Mouse models of dominant ACTA1 disease recapitulate human disease and provide insight into therapies

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Mutations in the skeletal muscle α-actin gene (ACTA1) cause a range of pathologically defined congenital myopathies. Most patients have dominant mutations and experience severe skeletal muscle weakness, dying within one year of birth. To determine mutant ACTA1 pathobiology, transgenic mice expressing ACTA1(D286G) were created. These Tg(ACTA1)D286G mice were less active than wild-type individuals. Their skeletal muscles were significantly weaker by in vitro analyses and showed various pathological lesions reminiscent of human patients, however they had a normal lifespan. Mass spectrometry revealed skeletal muscles from Tg(ACTA1)D286G mice contained ~25% ACTA1(D286G) protein. Tg(ACTA1)D286G mice were crossed with hemizygous Acta1+/- knock-out mice to generate Tg(ACTA1)D286G+/-, Acta1+/- offspring that were homozygous for the transgene and hemizygous for the endogenous skeletal muscle α-actin gene. Akin to most human patients, skeletal muscles from these offspring contained approximately equal proportions of ACTA1(D286G) and wild-type actin. Strikingly, the majority of these mice presented with severe immobility between postnatal Days 8 and 17, requiring euthanasia. Their skeletal muscles contained extensive structural abnormalities as identified in severely affected human patients, including nemaline bodies, actin accumulations and widespread sarcomeric disarray. Therefore we have created valuable mouse models, one of mild dominant ACTA1 disease [Tg(ACTA1)D286G], and the other of severe disease, with a dramatically shortened lifespan [Tg(ACTA1)D286G+/-, Acta1+/-]. The correlation between mutant ACTA1 protein load and disease severity parallels effects in ACTA1 families and suggests altering this ratio in patient muscle may be a therapy for patients with dominant ACTA1 disease. Furthermore, ringbinden fibres were observed in these mouse models. The presence of such features suggests that perhaps patients with ringbinden of unknown genetic origin should be considered for ACTA1 mutation screening. This is the first experimental, as opposed to observational, evidence that mutant protein load determines the severity of ACTA1 disease.
Introduction

Humans express six different actin proteins, each encoded by separate genes. Human disease is known to result from mutations in five of the six actin genes (Olson et al., 1998; Nowak et al., 1999; Nuno et al., 1999; Zhu et al., 2003; Guo et al., 2007), with the tissues affected dependent on where the mutant actin gene is normally expressed, e.g. heart diseases are caused by mutations within the cardiac actin (ACTC) gene. Enteric γ-actin is the only actin gene not presently associated with disease, however this may simply be due to patients with the resulting disease phenotype not having been screened for defects in this gene. Mutations were first described in the skeletal muscle α-actin (ACTA1) gene in 1999 (Nowak et al., 1999), and to date over 180 different ACTA1 mutations have been described (Laing et al., 2009; http://www.waimr.uwa.edu.au/research/lovd.html).

Skeletal muscle α-actin is the predominant actin isoform in postnatal skeletal muscles but is also expressed at lower levels in the postnatal heart (Ilkovski et al., 2005; Ravenscroft et al., 2008; Copeland et al., 2010). Patients with ACTA1 mutations present with congenital myopathies, which are almost always restricted to their skeletal musculature. Congenital myopathies are a heterogeneous group of skeletal muscle diseases usually present at birth, characterized by weakness and pathology of skeletal muscles. Although a range of clinical severities exist for patients with ACTA1 mutations, the vast majority are affected at birth by severe hypotonia and respiratory insufficiency, dying within the first year of life (Laing et al., 2009), however cases of childhood or adult onset with slow progression do occur (Jungbluth et al., 2001; Agrawal et al., 2004). No effective treatment for patients currently exists, though supportive interventions such as mechanical ventilation and naso-gastric feeding are highly beneficial (Wallgren-Pettersson et al., 2004).

As ACTA1 patients cannot be determined by clinical presentation alone, patients are categorized by the predominant structural lesions evident within their skeletal muscles. These lesions include actin accumulation (Nowak et al., 1999), caps (Hung et al., 2010), cores (Kaindl et al., 2004), core-like areas and rods (Jungbluth et al., 2001), fibre type disproportion (Laing et al., 2004), intranuclear rods (Koy et al., 2007), nemaline bodies (Nowak et al., 1999) and zebra bodies (Sewry et al., 2009). Some patients are reported to have exclusive presentation of only one of these features, whereas others have multiple features (e.g. Ravenscroft et al., 2010). The perspective that these morphological changes may exist as a continuous spectrum, with each patient displaying a portion of the spectrum, has gained considerable momentum in recent years (Sewry et al., 2008). Many of these abnormalities can also occur due to mutations within other genes, and as such genetic diagnosis can be complicated.

Only ~10% of ACTA1 mutations are recessive, with most cases of recessive disease resulting from homozygous genetic null mutations, meaning no skeletal muscle α-actin protein is produced, and the remaining recessive mutations being functional nulls (Nowak et al., 2007; Laing et al., 2009). An Acta1 knockout mouse model exists that recapitulates recessive ACTA1 disease, with complete absence of skeletal muscle α-actin protein leading to death within 9 days after birth (Crawford et al., 2002). However the majority of ACTA1 mutations are de novo dominant amino acid substitutions, and these are thought to cause disease due to the resulting mutant ACTA1 protein interfering with normal skeletal muscle function (Sparrow et al., 2003). Though there have been investigations of recombinant mutant actin proteins and mutagenesis studies in tissue culture (reviewed in Ilkovski et al., 2008), and studies in lower order organisms [e.g. yeast (Wen and Rubenstein, 2003) and Drosophila melanogaster (Haigh et al., 2010)] there are a lack of data from a mammalian model (Nguyen and Hardeman, 2008). Therefore, we sought to generate a mouse model of dominant ACTA1 disease, in order to allow detailed and comprehensive investigation of disease mechanisms, and importantly, evaluation of potential treatments.

