mice. Myofiber alignment was normal (*middle column*), but interstitial fibrosis (*red, right column*) was prominent in DN-TG mice. *B*, electron microscopic analysis before and after DOX injection. There was no significant difference between WT-TG and non-TG mice, but a loss of myofilaments and an increase in the mitochondria were observed in the ventricle of DN-TG mice. The injection of DOX (20 mg/kg) induced the cytoplasmic vacuolization and the myofibrillar loss of cardiac myocytes (*arrow*) in both non-TG mice and DN-TG mice, and these changes were more prominent in DN-TG mice. In contrast, there were few morphological changes in WT-TG mice even with DOX treatment. *Bar* = 1 μm. *C*, EM score. The EM scores were higher in DN-TG mice than in non-TG and WT-TG mice without DOX treatment. DOX induced an increase of EM score in non-TG mice and DN-TG mice but not in WT-TG mice, and EM scores were increased more in DN-TG mice than in non-TG mice. *, $p < 0.01$; †, $p < 0.05$. *H.E.*, hematoxylin-eosin.

results, we generated the transgenic mice that overexpress the dominant negative mutant of *Csx/Nkx2-5* to clarify the role of *Csx/Nkx2-5* in the adult heart. Unlike the previous reports showing that *Csx/Nkx2-5* mutations cause human congenital heart diseases and atrioventricular conduction delay (4, 5), the DN-TG mice had no congenital heart diseases. The later expression of the *LP* mutant, which is driven by the *α-MHC* promoter, may be a cause of the lack of congenital heart diseases and atrioventricular conduction delay in the transgenic mice.

DN-TG mice showed impaired contractile function and some histological abnormalities. These results suggest that inhibition of *Csx/Nkx2-5* function impairs the integrity of highly differentiated cardiomyocytes, which may lead to cardiac dysfunction. Injection of *Csx/Nkx2-5* mRNA into oocytes of *Xenopus* and Zebrafish induces enlargement of hearts and ectopic hearts, respectively (20, 21). We have reported that transgenic mice that overexpress *Csx/Nkx2-5* show up-regulation of some cardiac genes including *ANP*, *BNP*, and *CARP* (9). These results suggest that *Csx/Nkx2-5* functions as a transcriptional regulator in adult hearts as well as in embryonic hearts.

DOX has been known to have severe cardiotoxic effects. After DOX injection, cardiac function of DN-TG mice was markedly impaired, while WT-TG mice showed only slight impairment of cardiac function. Ultrastructural abnormalities induced by DOX were also more prominent in DN-TG mice than in WT-TG

