Epigenetic Allele Silencing Unveils Recessive *RYR1* Mutations in Core Myopathies

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Epigenetic regulation of gene expression is a source of genetic variation, which can mimic recessive mutations by creating transcriptional haploinsufficiency. Germline epimutations and genomic imprinting are typical examples, although their existence can be difficult to reveal. Genomic imprinting can be tissue specific, with biallelic expression in some tissues and monoallelic expression in others or with polymorphic expression in the general population. Mutations in the skeletal-muscle ryanodine-receptor gene (*RYR1*) are associated with malignant hyperthermia susceptibility and the congenital myopathies central core disease and multiminicore disease. *RYR1* has never been thought to be affected by epigenetic regulation. However, during the *RYR1*-mutation analysis of a cohort of patients with recessive core myopathies, we discovered that 6 (55%) of 11 patients had monoallelic *RYR1* transcription in skeletal muscle, despite being heterozygous at the genomic level. In families for which parental DNA was available, segregation studies showed that the nonexpressed allele was maternally inherited. Transcription analysis in patients' fibroblasts and lymphoblastoid cell lines indicated biallelic expression, which suggests tissue-specific silencing. Transcription analysis of normal human fetal tissues showed that *RYR1* was monoallelically expressed in skeletal and smooth muscles, brain, and eye in 10% of cases. In contrast, 25 normal adult human skeletal-muscle samples displayed only biallelic expression. Finally, the administration of the DNA methyltransferase inhibitor 5-aza-deoxycytidine to cultured patient skeletal-muscle myoblasts reactivated the transcription of the silenced allele, which suggests hypermethylation as a mechanism for *RYR1* silencing. Our data indicate that *RYR1* undergoes polymorphic, tissue-specific, and developmentally regulated allele silencing and that this unveils recessive mutations in patients with core myopathies. Furthermore, our data suggest that imprinting is a likely mechanism for this phenomenon and that similar mechanisms could play a role in human phenotypic heterogeneity.

Mutations in the coding sequence of genes have a well-established role in the determination of phenotypic diversity and pathological conditions. In the past few years, modification in the DNA or chromatin structure due to DNA methylation, the covalent modification of cytosine, and posttranslational modification of histones such as methylation, acetylation, phosporylation, and sumoylation have been recognized as contributing to allele expression.[1] Collectively, these mechanisms of gene regulation are termed "epigenetic modifications" and include genomic imprinting, germline epimutation, and epigenetic polymorphism secondary to or mediated by aberrant DNA methylation and/or histone modification.[2–4]

Genomic imprinting describes a parent-of-origin–dependent epigenetic mechanism through which a subset of genes is expressed from only one allele. For some imprinted genes, one parental allele is totally silenced in all or virtually all tissues.[2,5] Most imprinted genes contain differentially methylated regions (DMRs), which function as imprinting control regions (ICRs), in which the maternal and paternal alleles are differentially methylated. Deletions of these ICRs can disturb the transcription of imprinted genes, which can be located >1 million bp away.[6,7] For a restricted number of genes, the imprinting is tissue specific, which leads to biallelic expression in some tissues and monoallelic expression in others.[8] The allele-specific loss of expression can also be polymorphic; that is, it can vary between individuals. Examples of genes that are polymorphically imprinted include the Wilms tumor 1 gene (*WT1*) and serotonin 2A receptor genes.[9,10]

Some epigenetic changes can be transmitted unchanged through the germline (termed "epigenetic inheritance"). Evidence that this mechanism occurs in humans was recently provided by Suter et al.,[3] by the identification of individuals in whom one allele of the gene encoding the DNA mismatch-repair protein MLH1 was epigenetically silenced throughout the soma (implying a germline event).[3] These individuals are affected by hereditary nonpolyposis colorectal cancer but do not have any identifiable mutation in MLH1, even though it is silenced, which demonstrates that an epimutation can phenocopy a genetic disease.

Genomic imprinting, on the other hand, is reversible through successive generations. The inherited imprints are erased in primordial germ cells, and, within each genera-

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Table 1. PCR Primers and Restriction Enzymes Used for Genotyping SNPs

SNP[a]	dbSNP Allele	Heterozygosity (%)	Genomic DNA Primer Forward	Genomic DNA Primer Reverse	Genomic DNA Fragment Size (bp)	cDNA Primer Forward	cDNA Primer Reverse	cDNA Fragment Size (bp)	Restriction Enzyme
rs2229146	C/T	41.8	GTGGAGGGGGTAGAATGGAC	TTTTCTGCCAGTTGTTCTGC	294	CACAAAGTGCCCAGACCTATG	TAACCGCGTAGCCATTCATCT	285	*Cfo*I
rs2071089	A/G	41.4	GGGTGCAGTAGCATTCCAAC	ATCAGCTCTGGAATCGGAGA	282	TCCCCACATGAACAGGAGATT	TCAGGGCCTGACTTCATCACT	293	*Pvu*II
rs11083462	C/T	48.6	CCCCACACCATGTCTTCTCT	ACCGTCCCCAATCTCAATCT	211	GCAGTCACCACAGGCGAGATG	ATGGCAAAGAACCTCAGAGAG	368	*Mbo*I
rs2228069[b]	A/G	48.3	GCCCGCAGGTATATGATGAG	TCGATGTTGTAGCCGTAGCC	296	TCTGGGGAGACGCTCAAGACT	CTTCATCCAGCAGGCGGTAGG	316	…
rs2229139[b]	A/G	48.2	ACCCTTGATTTCTGGCCTCT	GTATGCCTGAAGGGCAACAT	250	CAGGAGGACGCAACAGGAGAG	AGCAGGGGAAATGGTCAGACA	297	…

