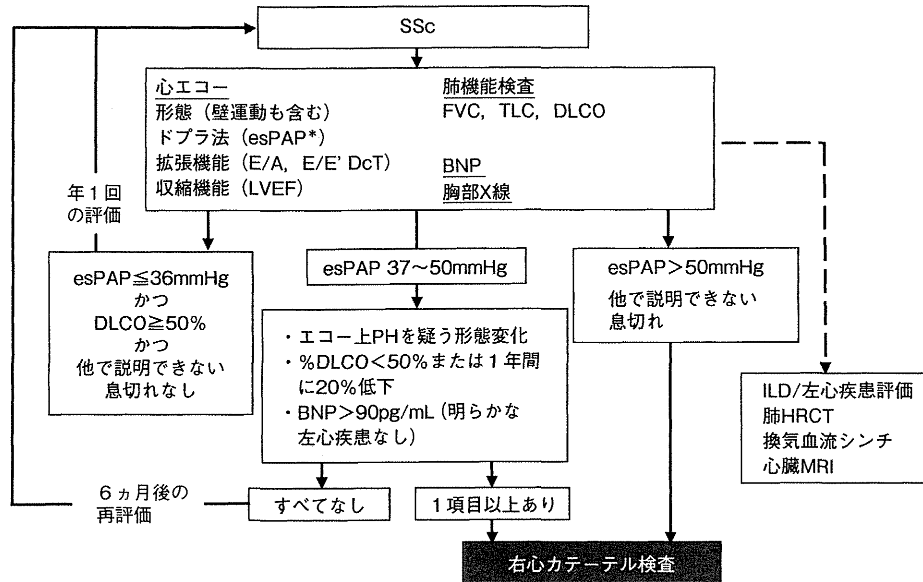

図1．SScにおけるPHスクリーニング
*esPAP：推定肺動脈収縮期圧（右房圧を5mmHgと仮定）

ングを実施することが望ましい。SSc関連PAHの多くは，長い罹病期間を経て顕性化することから，定期的なスクリーニングにより早期発見が可能である[14]。

PHの診断はスクリーニング，右心カテーテルによる確定診断，病態鑑別と機能評価の順に系統的に進める。肺性Ⅱ音亢進など身体所見，胸部X線や心電図による右心負荷所見は早期例での感度は高くない。PAHを早期に捉えるのに有用なスクリーニング検査として，肺機能検査による一酸化炭素拡散能（DLCO）と脳性ナトリウム利尿ペプチド（brain natriuretic peptide；BNP）またはその前駆体NT-proBNPが注目されている。ILDによる拘束性換気障害では％VC，％DLCOともに低下するが，PAHでは％VCに比べて％DLCO低下が顕著となる。一方，BNPはPAH患者で上昇するが，左心不全でも高値を示す。また，PAHのないSScの約半数で基準値を超えることから，単独での特異性は低い。そのため，％DLCOとBNPを組み合わせることで特異度が上昇し，％DLCO低下とNT-proBNP上昇の両者を有する例は，その後3年以内にPAHと診断されるリスクが47倍高いことが示されている[15]。

スクリーニングとして広く用いられている検査は経胸壁心エコーである。特に，ドプラ法により求めた三尖弁逆流ジェット速度から計算した推定収縮期肺動脈圧（esPAP）が汎用されている。ただし，ドプラ法によるesPAPと右心カテーテル検査で測定した平均肺動脈圧実測値の相関は必ずしも強くない。PHの基準となる平均肺動脈圧25mmHgに相当するドプラ法によるesPAPを設定することは困難である。経験的にドプラ法によるesPAP 50mmHgを超える例の90％以上が右心カテーテルでPHと診断されるが，37～50mmHgの境界値では20％程度である。PHの診断には右心カテーテル検査による肺動脈圧測定が必須だが，当院では自覚症状，心エコー，肺機能検査，BNPを組み合わせて，右心カテーテル検査を実施する症例を絞り込んでいる（図1）。

SScではPAH以外のPH病態をきたすことが高頻度なことから，治療開始前に病態の鑑別が必須である。右心カテーテル検査で肺毛細血管楔入圧の上昇がなければ左心疾患単独によるPHは否定される。ただし，心エコーで心筋壁運動異常や拡張障害がみられる場合は，心筋病変の併存を示唆する。慢性肺血栓塞栓症の除外のためには，肺換気血流シンチグラムや造影CTの実施が必要である。ILDが存在する場合は，PAH，ILDによるPH，両者の合併の鑑別は困難であるが，拘束性換気障害，CT所見に比して自覚症状，運動耐容能，酸素化が不良な場合はPAH病態の関与を疑う。

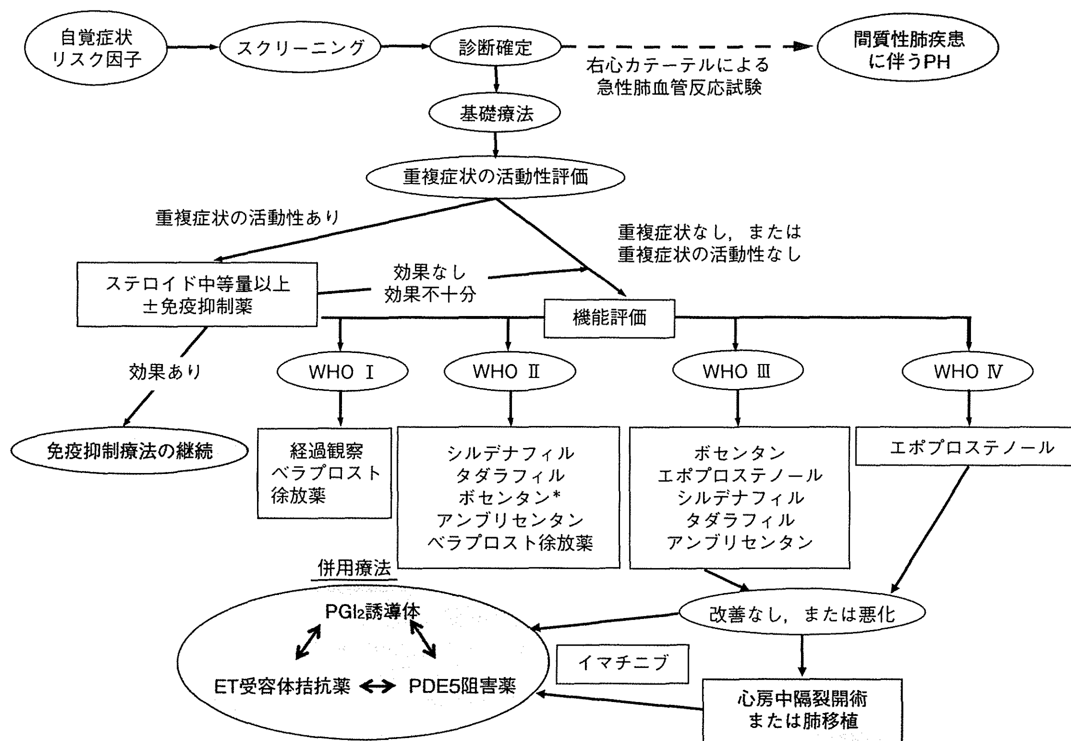

図2．SScに伴うPHの診療アルゴリズム（全身性強皮症診療ガイドライン2010）[16]
*2010年9月現在保険適応なし
PGI2：プロスタサイクリン，ET：エンドセリン，PDE5：ホスホジエステラーゼ5

Ⅳ. 治　療

最近，厚生労働省強皮症研究班でPHの診療アルゴリズムが作成された(図2)[16]。作成にあたってはエビデンスを重視し，それが十分でない場合は特発性PAHのエビデンスやエキスパートの意見を取り入れた。左心疾患，ILD，慢性肺血栓塞栓症によるPHに対しては，原因となる病態に対する治療が優先される。PAHであれば重症度，併存病態の有無（心筋障害，ILDなど），他の膠原病の重複の有無およびその活動性を勘案して治療方針を決定する。

SLEやMCTDに関連したPAHでは免疫抑制療法が効果を示す症例があるが，SSc単独症例では免疫抑制療法の効果を期待できない。ただし，SSc重複症候群でSLE，筋炎の疾患活動性を伴う場合や高度の免疫異常を伴うシェーグレン症候群合併例では，ステロイド大量療法にシクロホスファミドなど免疫抑制薬を併用する。しかし，免疫抑制療法のみでPAHの寛解維持は難しく，早期からPAH治療薬を併用すべきである。