The ACTA1(D286G) mutation utilized to generate this transgenic mouse model was identified in an isolated patient with severe nemaline myopathy. This patient had no movement or respiratory effort at birth and subsequently died at 9 days of age (Agrawal et al., 2004; Alan Beggs, personal communication). Actins are a highly conserved protein family with 89% identity between cytoskeletal actin in yeast and β-actin in humans (Sheterline et al., 1995) and the D286 residue is completely conserved. D286 has been directly implicated in filamentous-actin subunit–subunit interactions through its involvement in the formation of a salt bridge with R39 and E270 of neighbouring actin monomers (Sheterline et al., 1995). In yeast the ACT1 D286A substitution is lethal (Wertman et al., 1992). However, in vitro, the ACTA1(D286G) mutation does not appear to produce any defects in stability, folding or copolymerization (Costa et al., 2004), although a Myc-tagged ACTA1(D286G) fusion protein incorporates into abnormal ‘wavy’ stress fibres and cytoplasmic aggregates in NIH3T3 fibroblasts (Costa et al., 2004).

Our current study demonstrates that expression of the ACTA1(D286G) mutation in mouse skeletal muscles creates mouse models of mild and severe human ACTA1 disease. When Tg(ACTA1)D286G/+ mice express the mutant ACTA1(D286G) protein at ~25% of the total striated α-actin pool they exhibit a range of abnormal features within their skeletal muscle; the muscle is weak and mice are less active, yet, like mildly affected patients, show no overt phenotype and have a normal lifespan. Contrastingly, Tg(ACTA1)D286G/−/+ Acta1+/− mice, in which the dose of mutant ACTA1(D286G) is increased to ~45% of the total striated α-actin pool, display more dramatic skeletal muscle weakness, striking pathological features, and in strong parallel to most ACTA1 disease patients who have a severe disease phenotype, early lethality. Significantly, these models allow for longitudinal studies of disease pathogenesis, detailed investigation into the relationship between dosage of mutant ACTA1 proteins and disease...
severity and crucially can act as a test-bed for appraisal of various therapies.

Materials and methods
Generation and screening of transgenic lines
All animal procedures were approved by the Animal Experimentation Ethics Committee of The University of Western Australia. Transgenic mice were generated to express the ACTA1 protein containing the D286G mutation in their skeletal muscles. The human ACTA1 complementary DNA sequence was amplified from human skeletal muscle complementary DNA, reverse transcribed from RNA extracted from control human skeletal muscle. The D286G mutation was introduced by site-directed mutagenesis (Stratagene) and was confirmed by sequencing. Expression of the ACTA1(D286G) complementary DNA sequence was driven by a 2.2 kb fragment of the human skeletal muscle α-actin promoter (Brennan and Hardeman, 1993) and the 157 base pair slow human troponin I enhancer (Crawford et al., 1994), along with a 3 kb fragment of the human skeletal muscle Tg(ACTA1)D286G + / + .Acta1+/ – bodies [myosin heavy chain (MHC)I diluted 1:10, Novocastra; MHCIIA by standard techniques to create the transgenic mouse line Tg(α-actin)-actin promoter (Brennan and Hardeman, 1993) and was confirmed by sequencing. Expression of the ACTA1(D286G) complementary DNA sequence was driven by a 2.2 kb fragment of the human skeletal muscle α-actin promoter (Brennan and Hardeman, 1993) and the 157 base pair slow human troponin I enhancer (Corin et al., 1994), along with a 3 kb fragment of the human skeletal muscle α-actin 3’ UTR (all courtesy of Professor Edna Hardeman). The expression construct was used for injection into CBA x C57BL/6 mouse embryos by standard techniques to create the transgenic mouse line Tg(α-actin)-actin promoter. Positive offspring were detected by polymerase chain reaction on tail-tip DNA using primers designed against the human skeletal muscle α-actin promoter region.

Hemizygous skeletal muscle α-actin knock-out mice (Acta1+/–; Crawford et al., 2002) were bred with Tg(α-actin)-actin promoter to generate Tg(α-actin)-actin promoter offspring after a number of generations.

Histology and immunohistochemistry
Freshly excised skeletal muscles were frozen in optimum cutting temperature medium using liquid nitrogen cooled isopentane. Gomori tri-chrome staining was performed on 8–10 μm sections, cut on a Leica Jung CM cryocut 1800, according to standard procedures (Dubowitz and Sewry, 2007).

For fibre typing, sections were fixed, blocked and labelled as described previously (Nowak et al., 2009). Briefly, mouse IgG1 antibodies [myosin heavy chain (MHC)I diluted 1:10, Novocasta; MHCIIA diluted 1:5, DSMZ] were conjugated to Zenon®-XX-Biotin (Invitrogen). Primary mouse IgG1 antibodies conjugated to Zenon®-XX-Biotin and mouse IgM antibodies (MHCIIb diluted 1:1, DSMZ; MHCIIx diluted 1:3, a gift from J. Hoh) were then added to sections and incubated overnight at 4°C. Secondary antibody (anti-mouse IgM-biotin) and streptavidin-horseradish peroxidase were sequentially incubated for 1:20, Abcam) were conjugated to Zenon® AlexaFluor® (Invitrogen; either 350, 488 or 594), diluted in the foetal calf serum/bovine serum albumin solution and incubated overnight at 4°C. After three 5-min washes in phosphate buffered saline, the sections were mounted in Hydromount (National Diagnostics).

Muscle sections were stained following fixation with 2% paraformaldehyde with 1:1000 dilution of phalloidin-fluorescein isothiocyanate to label filamentous actin.

Electron microscopy
Skeletal muscles were removed and immediately cut into very thin strips before immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Samples were post-fixed with 2% osmium tetroxide for 30 min, dehydrated through an ascending series of ethanol, embedded in Spurr’s epoxy resin and cured for at least 3 days at 70°C. Ultra thin sections (70 nm) were cut with an LKB 8800 ultramicrotome and grids viewed and photographed with a JEOL 2100 transmission electron microscope and 11 megapixel Oriel digital camera.