[a] Cluster ID of SNP.
[b] No restriction enzyme was available.

tion, new imprints are reset during gametogenesis and are maintained throughout development.[11]

Core myopathies, including central core disease (CCD [MIM 117000]) and multiminicore disease (MMD [MIM 255320]), are characterized by corelike structures in patient skeletal-muscle biopsy samples, as a result of the lack of oxidative enzyme activity, and have been associated with mutations in the skeletal-muscle ryanodine receptor (*RYR1* gene [MIM 180901; GenBank accession number NP_000531]) on chromosome 19q13.1.[12–14] In addition, malignant hyperthermia susceptibility (MHS [MIM 145600]), a pharmacogenetic predisposition to develop a severe reaction after the administration of general anesthetics, is also due to mutations in the *RYR1* gene. Most patients with CCD and MHS carry dominant *RYR1* mutations, whereas MMD is recessively inherited.[15] Most *RYR1* mutations in CCD and MHS induce depletion of sarcoplasmic reticulum calcium stores, with resultant increase in cytosolic calcium levels ("leaky channels"), whereas recessive MMD mutations appear to affect RyR1 function in a more subtle way.[12,16]

During the mutation analysis of a cohort of patients with recessive core myopathies, we discovered that, in a proportion of them, *RYR1* was transcribed from only one allele (i.e., monoallelic expression) in skeletal muscle. The transcribed allele in skeletal muscle carried a recessive mutation. Surprisingly, transcription analysis of patient fibroblasts and lymphoblastoid cell lines indicated biallelic transcription, suggesting tissue-specific allele silencing. Transcription studies in normal human fetal tissues revealed a monoallelic transcription pattern for *RYR1* in a proportion (10%) of fetuses, whereas this phenomenon was not found in 25 unaffected adult skeletal-muscle samples. These results suggest that recessive *RYR1* mutations in a proportion of patients with core myopathies can be unveiled by lack of expression of the other, apparently wild-type, allele. Various lines of experimental evidence suggest that this is due to genomic imprinting in these patients, although other epigenetic modification of allele expression cannot be definitively excluded.

Material and Methods

Mutation Analysis

Total RNA was extracted from frozen skeletal-muscle biopsy samples and from myogenic differentiation antigen (MyoD)–transformed skin fibroblasts, with use of the RNeasy mini kit (QIAGEN). Total RNA (1–2 μg) was reversely transcribed using SuperScript III first-strand synthesis system kit (Invitrogen). The cDNA was subsequently used for the following amplification reactions: 27 overlapping fragments were amplified, with a range of 400–1,000 bp, covering the entire 15-kb coding sequence of the *RYR1* gene (primer sequences available on request). PCR amplification of exons 4, 12, 45, 57, 91, and 96 of *RYR1* were performed on genomic DNA with exon-specific primers. Platinum *pfx* DNA polymerase (Invitrogen) was used in the amplification. A touchdown program was used for all PCR conditions, with use of GeneAmp PCR System 9700 (Applied Biosystem [ABI]). Briefly, the annealing temperature started at 65°C and decreased, in steps of 0.5°C per cycle, to 55°C. After 20 cycles, another 15 cycles were performed with an annealing temperature of 55°C. All PCR products were gel-purified by using the QIAquick gel-purification kit (QIAGEN) and were directly sequenced, in forward and reverse directions, with use of an ABI 3730XL automated sequencer.

Haplotype Study

DNA samples extracted from peripheral blood were genotyped for five microsatellite markers (*D19S897, D19S421, RYR1* intragenic marker, *D19S422,* and *D19S417*) spanning the *RYR1* locus. PCR conditions were the same as those for mutational analysis. Amplified products were run on an ABI PRISM 3730 DNA sequencer machine, followed by analysis of the results with use of GeneMapper version 3.7 software.

SNP Analysis of Human Fetal and Adult Control Tissues

Five SNPs (*rs2229146, rs2071089, rs11083462, rs2228069,* and *rs2229139*) in the *RYR1* gene were used to assess heterozygosity. Primer sets and specific restriction enzymes of SNPs are listed in table 1. The PCR and amplification conditions are described in the "Mutation Analysis" section.

Cell Cultures

Myoblast and skin fibroblast cell lines were established from patient skeletal-muscle and skin biopsy samples. Myoblast cultures were purified using a commercially available system (Miltenyi Biotec) and primary antibody anti-CD56.[17] Myoblast cell cultures were maintained in skeletal-muscle growth medium supplemented with 5% fetal calf serum (FCS), 50 μg/ml fetuin, 1 ng/ml basic fibroblast growth factor, 10 ng/ml epidermal growth factor, 10μg/ml insulin, and 0.4 μg/ml dexamethasone (PromoCell). The muscle-cell phenotype was confirmed immunohistochemically in each culture, with use of antibodies to the muscle-specific protein desmin.