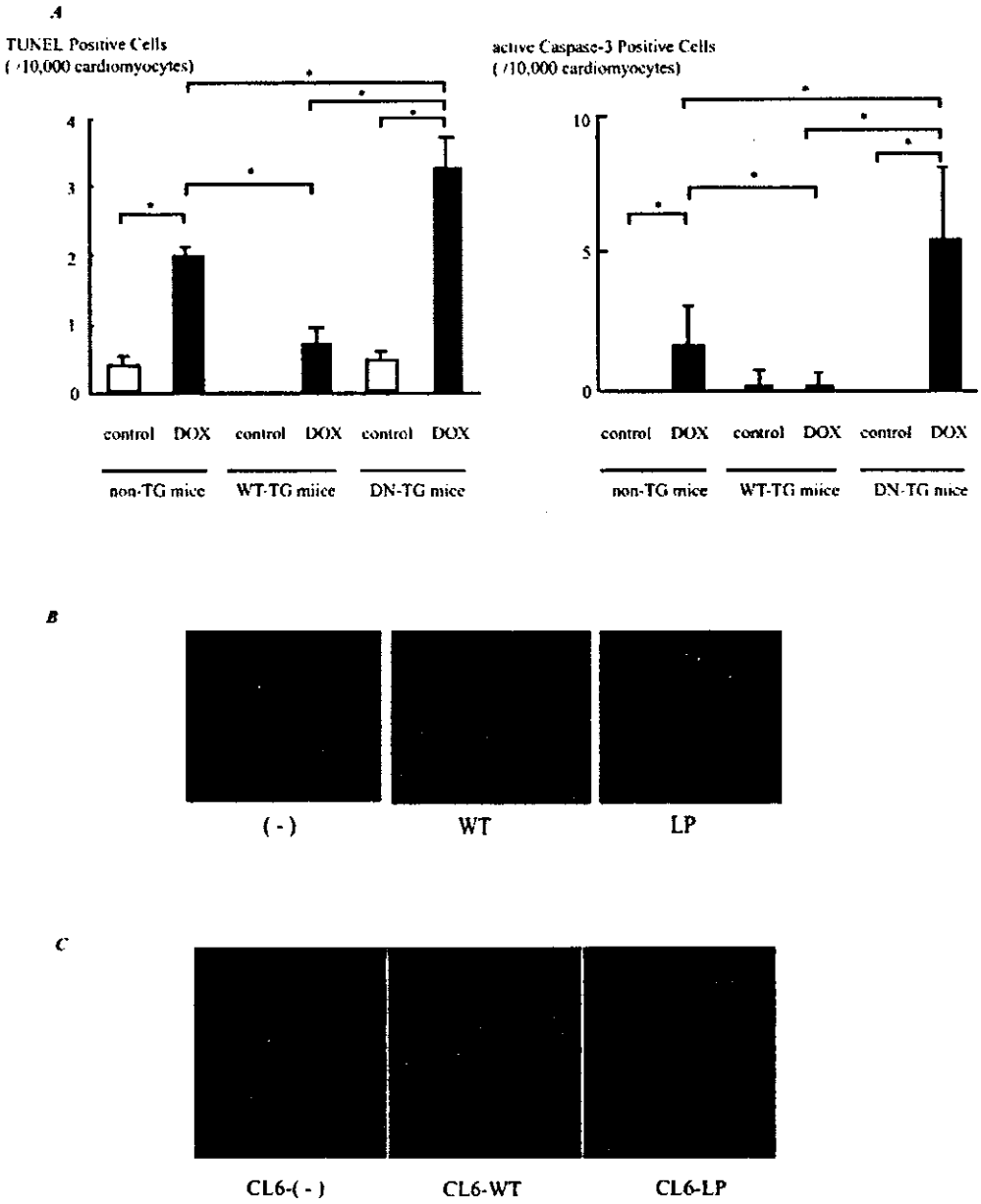

FIG. 5. **Apoptosis.** *A*, DNA fragmentation was analyzed *in situ* with the TUNEL method, and activation of an apoptosis-related protein was analyzed with immunohistochemistry using anti-active caspase-3 antibody in multiple sections at 24 h after injection of DOX. TUNEL-positive cells and active caspase-3-positive cells were counted out of 10,000 cells. There were few positive cells among the three groups before DOX injection. DOX significantly increased the number of apoptotic cardiomyocytes in non-TG mice and DN-TG mice but not in WT-TG mice. The positive cells were more abundant in DN-TG mice than in non-TG mice. *, $p < 0.01$. *B*, after transfection with Myc-tagged WT *Csx/Nkx2-5* and *LP* mutant, cardiomyocytes were stimulated by 1 μM DOX for 24 h and stained with TUNEL (*green*) and anti-Myc antibody (*red*). (−), empty vector; *WT*, WT *Csx/Nkx2-5*; *LP*, *LP* mutant. *C*, the CL6 cell lines were treated with 1 μM DOX for 24 h in the absence of serum. The number of surviving cells was lower in CL6-LP than in CL6-(−). In contrast, the number of surviving cells of CL6-WT was more than that of CL6-(−).

mice. These results indicate that *Csx/Nkx2-5* protects hearts from the cardiotoxic effects of DOX. Because we have previously demonstrated that DOX induces apoptosis in cultured cardiomyocytes (22), we further examined cardiomyocyte apoptosis in these mice after DOX injection. TUNEL and immunohistochemical analysis using anti-active caspase-3 antibody revealed that there were more apoptotic cells in the order of DN-TG mice, non-TG mice, and WT-TG mice. The protective function of *Csx/Nkx2-5* against DOX was also observed in cultured cardiomyocytes and CL6-derived cardiomyocytes. Although it remains to be determined whether such a small occurrence of cardiomyocyte apoptosis causes DOX-induced cardiac dysfunction, even a small rate of death might have eventually led to a cause of cardiac dysfunction over a longer time in this study. Several studies have also suggested the interaction of DOX with myofibrillar proteins as an etiology of DOX cardiotoxicity (23–25). Similarly, in the present study, the myofibrillar structure was shown to be damaged in a very early stage.

DOX induced expression of Bcl-2 in the three kinds of mice with different levels. The different expression levels of Bcl-2 after DOX injection among the three kinds of mice may reflect the different degrees of cardiac impairments, which may be dependent on the different expression of cardiac genes after

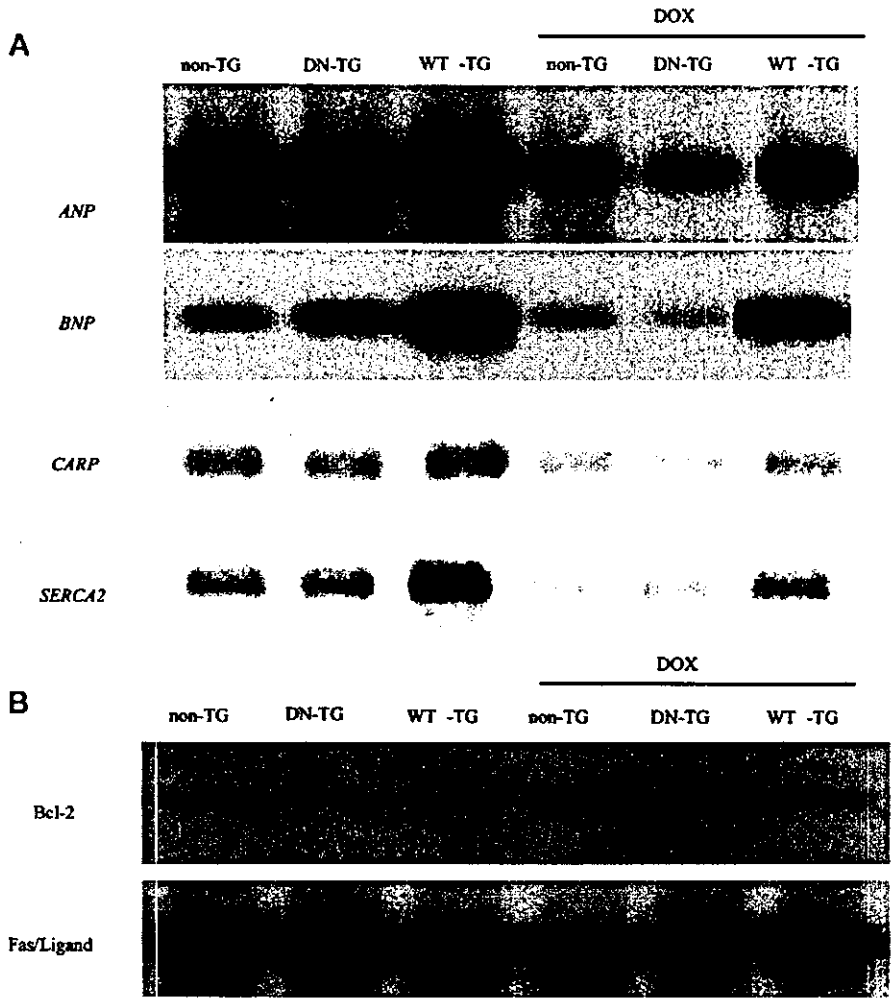

FIG. 6. **Gene and protein expression.** *A*, cardiac gene expression. RNA was prepared from the heart before and after DOX injection for 24 h. 10 μg of RNA from each sample was subjected to Northern blot analysis. Representative autoradiograms from three independent experiments are shown. *B*, death-related protein. At 24 h after DOX injection for 24 h, Bcl-2 and Fas/ligand protein levels were evaluated by Western blot analysis using each specific antibody. Representative autoradiograms from three independent experiments are shown.