現時点でPAH治療薬としてプロスタサイクリン誘導体（エポプロステノール，ベラプロスト），エンドセリン受容体拮抗薬（ボセンタン，アンブリセンタン），ホスホジエステラーゼ5阻害薬（シルデナフィル，タダラフィル）の3系統6剤が承認されている。特発性PAH，膠原病関連PAHを含めたPAH患者を対象とした臨床試験で，これら薬剤は3ヵ月までの6分間歩行距離，機能分類，血行動態をプラセボ群に比べて有意に改善し，症状悪化までの期間を延長する効果が示されている。ただし，SSc関連PAHに限定した少数例の解析では，エポプロステノール以外の治療薬で有効性に関する統計学的な有意差は得られていない。ただし，過去対照群と比較して生命予後を改善する効果が多くの前向きオープン試験で示されている[17]。

アルゴリズムではWHO機能分類Ⅲ度に対してベラプロストを除く5剤いずれも選択できるが，Ⅳ度にはエポプロステノールの経静脈的投与を優先的に用いる。Ⅲ度であっても右心不全徴候を有する例や肺動脈圧の高い例ではエポプロステノールの導入を考慮する。ただし，顕性のILDを併存する場合はエポプロステノールの使用は控える。ベラプロスト徐放薬は単剤でのエ

ビデンスに乏しいが，作用機序から他剤との併用で相乗効果が得られる可能性がある。本アルゴリズムはエビデンスに基づいていることから，単剤治療の導入後に定期的な治療効果の評価を実施し，不十分であれば異なる系統の薬剤を併用する段階的併用療法が推奨されている。しかし，表に示すように，PAH治療薬を使用してもSSc関連PAHの長期的予後はなお不良である。そのため，最近は早期から2剤以上をほぼ同時に開始する積極的な併用療法が行われるようになりつつある。

おわりに

PAH治療薬の導入にもかかわらず，SSc関連PAHが難治性病態である状況は解消されていない。さらなる予後改善のためには，SSc関連PAHの特殊性を理解し，早期発見と積極的併用療法を中心とした治療の最適化が必要である。

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ORIGINAL ARTICLE
Pediatric Cardiology and Adult Congenital Heart Disease

Genetic Analysis of Essential Cardiac Transcription Factors in 256 Patients With Non-Syndromic Congenital Heart Defects

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Background: The genetic basis of most congenital heart defects (CHDs), especially non-syndromic and non-familial conditions, remains largely unknown.

Methods and Results: DNA samples were collected from immortalized cell lines and original genomes of 256 non-syndromic, non-familial patients with cardiac outflow tract (OFT) defects. Genes encoding NKX2.5, GATA4, GATA6, MEF2C, and ISL1, essential for heart development, were analyzed using PCR-based bidirectional sequencing. The transcriptional activity of proteins with identified sequence variations was analyzed using a luciferase assay. A novel sequence variant (A103V in *MEF2C*) was identified, in addition to 4 unreported non-synonymous sequence variants in 3 known causative genes (A6V in *NKX2.5*, T330R and S339R in *GATA4*, and E142K in *GATA6*) in 5 individuals. None of these was found in 500 controls without CHDs. In vitro functional assay showed that all proteins with identified sequence variations exhibited significant changes in transcriptional activity and/or synergistic activity with other transcription factors. Furthermore, overexpression of the A103V MEF2C variant in a fish system disturbed early cardiac development.

Conclusions: New mutations in the transcription factors NKX2.5, GATA4, GATA6, and MEF2C that affect their protein function were identified in 2.3% (6/256) of patients with OFT defects. Our results provide the first demonstration of *MEF2C* mutation and suggest that disturbances in the regulatory circuits involving these cardiac transcription factors may cause a subset of non-syndromic and non-familial CHDs. (*Circ J* 2012; **76:** 1703–1711)

Key Words: Congenital heart defects; Genetics; Genotype; Pediatrics; Screening

Congenital heart defects (CHDs) account for the majority of human birth defects, with an incidence of 4–10 per 1,000 live births, and are the leading non-infectious cause of mortality in newborns.[1] Despite their clinical importance, little is known about the genetic basis of CHDs.

Results from recent studies in animal models suggest that changes in the functioning of transcription factors essential for cardiac progenitor lineages (eg, NKX2.5, MEF2C, ISL1, T-box, and GATA families) may result in various CHDs.[2–11] These transcription factors exhibit overlapping expression patterns, to some degree, and intricate cross-talk during heart development, suggesting that they constitute a core regulatory network for normal cardiac morphogenesis, and making them good candidates as the genetic cause of variable CHDs.[12–15]

Based on knowledge gained from linkage analyses and animal models, causative genes have been identified for several syndromes and/or familial conditions. For example, Basson et al identified mutations in *TBX5*, as autosomal dominant traits, that are frequently associated with atrial septal defects (ASD), ventricular septal defects (VSD), and upper limb defects in Holt-Oram syndrome.[16] Others have demonstrated that *TBX1* is a major genetic determinant of CHDs associated with 22q11.2 deletion syndrome.[17] In non-syndromic conditions, using linkage analysis of familial cases, Scott et al have iden-

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tified mutations in *NKX2.5* in four families with ASD and atrioventricular conduction disturbance,[18] whereas Garg et al have identified mutations in *GATA4* in 2 families with ASD and VSD.[19] However, the genetic basis of most types of CHDs is yet to be determined because the overwhelming majority of CHDs are non-syndromic with no segregation in Mendelian ratios.

Recently, we identified 2 mutations in *GATA6* by screening DNA from 21 non-syndromic patients with persistent truncus arteriosus (PTA), which is the most severe outflow tract (OFT) defect.[20] We also demonstrated a molecular basis for the pathogenesis of PTA, whereby mutations in *GATA6* resulted in a disturbance of direct regulation of semaphorin 3C (SEMA3C) and plexin A2 (PLXNA2), which mediate essential neurovascular signaling in the development of the OFT.[20] To further explore the genetic causes of non-syndromic and non-familial CHDs, we undertook a genetic analysis of selected essential cardiac transcription factors, focusing on lesions involving the OFT. OFT defects constitute approximately 30% of CHDs[21] and are often difficult to repair surgically because of complex morphological abnormalities, resulting in an unfavorable prognosis. In the present study, we report our findings in 256 non-syndromic patients with OFT defects.

Methods

A detailed description of the methodology used is available as online supplemental information for this paper (**Data S1**).

Mutation Analysis and Clinical Evaluation of Patients

The establishment of a genomic bank with different cell lines and the extraction of genomic DNA samples have been described previously.[22] All exons and flanking introns of *NKX2.5*, *GATA4*, *GATA6*, *MEF2C*, and *ISL1* were amplified by polymerase chain reaction (PCR) and were sequenced using direct, bidirectional sequencing (**Data S1**). The mutations identified were subsequently confirmed using original genomic DNA extracted from the patients' peripheral blood leukocytes. Primer sequences are given in **Table S2**. In all, 256 samples were collected from patients with CHDs involving OFT defects. Phenotypic data for the affected individuals and their family members were obtained from detailed clinical evaluations based on echocardiography, cardiac catheterization, and/or surgical findings. Genomic samples from available family members, 206 healthy Japanese volunteers with no CHDs (controls), and 294 samples of control genomic DNA (BioChain, Hayward, CA, USA) were analyzed using the same protocol. This investigation conformed with the principles outlined in the Declaration of Helsinki, and the clinical evaluations and genetic studies of the patients and their families were approved by the Internal Ethics Committee of Tokyo Women's Medical University and were undertaken only after the patients and their family members had provided informed consent.

Plasmid Construction and Site-Directed Mutagenesis

Site-directed mutagenesis was performed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. *GATA6*-pcDNA3.1,[20] *NKX2.5*-pcDNA3.1, *GATA4*-pcDNA3.1 or *Mef2c*-pcDNA were used for mutagenesis. *Mef2c*-pcDNA was kindly provided by Dr Brian Black (Cardiovascular Research Institute, University of California, San Francisco, CA, USA). Mouse Mef2c wild-type (WT) and A103V mutant cDNA were subcloned into a pCS2+ plasmid.[23] All vectors constructed were verified by sequencing.

Luciferase Assay

HeLa cells were transfected using Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA, USA) with 400 ng reporter vector, 800 ng expression vectors, and 0.25 ng pSV-*Rluc* internal control vector, as described previously.[20] Luciferase activity was measured 36 h after transient transfection by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Experiments were repeated at least 3 times.