Skin fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. To force myogenesis, fibroblasts were transfected with a MyoD-en-

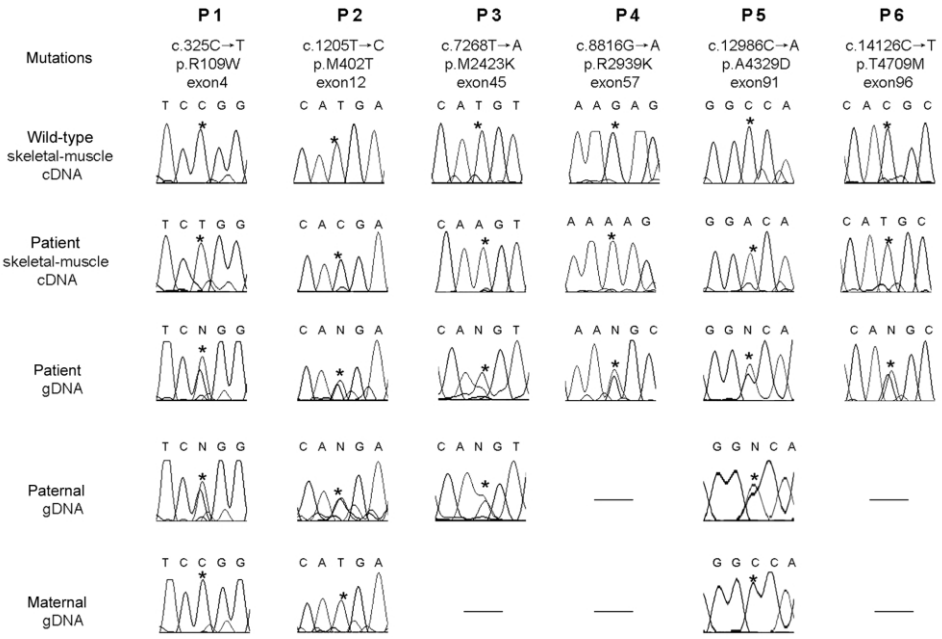

Figure 1. Monoallelic expression of the *RYR1* gene in six patients with core myopathies. Chromatograms for the sequencing of skeletal-muscle cDNA, genomic DNA (gDNA), and parental genomic DNA from six patients (P1–P6) with *RYR1* mutations. The details of six mutations, including nucleotide changes in cDNA sequences and amino acid substitutions, are presented at the top of individual patient lanes. In all six patients, there was monoallelic expression of the mutations in skeletal-muscle cDNA, despite the fact that all patients clearly were heterozygous at the genomic DNA level. Allele-transmission studies indicate that, in four cases (P1, P2, P3, and P5), the only allele transcribed was the paternal one, which carried the mutation, whereas the maternal allele was silenced at the transcriptional level. An asterisk (*) indicates the position of mutation; a dash indicates that there was no sample available. The nucleotide sequences are shown above the chromatogram peaks. For P4, the nucleotide change was on the last nucleotide residue in exon 57; therefore, the sequences after the mutation differed between DNA and cDNA.

coding adenovirus (Crucell). After incubation for 3 h, cells were washed with DMEM and then were allowed to differentiate into multinucleated myotubes by culturing in differentiation medium (DMEM supplemented with 2% horse serum) for 5–7 d.

Lymphoblastoid cell lines were established from patient peripheral-blood leukocytes and were transformed with Epstein Barr virus (EBV) by Health Protection Agency of European Collection of Cell Cultures. Cells were cultured in RPMI-1640 supplemented with 20% FCS.

5-Aza-Deoxycytidine (5-azaC) and Trichostatin A (TSA) Treatments

Purified myocytes were planted at a density of 4×10^5 cells/dish in four dishes containing skeletal-muscle growth medium. After 24 h, 10 μM 5-azaC was added to dishes 1 and 2. Growth medium with freshly added 5-azaC was changed on day 3. In the meantime, 50 nM TSA was added to dish 2 (in the presence of 5-azaC) and dish 3 on day 3.[18] At the doses employed (50 nM), TSA did not induce apoptosis or signs of cell toxicity.[19] Dish 4 was used as an untreated control. After 24 h, cells were collected, and total RNA was extracted, as described above, from all four dishes. Reverse transcription was performed, following the manufacturer's instructions. PCR was performed using the touchdown program described in the "Mutation Analysis" section. The primers used to amplify an 824-bp cDNA product were 5′-TCCAAGGAGAAGCTGGATGTG-3′ (forward) and 5′-TGCTTGTCCAGGAGGGAGATG-3′ (reverse). To exclude genomic DNA product contamination, the forward primer spanned the junction of exons 10 and 11, whereas the reverse primer was in exon 17. The same primers were used to amplify cDNA from skeletal-muscle tissue. After agarose-gel purification, PCR products were sequenced directly.

Bisulfite-Modified Genomic Sequencing

Skeletal-muscle DNA samples (2 μg) were treated with bisulfite by using EZ DNA Methylation-gold Kit (Zymo Research) and were diluted into 10-μl M-elution buffer. For the following PCR, 1 μl was used. Bisulfite-modified skeletal-muscle DNA was amplified with Hotstart Taq DNA polymerase (Qiagen). A touchdown program was used to amplify the products. PCR conditions were as follows: at 95°C for 15 min; followed by 20 cycles performed at 94°C for 30 s; with an annealing temperature, starting at 65°C and decreasing in steps of 0.5°C per cycle until 55°C, for 30 s; and an extension at 72°C for 1 min. After 20 cycles, another 20 cycles were performed as follows: denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The primer sets used to amplify three CpG islands were 5′-TTGTGAAATGGGAGAATGATGGTA-3′ and 5′-AATCTCAAAAACCACCAACAAACC-3′ for CpG I, 5′-GAGATGGGGGTTTTATTATGTATATTG-3′ and 5′-AAAAATCAAAATCCCTACCTTAACT-3′ for CpG II, and 5′-TTTGTGGTTTGTAGTATTTGTGGTA-3′ and 5′-CCAAAATTCTCTACCCCTTCAAAC-3′ for CpG III. Amplified fragments were gel-purified and