DOX injection. It has been reported that *ANP* and *BNP* are sensitive markers of cardiac impairments induced by DOX (26). In this study, DOX suppressed the transcription of *ANP*, *BNP*, *CARP*, and *SERCA2* in non-TG mice. The inhibition was more prominent in DN-TG mice. In WT-TG mice, although mRNA levels of these genes were also down-regulated, the levels were comparable to or still higher than basal levels of these genes in non-TG mice. A previous study also demonstrated that transcription of cardiac genes is suppressed rapidly and selectively by DOX (25). In skeletal muscle, the inhibition of gene transcription by DOX has been linked to a reduction of transcription factor *MyoD* activity (27), suggesting that the transcriptional repression of many cardiac-specific genes by DOX may be due to the reduced activity of cardiac transcription factors. Recently it has been reported that cardiac transcription factors such as *Csx/Nkx2-5*, myocyte enhancer factor-2C, and *dHAND* were down-regulated by exposure of cultured cardiomyocytes to DOX (12). These observations and results suggest that *Csx/Nkx2-5* protects heart from DOX through controlling transcriptional homeostasis of cardiac-specific genes. Further studies are necessary to elucidate whether overexpression of *Csx/Nkx2-5* generally protects the heart from various stresses.

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Peripheral-blood or bone-marrow mononuclear cells for therapeutic angiogenesis?

Sir—Eriko Tateishi-Yuyama and colleagues (Aug 10, p 427)[1] reported angiogenesis therapy for atherosclerotic occlusion and Burger's disease by use of mononuclear cells harvested from autologous bone marrow. We attempted the same procedure with autologous peripheral blood.

Seven patients were included in this study, all of whom were men with atherosclerotic occlusion. The mean age was 65·3 years (SD 8·0; range 60–74), and the mean bodyweight 58·6 kg (11·2; 53–69). After obtaining informed consent, the patients were given 5 μg/kg granulocyte colony-stimulating factor (G-CSF) for 4 days subcutaneously. Two patients were excluded owing to poor mobilisation of CD34 cells (less than 0·03% of peripheral blood mononuclear cells). Mononuclear cell apheresis was done on days 4–6 for the five patients in whom the CD34 ratio had increased to more than 0·1% of peripheral white cells.

The peripheral white-cell counts of the five patients just before harvest were 22 900–44 500/μL. For each patient, 10 L peripheral blood was treated with an apheresis machine (COBE Spectra, Gambro, CO, USA), and a mean of $6{\cdot}88\times10^{10}$ (SD $3{\cdot}26\times10^{10}$; range $2{\cdot}3$–$11{\cdot}6\times10^{10}$) mononuclear cells were harvested. The CD34 ratio was 0·13% (0·10; 0·07–0·29). To prevent muscle necrosis at the injection point, we purified the CD34 cells with Isolex-50 (Takara-Koden, Shiga, Japan) to reduce the cell numbers. $1{\cdot}37$–$14{\cdot}9\times10^{7}$ CD34 cells were obtained and frozen at −80°C until injection. The mean purity of the CD34 cells was 40·6% (35·3; 25·6–92·3), and the mean recovery rate was 28·2% (23·5; 15·6–96·0. These CD34-enriched cells were injected at 50 points of limb muscle, with the patient under spinal anaesthesia, within a week of harvest.

All five patients recovered. Pain relief was seen within 3 days, and a lengthening of maximum walking distance became apparent within a week of injection. These improvements continued more than a year later. However, objective tests of improvement, such as ankle-brachial pressure index and blood vessel change by angiography, were inconclusive, except for the disappearance of a heel ulcer in one patient. Our study indicated that autologous CD34 cells were effective as angiogenesis therapy. Although this method is expensive because of the high cost of the CD34 cell collection technique, it provides a valuable option if bone marrow is not available.

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Sir—Eriko Tateishi-Yuyama and colleagues[1] show the efficacy and safety of implantation of bone-marrow mononuclear cells compared with peripheral-blood mononuclear cells in ischaemic limbs, and conclude that autologous implantation of mononuclear cells is better for therapeutic angiogenesis. We disagree with some of their conclusions.

First, although implantation of peripheral-blood mononuclear cells was much less effective than that of bone-marrow mononuclear cells in their study, we have found that it is just as efficient in a murine model of hind-limb ischaemia. This efficacy was shown by increases in capillary density and blood-flow recovery, and a reduction in the rate of autoamputation, consistent with previous reports.[2] On the basis of our animal data, we did autologous implantation of peripheral-blood mononuclear cells in ischaemic limbs of three patients who progressively developed non-healing ulcers or gangrene with severe rest pain despite having undergone intensive treatments. 4 weeks after implantation, we saw a significant increase in ankle-brachial pressure index (>0·1) in all patients. Rest pain in legs was greatly reduced in two of three patients, in one of whom it was completely abolished. Additionally, substantial improvement of ischaemic ulcers was seen in two of three patients, suggesting that implantation of peripheral-blood mononuclear cells was similar to that of bone-marrow mononuclear cells.