Co-Immunoprecipitation Assays

COS-1 cells were transiently transfected with lipofectamine LTX (Invitrogen), collected after 48 h in lysis buffer (Tris-buffered saline (TBS), 0.4% Nonidet P-40 (NP-40), and EDTA-free complete protease inhibitor cocktail; Roche Diagnostics, Basel, Switzerland), incubated with monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) and Dynabeads protein G beads (Invitrogen), immunoblotted with monoclonal anti-GATA6 (R&D systems, Minneapolis, MN, USA) or monoclonal anti-FLAG M2 antibodies, and detected by western blotting.

Overexpression of Mef2c mRNA in Medaka Fish

The plasmid mouse *Mef2c* WT or A103V mutant-pCS2+ was digested with Not I, and RNA samples of the *Mef2c* with polyA were prepared using the mMessage mMachine Kit (Invitrogen) for injections. Medaka embryos were fertilized in vitro and injected at the 1-cell stage (215 eggs) as previously described.[24] Pressure was adjusted to inject approximately 1 nl at a concentration of 0.2 $\mu g/\mu l$. Injected embryos were transfered into heat type of container where they were held at 28.5 degrees celsius. Embryos were observed, scored daily, and investigated for cardiac phenotypes at 5 days post-fertilization.

Statistical Analysis

For luciferase assays, all experiments were performed at least in triplicate and data are reported as normalized relative light units (fold activation) together with the SEM. For promoter activity assays, all experiments were performed at least in triplicate and data are reported as the ratio of normalized relative light units for coexpression with NKX2.5, GATA4, GATA6, or Mef2c to that with mock (pcDNA3.1). In all **Figures**, error bars indicate the SEM. Data were analyzed by 2-tailed unpaired t-test or chi-square test. $P \le 0.05$ was considered significant. For non-synonymous GATA4 and GATA6 nucleotide changes found in patients and controls, frequencies were compared between patients and controls by chi-square test. $P \le 0.05$ was considered significant.

Results

Novel Sequence Variants of Genes Encoding Cardiac Transcription Factors Identified in Patients With Non-Syndromic CHDs

In our genetic analysis of selected essential cardiac transcription factors (*NKX2.5*, *GATA4*, *GATA6*, *MEF2C*, and *ISL1*) in 256 patients with non-syndromic OFT defects, including 125 with tetralogy of Fallot (TOF), 84 with pulmonary atresia with VSD, 23 with double outlet the right ventricle, and 24 with PTA, we identified 5 new sequence variants in 6 individuals (6/256; 2.3%). These variants included an *NKX2.5* variant (A6V), 2 *GATA4* variants (T330R and S339R), a *GATA6* variant (E142K), and a *MEF2C* variant (A103V; **Figure 1**) that were not found in the 500 normal controls without CHDs, suggesting that these variants are potentially involved in the etiol-

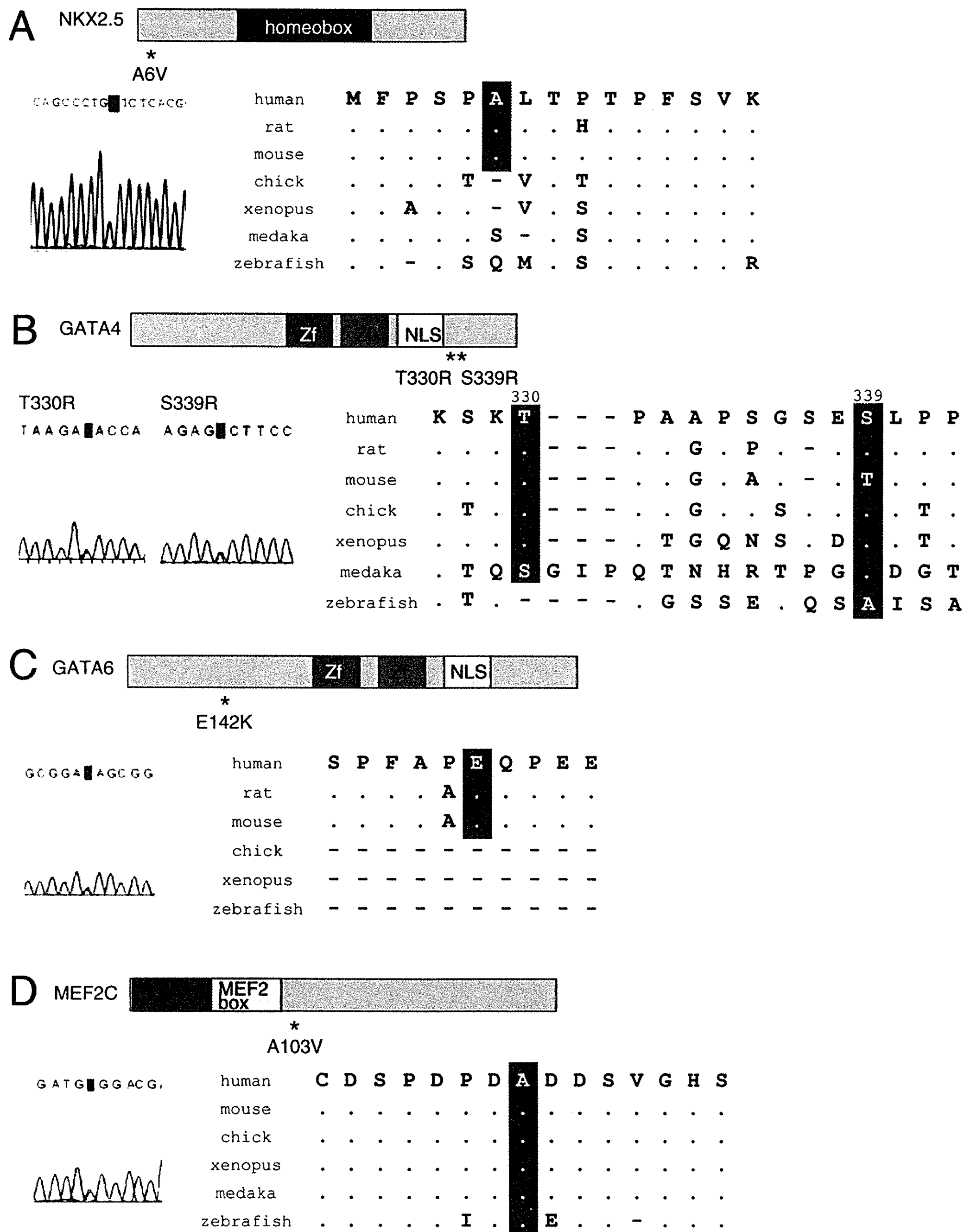

Figure 1. Identification of novel sequence variants in cardiac transcription factors in patients with outflow tract (OFT) defects. The structures of the human NKX2.5 (**A**), GATA4 (**B**), GATA6 (**C**) and MEF2C (**D**) genes, the positions of the novel variants (*), and the conservation of amino acids between species are shown. Zf, zinc finger; NLS, nuclear localization signal. Changes in amino acids are highlighted in red.

ogy of OFT defects. The results of the genetic analyses and the associated phenotypes are summarized in **Table 1**. As indicated in **Table 1**, the *GATA6* variant E142K was found in 2 unrelated patients with OFT defects whose phenotypes were somewhat variable. We also identified non-synonymous variations in the coding regions of *GATA4* and *GATA6* in both patients and controls (**Table 2**), as well as numerous known sequence polymorphisms in all genes, although none of these non-synonymous sequence variants showed significant difference in the frequency between patients and controls. We did not find any new sequence variants of *ISL1* in our patient group.

Table 1. Unique Non-Synonymous Sequence Variants Identified in Patients With Congenital Heart Defects

Gene	GenBank accession number	Patient no.	Nucleotide change	Amino acid change	Transcriptional activation	Cardiac phenotype	Extracardiac anomalies	Race	Sex
NKX2.5	NM_001166175	1	17C>Y	A6V	Loss of function	TOF	None	Japanese	Male
GATA4	NM_002052	2	989C>S	T330R	Loss of function	PTA	None	Japanese	Female
		3	1017C>S	S339R	Loss of function	PAVSD	None	Japanese	Female
GATA6	NM_005257	4	424G>R	E142K	Loss of function	PAVSD, MAPCA	None	Japanese	Female
		5	424G>R	E142K	Loss of function	DORV, VSD, PS, dextrocardia	None	Japanese	Male
MEF2C	NM_002397	6	308C>Y	A103V	Gain of function	PAVSD	None	Japanese	Female

TOF, tetralogy of Fallot; PTA, persistent truncus arteriosus; PAVSD, pulmonary atresia with ventricular septal defect (VSD); MAPCA, major aorticopulmonary collateral arteries; DORV, double outlet right ventricle; PS, pulmonary stenosis.

