



Defining α -skeletal and α -cardiac actin expression in human heart and skeletal muscle explains the absence of cardiac involvement in *ACTA1* nemaline myopathy

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Abstract

Mutations in α -skeletal actin (*ACTA1*) underlie several congenital muscle disorders including nemaline myopathy (NM). Almost all *ACTA1*-NM patients have normal cardiac function, and, even lethally affected congenital NM patients exhibit an unremarkable gestation with decreased foetal movement just prior to birth. Although α -skeletal actin is thought to be the predominant sarcomeric actin in human heart (Boheler KR, Carrier L, de la Bastie D, et al. Skeletal actin mRNA increases in the human heart during ontogenic development and is the major isoform of control and failing adult hearts. *J Clin Invest* 1991;88:323–30 [1]), *ACTA1*-NM patients almost never exhibit a cardiac phenotype. In this study, we define the relative expression of skeletal and cardiac actin proteins in human heart and skeletal muscle. We show that α -cardiac actin is the predominant sarcomeric isoform in human donor hearts and in early foetal skeletal muscle development. Skeletal actin is the predominant isoform from 25 to 27 weeks gestation and is the exclusive isoform expressed in muscle from infancy through to adulthood. These findings are consistent with clinical observations of NM patients and assist us to better understand the pathogenesis of inherited myopathies and cardiomyopathies with mutations in actin.

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1. Introduction

The actin family of proteins are involved in a variety of fundamental cellular processes including maintenance of the cytoskeleton, cell motility, mitosis and muscle contraction. There are six different actin isoforms that are highly conserved across species with >90% similarity in their amino acid sequence. β - and γ -Cytoplasmic actin are predominantly expressed in non-muscle cells [2], while the four muscle-specific isoforms are expressed in different

types of muscle tissues, and are functionally involved in muscle contraction. Smooth muscle γ - and α -actin are co-expressed in both visceral and vascular smooth muscle but the relative levels of each isoform vary in different tissues [3]. In cardiac and skeletal muscle, there is co-expression of two sarcomeric actins known as α -skeletal and α -cardiac actin. These two sarcomeric actins are highly homologous and their amino acid sequences differ by only four residues.

Disease-causing mutations have been identified in three of the actin isoforms to date. Mutations in the human α -cardiac actin gene underlie a subset of cases of dilated cardiomyopathy [4] and hypertrophic cardiomyopathy [5], while missense mutations have been identified in the cytoplasmic γ -actin gene in patients with autosomal dominant progressive deafness [6,7]. Mutations in the α -skeletal actin gene (*ACTA1*) are associated with a number of related congenital-onset myopathies, which are classified according to the predominant histopathological feature on muscle biopsy.

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To date, disease-causing mutations in *ACTA1* have been identified in patients with nemaline myopathy [8,9], actin myopathy [8,10], intranuclear rod myopathy [10,11], central core disease [12] and congenital fibre type disproportion (CFTD) [13]. The majority of mutations identified in *ACTA1* are sporadic (new dominant) [10].

Nemaline myopathy (NM) is the best characterised of all congenital myopathies, and the most common of the myopathies associated with mutations in *ACTA1*. The pathological hallmark of NM is the presence of rod bodies, a major constituent of which is the actin-binding protein, α -actinin-2 (reviewed in North et al. [14]). Clinically, patients with NM present with generalised muscle weakness affecting the limb, facial, bulbar and respiratory muscles. There is variation in age of onset and severity of weakness ranging from severe congenital NM with death in the first year of life due to respiratory insufficiency, to milder myopathy with survival into adulthood. In the majority of cases the diagnosis of NM is made at birth (due to breathing or swallowing difficulties) or during the first year or two of life (due to delay in attaining motor milestones).

We have previously characterised the clinical and pathological features of a series of patients with heterozygous missense mutations in *ACTA1* [9,11]. We demonstrated that mutations in *ACTA1* result in muscle disease through a dominant-negative effect in some cases [11], and evidence from genetic studies in patients with *ACTA1* mutations suggests that there is a 'dosage effect' of the mutant protein on muscle function [8,10]; that is, patients who are somatic mosaics for a mutation in *ACTA1* have a milder clinical phenotype than their offspring who carry the mutation in all cells and die early in the newborn period.