Liquid chromatography mass spectrometry-multiple reaction monitoring
Samples were prepared and run on 4–15% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis gels and then Comassie stained. The actin gel bands were excised and destained with 50 ml of 25 mM ammonium bicarbonate (Sigma) in 50% acetonitrile (Merck) for 45 min at 37°C. The gel pieces were dried and resuspended in dithiothreitol (10 mM, Sigma) for 15 min at 56°C. Then 50 ml of iodoacetamide (50 mM, Sigma) was added and incubated in the dark at room temperature for 20 min. The gel pieces were dried and a 100 mg/ml solution of chymotrypsin (Roche) in 5 mM ammonium bicarbonate was added with incubation for 16 h at 37°C. To each sample was added 10 ml of acetonitrile with 1% formic acid (Fluka) and the solution kept at room temperature for 20 min. The solution was removed to a fresh vial and a further 10 ml of acetonitrile with 1% formic acid was added to the gel pieces with incubation at room temperature for 20 min and then combined with the first extraction solution before drying.

Dried samples were resuspended in 20 ml of 2% acetonitrile (0.05% trifluoroacetic acid, Fluka) with 10 ml injected into an Ultimate 3000 nano-high performance liquid chromatography system (Dionex). The peptides were eluted with a gradient of 10–40% acetonitrile (0.1% formic acid) over 45 min through a Dionex C18 PepMap 100 column (3 mm, 100 Å, 75 μm ID × 15 cm) at 300nl/min into the mass spectrometer via a PicoTip emitter (FS360-75-15-N-5-C15, New Objective, Woburn, MA, USA).

A 4000 Q TRAP triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) was interfaced with a nanospray source. Multiple reaction monitoring transitions for the carbamidomethyl modified (asterisks) peptides were as follows:

(i) D286 wild-type, transition 950.1/1150.6, NSIMKC*DIRKDLY, y9 ion;
(ii) D286 wild-type, transition 950.1/1310.6, NSIMKC*DIRKDLY, y10 ion;
(iii) D286 wild-type, transition 950.1/1438.7, NSIMKC*DIRKDLY, y11 ion;
(iv) G286 mutant, transition 921.5/1092.6, NSIMKC*GIDIRKDLY, y9 ion;
(v) G286 mutant, transition 921.5/1252.6, NSIMKC*GIDIRKDLY, y10 ion;
(vi) G286 mutant, transition 921.5/1380.7, NSIMKC*GIDIRKDLY, y11 ion;
(vii) common reference peptide, transition 844.1/836.4, ELPDGQVITIGNERF, y7 ion;
(viii) common reference peptide, transition 844.1/949.5, ELPDGQVITIGNERF, y8 ion; and
(ix) common reference peptide, transition 844.1/1048.4, ELPDGQVITIGNERF, y9 ion.

Source temperature was set at 150°C, source voltage at 2650 V and collision energy at 45 V. The declustering potential was set at 50 V, exit potential to 10 V, collision cell exit potential to 10 V, the resolution for Q1 and Q3 to low and the time for all transitions was kept at 120 ms. For quantitative measurement, the area under the curve was calculated for all transitions using the quantization module of Analyst 1.5.1 (Applied Biosystems).

Analysis was conducted by determining the ratio of the area under the curve for the D286 wild-type peptide transitions and G286 mutant transitions relative to the common peptide. The percentage of mutant actin was then calculated by dividing the ratio of the mutant specific transitions to the common peptide transition by the sum of the ratios between the D286 and G286 actins (e.g. each of the wild-type and mutant specific daughter ion transitions) to the common peptide transition.

Skinned fibre analysis

Analysis of the contractility of mechanically-skinned extensor digitorum longus (EDL) fibres from 3- and 6-month old Tg(ACTA1)D286G and wild-type mice was performed as previously described (Nowak et al., 2009).

Whole muscle contractility

The contractile properties of excised EDL and soleus muscles from 1-month-old male Tg(ACTA1)D286G and wild-type mice were examined using an Intact Muscle Test System (Aurora Scientific Inc.) as previously described (Nowak et al., 2009). Twitch force (force developed in response to a single action potential) and tetanic force response elicited in response to trains of stimulation pulses at different frequencies (10, 20, 40, 60, 80, 100 and 120 Hz) were recorded. A 3 min interval was allowed between trains of stimuli, to prevent fatigue. The tetanic responses were normalized to percentage of maximal force to examine the specific effects of stimulation frequency on force production.

Force per muscle cross-sectional area (specific force) was determined using the method of Brooks and Faulkner (1988). At completion of functional testing, muscles were trimmed, blotted and weighed to determine muscle mass. Muscle cross-sectional area was determined by dividing muscle mass by the product of fibre length and the density of mammalian skeletal muscle (1.06 mg/mm^3; Mendez and Keys, 1960). Fibre length was calculated by multiplying the optimal muscle length by a previously determined EDL fibre length-to-optimal muscle length ratio of 0.45 and soleus fibre length-to-optimal muscle length ratio of 0.71 (Brooks and Faulkner, 1988). The maximum specific forces and twitch-specific forces were then determined using the formula described in Plant et al., 2005.

Accelerating Rota-rod test

Eight-month old wild-type and Tg(ACTA1)D286G mice were trained to stay on the Rota-rod by undergoing three 2 min training trials, with a 15 min break between trials. In the first two trials, the Rota-rod was set to rotate at a constant speed of 4 rpm. For the third trial, the Rota-rod operated at an accelerating speed from 4 rpm to a maximum of 40 rpm. The test consisted of two 2-min and a final 5-min trial, all of which were at an accelerating speed (4–40 rpm over 5 min), with a 5 min break between tests. The latency to fall was measured for each trial and the data are expressed as the percentage of the total duration of each test.