Second, whereas local inflammatory reactions or oedema were not detected in Tateishi-Yuyama and colleagues' study, we noted inflammatory responses such as oedema, swelling, and increased rest pain in injected legs 2 days after implantation in all cases, which were sustained for a week. Serum markers for inflammation including C-reactive protein were increased; such markers peaked 2 weeks after implantation and declined to nearly normal values at 4 weeks without the need for antibiotics. High concentrations of C-reactive protein were detected in all cases before implantation and were not improved by the long-term treatments with antibiotics, implying possible antipyrogenic properties of implantation of peripheral-blood mononuclear cells.

Given this efficacy, there are several advantages over implantation of bone-marrow mononuclear cells. For example, since the collection of peripheral-blood mononuclear cells does not require general anaesthesia, it is safe to apply to patients in whom anaesthesia is contraindicated. Moreover, such implantation can be repeated easily, which could enhance the effects of the first implantation on vascularisation. Taken together with our results, we believe that implantation of peripheral-blood mononuclear cells could be an alternative strategy for therapeutic angiogenesis in peripheral-artery disease.

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Author's reply

Sir—Shoichi Inaba and colleagues report the efficacy of angiogenic therapy with implantation of CD34 cells sorted from peripheral-blood mononuclear cells. Although Asahara and colleagues[1] reported that adult blood-derived CD34 angioblasts, but not CD34-negative leucocytes, differentiate into endothelial cells, further findings have suggested that peripheral-blood-derived mature mononuclear cells can also transdifferentiate into endothelial-lineage cells and play an essential part in neovascularisation in ischaemic limbs via leucocyte-leucocyte interaction with CD34 cells.[2]

We reported that the CD34-negative fraction in bone-marrow mononuclear cells supplies angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, and that their receptors are expressed in the CD34 fraction. Although the purity of CD34 cells was about 41% in our study and frozen until implantation, CD34 mononuclear cells might exert more potent angiogenic action when combined with CD34-negative mononuclear cells.

Inaba and colleagues treated patients with a low dose of G-CSF for 4 days to mobilise CD34 cells from bone marrow. However, we found reports that angina pectoris or acute arterial thrombosis can occur in patients receiving G-CSF due to leucocytosis or hypercoagulability.[3] Since most of the patients eligible for our trial were predicted to have severe atherosclerotic lesions in coronary or cerebral arteries, pretreatment with G-CSF might have caused deleterious vascular events before angiogenic cell therapy.

Because marrow cells include cells of various lineages, such as fibroblasts, osteoblasts, and myogenic cells as well as endothelial cells, such mixed populations might differentiate into various mesenchymal cells. We have shown in animal and human studies that injected bone-marrow mononuclear cells are unlikely to differentiate into other lineages in ischaemic tissues.[4] However, transdifferentiation into cells of other lineages was not completely excluded. Implantation of CD34 cells might be a more suitable strategy for angiogenic therapy in patients with ischaemic heart diseases.

In our study, we showed that implantation of peripheral-blood mononuclear cells substantially decreased the extent of rest pain and ischaemic ulcers in 20 patients, which is consistent with the observation by Tohru Minamino and colleagues, although we found that an increase in ankle-brachial pressure index was significantly lower than with implantation of bone-marrow mononuclear cells, and that collateral vessel formation detected by angiography was less evident.

We have recently studied the molecular mechanism responsible for angiogenic action by implantation of human peripheral-blood mononuclear cells in nude rats.[5] VEGF released from peripheral-blood mononuclear cells had a key role in the induction of angiogenesis, and no incorporation of implanted peripheral-blood mononuclear cells into neocapillaries was seen. Other angiogenic factors were also involved in the angiogenic action, and inclusion of polymorphonuclear leucocytes attenuated angiogenic action by releasing neutrophil elastase.[5]

Minamino and colleagues also noted that C-reactive protein concentrations, which were not improved by long-term treatment with antibiotics, were lower than normal after implantation. Thus, implantation of peripheral-blood mononuclear cells could be a novel strategy for angiogenic cell therapy for patients with peripheral-artery diseases who are not suitable for general anaesthesia due to impaired cardiac function or other potential risks. Alternatively, it could be used to enhance the effects of an implantation of bone-marrow mononuclear cells or of vascularisation.

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IL12B promoter polymorphism and asthma

Sir—Andrew Sandford and Peter Paré's Commentary (Aug 10, p 422)[1] on the study by Grant Morahan and colleagues (p 455)[2] prompts me to ask whether it is useful or instructive to look for asthma genes? The investigators report that heterozygosity for the *IL12B* promoter polymorphism is associated with increased severity of asthma in childhood. However, is it appropriate to apply genetics to a variable airway reactivity that has a normal distribution within the population? From Gregor Mendel's first experiments, genetically determined variation has been conceived as distinct from a comparison population—a red flower distinguished from a blue, male from female. Diseases caused by genetic variation are characterised by pathology that separates them from the healthy, or normal, population. Even when there is an unequivocal separation of the pathology from the rest of the population—for example in cystic fibrosis—the genetic basis of the disease is complex enough.

Asthma is not a distinct disease, merely a cut off point on the normal distribution of airway reactivity within a population. Someone with airway reversibility of 15% will not have asthma genes that explain why they differ from someone with airway reversibility of 10%. To find a genetic cause for a disorder that is one tail of a smooth distribution curve would need the discovery of multiple genes to explain the smoothness of the curve. A susceptibility to asthma would then be predicted from the number of asthma-prone genes the person inherited. Not only would the number of genes associated with asthma be important, but it seems logical that there would also be genes that have a protective effect against asthma. Knowledge of genes that afford protection would be needed to explain the other tail of the distribution curve—those individuals whose airways are unusually unresponsive.

With the expensive complexity of research into the genetic basis of asthma, are we any further on from knowledge derived from the spirometer?