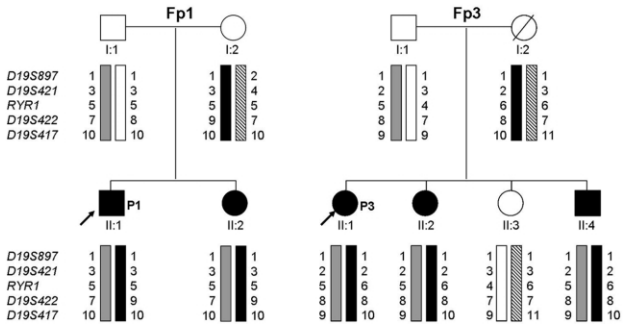

Figure 2. Pedigrees of two affected families, Fp1 and Fp3. Blackened symbols denote symptomatic patients with core myopathy; unblackened symbols indicate asymptomatic individuals. Haplotypes are derived from DNA satellite markers, listed on the left. Arrows indicate the probands. The deceased individual is marked with a diagonal bar. The maternal haplotype in Fp3 is reconstructed. Individuals II:1 and II:2 in Fp1 and II:1, II:2, and II:4 in Fp3 are affected by the core myopathy, whereas the parents are completely asymptomatic.

were sequenced directly. The purified products were cloned using the TOPO TA cloning system (Invitrogen). Individual clones were isolated using a Qiaprep minispin kit (Qiagen) and were sequenced with sense and antisense M13 primers by using standard ABI sequencing technology to determine the methylation status of the CpG dinucleotides. Only DNA strands that were >95% converted were used for analysis.

Results

Identification of Monoallelic RYR1 *Expression in Patients with Core Myopathies*

Investigation of the *RYR1* gene in a cohort of patients with clinical and pathological features evocative of recessive core myopathies revealed an unexpected phenomenon. Sequencing of skeletal-muscle cDNA showed that 6 (55%) of 11 patients had apparent homozygous *RYR1* missense mutations, yet subsequent analysis unequivocally demonstrated that all patients were heterozygous at the genomic DNA level (fig. 1); further studies showed that this apparent homozygosity for the mutant allele was the result of silencing of the other allele (see below). None of the patients were from consanguineous families; four were sporadic cases, and two (P1 and P3) were familial cases. RT-PCR was repeated by using different combinations of primers, and no additional bands were seen when products were run on the agarose gel. The mutations identified in these six patients are presented in figure 1. Recessive inheritance was suggested by linkage studies in two families with multiple affected individuals and by the clinical analysis of their unaffected parents (fig. 2). The mutations found in these patients are likely to be pathogenic, since they affect highly conserved residues across RyR isoforms and species and are not present in a white control population (>200 chromosomes). In addition, three of these patients were informative for other coding-region SNPs located at variable distances from the mutated exons, which also showed monoallelic expression (data not shown). Promoter and 3′ UTR mutations were excluded, in all six patients with core myopathies, by direct sequencing of the *RYR1* promoter and 3′ UTR regions.[20]

Recessive mutations were identified in the remaining 5 (45%) of the 11 patients with core myopathies, all of whom showed biallelic *RYR1* expression in skeletal muscle. These were three homozygous missense mutations, which included two mutations identified in consanguineous families and one homozygous change in a family without apparent consanguineous background, as well as two pairs of compound heterozygous mutations, which included three missense mutations and one splice-site mutation (H. Zhou, H. Jungbluth, E. Bertini, K. Bushby, V. Straub, H. Roper, M. R. Rose, M. Brockington, L. Feng, C. R. Müller, A. Manzur, S. Robb, S. Messina, S. Brown, S. Treves, C. A. Sewry, and F. Muntoni, unpublished data).

Parent-of-Origin–Dependent Monoallelic RYR1 *Expression*

In the four (of six) patients for whom parental genomic DNA samples were available, we could demonstrate that the mutated allele was invariably inherited from the clinically unaffected father (fig. 1). In one family (P2), we were able to study the skeletal muscle from the clinically unaffected mother, who had transmitted the untranscribed allele; analysis of two SNPs in both genomic DNA and skeletal-muscle cDNA showed that both her alleles were transcribed (fig. 3). These results clearly showed allele-specific silencing inherited from the parent(s), which raises the possibility of genomic imprinting.

Tissue-Specific RYR1 *Silencing in Patients with Core Myopathies*

Since the *RYR1* gene is also transcribed in tissues other than skeletal muscle—including lymphocytes[21] and, to a lesser degree, fibroblasts—we studied its transcription in

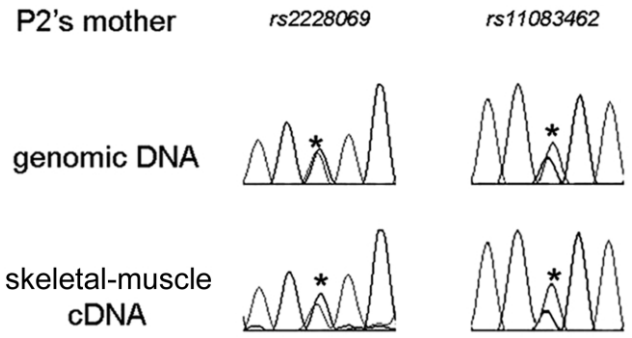

Figure 3. Allelic-expression study of an unaffected mother. Chromatograms for the sequencing of SNPs *rs2228069* and *rs11083462* show that both SNPs are heterozygous in patient P2's maternal genomic DNA, and the corresponding skeletal-muscle cDNA shows consistent biallelic transcription.

skin fibroblasts and EBV-immortalized lymphoblastoid cell lines from two (P1 and P6) of the six patients with monoallelic skeletal-muscle transcription. The results showed biallelic expression in fibroblasts from patient P1 and lymphoblastoid cell lines from patient P6 (fig. 4*A*), which suggests that the monoallelic expression is tissue specific.