Locomotor activity test

Spontaneous activity of 8-month old wild-type and Tg(ACTA1)D286G mice was measured during a 30 min interval in a novel environment. This test was conducted in the Tru-scan locomotor system that consisted of a plexiglass cage with two sensors to detect movement in both the horizontal and vertical planes. A single mouse was placed in each locomotor cell and recordings were made every 500 ms for a 30 min period. Parameters measured included time spent moving, distance moved and the number of entries into the vertical plane (rearing).

Grip-strength test

Eight-month old wild-type and Tg(ACTA1)D286G mice were allowed to grasp the grid with forelimbs and pulled gently until they released the grid. The maximum amount of force exerted (g) was recorded. This was repeated five times and the average reading calculated. Mice were then allowed to grasp the grip with all limbs. Again, the maximum amount of force exerted was recorded. This was repeated five times with a 30 s interval between trials and the average reading calculated.

Voluntary running wheel experiments

One- and 4-month old wild-type and Tg(ACTA1)D286G mice were housed singularly in cages containing a standard mouse running wheel connected to a speedometer that recorded distance travelled, time spent moving, average and maximum speeds. Activity was monitored every 24 h over a 7 day period and the average distance travelled per day calculated.

Statistics

GraphPad Prism 4 was used to conduct two-tailed t-tests with Welch’s correction for all the statistical analyses of two data sets, except for the force-frequency datasets. Multivariate analyses of variance were conducted using Statistica (StatSoft Pacific Pty. Ltd) with main effects of genotype and repeated measures of frequency. When these statistical test results indicated significant main effects and interaction, individual means were compared with a Student–Newman–Keuls post hoc test. A one-way analysis of variance was conducted for the voluntary running wheel data sets and body mass data sets. All data are presented as the mean ± standard error of the mean, with n in parenthesis.
Results

**Tg(ACTA1)D286G** mice are a suitable model of patients with mild dominant ACTA1 disease, demonstrating skeletal muscle weakness, various skeletal muscle pathologies and functional impairment

One-month old **Tg(ACTA1)D286G** mice were significantly less active when housed with a voluntary running wheel (**Tg**+/−, P < 0.01; **Tg**+/+, P < 0.05); travelling a reduced distance over a 1-week period (Fig. 1A), however their average (Fig. 1B) and maximum speeds (Fig. 1C) were not altered. **Tg(ACTA1)D286G** mice at 4 months of age were also less active on voluntary running wheels compared with wild-type mice [wild-type: 6.1 ± 1.0 km/day (14); **Tg**+/−: 4.1 ± 0.5 km/day (13); P < 0.05]. **Tg(ACTA1)D286G** mice had a reduced body mass at 1 month of age (Fig. 1D, P < 0.001), however this was no longer significant by 4 months of age [wild-type: 30.0 ± 0.8 g, (17); **Tg**: 28.5 ± 0.4 g, (13); P > 0.05]. Thus the deficit in voluntary running activity of the **Tg(ACTA1)D286G** mice is not due to their reduced body mass. Eight-month old **Tg(ACTA1)D286G** mice did not perform as well as wild-type mice during a 5 min trial on an accelerating Rota-rod (P < 0.01), but did during shorter 2 min trials (Fig. 1E). Spontaneous activity was measured during a 30 min interval in a novel environment, during which 8-month old **Tg(ACTA1)D286G** mice spent significantly less time travelling, covered a reduced distance and their average speed was diminished (Fig. 1F–H). Whole body force as measured with a grip-strength apparatus for these mice was similar to wild-type mice when housed with a voluntary running wheel (**Tg**+/+, P < 0.02; **Tg**+/−, P < 0.01), but did not perform as well as wild-type mice (Supplementary Fig. 1). Their presence was subsequently confirmed in serial sections using a SERCA1 antibody that detects the sarcoplastic Ca2+ -ATPase pump (Fig. 3C), a known marker of tubular aggregates. Notably tubular aggregates are not a feature normally seen in patients with ACTA1.

Fast twitch muscles from adult (4 months or older) **Tg(ACTA1)D286G** mice contained ringbinden fibres (Fig. 3D and Supplementary Fig. 1). Immunohistochemistry using antibodies specific to MHC types I, IIA or IIB showed that all ringbinden positive fibres were MHC type IIB (Fig. 3D and Supplementary Fig. 1); correspondingly, ringbinden fibres were not observed in soleus muscle, which does not have MHC type IIB fibres (data not shown). Ringbinden were not observed in muscle from **Tg(ACTA1)D286G** mice at 1 month of age.

By electron microscopy, Z-band widening, slipping and fragmentation were frequently observed in both fast- and slow-twitch predominant muscles (Fig. 3E), as were regions with complete loss of sarcomeric organization. Withgomor trichrome staining, dark regions reminiscent of myofibrillar disorganization were observed (Fig. 3A). Abnormal distributions of αB-crystallin and desmin were evident in **Tg(ACTA1)D286G** muscle by immunohistochemistry (Fig. 3F), but not in wild-type muscles. These aggregates of αB-crystallin and desmin are shown for quadriiceps muscle, but were also evident in **Tg(ACTA1)D286G** gastrocnemius and soleus muscles.