Table 2. Non-Synonymous GATA4 and GATA6 Nucleotide Changes Found in Patients and Controls

Gene	Nucleotide change	Amino acid change	No. of alleles with nucleotide changes		Significance*	Reference SNP
			Patients	Controls		
GATA4	1128C>S	H376Q	2/256	1/500	NS	rs116414842
	1138G>R	V380M	5/256	6/500	NS	rs114868912
	1220C>M	P407E	8/256	18/500	NS	rs115099192
GATA6	43G>S	G15R	38/256	30/500	NS	Unreported
	151G>R	E51K	1/256	1/519	NS	Unreported
	551G>R	S184N	3/256	4/500	NS	Unreported
	584G>R	G195A	1/256	1/500	NS	Unreported

NS, not significant.
*Statistical significance in frequency between patients and controls.

Functional Analyses of Cardiac Transcription Factors With the Identified Sequence Variations

To examine the clinical relevance of the identified sequence variants to non-syndromic OFT defects, luciferase assays were performed to evaluate the transcriptional activity of each factor exhibiting a sequence variation. First, we analyzed basal transcription activities using the common cardiac-specific promoter *NPPA*. The NKX2.5 variant A6V exhibited a 50% decrease in transcription activity compared with the WT protein (**Figure 2A**; P=0.0066, 2-tailed unpaired t-test; n=3). In addition, this variant exhibited a significant decrease in synergistic effects when it was cotransfected with *GATA4* compared with WT *NKX2.5* cotransfected with *GATA4* (**Figure 2A**; P=0.011, 2-tailed unpaired t-test; n=3).

The 2 GATA4 variants T330R and S339R exhibited significant decreases in synergistic effects on the *NPPA* promoter compared with WT GATA4 when they were cotransfected with *NKX2.5* (**Figure 2B**; P=0.015 and P=0.026, respectively 2-tailed unpaired t-test; n=3), although, alone, neither variant exhibited any significant decrease in transcription activity on the *NPPA* promoter. The GATA4 variant S339R also exhibited a significant decrease in its synergistic effect with GATA6 (**Figure 2B**; P=0.044, 2-tailed unpaired t-test; n=3), whereas the GATA4 variant T330R did not.

The GATA6 variant E142K exhibited significantly decreased transcriptional activity on the *NPPA* promoter (**Figure 2C**; P=0.0045, 2-tailed unpaired t-test; n=3). Furthermore, the GATA6 variant E142K exhibited significantly decreased synergistic activity with GATA4 on the *NPPA* promoter compared with WT GATA6 (**Figure 2C**; P=0.0010, 2-tailed unpaired t-test; n=3). In addition to synergistic effects between GATA6 and GATA4 on the *NPPA* promoter, we also found synergistic effects for GATA6 with TBX5 on the *NPPA* promoter (Figure 2C; P=0.034 for GATA6 WT with TBX5 v. GATA6 alone; P=0.031 for TBX5 alone, 2-tailed unpaired t-test; n=3). Furthermore, results from the co-immunoprecipitation assay revealed that the GATA6 protein was able to physically associate with the TBX5 protein (**Figure 2D**; WT), suggesting that the strong synergistic effect on the expression of *NPPA* was due to a direct interaction between GATA6 and TBX5. The GATA6 variant E142K exhibited significantly decreased synergistic activity with TBX5 on the *NPPA* promoter compared with WT GATA6 (**Figure 2C**; P=0.00092, 2-tailed unpaired t-test; n=3), although the interaction between the GATA6 variant E142K and TBX5 was unaffected (**Figure 2D**; E142K). Moreover, compared with WT GATA6, the GATA6 variant E142K exhibited significantly decreased transcription activity on the *SEMA3C* promoter (**Figure 2E**; P=0.029, 2-tailed unpaired t-test; n=3) and the *WNT2* promoter (**Figure 2F**; P=0.040, 2-tailed unpaired t-test; n=3), both of which are downstream targets of GATA6.

Although no disease-associated mutation in *MEF2C* has been reported to date, we identified a non-synonymous *MEF2C* sequence variant (A103V) in the present study. A recent animal study reported that Mef2c directly regulates *Smydl*, which is essential for early cardiomyocyte differentiation during development of the OFT.[25] To investigate whether the *MEF2C* variant A103V results in a disruption of *Smydl* regulation, we used a *Smydl* promoter that included an Mef2c-dependent consensus sequence[25] (**Figure 2G**) in our luciferase assay. The results suggest that the MEF2C variant A103V has a significant gain-of-function activity on the *Smydl* promoter compared with WT MEF2C (**Figure 2H**; P=0.040, 2-tailed unpaired t-test; n=3). The results of all functional analyses are summarized in **Table 1**.

Abnormal Heart Development in Medaka Fish With Overexpression of Mef2c

MEF2C is a highly conserved protein across species (**Figure 3A**)