In our series of patients with mutations in *ACTA1*, all NM patients were delivered at or close to term, with unremarkable pregnancies. Since the majority of patients with NM survive until birth, this suggests that expression of cardiac actin in the fetus is able to partially compensate for mutant α -skeletal actin. This suggestion is based on mRNA analyses that have shown that α -cardiac actin is the major sarcomeric actin isoform in skeletal muscle of the developing mouse embryo [15]. At embryonic day 14.5 α -cardiac actin is expressed at a ratio of 1.6/1.0 with skeletal actin and represents the predominant developmental sarcomeric actin. Interestingly, β -actin is also expressed at significant levels at this time point (1.0 α -skeletal actin:1.2 β -actin) [15]. In contrast, in postnatal murine skeletal muscle (12.5 days after birth) α -skeletal actin is the major sarcomeric actin isoform (4.5 α -skeletal actin: 1.0 cardiac actin) [15]. These findings are supported by studies in the α -skeletal actin knock-out mice [16] that survive until birth and are initially indistinguishable from their normal littermates. Analysis of skeletal muscle samples from newborn *ACTA1*^{-/-} mice demonstrates normal sarcomere formation. However, *ACTA1*^{-/-} mice deteriorate soon after at birth (when cardiac actin expression is 'switched off') and die by nine days of age.

α -Skeletal actin is reported to be the predominant sarcomeric actin isoform expressed in adult human heart by RNA analysis [1], and yet, surprisingly, the heart is rarely affected in NM patients with mutations in *ACTA1* [17]. None of the NM patients in our series had cardiac dysfunction clinically, by echocardiography or, in one case, at autopsy [9,11].

Accurate definition of the developmental expression of the sarcomeric actins, and of the relative levels of the different isoforms in different tissues, is crucial to understanding the pathogenetic mechanisms underlying the skeletal myopathies and cardiomyopathies associated with mutations in the α -cardiac and α -skeletal actin genes. This data is not available for human skeletal and cardiac muscle. To date, defining the relative levels of α -cardiac and α -skeletal actin expression in skeletal muscle has been performed in mice [18], rat [19] chicken [20], pig [21], bovine [21], cultured muscle cells [22] and humans [21,23]. Most studies have looked at RNA rather than protein levels, as the only antibodies previously available recognised two or more actin isoforms. Separation of the two sarcomeric actin isoforms is difficult as α -cardiac and α -skeletal actin are highly homologous and share the same molecular weight, isoelectric point and only differ by four amino acids. Recently, isoform-specific α -skeletal [24] and α -cardiac [25] actin antibodies have been developed. On the basis of clinical observations made in NM patients and the current knowledge of sarcomeric actin expression profiles, we sought to determine the levels of protein expression of the α -cardiac and α -skeletal actin isoforms in rodent and human cardiac and skeletal muscle, and in human skeletal muscle during development.

2. Methods

2.1. Ascertainment of skeletal muscle and heart samples

We studied archived frozen human fetal skeletal muscle biopsies from fetuses delivered at 14, 16, 19, and 22 weeks gestation. All specimens were de-identified in accordance with hospital ethical guidelines. In addition we studied skeletal muscle biopsies from individuals of the following ages: 25, 27, 28, 36, 37, 38 and 41 weeks gestation, 7 days, 8 days, 25 days, 6 months, 5 years, 28 years and 38 years. All of the samples were from patients with no known form of neuromuscular disease and showed normal muscle histology. Human ventricular myocardium was obtained from three patients with structural, congenital heart disease at 2, 5 and 11 years of age (i.e. for repair of tetralogy of Fallot). The samples were obtained while the patient was on cardiopulmonary bypass after cardioplegic arrest of the heart. Ventricular myocardium samples were also obtained from two donor hearts at 10 and 23 years of age that were not used for transplantation and approved for research use. The human heart samples were de-identified according to

the Children's Hospital at Westmead ethical guidelines and kindly provided by Dr David Winlaw. For comparison, samples from normal mouse hearts at six months of age, adult rat heart and mouse skeletal muscle at postnatal day 7 and six months of age were used. These samples were kindly provided by Dr Edna Hardeman at the Children's Medical Research Institute in accordance with ethical guidelines.