To assess whether the monoallelic expression at the *RYR1* locus could be induced by forcing myogenesis of patients' fibroblasts, we transfected the fibroblasts from patient P1 with a MyoD-adenoviral vector.[22] Transcriptional analysis of MyoD-transformed fibroblasts from this patient with skeletal-muscle monoallelic transcription showed that *RYR1* transcription retained the biallelic pattern (fig. 4*A*), irrespective of the expected up-regulation of *RYR1* transcription that followed the MyoD transfection (fig. 4*B*). The expression of desmin and myosin confirmed the myogenicity of these cells (not shown).

To rule out the unlikely possibility of chain-terminating mutations giving rise to a tissue-specific nonsense-mediated RNA decay, we sequenced the entire cDNA derived from MyoD-transfected fibroblasts from individual P1 of family Fp1, since we had confirmed biallelic expression in his fibroblasts. No additional mutation was identified in this patient.

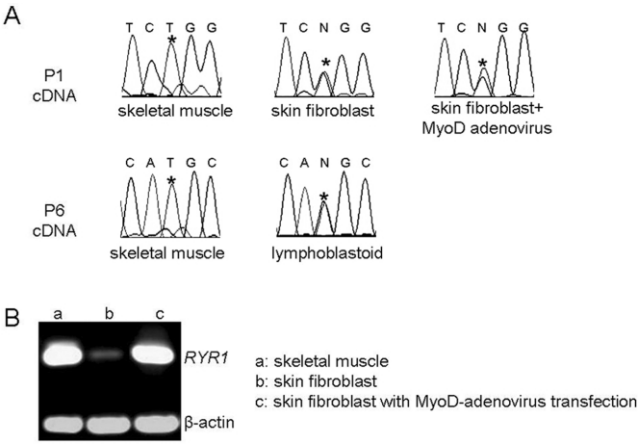

Figure 4. Tissue-specific maternal-allele silencing of the *RYR1* gene in patients with core myopathy. *A,* Chromatograms of the sequencing of cDNA derived from skeletal muscle, skin fibroblast, and lymphoblastoid cell lines. In contrast to the monoallelic expression in skeletal muscle, the cDNA from P1's skin fibroblast and MyoD adenovirus–transfected skin fibroblast and P6's lymphoblastoid cell lines show biallelic expression. An asterisk (*) indicates the mutation position. *B,* Increased expression of *RYR1* after forced myogenesis of skin fibroblast–derived myoblasts. RT-PCR shows weak expression of *RYR1* in skin fibroblasts. The level of *RYR1* transcription is noticeably higher after the MyoD transfection, because of the induced myogenicity.

RYR1 *Transcription in Human Fetal Tissues*

To better understand the mechanism leading to *RYR1* monoallelic expression, we investigated multiple tissues from unaffected human fetuses. Informative fetuses were first identified by studying five highly polymorphic SNPs located in the coding sequence of *RYR1* (table 1). Of the 57 fetuses, 39 were heterozygous for at least one SNP in genomic DNA; these fetuses were further characterized at the transcription level. In 4 (~10%) of these 39 fetuses, monoallelic expression was detected in skeletal muscle as well as in intestine, eye, brain, and spinal cord, whereas, in other tissue samples, including lung, placenta, heart, spleen, adrenal, and pancreas, the expression was biallelic (fig. 5). This pattern of expression was confirmed by direct sequencing and restriction-enzyme digestion. Parental DNA samples were not available for confirmation of the origin of the silenced *RYR1* allele in these fetuses. The frequency of *RYR1* monoallelic expression (10%) in unaffected fetuses was lower than the 55% found in patients with recessive core myopathies, most likely reflecting the ascertainment bias of the patient group. These results, therefore, strongly suggest that *RYR1* monoallelic expression is polymorphic and follows a tissue-specific pattern during human fetal development.

RYR1 *Transcription in Control Adult Human Muscle*

To determine whether some *RYR1* alleles are silenced in the general adult population, we obtained 39 adult skeletal-muscle biopsy samples and analyzed the five SNPs previously used. We identified 25 heterozygous individuals. The *RYR1* gene was found to be biallelically expressed in all individuals (data not shown). The data from the general adult population, together with the fetal studies, suggest that the epigenetic *RYR1* silencing is developmentally regulated.

Methylation Study of RYR1 *Monoallelic Expression*

Patient P2 carries a paternally derived missense mutation (c.1205T→C), which affects the only allele expressed in her skeletal-muscle cDNA. Transcription studies of cultured primary myoblasts from the patient showed a pattern of monoallelic expression identical to that in her skeletal muscle (fig. 6*A*). Since epigenetic modification of transcription is due mainly to differential DNA methylation or histone deacetylation, we applied the DNA methyltransferase inhibitor 5-azaC and the histone deacetylase inhibitor TSA to cultured myoblasts.[18] Administration of 5-azaC reactivated transcription of the silenced maternal allele when applied alone or in combination with TSA, whereas treatment with TSA alone did not show reactivation of the silenced maternal allele (fig. 6*A*), which suggests that DNA methylation plays the major role in the silencing of the maternal *RYR1* allele.