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Original article

A variant of congenital muscular dystrophy

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Abstract

We analyzed three Japanese patients (two boys and a girl) from two families with congenital muscular dystrophy (CMD) and brain involvement. One of the two families had two affected siblings of different sexes. Parental consanguinity was not documented in either family. All patients showed generalized hypotonia and weakness from infancy, delayed psychomotor development, facial muscle involvement, and joint contractures. Serum creatine kinase levels were markedly elevated. The histological change seen on muscle biopsy was characteristic of a dystrophic process, although dystrophin and merosin staining were normal. On MR imaging, cortical dysplasia and cerebral white matter abnormalities were observed. Although these clinical, myopathological and neuroradiological findings were typical of Fukuyama-type CMD (FCMD), full mutational analysis of the fukutin gene revealed neither a 3 kb insertion (Japanese founder mutation) nor point mutations. RT-PCR analysis of RNA isolated from lymphoblasts of a patient revealed normal expression of the FCMD transcript. As classification of CMD should be based on genetic background, our present cases with typical clinical, myopathological and neuroradiological findings of FCMD without mutation of the fukutin gene may represent a new variant (or variants) of CMD that is different from FCMD.
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Keywords: Congenital muscular dystrophy; Central nervous system involvement; Fukuyama-type congenital muscular dystrophy; Fukutin gene; Variant of congenital muscular dystrophy

1. Introduction

Congenital muscular dystrophies (CMD) are characterized by hypotonia and muscle weakness from early infancy and dystrophic changes in skeletal muscle pathology [1]. The classical form of CMD is an autosomal recessive disorder characterized by the above symptoms, but with no mental retardation. A deficiency in merosin in the basement membrane was shown to cause the classical CMD linked to chromosome 6q2 [2]. Merosin-deficient CMD is prevalent in white populations, while in Japan, most CMD patients have Fukuyama-type CMD (FCMD). FCMD is an autosomal recessive disorder characterized by severe CMD associated with brain and eye malformations [3]. At least two further forms of CMD with brain and eye involvement are nosologically well defined: muscle-eye-brain disease (MEB) in Finland [4] and Walker–Warburg syndrome (WWS) prevalent in other white populations [5].

Recently, the gene responsible for FCMD was identified on chromosome 9q31, which encodes a novel 461 amino acid protein termed fukutin [6]. Most FCMD-bearing chromosomes (87%) derive from a single ancestral founder, whose mutation consisted of a 3-kb retrotransposal insertion in the 3′ non-coding region of the fukutin gene. Nine non-founder mutations were later identified [7,8]. However, there have been no FCMD patients with non-founder (point) mutations on both alleles of the gene, which could be embryonic-lethal. This could explain why few FCMD cases are reported in non-Japanese populations.

Here, we report three Japanese patients from two families with the FCMD-phenotype, in whom full mutational analysis, including all exons and flanking introns in the fukutin gene were not clarified abnormalities. The possibility that these patients suffer from a different type of CMD from FCMD is discussed.

2. Case reports

2.1. Family 1 (cases 1 and 2)

Both parents were healthy and non-consanguineous.

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Their serum creatine kinase (CK) levels were within normal limits. There had been two miscarriages before the birth of case 1. Case 1 was a male infant who died of pneumonia at 23 months of age. He was born at 42 weeks' gestation by cesarean section due to fetal distress. His birth weight was 3300 g and Apgar score after 1 min was 9. Based on severe hypotonia since birth, elevated serum CK levels (1025 IU/L, normal range <130 IU/L), myopathic EMG findings and a low density area in the cerebral white matter on CT (Fig. 1), he was diagnosed as having FCMD. Psychomotor development remained poor during subsequent months. He had neither shown head control, nor said any meaningful words until his death at the age of 23 months.

Case 2 was a female infant born uneventfully after 39 weeks' gestation and her birth weight was 3088 g. At the age of 3 months, she was first referred to our clinic due to severe hypotonia. On physical examination, she showed general muscle weakness and deep tendon reflexes were hypoactive. Her serum CK level was markedly elevated (5350 IU/L). Brain CT at age 10 months revealed similar findings to her brother (Fig. 1). A tentative diagnosis of FCMD was given. At age 19 months, she was admitted to our hospital for further examinations. She showed facial muscle involvement, contractures of the ankle, knee and hip joints, and calf muscle pseudohypertrophy. Serum CK (2874 IU/L) and aldolase (23.7 mU/ml, normal range 1.2–7.6 mU/ml) were still high and EMG was myopathic. Motor nerve conduction velocities and EEG were normal. Ophthalmologically, she showed no abnormalities. She did not exhibit afebrile or febrile convulsions. MRI showed diffuse periventricular hyperintensity on T2-weighted images, and thick and bumpy cortices with shallow sulci corresponding to polymicrogyria over the frontal and parietal lobes (Fig. 2). These findings did not change during follow-up. Disorganized cerebellar folia and clusters of intraparenchymal cysts in the cerebellum were also found (Fig. 3).

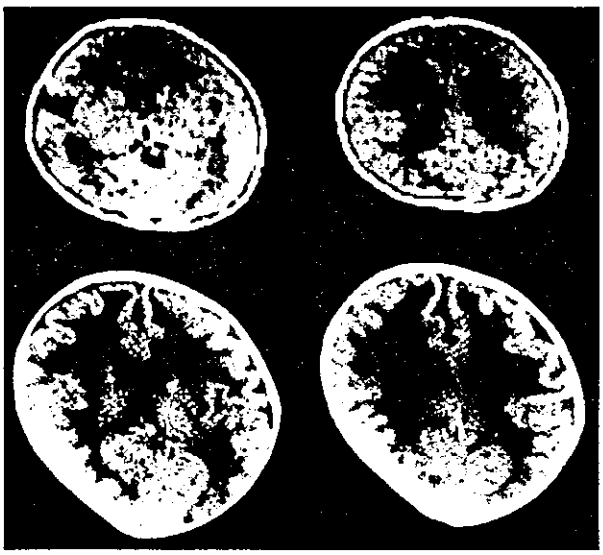

Fig. 1. CT scans of cases 1 and 2. The upper line shows scans of case 1 at the age of 6 months and the lower line shows those of case 2 at the age of 10 months. Note the marked hypodensities in the cerebral white matter in both cases.