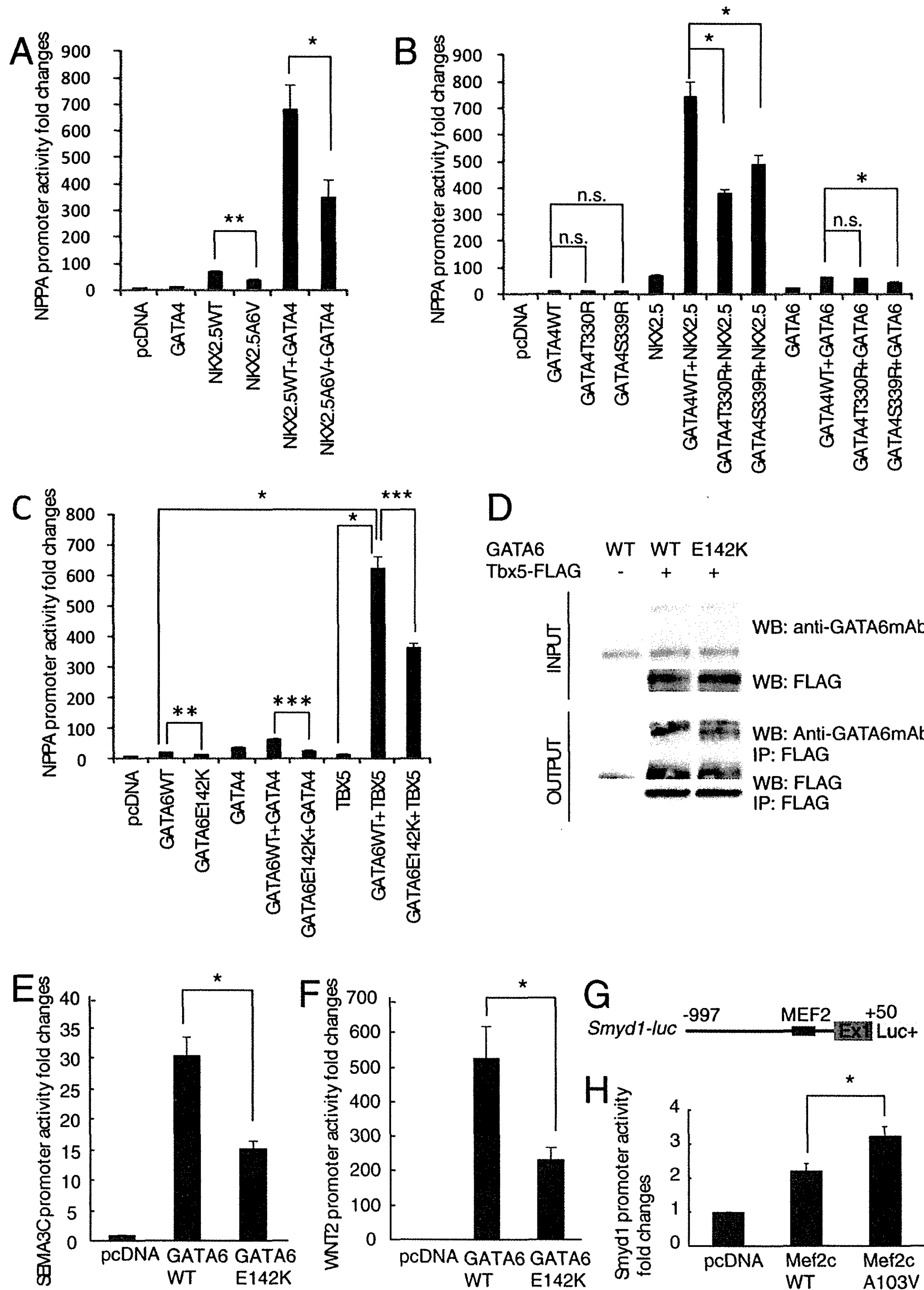

Figure 2. Transcriptional activities of proteins with identified sequence variants. (**A**) Relative luciferase activity in HeLa cells transfected with NKX2.5 wild-type (WT) or NKX2.5 variant A6V expression constructs and *NPPA-luc* with or without coexpression of GATA4 WT. (**B**) Relative luciferase activity in HeLa cells transfected with GATA4 WT or the GATA4 variants T330R or S339R expression constructs and *NPPA-luc* with or without coexpression of NKX2.5 WT or GATA6 WT. (**C**) Relative luciferase activity in HeLa cells transfected with GATA6 WT or GATA6 mutant (E142K) expression constructs and *NPPA-luc* with or without coexpression of GATA4 WT or TBX5. (**D**) Interaction between TBX5 and GATA6 WT or mutant protein (E142K). Co-immunoprecipitation of GATA6 demonstrates an association of both WT and E142K with FLAG-tagged TBX5. (**E,F**) Relative luciferase activity in HeLa cells transfected with GATA6 WT or GATA6 mutant (E142K) expression constructs and *SEMA3C-luc* (**E**) or *WNT2-luc* (**F**). (**G**) Structure of *Smyd1-luc* with a consensus MEF2 binding site. Genomic organization of a 1-kb section of the 5′ end of the mouse *Smyd1* locus subcloned upstream of *luc*+ reporter gene. (**H**) Relative luciferase activity in HeLa cells transfected with Mef2c WT or Mef2c mutant (A103V) expression constructs and *Smyd1-luc*. *P<0.05, **P<0.01, ***P<0.005.

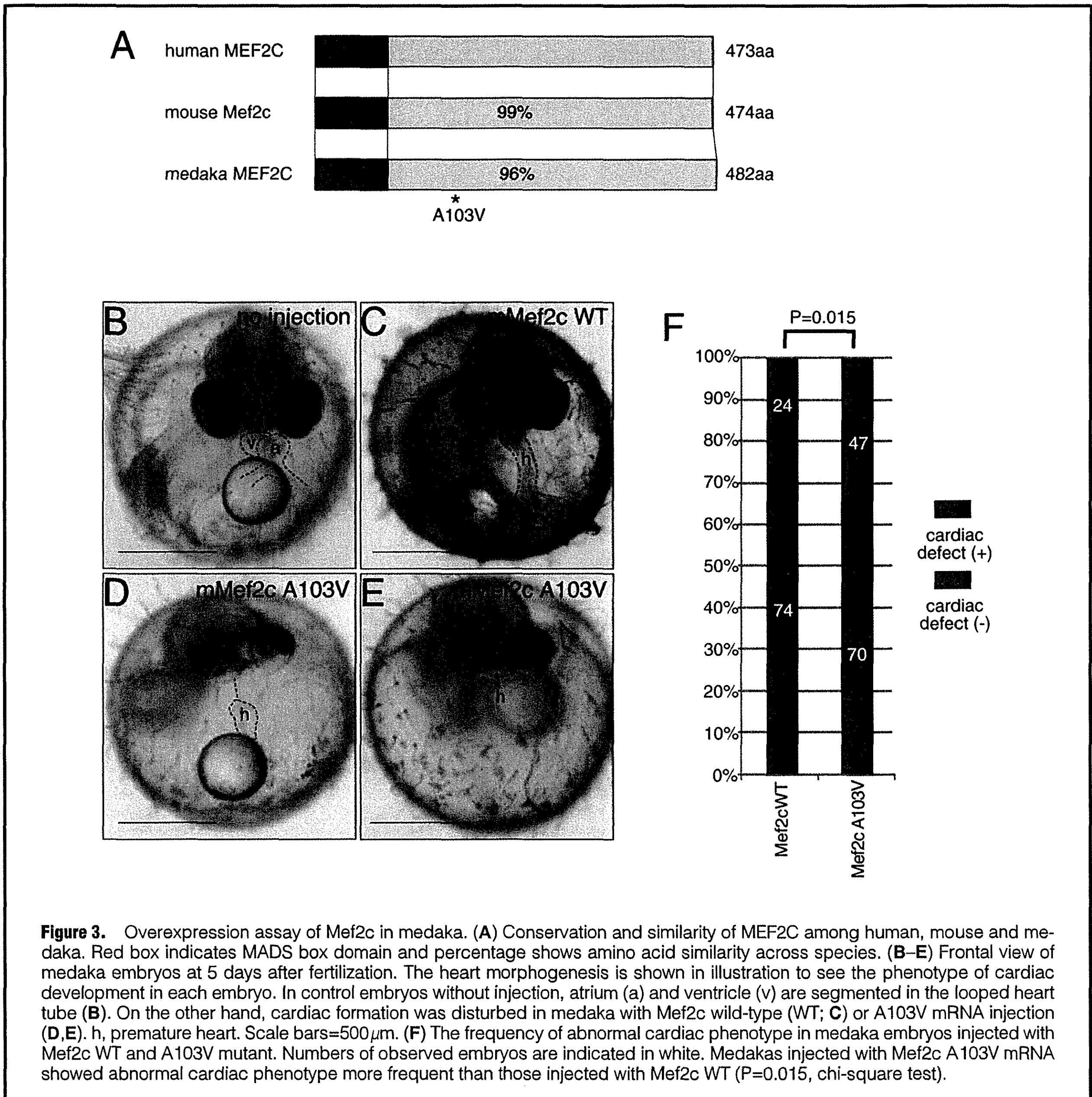

Figure 3. Overexpression assay of Mef2c in medaka. (**A**) Conservation and similarity of MEF2C among human, mouse and medaka. Red box indicates MADS box domain and percentage shows amino acid similarity across species. (**B–E**) Frontal view of medaka embryos at 5 days after fertilization. The heart morphogenesis is shown in illustration to see the phenotype of cardiac development in each embryo. In control embryos without injection, atrium (a) and ventricle (v) are segmented in the looped heart tube (**B**). On the other hand, cardiac formation was disturbed in medaka with Mef2c wild-type (WT; **C**) or A103V mRNA injection (**D**,**E**). h, premature heart. Scale bars=500μm. (**F**) The frequency of abnormal cardiac phenotype in medaka embryos injected with Mef2c WT and A103V mutant. Numbers of observed embryos are indicated in white. Medakas injected with Mef2c A103V mRNA showed abnormal cardiac phenotype more frequent than those injected with Mef2c WT (P=0.015, chi-square test).

and alanine 103 is well conserved (**Figure 1D**). In order to assess the gain-of-function of *MEF2C* A103V variant protein during heart development in vivo, we used a fish system. Overexpression of Mef2c WT or A103V protein in medaka by mRNA injections showed a range of abnormal cardiac development (**Figures 3B–E; Movies S1,S2**). Approximately 25% of embryos injected with WT Mef2c and 40% of embryos injected with A103V variant Mef2c developed cardiac defects ranging from disorganized cardiac chamber formation in the straight heart tube to ectopic beating tissues with no formation of the heart tube, suggesting that overexpression of Mef2c may disturb early cardiac development. Consistent with A103V variant having a gain-of-function effect on the transcriptional activity of Mef2c, abnormal cardiac development was observed more frequently in transgenic medaka embryos injected with the variant Mef2c than those with the WT (**Figure 3F**; P=0.015, chi-square test).