Methylation of CpG Islands in the 5′ Region of RYR1 *Gene*

To seek the DMR associated with *RYR1* silencing, we studied the methylation status of the 5′ region of the *RYR1* gene in the patients with monoallelic expression. This re-

A

Fetus	SNP	GDNA	cDNA										
			brain	Spinal cord	limb	eye	intestine	lung	placenta	heart	spleen	adrenal	pancreas
1	*rs2229146*	T/C	C	C	C	-	-	C/T	C/T	-	-	-	-
2	*rs2229146*	T/C	C	C	C	C	-	-	-	C/T	-	-	-
3	*rs11083462*	T/C	-	-	-	-	T	-	-	-	-	T/C	T/C
4	*rs2071089*	A/G	-	-	-	-	G	-	-	-	A/G	-	-

B

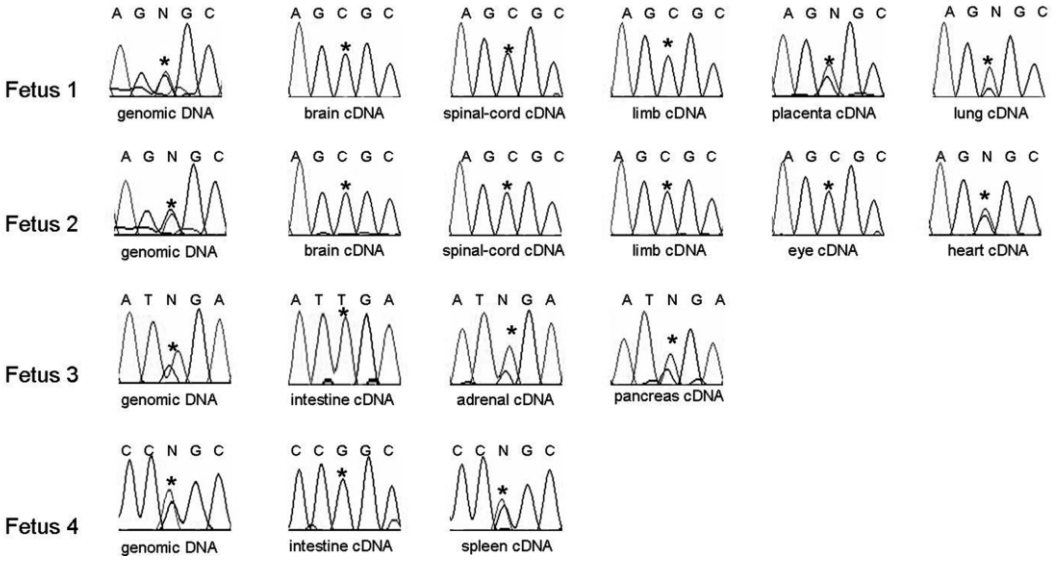

Figure 5. Monoallelic expression of the human *RYR1* gene in unaffected fetuses. *A,* Monoallelic expression of the human *RYR1* gene, which is tissue specific. Highly polymorphic SNPs were studied in both genomic DNA and cDNA from human fetuses. In four heterozygous fetuses, cDNA from limb (skeletal muscle), brain, spinal cord, eye, and intestine show monoallelic expression, whereas lung, placenta, heart, spleen, adrenal tissue, and pancreas cDNA show biallelic transcription. Dashes indicate that samples were not available. *B,* Sequencing chromatograms of SNP analysis. An asterisk (*) indicates the position of the SNP.

gion contains the promoter, exon 1, intron 1, and exon 2 (nucleotides −2730 to +7035). The presence of three CpG islands was identified by using Methprimer software (fig. 6*B*). CpG I contains 17 CpG dinucleotides (nucleotides −177 to +61), which occupy a small part of the promoter region, the whole 5′-UTR, and the entire exon 1. CpG II is located in intron 1 (nucleotides 4815–4985) and contains 10 CpG dinucleotides. CpG III includes the entire exon 2 (nucleotides 6890–6998) and contains 13 CpG dinucleotides. We used bisulfite sequencing to screen genomic DNA isolated from the skeletal muscle of five patients with monoallelic *RYR1* expression and eight control skeletal-muscle samples. No differential methylation was found in CpG I, either in the patient group or in controls (fig. 6*C*). Hypermethylation was identified in CpG II and CpG III, although there was no difference between the patient group and controls (fig. 6*C*). This suggests that methylation of CpG islands I, II, and III is not responsible for the monoallelic expression of the *RYR1* gene.

Ryr1 *Transcription in Mouse*

To see whether expression of the *Ryr1* gene (GenBank accession number NP_033135) is monoallelic in mouse, we sequenced the cDNA from C57BL/6 and CAST/Ei mice and identified an A/G SNP between the two species. Analysis of this SNP in reciprocal F1 crosses clearly showed that *Ryr1* expression was biallelic in whole E16.5 embryos as