Psychomotor development remained poor during subsequent years. She obtained head control after 3 years 6 months and learned to sit unsupported at 4 years 10 months, while the ability to sit alone was lost at the age of 8 years. She spoke her first word at 18 months, and spoke two-word sentences at 6 years 5 months. Later, nocturnal hypoventilation became evident, and she received respiratory support at night from 9 years of age.

2.2. *Family 2 (case 3)*

A 15-month-old boy was first referred to our clinic due to floppiness and developmental delay. He was born uneventfully at 40 weeks' gestation and his birth weight was 3232 g. Both parents were healthy and non-consanguineous, and had no family history of neuromuscular disease. There had been neither miscarriage nor stillbirth before his birth. He was the first-born child.

On physical examination, he showed hypotonia and calf muscle pseudohypertrophy. Deep tendon reflexes were preserved and joint contractures were not found. Delayed psychomotor development was evident: he obtained head control at 4 months, rolled over at 9 months and learned to sit unsupported at 12 months. Serum CK was 2776 IU/L, while dystrophin gene deletions were not detected. A muscle biopsy revealed variation in fiber size, areas of muscle fiber necrosis with regeneration, and increased endomysial and perimysial connective tissues compatible with dystrophic changes (Fig. 4). Dystrophin and merosin immunostainings were normal (Fig. 5). He developed a generalized tonic-clonic seizure with fever just after the muscle biopsy, while serum CK was not so elevated compared to the previous levels. An EEG showed no paroxysmal discharges. No febrile or afebrile convulsions has been observed since then. Brain MRI showed diffuse periventricular hyperintensity on T2-weighted images (Fig. 6), which decreased later during follow-up.

Psychomotor development remained poor during subsequent years. He crept at 19 months and stood with support at 26 months of age. Joint contractures developed in the hips and knees and deep tendon reflexes disappeared. He spoke his first word at 19 months. At age 2 years 3 months the face was apparently myopathic.

3. Investigations and results

Genomic DNA was extracted from the white blood cells of cases 2 and 3 and their parents. Haplotype analysis using polymorphic microsatellite markers flanking the FCMD gene was done. Our results did not reveal the founder haplotype (138-192-147-183 in D9S2105-D9S2170-D9S2171-D9S2107) [7] in any of them. In addition, we could not

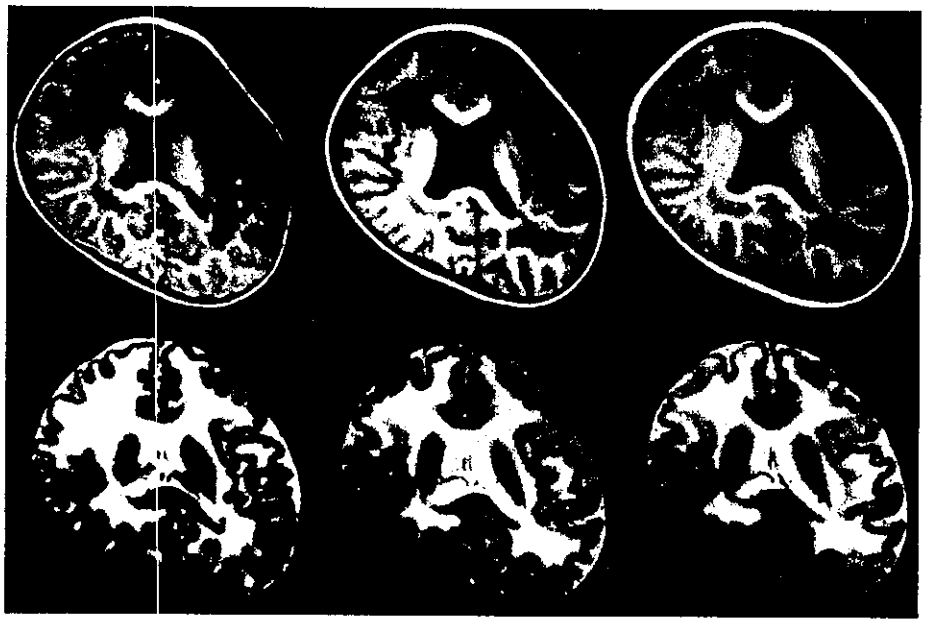

Fig. 2. Axial T1-weighted (the upper line) and T2-weighted (the lower line) sequences of case 2. On the left two images at age 19 months, myelination is present in the internal capsule, corpus callosum, and occipital region. The cerebral white matter shows symmetric low intensity on T1-weighted and high intensity on T2-weighted sequences. This is an immature pattern of myelination for this age. Thick and bumpy cortices with shallow sulci are seen over the frontal and parietal lobes. Although myelination progresses with age, abnormal intensities in the white matter did not change during follow-up at 3 years 1 month of age (middle two images) and at 4 years 7 months of age (right two images).

detect any abnormal 3-kb insertion bands (Japanese founder mutation). We also performed full mutational analysis on cases 2 and 3, including all exons and flanking introns in the fukutin gene. There was neither abnormal band shift by single-stranded conformation polymorphism analysis nor mutations by sequencing.

RT-PCR analysis of RNA isolated from the lymphoblasts of case 2 was performed. The level of amplified product was normal compared to those from FCMD patients who carry the insertion homozygously or heterozygously [7].