Discussion

Herein we report on the identification and characterization of multiple sequence variants of the transcription factors, that are essential for cardiac development, in non-syndromic patients with cardiac OFT defects. Each variant exhibited changes, to varying degrees, in the transcriptional activity of its protein products on downstream target genes involved in heart development. The results suggest that the sequence variations identified in *NKX2.5*, *GATA4*, *GATA6*, and *MEF2C* in the present study are likely to be involved in some aspect of the etiology of OFT defects, accounting for approximately 2.3% of cases of non-syndromic OFT defects (6 cases of 256 patients in the present series). These findings suggest that a subset of CHDs

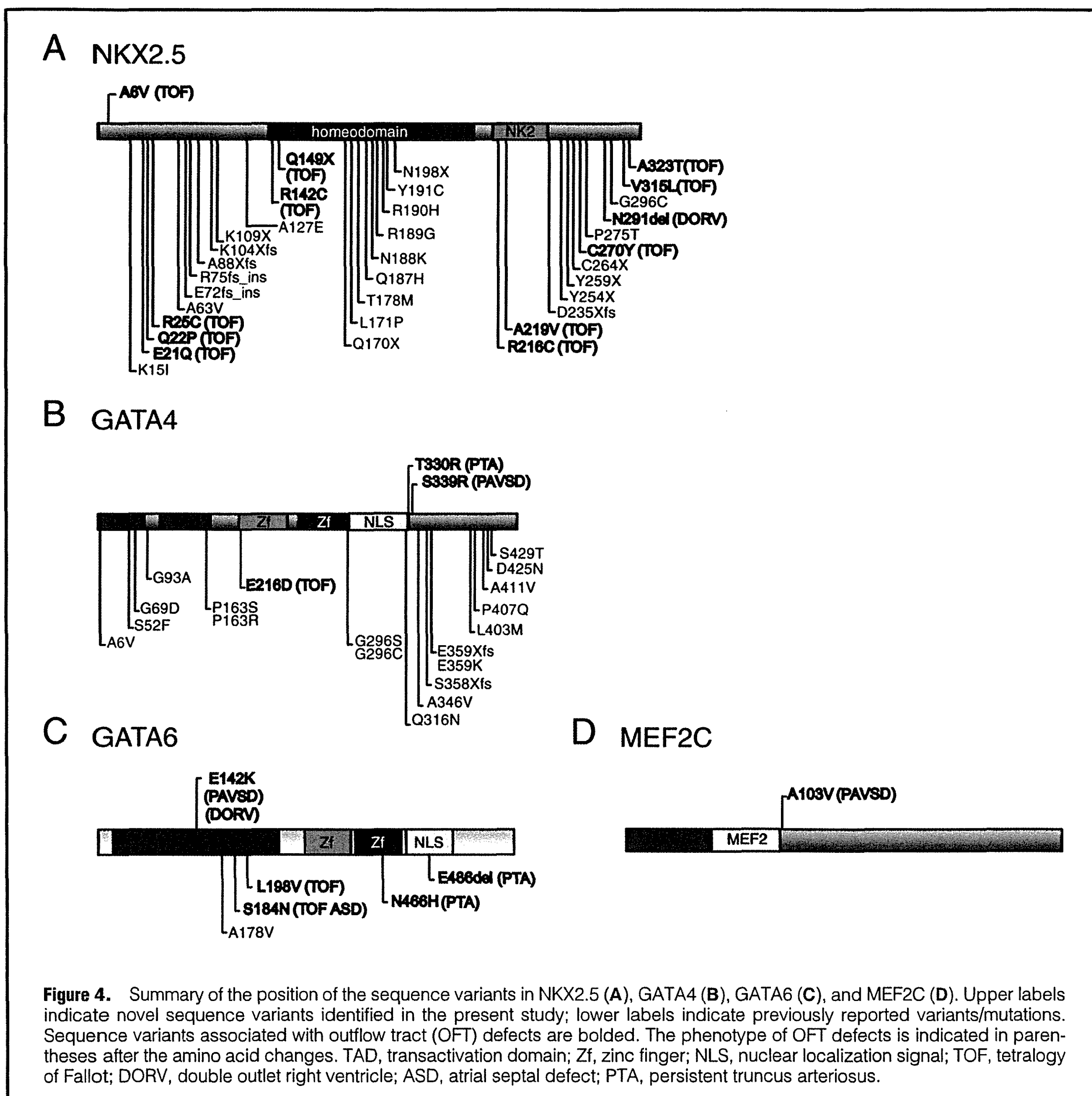

Figure 4. Summary of the position of the sequence variants in NKX2.5 (**A**), GATA4 (**B**), GATA6 (**C**), and MEF2C (**D**). Upper labels indicate novel sequence variants identified in the present study; lower labels indicate previously reported variants/mutations. Sequence variants associated with outflow tract (OFT) defects are bolded. The phenotype of OFT defects is indicated in parentheses after the amino acid changes. TAD, transactivation domain; Zf, zinc finger; NLS, nuclear localization signal; TOF, tetralogy of Fallot; DORV, double outlet right ventricle; ASD, atrial septal defect; PTA, persistent truncus arteriosus.

may be caused by disruption of cardiac transcription factor regulation. The approach we used in the present study may prove useful in identifying new genetic causes and exploring unknown molecular mechanisms underlying non-syndromic and non-familial CHDs, despite the known difficulties in determining the genetic basis of most such cases of CHDs.

A previous study has indicated that mutations in the homeodomain of *NKX2.5* are likely to cause ASD, whereas mutations located outside the homeodomain may be associated with OFT defects.[26] Benson et al first identified mutations in the N-terminal region of *NKX2.5* in patients with TOF, and this region may be a hot spot for mutations responsible for TOF[27] (**Figure 4A**). Consistent with this notion, in the present study we identified the novel sequence variant A6V in the N-terminal region of *NKX2.5* in a patient with TOF. It is known that the N-terminal region is required for NKX2.5 activity essential for cardiomyogenesis.[28] The NKX2.5 variant A6V exhibited significantly decreased transcriptional activity, which likely resulted in TOF in our patient.

Mutations in *GATA4* are known to cause intracardiac septal defects, probably as a result of disruptions to interactions with TBX5.[19] Although only a few reports have demonstrated an association between GATA4 mutations and OFT defects,[29] in the present study we identified 2 novel GATA4 sequence variants in patients with OFT defects (**Figure 4B**). Both *GATA4* variants (T330R and S339R) were located in a common basic region of the GATA transcriptional factors and both variants resulted in a disruption of the synergistic activity between GATA4 and NKX2.5 or GATA6, although the correlation between the genotype and phenotype was unclear. It is of note that each variant alone did not exhibit any significant decrease in transcription activity. It has been reported that reciprocal

regulation between GATA4 and NKX2.5, as well as a synergistic activity between them, is essential for several cardiac-specific genes.[30–34] Other studies have indicated that GATA4 plays a role in cardiovascular development in collaboration with GATA6.[9,35,36] Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation, resulting in acardia in mice.[37] GATA4 and GATA6 regulate MEF2C by binding directly to its enhancer and via calcium-dependent pathways;[38,39] in turn, MEF2C plays a role in the differentiation of the OFT myocardium and vascular smooth muscle cells from their progenitor pool (ie, the second heart field).[10] These observations suggest that GATA4 and GATA6 may function redundantly as well as cooperatively in multiple steps of cardiovascular development, ranging from the differentiation of cardiac progenitor cells to the development of the OFT. On the basis of the results obtained in the present study, we believe that a decrease in the synergistic activity of GATA4 with NKX2.5 or GATA6 may result in some type of OFT defect.

The novel GATA6 variant E142K exhibited significantly decreased transcriptional activity, as well as decreased synergistic activity with other cardiac transcription factors, making this variant a strong candidate for a subset of OFT defects. Since our initial report of the first mutations in *GATA6* associated with OFT defects,[20] a further 3 mutations have been reported, with 2 of them associated with OFT defects[40,41] (**Figure 4C**). These findings indicate that mutations in *GATA6* may be mainly associated with OFT defects, probably as a result of the dysregulation of the SEMA3C–PLXNA2 pathway.[20]

The present study revealed that GATA6 binds directly to TBX5, which is the first demonstration of a direct physical interaction between GATA6 and TBX5 leading to synergistic activity. The GATA6 variant E142K exhibited decreased synergistic activity with TBX5; however, its direct interaction with TBX5 was not altered. These results suggest that TBX5 binds to GATA6 normally via the zinc finger domain, like other cofactors, but the sequence variation E142K in the N-terminal domain of *GATA6* changes transactivation on the target gene independent of binding to TBX5.

