Alterations in Notch signalling in skeletal muscles from *mdx* and *dko* dystrophic mice and patients with Duchenne muscular dystrophy

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Abstract

In Duchenne muscular dystrophy (DMD), muscle damage and impaired regeneration leads to progressive muscle wasting, weakness and premature death. The Notch signalling pathway represents a central regulator of gene expression and is critical for cellular proliferation, differentiation and apoptotic signalling during all stages of embryonic muscle development. Notch activation improves muscle regeneration in aged mice, but its potential to restore regeneration and function in muscular dystrophy is unknown. We performed a comprehensive examination of several genes involved in Notch signalling in muscles from dystrophin-deficient *mdx* and *dko* (utrophin/dystrophin null) mice and DMD patients. A reduction of Notch1 and Hes1 mRNA in TA muscles of *dko* mice and quadriceps muscles of DMD patients, and a reduction of Hes1 mRNA in the diaphragm of the *mdx* mice were observed, with other targets being inconsistent across species. Activation and inhibition of Notch signalling, followed by measures of muscle regeneration and function, were performed in the mouse models of DMD. Notch activation had no effect on functional regeneration in C57BL/10, *mdx* or *dko* mice. Notch inhibition significantly depressed the frequency-force relationship in regenerating muscles of C57BL/10 and *mdx* mice after injury indicating reduced force at each stimulation frequency, but enhanced the frequency-force relationship in muscles from *dko* mice. We conclude that while Notch inhibition produces slight functional defects in dystrophic muscle, Notch activation does not significantly improve muscle regeneration in murine models of muscular dystrophy. Furthermore, the inconsistent expression of Notch targets between murine models and DMD patients suggests caution when making inter-species comparisons.

Introduction

Duchenne muscular dystrophy (DMD), an X-linked recessive disorder affecting ~1 in 3500 live births, is caused by mutations in the dystrophin gene that result in the absence of the membrane stabilizing protein dystrophin (Koenig *et al.*, 1988; Ervasti & Campbell, 1991; Emery, 2002; Allen *et al.*, 2010). Dystrophic muscles are fragile, susceptible to damage, and have impaired regeneration, leading to progressive wasting and weakness. There is currently no cure for DMD and existing therapies are ineffective. Although gene therapies are likely to eventually provide a cure for this devastating disease, there are significant obstacles that currently limit their application. In the interim, other approaches including pharmacologic therapies are needed to improve the pathophysiology of DMD, especially identifying ways to enhance muscle regeneration (Gehrig *et al.*, 2012).

The Notch signalling pathway represents a central regulator of gene expression (Fischer & Gessler, 2007) and is critical for cellular proliferation, differentiation and apoptotic signalling during all stages of embryonic muscle development (Artavanis-Tsakonas *et al.*, 1999). In mammals Notch signalling is initiated by extracellular expression of the ligands Delta and Jagged (Conboy & Rando, 2002; Lai, 2004). Ligand binding to the Notch family of transmembrane receptors results in enzymatic cleavage of the Notch intracellular domain (NICD) where it is sequestered to the nucleus.(Jarriault *et al.*, 1995; Shinin *et al.*, 2006; Carlson & Conboy, 2007; Wen *et al.*, 2012) Nuclear NICD binds to members of the CSL (named for CBF1, Suppressor of Hairless, and Lag-1) family of transcription repressors (Fischer & Gessler, 2007), causing the CSL members to become transcriptional activators. In vertebrates the most extensively studied of the CSL target genes are transcription factors belonging to the Hairy and Enhancer of Split (*Hes)* and Hes-related (*Hey*) families (Jarriault *et al.*, 1995; Maier & Gessler, 2000). While the regulation of *Hes* and *Hey* in skeletal muscle

is still not understood completely, it is clear they play an important role in satellite cell activation and differentiation during muscle regeneration (Hansson *et al.*, 2004). Upregulation of *Hes* and *Hey* via activation of Notch1 promotes satellite cell proliferation (Conboy & Rando, 2002).