**Tg(ACTA1)D286G**+/+.Acta1+/− mice recapitulate severe ACTA1 disease

**Tg(ACTA1)D286G** mice were crossed with hemizygous skeletal muscle α-actin knock-out (Acta1+/−) mice in an attempt to increase the mutant ACTA1(D286G) protein load, and as a result to produce a more severe disease phenotype. The majority (71 of 87 individuals from >20 litters, ∼82%) of the **Tg(ACTA1)D286G**+/+.Acta1+/− offspring resulting from this breeding regime presented with severe hindlimb paralysis and immobility between postnatal Days 8 and 17 (average = 12 ± 3 days, Fig. 4A and Supplementary Video 1), with the severity of the phenotype requiring affected pups to be euthanized. Between postnatal Days 10 and 12, these pups had a
Figure 1 Transgenic mice are less active, show impaired performance on functional tests and have reduced body mass. (A–C) Voluntary wheel running activity was monitored over a 7-day period. (A) The average distance travelled per day was significantly reduced for Tg(ACTA1)D286G +/– (n = 13) and Tg(ACTA1)D286G +/+ (n = 14) mice compared with wild-type (n = 13). Average (B) and maximum (C) speeds were similar for all groups. (D) Body mass of Tg(ACTA1)D286G +/– (n = 19) and Tg(ACTA1)D286G +/+ (n = 14) mice was significantly reduced compared with wild-type (n = 24). (E) Tg(ACTA1)D286G +/– mice performed worse than wild-type mice in a 5 min trial on an accelerating Rota-rod apparatus. Tg(ACTA1)D286G +/– mice were less active when exposed to a novel environment, spending less time moving (F), covered less distance (G) and had reduced speed (H) compared with wild-type. (I) Whole body force as measured by grip-strength was similar for wild-type and Tg(ACTA1)D286G +/– mice. (E–I) 8-month old mice (wild-type: n = 12, transgenic: n = 13). *P < 0.05, **P < 0.01, ***P < 0.001.
significantly reduced body mass compared with age-matched wild-type pups (wild-type: 6.3 ± 0.1 g, n = 37; Tg(ACTA1)D286G/–.Acta1+/–: 4.6 ± 0.1 g, n = 21; P < 0.0001). The ~18% of Tg(ACTA1)D286G/+ .Acta1+/– mice that did survive to sexual maturity were often poor breeders, producing no, or only a few small litters.

Skeletal muscles from Tg(ACTA1)D286G+/+ .Acta1+/– mice contained prominent pathological changes by light and electron microscopy. Phalloidin staining showed numerous intense aggregates of filamentous actin that co-stained with α-actinin (a component of the Z-band and indicative of the presence of nemaline bodies; Fig. 4B) in gastrocnemius and quadriceps muscles, and this was
confirmed by gomori trichrome staining (Fig. 4C). Large variations in myofibre size were also present.

At the ultrastructural level, structural lesions including nemaline bodies (Fig. 5A) and Z-band fragmentation and streaming were common. Approximately 20% of muscle fibres displayed widespread myofibril disorganization (Fig. 5B). Subsarcolemmal accumulations of fine filaments (actin; Fig. 5C) and atrophic fibres (Fig. 5D) were also observed.

Examination of both fast-twitch predominant (flexor digitorum brevis, EDL, quadriceps and gastrocnemius) and the slower-twitch soleus muscle from those Tg(ACTA1)D286G+/+.Acta1+/− mice that survived to adulthood (analysed at 9- to 12-months old) showed large aggregates that co-stained with phalloidin and a sarcomeric α-actinin antibody and these corresponded to regions of nemaline bodies by gomori trichrome staining (Fig. 6A and B). Large variations in myofibre diameter were also observed (Fig. 6A–C). Ringbinden were a prominent feature of fast-twitch predominant muscle (Fig. 6C), however these were never observed in muscle of the severely affected Tg(ACTA1)D286G+/+.Acta1+/− pups.
The load of mutant ACTA1(D286G) determines disease severity

The percentage of ACTA1(D286G) in the actin pool was similar in limb skeletal muscle for heterozygous and homozygous Tg(ACTA1)D286G mice, specifically ~25% (Fig. 5E). In comparison, ACTA1(D286G) accounted for ~40–45% of the actin pool in Tg(ACTA1)D286G+/− Acta1+/− limb muscle, a similar mutant protein load to the majority of patients with dominant ACTA1 disease (Ilkovski et al., 2004; Clarke et al., 2007). ACTA1(D286G) protein was also present in the hearts of Tg(ACTA1)D286G+/− Acta1+/− pups (~20% of the total actin pool).

The total actin protein levels were similar in skeletal muscles from all Tg(ACTA1)D286G mice compared with wild-type (Supplementary Fig. 2), indicating that expression of the transgene did not increase the total actin protein content. Nor were these levels decreased in the Tg(ACTA1)D286G+/− Acta1+/− mice, therefore indicating that alterations in total actin content did not underlie the muscle pathology and weakness observed. Previously,

Table 1 Properties of electrically mediated twitch responses in 1-month old Tg(ACTA1)D286G+/− and wild-type EDL and soleus muscles

<table>
<thead>
<tr>
<th>Parameters</th>
<th>EDL Wild-type (n = 14)</th>
<th>Transgenic (n = 11)</th>
<th>Soleus Wild-type (n = 9)</th>
<th>Transgenic (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twitch specific force (N/cm²)</td>
<td>4.3 ± 0.3</td>
<td>2.3 ± 0.2**</td>
<td>2.1 ± 0.1</td>
<td>1.3 ± 0.1**</td>
</tr>
<tr>
<td>Time to peak (ms)</td>
<td>22.0 ± 0.5</td>
<td>19.7 ± 0.5**</td>
<td>42.0 ± 1.0</td>
<td>36.5 ± 1.5**</td>
</tr>
<tr>
<td>Time to peak/twitch specific force</td>
<td>4.0 ± 0.3</td>
<td>8.5 ± 1.1**</td>
<td>12.6 ± 0.6</td>
<td>47.6 ± 2.8**</td>
</tr>
<tr>
<td>Half relaxation time (ms)</td>
<td>28.7 ± 2.0</td>
<td>9.9 ± 2.0NS</td>
<td>55.2 ± 4.3</td>
<td>49.3 ± 1.5NS</td>
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<tr>
<td>Maximum rate of force development (g/s)</td>
<td>610 ± 33</td>
<td>333 ± 41***</td>
<td>97 ± 11</td>
<td>54 ± 3**</td>
</tr>
</tbody>
</table>