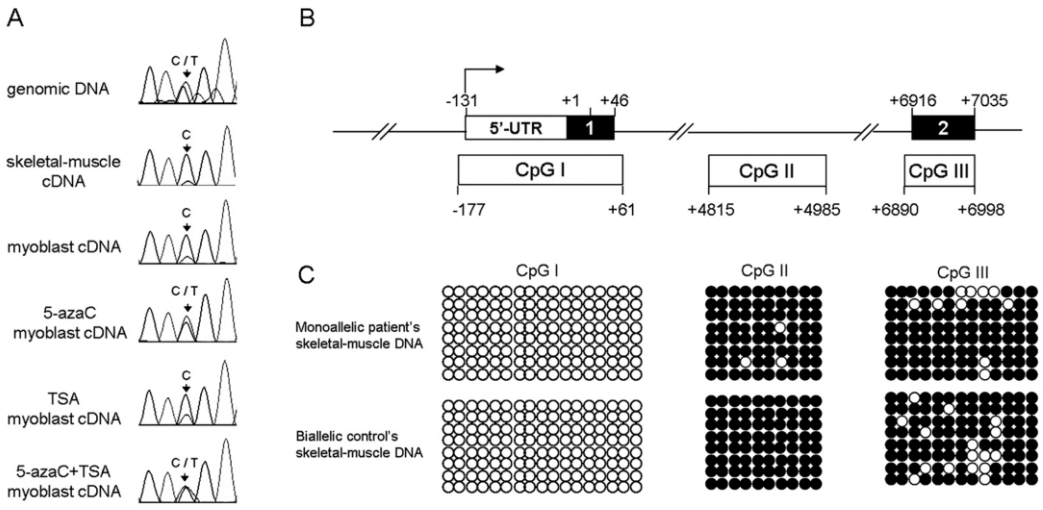

Figure 6. Mechanism of *RYR1* monoallelic expression. *A,* Analysis of the silenced allele in cultured skeletal myocytes from patient P2 after inhibition of DNA methyltransferase (by 5-azaC) and histone deacetylase (by TSA) activity. Chromatograms of the sequenced RT-PCR products spanning mutation c.1205T→C show that the cultured primary skeletal myoblasts have the same monoallelic expression as do skeletal-muscle tissue. The mutant C peak (*arrow*) indicates the paternal allele, and the T peak indicates the maternal allele. Only 5-azaC significantly increased the expression of the silenced maternal allele, either alone or in combination with TSA. TSA had no effect on its own. *B,* Schematic representation of the 5′ region of the human *RYR1* gene. Exons are indicated by blackened boxes. The bent arrow indicates transcription start site, and CpG islands are as indicated by numbered position. All three CpG islands were analyzed by bisulfite sequencing. *C,* Cloning and sequencing of bisulfite PCR products from skeletal-muscle DNA of monoallelic patients and biallelic controls. Complete absence of methylation and hypermethylation of CpG dinucleotides is indicated as unblackened and blackened dots, respectively. After the quantification of methylation by cloning and sequencing, no difference in the methylation status between patient and controls was found in the three CpG islands.

well as in brain and skeletal-muscle cDNA from newborn animals (data not shown). These data indicate that the *Rry1* gene is not expressed monoallelically in mouse.

Discussion

In this study, we describe—to our knowledge, for the first time—that *RYR1,* encoding the skeletal-muscle ryanodine receptor, undergoes epigenetic allele silencing during normal human development in a tissue-specific fashion. Moreover, we were able to demonstrate that, of the patients with core myopathies we studied—a group of patients with clinicopathological features suggestive of recessive *RYR1* involvement—55% had monoallelic expression in skeletal muscle but not in other tissues. The monoallelic expression unveiled the presence of the apparently recessive mutation. The unmasking of recessive mutations in patients with core myopathies is a phenomenon similar to the discovery of isodisomy in cystic fibrosis, in which patients inherit a recessive allele from one parent only.[23] Various lines of evidence suggest that the monoallelic *RYR1* expression observed in the skeletal muscle of our patients is the result of epigenetic modification. We excluded changes in the nucleotide sequence of *RYR1* in these patients by studying not only the coding region but also the promoter and the 3′ UTR regions. We have demonstrated, by studying intragenic SNPs, that these patients do not carry large-scale deletions. We have also clearly demonstrated biallelic expression in several tissues other than skeletal muscle. This makes it very unlikely that chain-terminating mutations could be present on the silenced allele, a possibility that was further excluded by direct sequencing of the entire cDNA from biallelic fibroblasts of a patient with monoallelic skeletal-muscle transcription. Although a tissue-specific splicing defect has been recently reported in one patient with core myopathy,[24] our patients' RT-PCR studies failed to identify any additional products, despite study of different combinations of primers, which suggests that a mutation affecting tissue-specific splicing is very unlikely. Finally, the restoration of biallelic expression after treatment with the DNA methyltransferase inhibitor 5-azaC in one patient's primary myoblasts provides evidence toward epigenetic modification of *RYR1* transcription in these patients with monoallelic expression.

Which epigenetic mechanisms could be responsible for the observed monoallelic expression? The phenomenon of monoallelic expression shares several characteristics with epimutation, epigenetic polymorphism, and imprinting. The role of germline epimutations is increasingly being recognized in cancer, since the demonstration of epimutations of the DNA mismatch-repair gene *MLH1* in two individuals with multiple primary tumors.[3] In addition, variations in gene expression secondary to epigenetic poly-

morphisms are likely to play a major role in human variability, as suggested by the surprising finding that allelic imbalance affected 6 of 13 genes studied by Yan et al.,[25] and ratios for those alleles differentially expressed varied from 1.3:1.0 to 4.3:1.0. These studies were extended by Lo et al.,[26] who found that >50% of 602 studied genes showed preferential expression of one allele in at least one individual, and ~55% of those showed greater than fourfold difference between the two alleles; most of these genes were distributed throughout the genome, in areas not located in known imprinting domains. Several indirect lines of evidence suggest that the epigenetic mechanism leading to *RYR1* monoallelic expression in a proportion of cases is likely to be the result of genomic imprinting. First, there is an association with the sex of the nontransmitting parent, the mother in all informative cases studied. This phenomenon is a unique feature of genomic imprinting. Second, the *RYR1* monoallelic expression followed a tissue-specific pattern, which would not be expected in other instances of epigenetic modification. Third, studies of unaffected fetuses confirmed both the occurrence of silencing of *RYR1* in an appreciable proportion (10%) of cases and the tissue-specific nature of the silencing. This, together with the biallelic expression found in the unaffected adult control population, suggests a developmental pattern of allele silencing that is also a feature of genomic imprinting.