2.2. Western Blot analysis

One 8 μm frozen muscle biopsy section, measuring approximately 10 mm^2 was resuspended in 15 μl of 2% SDS, 62.5 mM Tris pH 6.8, 10% glycerol, 50 mM DTT, and protease inhibitor cocktail (1:500 dilution) (Sigma), sonicated, heat inactivated at 94 $^{\circ}\text{C}$ for 4 min and solubilised on ice for \sim 20 min. The samples were separated through a discontinuous polyacrylamide gel (5%/9%) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed as described [26]. The PVDF membranes were immunoblotted using a polyclonal anti- α -skeletal actin specific antibody (anti-SKA @1:2000) [24] and a monoclonal anti- α -cardiac actin (anti-CA @1:1500) (American Research Products) specific antibody prepared in 5% skim milk powder and phosphate buffered saline with 0.1% tween 20 (PBST) and incubated for 2–3 h at room temperature (RT). To demonstrate equal loading for total sarcomeric actin, the PVDF membranes were re-probed with the α -sarcomeric actin antibody (5c5 antibody @ 1:2000, Sigma, which recognises both α -skeletal and α -cardiac actin isoforms).

2.3. Immunohistochemistry

Frozen muscle biopsy sections (8 μm thickness) were fixed in 3% paraformaldehyde for 3 min at RT, followed by extraction in methanol at -20 $^{\circ}\text{C}$ for 5 min. The samples were washed three times in either PBS (anti-SKA antibody) or PBS containing 0.5 M NaCl (anti-CA antibody), blocked in blocking buffer (2% bovine serum albumin prepared in PBS) for 10 min and then incubated with anti-SKA (1:100) or anti-CA (1:250) diluted in blocking buffer for 1–2 h at RT. The samples were washed and blocked as described above and incubated with either a CY3-conjugated donkey anti-mouse IgG (1:250) or CY3-conjugated donkey anti-rabbit IgG (1:250) for 1 h at RT. Samples were washed three times in PBS and mounted with glass coverslips using FluorosaveTM mounting reagent (Calbiochem). Confocal microscopy was performed using a Leica SP2 Scanning Lase Confocal Microscope.

3. Results

3.1. α -Cardiac actin is the major isoform expressed in human heart muscle

RNA studies performed in rodents have shown that α -cardiac actin is the prevalent isoform in heart whereas α -skeletal actin is the major isoform in adult skeletal muscle [21,27,28]. In this study we examined the protein expression of the sarcomeric actin isoforms in mouse and rat skeletal and heart tissue. α -Skeletal actin is the major sarcomeric actin isoform expressed in postnatal (day 7) and adult (six-