**P < 0.005, ***P < 0.0001.
NS = not significant.

Figure 4 The majority of Tg(ACTA1)D286G+/− Acta1+/− mice present with severe immobility and splayed hindlimbs and the musculature shows lesions consistent with nemaline myopathy. (A) A photograph of three littermate pups taken at postnatal Days 2, 5 and 10. Two of these pups were Tg(ACTA1)D286G+/− Acta1+/− and developed the splayed leg phenotype, while the other was Tg(ACTA1)D286G+/− Acta1+/+ and developed normally. This photograph highlights the splayed leg appearance, reduced overall size and the decreased bulk of the hind limbs of the Tg(ACTA1)D286G+/− Acta1+/− pups. (B) Tg(ACTA1)D286G+/− Acta1+/− muscle contained filamentous actin (green) and α-actinin (red) positive protein aggregates. Filamentous actin aggregates co-localized with abnormal accumulations of α-actinin as can be seen in the merged micrographs. (C) Gomori trichrome staining revealed the presence of nemaline bodies, sarcomeric disorganization and large variations in fibre size. Scale bars represent 50 µm.
Figure 5  Tg(ACTA1)D286G +/+ .Acta1 +/- muscle contains widespread structural lesions which are evident at both the light and ultra-structural level and an increased mutant ACTA1 load. (A) Electron-dense nemaline bodies, (B) widespread sarcomeric disarray, (C) thin filament (actin) accumulations (asterisk) and (D) atrophic fibres (arrow) in which the Z-bands appear condensed were frequently observed. Scale bars represent 2 μm. (E) Percentage mutant actin in muscle from transgenic and Tg(ACTA1)D286G +/+ .Acta1 +/- mice determined by mass spectrometry. Data shown for muscles from three different mice (except for Tg(ACTA1)D286G +/+ gastrocnemius and Tg(ACTA1)D286G +/+ .Acta1 +/- gastrocnemius where muscles from six mice were analysed). G = gastrocnemius; H = heart; Q = quadriceps, WT = wild-type.
we have shown that total actin and skeletal muscle α-actin levels are similar in wild-type and Acta1+/− muscle (Nowak et al., 2009).

One outcome of the breeding regime instigated should have been the production of Tg(ACTA1)D286G +/+ .Acta1−/− mice, however none of the resulting offspring were determined as having this genotype. This finding probably indicates that expression of the ACTA1(D286G) protein in the absence of wild-type ACTA1 protein was embryonic lethal.

**Discussion**

With so many different dominant mutations identified within the ACTA1 gene in an ever-growing number of patients worldwide, we sought to generate a transgenic mouse model of dominant human ACTA1 disease to assist with understanding the pathogenesis and to facilitate direction and testing of possible therapies. Very little patient muscle tissue exists for experimentation, especially since most patients do not undergo more than one biopsy, and many patients are not genetically diagnosed until after their death. As such, a mouse model would provide crucial in vivo and in vitro analytical possibilities, especially longitudinal studies. We chose to express a version of the ACTA1 protein containing the D286G amino acid substitution, which was identified in a severely affected patient with nemaline myopathy, one of eight currently identified skeletal muscle diseases resulting from ACTA1 mutations. Our aim was to generate a mouse model that mimicked the most common presentation of patients—severe skeletal muscle weakness, the presence of the pathological entities known as nemaline bodies and a shortened lifespan. The D286G substitution, being an amino acid charge change, would also facilitate proteomic analysis in order to determine the ratio of mutant ACTA1 protein compared with wild-type. The human ACTA1 complementary DNA sequence was utilized as the transgene rather than the mouse sequence to enable the mutant ACTA1 transcript to be distinguished from the endogenous Acta1 transcript if necessary, however, it should be noted that the human and mouse skeletal muscle α-actin proteins are identical at the amino acid level.

The force and functional deficits exhibited by Tg(ACTA1)D286G mice recapitulated the weakness observed in patients with ACTA1 mutations. Tg(ACTA1)D286G mice were less active than wild-type mice on voluntary running wheels at both ages tested and also had reduced body mass at 1 month of age. Behavioural deficits (e.g. poorer performance on an accelerating Rota-rod) were still evident at 8 months of age, despite there being no significant difference in body mass at 4 months and time points beyond, indicating that the behavioural manifestations also observed at 1 month of age were not merely due to their reduced body mass.

In order to examine the effect of the ACTA1(D286G) protein on the function of the thin filament, mechanically-skinned myofibres were examined in vitro. Analysis of Tg(ACTA1)D286G EDL fibres demonstrated that they produced ∼40% less maximal specific force at all time points examined (3, 6 and 9 months of age),

**Figure 6** Muscle from surviving Tg(ACTA1)D286G +/+ .Acta1+/− mice show extensive structural lesions and ringbinden. (A) Flexor digitorum brevis and (B) soleus muscle from surviving Tg(ACTA1)D286G +/+ .Acta1+/− mice showed numerous and large aggregates co-staining for filamentous actin and α-actinin which corresponded to regions of deep purple staining by gomori trichrome (GT). (C) Ringbinden were a prominent feature of fast-twitch muscle of surviving Tg(ACTA1)D286G +/+ .Acta1+/− mice. Asterisk denotes ringbinden. Scale bars represent 50 μm. FDB = flexor digitorum brevis; G = gastrocnemius.
revealing that the ACTA1(D286G) protein interferes directly with the ability of the sarcomeres to produce force. Maximal specific force produced by both whole isolated EDL (~35% deficit) and soleus (~37% deficit) muscles from 1-month old Tg(ACTA1)D286G mice were also significantly reduced, which is consistent with the skinned fibre results and also indicates that the mutant protein influences contractile function of both fast- and slow-twitch muscles.

The skinned fibre results also showed a significant rightward shift of the pCa-force curve in the Tg(ACTA1)D286G fibres, indicating a reduced sensitivity of the contractile apparatus to Ca2+. This result suggests that the Tg(ACTA1)D286G mutation affects the ability of thin filament troponin C to bind activating Ca2+. This decrease in the sensitivity to Ca2+ of the mutant actin filaments explains the slower rates of force production and the rightward shift in the force-frequency curves observed in the intact Tg(ACTA1)D286G muscles.