Other genes have been reported in which tissue-specific imprinting leads to monoallelic transcription that is limited to a subset of tissues.[8] These include *UBE3A, KCNQ1,* and *GNAS1*. The *UBE3A* gene, involved in Angelman syndrome, is imprinted in a subset of regions in the CNS, with expression only from the maternal allele.[27] Fetal-tissue studies demonstrated that imprinted expression of *UBE3A* is limited to the brain.[28] The nearby *ATP10C* gene is also preferentially maternally expressed in brain and lymphoblasts.[29,30] The *KCNQ1* gene within the Beckwith-Wiedemann syndrome–imprinted cluster on 11p15.5 is expressed from only the maternal allele in most tissues and also carries an associated antisense transcript (LIT1) that is expressed only from the paternal allele[31]; *KCNQ1* undergoes both tissue-specific and developmentally regulated imprinting, with maternal-specific expression occurring during embryonic development and subsequent (postnatal) loss of imprinting, which then results in biallelic expression.[32] The *GNAS1* gene, a complex imprinted locus on chromosome 20q13, encodes multiple gene products through the use of alternative promoters and first exons that are differentially imprinted in a tissue-specific manner.[5,33,34]

Polymorphic and tissue-specific imprinting has also been described in humans. Examples include the Wilms tumor 1 gene (*WT1*), the serotonin 2A receptor (*5-HT2A*), and the insulin-like growth factor II receptor gene (*IGF2R*).[9,10,35,36] However, the imprinting in all these examples has no phenotypic effect, because of the lack of allele silencing in affected tissues. In particular, *KCNQ1,* mutated in different forms of long–Q-T syndromes, is not imprinted in the heart.[32] Similarly *WT1,* mutated in some patients with Wilms tumor and other complex conditions with renal tumors, is imprinted in a proportion of patient fibroblasts but not in kidneys.[9] Although the relevance of tissue-specific and polymorphic imprinting for human diseases has been hypothesized in the past,[8] a clear example of this mechanism has never been documented before. We cannot be absolutely certain that the monoallelic expression we observed is the result of imprinting, as opposed to other epigenetic modification of gene expression; however, imprinting seems the most plausible hypothesis.

The *RYR1* gene, involved in congenital core myopathies, MMD, and MHS, had never been considered in terms of imprinting or other epigenetic modification. However, reduced penetrance of dominant *RYR1* mutations is a well-recognized phenomenon.[37] Furthermore, marked "anticipation" of severity has also been reported in families with rare dominant mutations, because severely affected children have invariably inherited the phenotype from a mildly affected father[38] (F.M., personal observation).

The identification of the *RYR1* monoallelic transcription in our study was facilitated by the use of cDNA derived from skeletal muscle, not the genomic DNA used in the majority of previous reports.[39] In the case of the recessive *RYR1* mutations in patients with silenced maternally inherited alleles, genomic DNA studies might indeed be misleading. The fact that skeletal muscle is a target tissue for *RYR1* mutations has led to the clustering of individuals with monoallelic transcription in our patient population.

Our studies of cultured myoblasts treated with the DNA methyltransferase inhibitor 5-azaC suggest that the monoallelic expression of *RYR1* is associated with DNA methylation. Imprinted genes are usually associated with a CpG island near or inside their promoters showing differential methylation between two parental alleles. However, sequencing of the CpG islands located within the 5′ end of the *RYR1* gene—including the first intron containing sequences involved in directing porcine skeletal-muscle tissue-specific *RYR1* transcription[40]—did not reveal differential methylation between patients and controls, which suggests that the regulatory elements of *RYR1* monoallelic expression are localized outside this region. It is possible that the *RYR1* gene lies within a cluster of imprinted genes regulated by a *cis*-acting element. Interestingly, both *PEG3* and *ZIM2,* which are located on chromosome 19q13.4, are also subject to maternal imprinting.[41] However, the physical distance between these genes and *RYR1* (>14 Mb) makes it unlikely that they belong to a single cluster.

Our finding that the mouse *Ryr1* orthologue was not imprinted was surprising and will limit the value of animal models in further investigation of the monoallelic *RYR1*-expression mechanism. A large number of imprinted transcriptional units show imprinting-status discordance between human and mouse, with most imprinted in mouse but not in human.[41] Nevertheless, another gene, *L3MBTL,* is imprinted in human but not in the mouse orthologue.[42]

In conclusion, we have identified the tissue-specific silencing of the *RYR1* that unveils paternally inherited recessive mutations, a novel mechanism responsible for autosomal recessive core myopathies. The polymorphic nature of this phenomenon, together with its developmental regulation in human, suggests that this is likely the result of a genomic-imprinting mechanism mediated by DNA methylation. It is possible that similar epigenetic modification of *RYR1* expression accounts for both the reduced penetrance and the "anticipation" of dominant *RYR1* mutations. It is also likely that similar mechanisms play a role in phenotypic heterogeneity of other known cell- or tissue-specific human gene defects.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
EuroBioBank, http://www.eurobiobank.org/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *RYR1* [accession number NP_000531] and *Ryr1* [accession number NP_033135])
Methprimer, http://urogene.org/methprimer/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CCD, MMD, *RYR1,* and MHS)

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