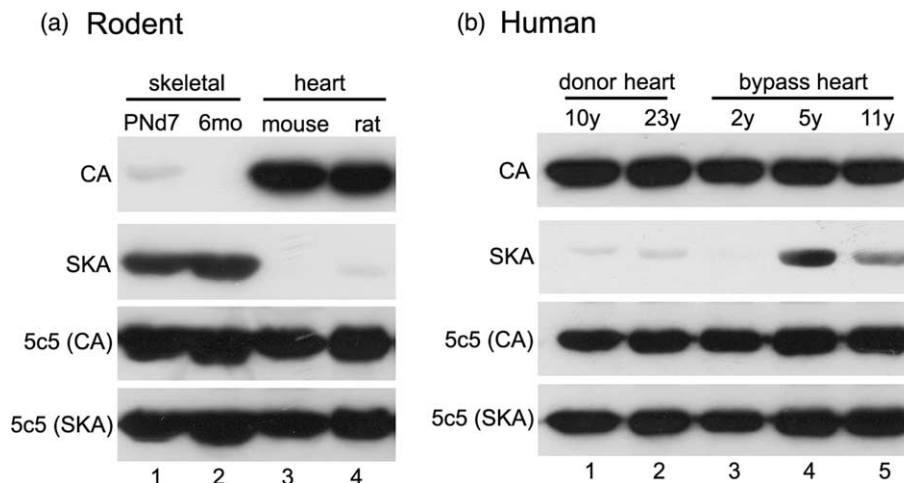


Fig. 1. Expression of α -cardiac and α -skeletal actin in heart and skeletal muscle. Protein extracts were prepared from the following tissues: (a) mouse skeletal muscle at postnatal day 7 (PNd7, lane 1) and 6 months (lane 2), adult mouse heart (6 months, lane 3), adult rat heart (1 month, lane 4) and (b) control human donor hearts at 10 years (y) and 23 y (lanes 1 and 2), and diseased hearts which have undergone bypass surgery at 2 y (lane 3), 5 y (lane 4) and 11 y (lane 5). The protein samples were separated by SDS-PAGE and the membranes probed with α -cardiac (CA) and α -skeletal (SKA) actin antibodies. To determine protein loading for total sarcomeric actin, each membrane was re-probed with a sarcomeric actin antibody (5c5, Sigma). (a) α -Skeletal actin is the predominant isoform in mouse skeletal muscle (SKA, lanes 1 and 2) whereas α -cardiac actin is the major isoform in murine heart (CA, lanes 3 and 4). (b) α -Cardiac actin is the major isoform in control (donor) human heart. There are increased levels of α -skeletal actin in diseased hearts undergoing bypass (SKA, lanes 4 and 5) compared with control hearts (SKA, lanes 1 and 2).

month old) mouse skeletal muscle (Fig. 1a, lanes 1 and 2). A faint immunoreactive band is detected for cardiac actin at postnatal day 7 (lane 1) but not in adult mouse skeletal muscle even with long exposures (lane 2). The postnatal down-regulation of the cardiac actin isoform is in accordance with previous RNA based studies [27,29]. α -Cardiac actin is the major sarcomeric actin isoform in adult mouse and rat heart with absent or low levels of α -skeletal actin (Fig. 1a, lanes 3 and 4). Fig. 1a also demonstrates the specificity of the α -skeletal (SKA) and α -cardiac actin (CA) antibodies, which only detect the appropriate sarcomeric actin isoform.

A previous RNA study performed in human heart samples showed that α -skeletal actin is the prevalent isoform in the heart [1]. However, NM patients with mutations in α -skeletal actin do not exhibit a cardiac phenotype. Hence, we sought to determine the protein expression of the sarcomeric actins in cardiac muscle using protein extracts prepared from control (donor) human hearts. For comparison, we have also shown data from human heart samples from patients with heart disease who had undergone bypass surgery (Fig. 1b). α -Cardiac actin is the major sarcomeric actin isoform in human donor heart samples with low relative levels of α -skeletal actin (Fig. 1b, CA, lanes 1 and 2). Increased levels of α -skeletal actin were observed in two diseased hearts, compared to control samples (Fig. 1b, SKA, compare lanes 4 and 5 with lanes 1 and 2). One of the diseased hearts in this study (Fig. 1b, lane 3) did not express elevated levels of α -skeletal actin but showed similar levels to that observed in control hearts. The up-regulation of α -skeletal actin in diseased hearts is consistent with a previous study [30]. Suurmeijer et al. [30], also showed that the amount of α -skeletal actin in healthy human hearts varied markedly in different locations of the myocardium at the protein level using the same α -skeletal actin specific antibody (anti-SKA).

3.2. Skeletal actin protein is expressed throughout human development and is the prevalent isoform from late gestation to adulthood

We characterised the expression of skeletal and cardiac actin proteins in human skeletal muscle from early gestation to adulthood by Western blotting and immunohistochemistry (Figs. 2 and 3). These studies represent analysis of a single biopsy/autopsy specimen from control patients, with normal muscle histology and no form of neuromuscular disease. In early gestation (14–19 weeks) cardiac actin is the predominant sarcomeric actin isoform in skeletal muscle (Fig. 2a). However, skeletal actin is readily detected as early as 14 weeks gestation, and is expressed strongly by the end of the second trimester. The expression of α -cardiac actin is down-regulated around birth, and is undetectable from 6 months of age to adulthood.

To more accurately define the period of cardiac actin down-regulation, we examined nine skeletal muscle

samples from 27 weeks gestation to one month postnatal (Fig. 2b). In all nine samples, skeletal actin was the predominant sarcomeric actin isoform, with only low levels of cardiac actin detected. Hence, α -skeletal actin becomes the major sarcomeric actin isoform at the end of the second trimester (between 25 and 27 weeks gestation in this study) and is the predominant sarcomeric actin isoform in postnatal human muscle.