The Tg(ACTA1)D286G model also mimicked the hallmark pathological features of ACTA1 disease and the associated disruption of normal skeletal muscle structure. Staining of serial Tg(ACTA1)D286G skeletal muscle sections with gomori trichrome and phalloidin indicated that the dark blue aggregates observed with gomori trichrome were due to abnormal accumulations of filamentous actin. The presence of nemaline bodies was confirmed with a SERCA1 antibody to ACTA1(D286G) mutation had nemaline myopathy. In addition, lesions consistent with other patients with ACTA1 disease were seen, including sarcomeric disorganization, and Z-band fragmentation and streaming.

Both heterozygous and homozygous Tg(ACTA1)D286G mice expressed a similar amount of mutant skeletal muscle α-actin (D286G; ~25% of total actin) in their musculature by mass spectrometry. However, since patients with dominant ACTA1 disease most frequently express closer to 50% mutant ACTA1 protein in their skeletal muscle (Ilkovski et al., 2004) we sought to increase the mutant protein load in our transgenic mouse model in an attempt to more closely mirror human disease. Therefore, we crossed Tg(ACTA1)D286G mice with hemizygous Acta1 knockout mice to produce Tg(ACTA1)D286G+/– Acta1+/- offspring. Approximately 45% of the actin pool was ACTA1(D286G) in skeletal muscle from Tg(ACTA1)D286G+/–.Acta1+/- mice, in a similar fashion to the mutant protein load of the vast majority of patients with dominant ACTA1 disease (Ilkovski et al., 2004; Clarke et al., 2007). Remarkably, ~80% of the Tg(ACTA1)D286G+/–.Acta1+/- offspring appeared smaller than their Tg(ACTA1)D286G+/+ .Acta1+/- littermates shortly after birth, and then presented with a severe spayed leg phenotype and hind limb immobility between postnatal Days 8–17, thus debilitating the pups and requiring them to be euthanized. As a result, Tg(ACTA1)D286G+/–.Acta1+/- mice recapitulate the extremely severe phenotype of most patients with ACTA1 disease.

By Mendelian genetics, ~25% of the offspring from this cross-breeding should also have been of the Tg(ACTA1)D286G+/–.Acta1+/- genotype, however, after genotyping numerous litters, no mice with this genotype were detected, suggesting that they had not survived to birth. This finding reflects that the presence of some wild-type ACTA1 protein is necessary during in utero development. It is intriguing that ~18% of Tg(ACTA1)D286G+/+ .Acta1+/– mice survived to adulthood and did not develop the spayed leg phenotype that occurs in the majority of these mice. This variation in phenotype suggests the existence of modifying factors. Kumar et al. (1997) showed that ~56% of mice that were cardiac α-actin null (Actc1–/–) did not survive to birth and that only 32% of Actc1–/– mice could be rescued to adulthood by transgenic over-expression of enteric γ-actin (Kumar et al., 1997). Similarly, this dichotomy in phenotype has been reported with ACTA1 patients, as some individuals who are severely affected initially are able to survive the early postnatal period and then appear to do comparatively well (Ryan et al., 2001; Agrawal et al., 2004). Despite some Tg(ACTA1)D286G+/+.Acta1+/- mice surviving to adulthood, those that did bred poorly, producing either only small litters, or no litters at all, even when placed with different mates. Microarray analysis on skeletal muscle from surviving and spayed leg Tg(ACTA1)D286G+/+.Acta1+/- mice may identify genes regulating phenotype severity and may thus lead to novel therapeutic avenues for ACTA1 disease.

Like Tg(ACTA1)D286G+/+ mice, the Tg(ACTA1)D286G+/+.Acta1+/- model also recapitulated various structural hallmarks of ACTA1 disease skeletal muscle pathology. Fluorescent microscopy showed accumulations of filamentous actin that co-labelled with an antibody to α-actinin. α-Actinin localizes to nemaline bodies in patient biopsies and the characteristic electron dense nature of nemaline bodies is due to the presence of Z-band proteins, including α-actinin (Jockusch et al., 1980). By electron microscopy, nemaline bodies were confirmed as being abundant, and widespread sarcomeric disorganization was frequently observed. Subsarcolemmal accumulations of fine filaments were also noted, reminiscent of actin accumulation myopathy. Additionally, Tg(ACTA1)D286G muscle contained a number of other structural lesions not usually associated with ACTA1 disease. Precocious tubular aggregates as indicated by large regions of magenta coloured stain by gomori trichrome and labelling with a SERCA1 antibody were frequently observed in gastrocnemius and quadriceps muscles of young (4-month old) Tg(ACTA1)D286G mice. These were not seen in skeletal muscles from age- and sex-matched wild-type mice, although it is known that tubular aggregates are common in aged (<10-months old) inbred male mice (Agbolut et al., 2000). A transgenic mouse model expressing the nemaline myopathy M9R mutation in the slow α-tropomyosin gene (TPM3) also developed premature tubular aggregates (Corbett et al., 2001), with these authors speculating that their early appearance reflects premature muscle ageing.

Evidence of myofibrillar disorganization was seen by gomori trichrome staining and abnormal distribution of αB-crystallin and desmin were observed in Tg(ACTA1)D286G muscle. Both of these proteins are known to comprise the pathological hallmarks of the myofibrillar myopathies and both genes are mutated in this disease entity (reviewed in Schroder and Schoser, 2009). However, abnormal desmin and αB-crystallin accumulations have been shown to occur non-specifically in a number of other myopathies (Bonnemann et al., 2003). Nemaline bodies have been seen in combination with disturbed desmin distribution in patients with
severe neonatal presentations of nemaline myopathy (van der Ven et al., 1995).