The present study also reports the first sequence variation in *MEF2C* associated with CHDs (**Figure 4D**). MEF2C is essential for the development of the right ventricle and the OFT interacting with NKX2.5.[42] Loss of *Mef2c* in mice results in hypoplasia of the right ventricle and the OFT.[10,11] Furthermore, Mef2c is thought to have an essential role in ventricular cardiomyocyte differentiation,[43] and, recently it was reported that a transduction of Mef2c together with Gata4 and Tbx5 rapidly and efficiently reprogrammed postnatal fibroblasts directly into differentiated cardiomyocyte-like cells.[44] Intriguingly, Mef2c overexpression assay using fish in this current study showed defects of cardiac development, including disorganized cardiac chamber formation and ectopic cardiomyocyte differentiation. These findings indicate that the level of transcriptional activity of Mef2c should be critically controlled for normal cardiac development. In our study, the A103V variant of Mef2c identified in a patient with OFT defect showed gain-of-function in transcriptional activity on the promoter of its downstream target, *Smyd1*, which is essential for early cardiomyocyte differentiation of development of the right ventricle and OFT.[25] Consistently, abnormal cardiac development was observed more frequently in transgenic fish embryos overexpressing the A103V variant of Mef2c than in those with the WT Mef2c. Taken together, our findings suggest that the gene mutation causing the A103V variation of MEF2C is likely to be implicated in the OFT defect as a result of altered transcriptional activity of MEF2C.

Of note, we did not identify any sequence variants in *ISL1*. During development, *Isl1* is expressed in the splanchnic mesoderm comprising the second heart field, which gives rise to the OFT, right ventricle, and most parts of the atria, but is downregulated in the heart proper.[45] In contrast, *Nkx2.5*, *Gata4*, *Gata6*, and *Mef2c* are expressed continuously in the heart during development.[12–14] One could speculate that this may be why no CHD-associated sequence variant was found in *ISL1* in the present study, although this finding does not rule out the possibility that *ISL1* is responsible for some type of OFT defect in humans. Clearly, in the present study we did not attempt to incorporate the effects of all factors relevant to heart development. Further genetic screening and functional assays for a comprehensive range of transcription factors essential for heart development in patients with all types of CHDs would reveal more precise genotype–phenotype correlations, and eventually provide new insights into the molecular mechanisms underlying CHDs.

Conclusions

Genetic and functional analyses of essential cardiac transcription factors in 256 patients with non-syndromic and non-familial CHDs that could not be evaluated using conventional chromosome and/or linkage analysis identified new gene mutations of *NKX2.5*, *GATA4*, *GATA6*, and *MEF2C* in 6 patients. These mutations may account for approximately 2.3% of OFT defects.

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Supplementary Files

Supplementary File 1

Data S1. Materials and Methods

Table S1. Human MEF2C Primers for PCR and Sequence Reactions

Table S2. Human ISL1 Primers for PCR and Sequence Reactions

Supplementary File 2

Movie S1. Abnormal cardiac phenotypes of medaka injected with Mef2c A103V mRNA at 5 days after fertilization. Beating premature heart tube was seen in the embryo and blood flow was severely disturbed.

Supplementary File 3

Movie S2. Only beating tissue was seen in ectopic position and no blood flow was detected.

Please find supplementary file(s);
http://dx.doi.org/10.1253/circj.CJ-11-1389

CHAPTER FOUR

Factors Involved in Signal Transduction During Vertebrate Myogenesis

Yohtaroh Takagaki,*,† Hiroyuki Yamagishi,‡ and Rumiko Matsuoka*,§,}

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Abstract

Muscle is a contractile tissue of animals, dedicated to produce force and cause motion. In higher animals, there are two types of muscle tissue: (a) striated muscle, including all voluntary skeletal muscles and involuntary cardiac muscle, and (b) smooth muscle consisting of involuntary muscles, including those of the viscera, blood vessels, and uterus. Although muscle growth and regeneration take place throughout vertebrate life, the heart is the first organ to start functioning, with continued development until delivery. Skeletal muscles, on the other hand, develop in four successive, temporally distinct phases of embryonic, fetal, neonatal, and adult muscle with the postnatal phase being basically hypertrophy. Unlike terminally differentiated skeletal and cardiac muscles in adults, smooth muscle cells retain their plasticity and the phenotype can change reversibly in response to environmental changes. For the past 20 years, the availability of gene recombination technology directed the focus of studies on transcription factors and signaling molecules, and we would like to review what has been explored by recent studies on myogenesis.

Key Words: Myogenic regulatory factor, Serum response factor, CArG box, Skeletal muscle,Cardiacmuscle,Smoothmuscle,Head muscle. 2012ElsevierInc.

1. Introduction

Muscle is a contractile tissue of animals, dedicated to produce force and cause motion by converting chemical energy into mechanical energy. In higher vertebrates, there are two kinds of muscle tissue: striated or striped muscle and smooth or plain muscle. Skeletal muscle and cardiac muscle have rod-like contracting structures arranged in units called sarcomeres aligned to give a striated appearance. A sarcomere consists of a bipolar myosin (thick) filament and two actin (thin) filaments, in which

the thin filaments are pulled by the thick filament such that the sarcomere shortens and generates force during contraction (Huxley, 1969). In smooth muscle (SM), myosin II and actin filaments form less organized contractile bundles without a striated appearance.

Myogenesis is the formation of muscle tissue. In the last century, grafting experiments were instrumental for prospective cell lineage analysis using chicken embryos. Recombinant technology in recent years has provided

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different tools with respect to the understanding of factors governing the development of each muscle type. Myogenic regulatory factors (MRFs) in skeletal muscle(myf5, MyoD, myogenin,and MRF4) havebeen instrumental in forming the basis of a paradigm for the existence of a "master gene" specific for lineage development (Olson, 1990). This paradigm has been extensively studied in cardiac muscle (e.g., dHAND) (Olson and Srivastava, 1966) and subsequentlyin SM (e.g., serum responsefactor(SRF)and myocardin) (Wang et al., 2003). Over the past two decades, this paradigm has been put to the test byexperimental depletionofthese genes, andthese experiments have not only advancedourunderstandingofmyogenicprocesses butalsouncoveredhidden compensatory and redundant pathways (Tohsato et al., 2010). Furthermore, the technological advancement in fluorescent markers and sophisticated imaging allowed for more refined means for tracking cell lineage in developmental biology (Buckingham and Meilhac, 2011).

Two epoch-making accomplishments in the first decade of the present century are changing the scope of biological investigation. The first impact is the completion of the human genome project (IHGSC, 2004), followed by genome sequencing of many biological species. The genome sequence information of a variety of bioorganisms has enabled "Omics," which takes advantage of computer technology to try and grasp the entirety of the biological processes under investigation. "System Biology" or the integrative approach has been proposed to overcome the limitation of reductionism (Kitano, 2002). Although present analytical and computation technologies require further development to fulfill expectations, the approach is already boosting a new horizon of studies such as epigenetics and regulatory RNA. Notably, noncoding RNAs (ncRNAs), such as micro-RNAs (miRNA), short interfering RNAs, and long ncRNAs, have been revealed to be important members of posttranscriptional regulators in gene expression (Amaral and Mattick, 2008).

The second impact was introduced by Yamanaka and his colleagues, who demonstrated that the developmental fate of cells could be reversed to pluripotent stem cells by introducing a limited number of factors into

mouse and human cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). The induction of stem cells from several differentiated cell types revealed the remarkable plasticity retained in differentiated cells, and this raised the expectation for cell fate manipulation to developing "regenerative medicine" and drawing concomitant enthusiasm in the economy and politics. The social demands for the field of muscle development are high, such as transplantable organs to extend life, increasing meat production in the domestic animal industry, and boosting muscle efficiency in sports medicine. The technology for cell fate manipulation requires an understanding of the key factors for the induction of specific cell types.

There are extensive research reports and many excellent reviews available in the field of skeletal myogenesis and cardiomyogenesis. Traditionally, reviews on myogenesis covered the developmental process of skeletal muscle formation. Recently, however, the commonalities between three principal muscle types are gaining attention: mechanisms such as the involvement of SRF as the master regulator of all muscle types (Li et al., 2005; Miano et al., 2007), and the common lineage relationships between head skeletal muscles and the second heart field (SHF), an origin of cardiac muscle (Lescroart et al., 2010). By compiling all three muscle types, we hope that an integrative approach will become feasible leading to new insights and views. Due to limitations in our ability, this review covers cardiac development with a brief coverage of skeletal and SM development, mostly on murine myogenesis with some reference to that

2. Cells Involved in Myogenesis

of humans. Readers are asked to refer to other reviews for invertebrate (Hooper and Thuma, 2005) and zebrafish myogenesis (Chong et al., 2009).