These results are supported by immunohistochemical staining for α -skeletal and α -cardiac actin in frozen

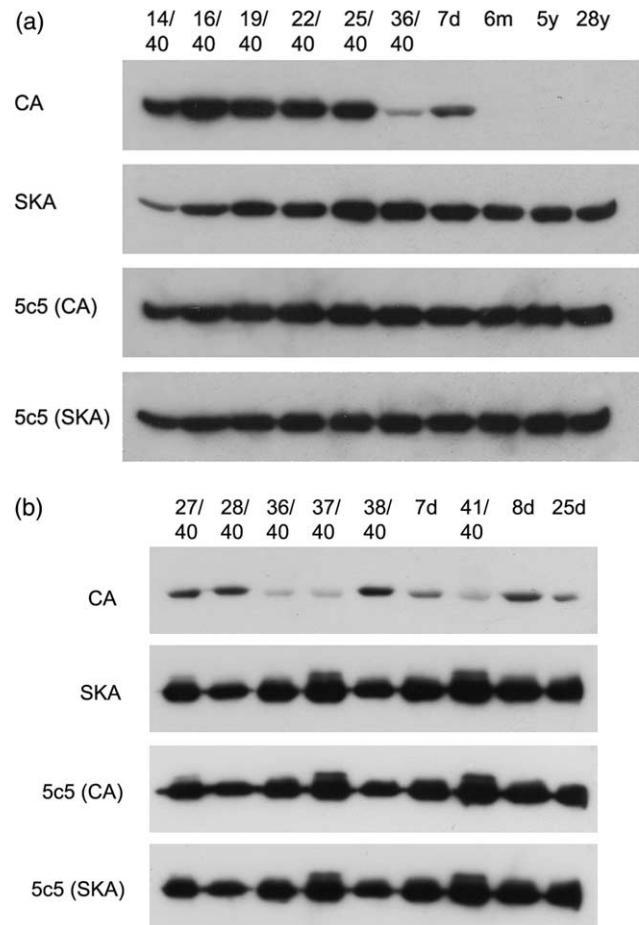


Fig. 2. Protein expression of α -skeletal and α -cardiac actin during human skeletal muscle development. (a) Protein extracts from control frozen muscle biopsies ranging in age from 14 weeks gestation (14/40) to 28 years (y) were prepared and separated on duplicate SDS-PAGE gels. The PVDF membranes were probed with antibodies recognising α -cardiac (CA) or α -skeletal (SKA) actin. Equal loading for sarcomeric actin was determined by re-probing the PVDF membranes with a total α -sarcomeric actin antibody (5c5, Sigma, recognises both α -cardiac and α -skeletal isoforms). α -Cardiac actin is expressed throughout embryonic development until birth (36/40 sample) where expression is markedly downregulated. α -Cardiac actin is not detected in skeletal muscle at 6 months (m), 5 years (y) and in adulthood (28 y sample). α -Skeletal actin is expressed during embryonic development, the neonatal period and throughout adulthood. (b) Analysis of nine skeletal muscle samples from 27 weeks gestation to one month postnatal demonstrates that down-regulation of cardiac actin occurs around the third trimester of pregnancy (between 25 and 27 weeks gestation) when α -skeletal actin increases, becoming the predominant sarcomeric actin isoform in postnatal human muscle.