4. Discussion

Our present cases had characteristic clinical findings of typical FCMD, including generalized hypotonia and weak-

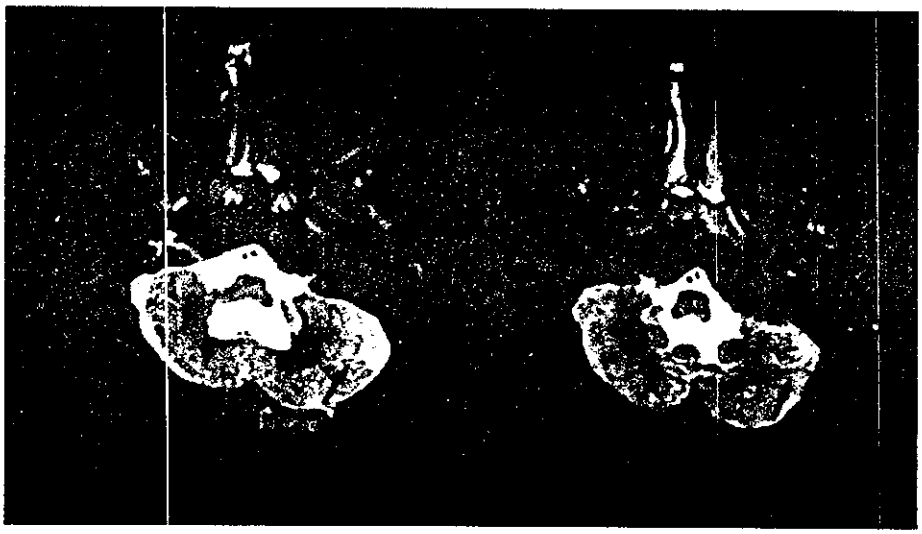

Fig. 3. Axial T2-weighted images of the midportion of the cerebellum of case 2 at 10 years 3 months. The left portion of the cerebellum reveals disorganized folia (thick arrow). There are some round cystic lesions (thin arrows).

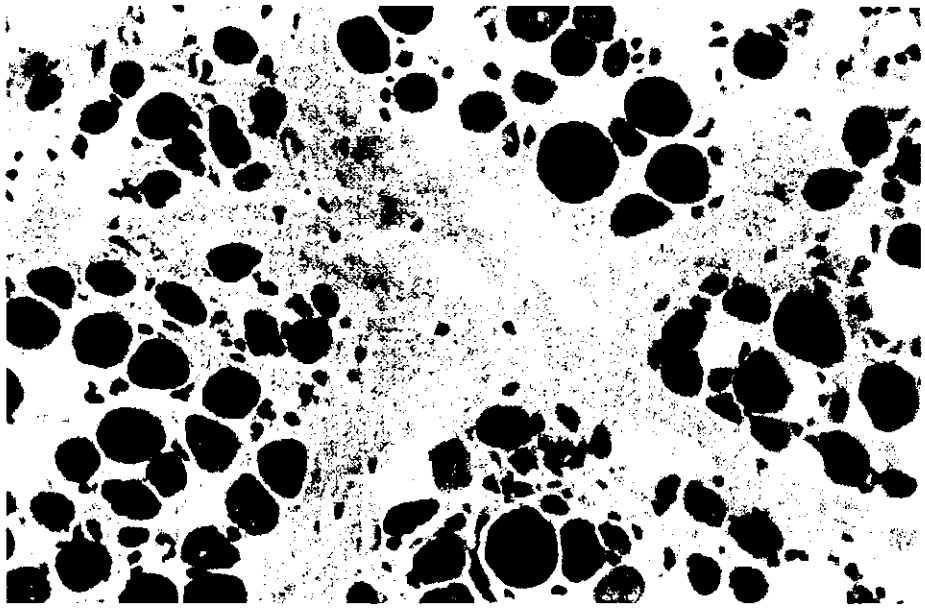

Fig. 4. Muscle biopsy of case 3. A variation in muscle fiber diameter, areas of muscle fiber necrosis with regeneration, and increased endomysial and perimysial connective tissues are seen. HE X100.

ness from infancy, delayed psychomotor development, facial muscle involvement, and joint contractures [3,9]. On MR imaging, cortical dysplasia and cerebral white matter abnormalities were identical to those seen in FCMD [10], although the abnormal intensities in the white matter did not change during follow-up in case 2. Full mutational analysis of the fukutin gene, however, revealed neither a 3 kb insertion nor point mutations. RT-PCR analysis of RNA isolated from the lymphoblasts of patient 2 revealed normal expression of the FCMD transcript. On muscle histology, early-onset dystrophinopathy and merosin-deficient CMD could be excluded, because dystrophin and merosin were positive in a muscle specimen from case 3. In addition, normal staining for merosin made