2.1. Cardiomyogenesis

The vertebrate heart is an organ with four chambers, constituted by a multilayered striated myocardium with an external epicardium and an internal endocardium. This is in contrast to the invertebrate heart which usually consists of a single tubular structure composed of myoepithelium with peristaltic contraction (Pe´rez-Pomares et al., 2009). In cardiac myogenesis in vertebrates, presumptive heart cells originate in the early primitive streak, migrate to form the cardiogenic mesoderm, and develop into cardiomyocytes. Cardiomyocytes adhere to each other forming linear myofibrils with intercalated discs (IDs), which connect single cardiomyocytes to form myofibrils with an electrochemical syncytium. IDs contain gap junctions for metabolic coupling and permitting the

passage of ions between cells. Cardiac muscle cells, however, remain mononuclear with IDs responsible for force transmission generated by synchronized contraction. The resulting cardiac tube is the first organ to exhibit its specific function by beating, at day 9 postcoitus (E9) in mice and at day 22 in humans, to meet the need to deliver nutrients throughout the developing embryo. From the beating tubal heart, the shape changes to create a cardiac loop, followed by steps to create a four-chambered structure in vertebrate animals in harmony with concomitant vasculogenesis. The cardiac tube is shaped with progenitor cells from the first heart field (FHF). During chamber formation, progenitor cells from the SHF are recruited at the poles of the tube. The left ventricle is derived from the FHF while the right ventricle and outflow tract are derived from a distinct SHF, thus the original straight heart tube constitutes a part of the developed heart (Buckingham et al., 2005). Maintaining pumping function with concomitant shape changes during

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development may make the cardiovascular system relatively susceptible to a variety of mistakes. It is known that morphological anomalies of heart are the highest among congenital diseases, affecting about 1% of delivered children. Immediately after delivery, usually around 270 days after conception in humans and at about 20 days in mice, the cardiovascular system switches the course of blood circulation for an oxygen source from the placenta to the lung, which involves closure of the ductus arteriosus, ductus venosus, and foramen ovale. Cardiac muscle cells function in response to its pace makers residing within the heart without the direct involvement of the central nervous system, and therefore, are called involuntary muscle.

The mammalian heart after delivery has been regarded as a terminally differentiated organ. Recent evidence, however, indicates that it has resident stem cells with a self-renewing capacity, which could replenish cells damaged by heart failure or myocardial infarction (Beltrami et al., 2003). Bergmann and colleagues used samples of heart muscle from people born before the atmospheric radiocarbon increase due to nuclear bomb tests during the Cold War. By tracking the amount of ^{14}C fixed in cardiomyocyte DNA, the authors estimated that 20 year olds renew about 1% of heart muscle cells per year and that about 45% of the heart muscle cells of a 50-year-old were generated after birth (Bergmann et al., 2009) ,

2.2. Skeletal muscle development

All skeletal muscle is derived from progenitors originating in the somite, except for head muscles. Somite formation depends on a "clock and wave"

signal, provided by the Notch and Wnt pathways for "clock," and fibroblast growth factor (Fgf) for "wave." The progenitor cells for all trunk and limb skeletal muscles are derived from the dorsal portion of the somite, the dermomyotome, and they migrate into their destinations and proliferate to give muscle and endothelial cells (Buckingham and Vincent, 2009 ; Buckingham et al., 2003). Cells destined for skeletal muscle are specified to become myoblasts, and differentiate into mononuclear myocytes, which subsequently fuse with each other to form multinucleated myofibers or myotubes. Four distinctive stages of skeletal myogenesis are observed in mice, namely embryonic (E10.5–E12.5), fetal (E14.5–P0; P, postnatal day), neonatal (P0–P21), and adult stages (after P21) (Biressi et al., 2007a,b; Murphy and Kardon, 2011; Tajbakhsh, 2005). The postnatal increase in muscle mass is achieved by an increase in fiber diameter and an increase in nuclear number per fiber. Consequently, there is no net increase in the number of muscle fibers, and maturation of skeletal muscle takes place after birth by instinctive and arbitrary training (Saccone and Puri, 2010). The exception to this is a population of muscle progenitors called myosatellite cells or satellite cells that does not differentiate into skeletal muscles during embryogenesis. Satellite cells are small mononuclear cells found sandwiched between individual skeletal myofibers. These cells are typically quiescent in adult muscle and form a pool of "reserve" muscle stem cells. In response to damage, they proliferate, give rise to myoblasts, progress through differentiation steps, and fuse with damaged myofibers or generate new myofibers. When regeneration fails, however, adipocytes infiltrate damaged cells and generate a fibrotic scar (fatty degeneration) (Natarajan et al., 2010).

Muscle fibers are composed of aligned functional units, sarcomeres, which are bundled together to form myofibrils consisting of multinucleated long fiber-like cells anchored by tendons to bones at both ends (Clark et al., 2002). Previously, muscle fibers were classified as fast-twitch muscles, characterized by glycolytic metabolism also called white muscles, and slow-twitch muscles, characterized by oxidative enzymes, rich in myoglobin and were also called red muscles (Needham, 1926). More recently, fibers are typed using histochemical staining for the identification of myosin heavy chain (MyHC) isoforms (Table 4.1). This typing led to the original division of muscle fibers into Type I (slow-twitch) and Type II (fasttwitch), with further divisions of three subpopulations of Type II skeletal muscle fibers, referred to as Type IIA, IIB, and IIX, in rat skeletal muscle (Gorza, 1990). A fiber type profile similar to that present in rat skeletal muscle has been observed in different mammalian species including mouse, rabbit, and guinea pig (Gorza, 1990). Human muscles are different from rat, however, with no detection of Type IIB (Scott et al., 2001). The four major fiber types in mammalian skeletal

muscles, together with several minor types, are reviewed extensively by Schiaffino and Reggiani (2011).

An important characteristic of skeletal muscle types is plasticity. Muscle is able to change its fiber type profile to adapt to physiological and functional demands. When skeletal muscle changes its fiber type profile, MyHC isoform transformation follows the pathway: Type I$Type IIA$TypeIIX/D$Type-IIB. Hybrid muscles are detected with coexpression of different MyHC chains in a single fiber (Erzˇen et al., 2001).

Skeletal muscle is the most abundant tissue in the vertebrate body. The average adult human is made up of roughly 40% skeletal muscle as a percentage of body mass (Marieb and Hoehn, 2007). Individual muscles perform different tasks, and muscles are comprised of a variable number of contracting myofibers, with variable proportions of the different types of fiber, determining the appropriate force and contraction (Biressi et al., 2007a,b). The fetal stage is crucial for skeletal muscle development, and maternal nutritional status is decisive in determining postnatal muscle quality, a very important factor in the livestock industry (Du et al., 2011). (See
Table 4.1 for a list of actomyosin components.)

Table 4.1 The genes for muscle actomyosin components in vertebrates and their chromosome locations in human and mouse

	Myosin II heavy chain type	Human	OMIM	Mouse
MYH 1	Adult sketetal muscle type IIX fibers	Chr 17p13.1	160730	Chr 11
MYH 2	Adult sketetal muscle type IIA fibers	Chr 17p13.1	160740	Chr 11
MYH 3	Fetal sketetal muscle	Chr 17p13.1	160720	Chr 11
MYH 4	Adult skeletal muscle type IB, fibers[a]	Chr 17p13.1	160742	Chr 11
MYH 6	Cardiac muscle, a; fast isoform some jaw muscle[a]	Chr 14q12	160710	Chr 14
MYH 7	Cardiac muscle, b; slow-twitch Type I (slow) muscle fibers	Chr 14q12	160760	Chr 14
MYH 8	Neonatal skeletal muscle	Chr 17p13.1	160741	Chr 11
MYH 9	Nonmuscle; kidney	Chr 22q12–13	160775	Chr 15
MYH 10	Nonmuscle; postsynaptic	Chr 17p13	160776	Chr 11
MYH 11	Smooth muscle; intestinal	Chr 16p13	160745	Chr 16
MYH 13	Eye muscle (extraocular)	Chr 17p13–p12	603487	Chr 11
MYH 14 (7b)	Heart, skeletal slow, extraocular	Chr 19q13.33	608568	Chr 7
MYH 15	Adult eye muscle (extraocular)	Chr 3q13.13	609929	Chr 16
MYH 16	Jaws (Temporalis and Masseter)[a]	Chr 7q22	608580	N/A
	Myosin light Chain type	Human	OMIM	Mouse
MYL 1	Alkali, skeletal, fast	Chr 2q34	160780	Chr 1
MYL 2	Smooth muscle, Cardiac regulatory	Chr 12q24.11	160781	Chr 5
MYL 3	Alkali, ventricular, skeletal, slow	Chr 3p21.31	160790	Chr 9
MYL 4	Alkali, atrial, embryonic	Chr17q21.32	160770	Chr 11
MYL 5	Regulatory,	Chr 4p16.3	160782	N/A