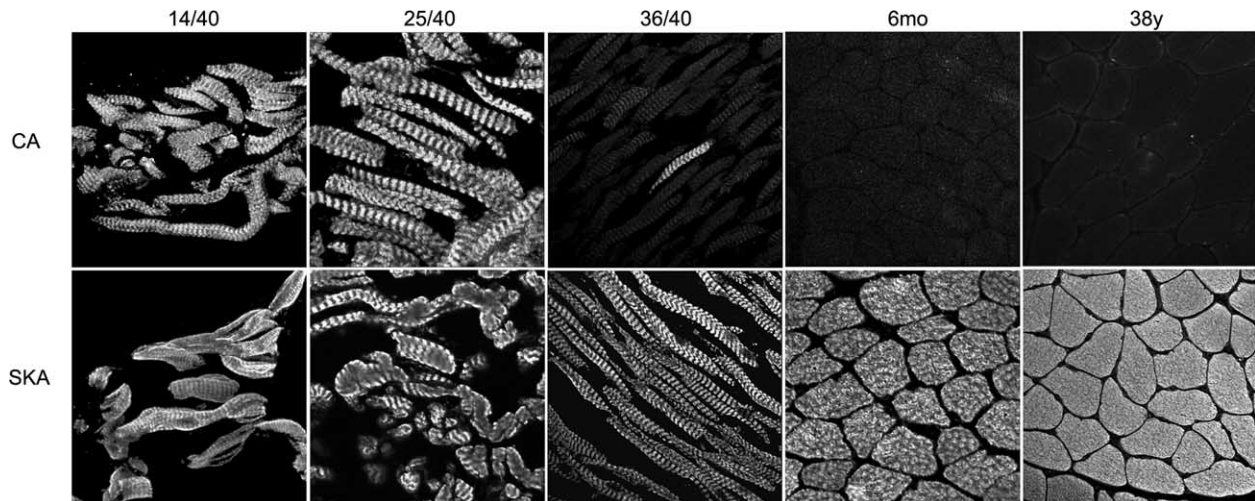


Fig. 3. Localisation of α -skeletal and α -cardiac actin in human skeletal muscle development. Frozen muscle cryosections (8 μ m) were stained with antibodies recognising α -cardiac actin (CA) and α -skeletal actin (SKA), followed by a CY3-conjugated secondary antibody. Cardiac actin showed striated labelling in all fibres of biopsy samples at 14, 16, 19, 22 and 25 weeks gestation (representative images are shown for 14 and 25 weeks gestation samples only, 100 \times mag, 2 \times zoom). At 36 weeks gestation and 7 days after birth <1% of fibres show positive staining for cardiac actin (shown for 36 week sample only, 100 \times mag), with absent staining detected at 6 months (100 \times mag, 1.37 \times zoom) and in adult muscle (shown for 38 years, 40 \times mag). Skeletal actin shows striated staining in all gestational samples and homogenous staining in 6 month and adult samples due to cross-sectional orientation. Confocal microscopy was performed using a Leica SP2 Scanning Laser Confocal Microscope using 100 \times (HCX PL APO 1.40) and 40 \times (HCX PL APO 1.25) oil lenses. Mag, magnification; mo, month, y, year.

cryosections of skeletal muscle samples taken at 14 weeks gestation though to adulthood (Fig. 3). Immunohistochemical analysis of muscle biopsies at gestational age 14, 16, 19, 22 and 25 weeks showed a striated staining pattern in all fibres with the cardiac actin antibody (Fig. 3, shown for samples 14 and 25 weeks). At 36 weeks gestation, <1% of fibres showed positive staining for α -cardiac actin, with similar results observed in samples obtained at 37 and 38 weeks gestation (not shown). α -Cardiac actin staining was undetectable by immunohistochemistry in skeletal muscle biopsies at 6 months, 5, 28 and 38 years of age (Fig. 3, shown for 6 months and 38 years). Staining with the α -skeletal actin antibody demonstrated a striated staining pattern in all samples from 14 weeks gestation to adulthood. A uniform homogenous staining pattern is observed in the 6 month and 38 years sample due to the cross-sectional orientation of the muscle biopsy. Striated labelling was observed in some fibres but this was dependent on the orientation of muscle biopsy.

4. Discussion

This study was prompted by clinical observations in patients with NM that were difficult to explain based on current knowledge of the expression of sarcomeric actins during development and the relative levels of α -skeletal and α -cardiac actin in postnatal cardiac and skeletal muscle. We have previously demonstrated that mutant α -skeletal actin exerts a dominant negative effect in skeletal muscle [11]. However even patients with severe lethal forms of NM

usually survive until birth and exhibit an unremarkable gestation with decreased foetal movement reported only just prior to birth. This suggests that co-expression of cardiac actin in skeletal muscle during development reduces the deleterious effect exerted by the mutant skeletal actin isoform. Secondly, cardiac abnormalities are rarely observed in NM. In a large series of NM patients, congestive heart failure in first months of life was reported in only six of the 143 patients, and in most of these cases the cardiac decompensation was thought to be secondary to respiratory insufficiency [31]. This suggests that mutant α -skeletal actin is not expressed at sufficient levels in cardiac muscle to affect its function. This is in agreement with previous RNA-based studies performed in adult mouse [18], pig [21] and bovine [21] heart which showed that α -cardiac actin is the predominant sarcomeric actin isoform.