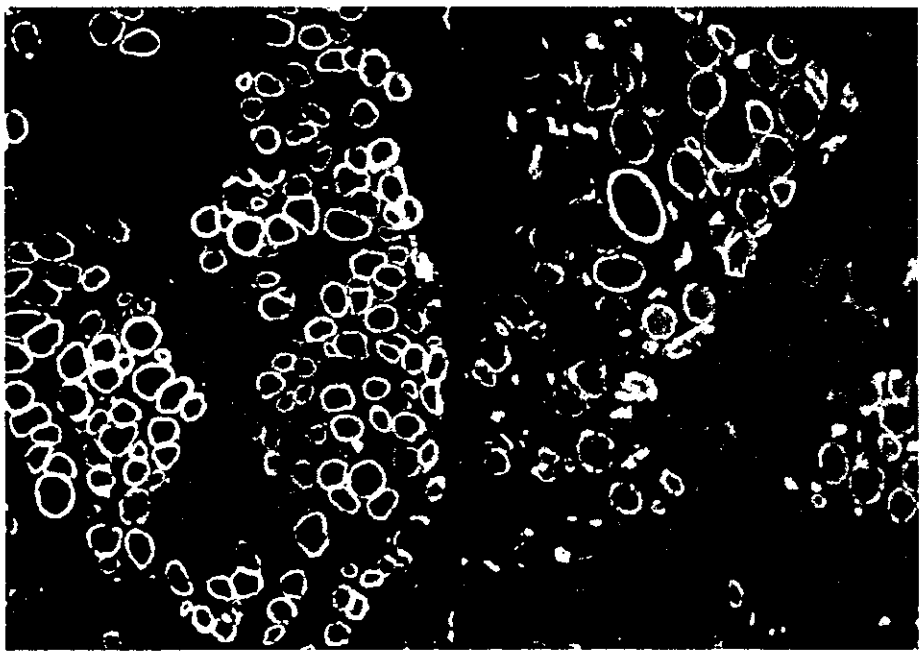

Fig. 5. Frozen sections of biopsied muscle tissue from case 3, immunoreacted for dystrophin (on the left) and merosin (on the right). Note the normal immunostaining patterns for both.

FCMD and MEB unlikely, because weak staining for merosin was reported in FCMD [11] and MEB [12]. The normal nerve conduction velocities in case 2 made merosin- deficient CMD unlikely [13]. No eye involvement was noticed unlike WWS or MEB [4,5]. Family 1 had two affected siblings of different sexes, suggesting autosomal recessive inheritance.

A systematic analysis of the FCMD gene in 107 unrelated Japanese CMD patients revealed that the 3 kb insertion was found to be present on 85.6% of disease chromosomes examined [7]. In addition, there were eighty probands (75%) homozygous for the 3 kb insertion, while 25 (23%) were heterozygous, and only two lacked the 3 kb insertion on either allele. However, there were no FCMD patients with non-founder (point) mutations on both alleles of the gene, which could be embryonic-lethal. This could explain why few FCMD cases are reported in non-Japanese populations. One of two probands without the 3 kb insertion in this report [7] was case 2, and another was reported by Miura et al. [14]. Thus, until now, four Japanese patients including our three cases have shown almost the same clinical findings to those with typical FCMD, yet full mutational analysis of the fukutin gene revealed neither a 3 kb insertion nor a point mutation. From these observations, three possibilities are considered: (i) mutations in the promotor region or introns of the FCMD gene; (ii) genetic heterogeneity (second locus?) in FCMD; or (iii) a different type of CMD from FCMD. RT-PCR analysis revealed normal expression of the FCMD transcript in patient 2 supports (ii) or (iii).

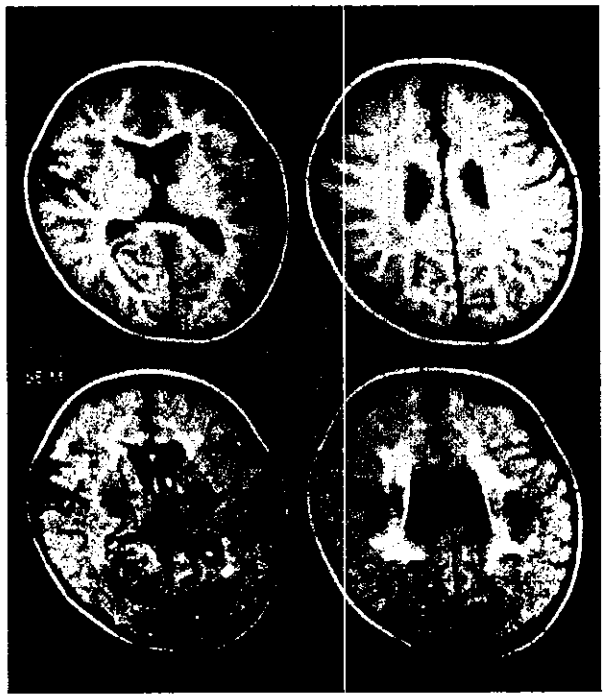

Fig. 6. MR imaging of case 3 at 15 months of age. Symmetric low intensity on T1-weighted (the upper line) and high intensity on T2-weighted (the lower line) sequences in the cerebral white matter.

Although FCMD seems to be very rare in ethnic groups other than the Japanese population, an increasing number of cases, all resembling or sharing features with FCMD, have been reported from countries other than Japan [15–19]. Among them, genetic studies were done only in cases reported from Taiwan [15]. Three Chinese patients were afflicted with the typical clinical, myopathological and neuroradiological findings of FCMD with neither a 3 kb insertion nor a point mutation on the fukutin gene. While genetic studies have not been done in other cases reported from western countries [16–19], they may be genetically different from FCMD, because there were no FCMD patients with non-founder (point) mutations on both alleles of the gene and the 3-kb insertion allele is unique to the Japanese.

Three forms of CMD with brain and eye involvement have been described: FCMD, WWS and MEB. However, it is sometimes difficult to differentiate these three forms on clinical grounds alone [9,20]. The neuropathological difference between them also reflects variation within the same disease [21]. Why do the phenotypes of these diseases resemble each other or overlap? These features may result from disturbances in basement-membrane components that are common to the muscle, eye, and brain, since several investigators have observed aberrations in extracellular matrix proteins in these diseases [11,12,22]. Nowadays, classification of CMD should be based on the genetic background. The FCMD gene is located on 9q31, and the gene underlying MEB has recently been mapped to chromosome 1p32-34 [23], while the chromosomal location of the WWS gene is still unknown; WWS is likely to be a heterogeneous disorder. This indicates that these conditions are separate disease entities from a genetic point of view.

From these findings, our present cases with clinical phenotypes similar to, but genetically different from FCMD may be a new variant (or variants) of CMD that is different from FCMD, WWS or MEB.

Acknowledgements

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