Ringbinden fibres [where approximately 3 or 4 of the peripheral myofibrils of a muscle fibre circle the periphery of the muscle fibre (Morris, 1959)] were observed in fast-twitch muscles of adult (>4 months of age) Tg(ACTA1)D286G and surviving Tg(ACTA1)D286G +/+ .Acta1+/- mice, but were never observed in wild-type muscle. Labelling of muscle sections with antibodies specific to MHC subtypes revealed that ringbinden were exclusively MHC IIB fibres, supporting the absence of ringbinden in the soleus, as the soleus expresses mostly MHC type I and IIA fibres and some IIX fibres, but no MHC IIB (Agbulut et al., 2009). Numerous groups have demonstrated that skeletal muscle promoters preferentially express in MHC IIB fibres, including the human skeletal muscle α-actin promoter used to make the Tg(ACTA1)D286G model (Tinsley et al., 1998; Corbett et al., 2001), the myosin light chain promoter (Neville et al., 1996) and the muscle creatine kinase promoter (Dunant et al., 2003; Wang et al., 2008). We have also seen preferential expression of ACTA1(D286G) fused to enhanced green fluorescent protein in another transgenic mouse model (Ravenscroft, unpublished data). Perhaps this is the reason for the ringbinden only occurring in MHC type IIB fibres, as the transgene is expressed more strongly in these myofibrils. Ringbinden have not been reported in patients with ACTA1 disease, but are a frequent feature of muscular dystrophies and myopathies (Engel and Franzini-Armstrong, 2004), as well as being prevalent in muscles that do not extend between bones [e.g. the diaphragm and extraocular muscle (Bethlem and Vanwijngaarden, 1963)].

Studies of regenerating and/or tenotomized muscle (Morris, 1959; Pena et al., 2001) or muscle prone to myotendinous junction disruption (Banks et al., 2008) have shown that ringbinden fibres can be experimentally induced. Banks et al. (2008) propose that in response to chronic myotendinous strain, myofibrils adapt to form ringbinden and that this then prevents the transmission of lateral forces between fibres to minimize transmission of force to the tendon and protect the muscle from eccentric strain. The presence of ringbinden in Tg(ACTA1)D286G muscle at 4 months of age and older, but not 1 month of age, may be due to either muscle growth under reduced strain, or myotendinous strain, or another yet unknown mechanism. Ringbinden seem to appear with time and do not appear to be dependent on the mutant protein load, since muscle from severely affected Tg(ACTA1)D286G +/+ .Acta1+/- pups did not contain ringbinden. Thus, screening of ACTA1 in patients with genetically undiagnosed ringbinden may also be worthwhile given the findings in our mouse model.

The increased severity of the phenotype of this mouse model from a mild phenotype in which Tg(ACTA1)D286G mice showed skeletal muscle weakness, pathological features and functional impairment, yet no overt phenotype, to a severe phenotype (with splayed hindlimbs and substantially impaired mobility) in the Tg(ACTA1)D286G +/+ .Acta1+/- mice, with increased mutant protein load from ~25 to 45% indicates that ‘dilution’ of mutant protein load could be a viable therapeutic avenue for dominant ACTA1 disease. Studies of a number of mutations in the Act88F gene, the actin expressed in the indirect flight muscle of D. melanogaster, have shown that there is a dosage effect on the extent of flight impairment for some mutants, but not others (Hiromi et al., 1986; Drummond et al., 1991). Further evidence of a dosage effect stems from the lack of involvement of the extracardiac muscles and lack of involvement of the heart in almost all patients with ACTA1 mutations (Ryan et al., 2003). It has long been known that ACTA1 is only present at low levels in the postnatal heart (Ikkovski et al., 2005) and we have recently demonstrated high levels of cardiac α-actin, similar to those in the heart, in extraocular muscle (Ravenscroft et al., 2008). Additionally, in a multigenerational family with intranuclear rod myopathy disease severity correlated with the mutant protein [ACTA1(V163M)] load (Domazetovska et al., 2007). Finally, in a number of families where there is a mildly affected parent of one or more severely affected children, it has been shown that the mildly affected parent is a somatic mosaic for the mutation (Nowak et al., 1999).

The relative mutant protein load, expressed from the randomly inserted transgenes, was elevated in the absence of one of the endogenous Acta1 alleles. It has long been suggested that the total pool of actin proteins is tightly regulated to ensure that actins meet their important structural roles and that the stoichiometry of sarcomeric proteins in preserved (Kumar et al., 2004; Jaeger et al., 2009; Nowak et al., 2009). The exact mechanisms of the auto-regulation and feedback mechanisms that ensure that the amount of total actin expressed remains constant is not known, though it has been suggested that the feedback involves multiple regions of the cardiac α-actin gene (Dunwoodie et al., 1994). The mutant protein load was similar in muscle from mice that were heterozygous or homozygous for the transgene. This perhaps suggested that if both endogenous Acta1 alleles are intact, the output from the transgenes is suppressed to a particular level. The transgenes appear less efficient at contributing to the actin protein pool than the endogenous genes.

We have shown that transgenically expressed cardiac α-actin (the foetal isoform of actin in skeletal muscle; Ikkovski et al., 2005) is able to functionally replace skeletal muscle α-actin in skeletal muscle and rescue the early lethality of Acta1-/- mice (Nowak et al., 2009). From this and the present results, increased levels of cardiac α-actin may alleviate the phenotype of many, though perhaps not all (for example mutants resulting in thin filament end-capping; Sparrow et al., 2003), dominant ACTA1 mutations. The auto-regulation of total actin within the actin protein families may help the outcome since increased wild-type cardiac α-actin in postnatal skeletal muscle should reduce the expression of endogenous skeletal muscle α-actin and thereby decrease the mutant protein load in patients with ACTA1 disease. This hypothesis will form the basis of future experiments with our Tg(ACTA1)D286G, Acta1+/- mouse model described herein, which mimics human disease by expressing a similar mutant protein load, being severely weak and displaying the hallmark structural lesions of ACTA1 disease.

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Supplementary material

Supplementary material is available at Brain online.

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Mouse models recapitulate ACTA1 disease


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