Our study was made possible due to the generation of α -cardiac actin [25] and α -skeletal actin [24] isoform-specific antibodies. We confirmed the specificity of these antibodies by Western analysis in skeletal and cardiac tissue. Analysis of control (donor) heart samples showed predominant expression of the α -cardiac actin isoform with low or undetectable levels of α -skeletal actin in adult human, mouse and rat hearts and postnatal day 7 mouse heart. This is consistent with previous studies which have shown that cardiac actin is the prevalent isoform in postnatal and adult mouse hearts at the RNA level [27–29]. However, previous RNA based studies of the relative expression of the sarcomeric actins in human heart report variable results. Vandekchove et al. [21] demonstrate α -cardiac actin is the predominant sarcomeric actin in the

heart, while other studies report α -skeletal actin predominates [1], or that the two sarcomeric actins are expressed at equal levels (in heart samples from a patient undergoing bypass [23]). The comprehensive analysis by Boheler and colleagues examined 21 control human hearts; including fetal (13–29 weeks gestation), paediatric (3 months to 12 years) and adult (17–40 years) heart samples, and demonstrate that transcripts encoding α -skeletal actin account for ~60% of the total sarcomeric actin pool [1]. The discrepancy between these results and those from our study may reflect posttranscriptional regulation of sarcomeric actin mRNA isoforms, and suggest that mRNA expression profiles may not necessarily reflect protein expression levels.

While mutations in α -cardiac actin result in dilated cardiomyopathy [4] and hypertrophic cardiomyopathy [5], none of the NM patients with mutations in α -skeletal actin have clinically detectable abnormalities of cardiac muscle contractility. Our data, demonstrating that α -skeletal actin is expressed at extremely low or undetectable levels in the postnatal human heart is consistent with the observation that mutations in *ACTA1* do not usually result in a clinically significant cardiomyopathy and suggest that expression of α -cardiac actin and the presence of one normal *ACTA1* allele is sufficient for normal cardiac-muscle function.

We show that α -cardiac actin is the predominant sarcomeric actin isoform expressed in early human development and is down-regulated in the third trimester of pregnancy (27–28 weeks). α -Skeletal actin becomes the predominant sarcomeric actin isoform around 27–28 weeks, and is maintained as the predominant postnatal sarcomeric actin isoform from birth to adulthood. These results are consistent with the clinical presentation of lethally affected NM patients, who exhibit an unremarkable gestation with decreased fetal movement noted only in the last few weeks of pregnancy. At birth, when skeletal actin is the prevalent isoform in skeletal muscle, the majority of patients exhibit signs of muscle weakness including limited anti-gravity movement with respiratory, feeding and swallowing difficulties.

We have examined sarcomeric protein expression profiles in muscle biopsies from NM patients with characterised mutations in *ACTA1* [11]. In these patients, the levels of α -skeletal actin in all NM patients did not differ significantly from age-matched controls. However, elevated levels of α -cardiac actin were observed in severely affected NM neonates, but not milder affected NM patients by immunohistochemistry [9] and Western blotting [11]. This may reflect compensatory up-regulation of α -cardiac actin, although it may also just be a consequence of altered maturation and fibre regeneration.

In *ACTA1* knockout mice and in humans with NM, the ‘switching off’ of α -cardiac actin in skeletal muscle is concomitant with deterioration in muscle strength. In addition, high levels of α -cardiac actin relative to α -skeletal actin likely protect the heart from the development of cardiomyopathy. The definition of actin isoform expression

during development in cardiac and skeletal muscle helps us to better understand the pathogenesis of inherited myopathies and cardiomyopathies associated with mutations in actin genes. More importantly, these studies suggest that manipulation of the expression of other actin isoforms may be able to be used in the therapy of these actin disorders.

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