The Notch pathway plays an important role in regeneration after myotoxic injury (Conboy & Rando, 2002; Sun *et al.*, 2007; Sun *et al.*, 2008; Kitamoto & Hanaoka, 2010; Wen *et al.*, 2012) and stimulation of the pathway improves muscle regeneration in aged mice (Conboy *et al.*, 2003). To date, the ability of the Notch signalling pathway to restore regeneration and improve muscle function in muscular dystrophy has not been investigated. Additionally, the regulation of members involved in the Notch signalling pathway has not been well characterised in muscular dystrophy. Skeletal muscle gene expression profiling studies have revealed alterations in some genes involved in the Notch pathway in both DMD patients and *mdx* mice (Turk *et al.*, 2005; Pescatori *et al.*, 2007). From the few investigations performed to date, the regulatory pattern of Notch signalling genes observed in *mdx* mice and DMD patients appears to show little similarity (Turk *et al.*, 2005), highlighting the need for comparisons between different dystrophic models in order to provide insight into the therapeutic potential of manipulating Notch signalling for DMD.

In the present study we performed a comprehensive examination of several gene targets involved in the Notch signalling pathway in tibialis anterior (TA) muscles from dystrophin-deficient *mdx* mice and dystrophin/utrophin null *dko* mice and in muscles from DMD patients. The diaphragm muscle from *mdx* mice was also investigated. Manipulation of Notch using an activator and inhibitor of Notch tested the hypothesis that Notch activation would enhance muscle regeneration and improve muscle function in muscular dystrophy.

Methods

Ethical approval. All procedures were approved by the Animal Ethics Committee of The University of Melbourne and conformed to the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council (Australia). C57BL/10 and *mdx* mice used were obtained from the Animal Resources Centre (Canning Vale, WA, Australia). The *dko* mice (Deconinck *et al.*, 1997) were originally kindly provided by Professor Dame Kay Davies (University of Oxford) and then established and bred in the Biological Research Facility (BRF) at The University of Melbourne (Gehrig *et al.*, 2012). All mice were housed in the BRF under a 12 hour light-dark cycle, with drinking water and standard chow provided *ad libitum*. Male mice aged 8-9 weeks were used for this study.

Myotoxic injury. Myotoxic damage was induced in the TA muscles of mice as described previously (Schertzer & Lynch, 2006). Briefly, mice were anaesthetised using a mixture of ketamine (76 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and a small portion of the tibialis anterior (TA) muscle of the right hindlimb was surgically exposed by a single incision through the skin. The muscle was filled to its maximal holding capacity (~40 μl) via a single intramuscular injection with the myotoxin, Notexin (1 μg/ml, Latoxan) using a 29-gauge needle. The wound was closed with Michel clips (Aesculap, Tuttlingen, Germany) and the mice were allowed to recover. After 3 days, the mice were anaesthetised again and given a single intramuscular injection of either saline, a Notch antibody (to activate Notch signalling), a Jagged-Fc fusion protein (to inhibit Notch signalling), or their respective controls. Mice were then allowed to recover for a further 4 or 11 days (corresponding to 7 and 14 days post the initial myotoxic injury).

Notch manipulation. Acute manipulation of the Notch signalling pathway was achieved as described previously (Conboy *et al.*, 2003). For Notch activation, mice received 35 μl of a 1:4 dilution of anti-Notch-1 antibody (α-Notch; hybridoma clone 8G10, Upstate

Biotechnology, Lake Placid NY New York) via a single injection into the TA muscle. Hamster IgG was used as a vehicle control for α-Notch. For Notch inhibition, we prepared a Jagged-1Fc/anti-Fc complex as described previously.(Conboy *et al.*, 2003) Jagged-1Fc (10 μg; R&D systems, Minneapolis, MN) was incubated for 1 hour on ice with anti-human Fc antibody (5 μg; Sigma, Castle Hill, New South Wales) to give a 2:1 stoichiometric ratio and a final concentration of Jagged-Fc of 100 μg/ml. Mice received 35 μl of the Jagged-1Fc/anti-Fc complex (referred to henceforth as Jagged-Fc) via a single injection into the right TA muscle. Anti-Fc antibody alone was used as the vehicle control for Jagged-Fc. In our initial experiments, we treated uninjured C57BL/10 mice using this protocol and found α-Notch resulted in a 69% increase in Hes1 expression within 1 hour of treatment when compared with IgG, while Jagged1 did not affect Hes1 expression in the uninjured muscle when compared with anti-Fc (data not shown). Mice were then allowed to recover for a further 4 or 11 days (corresponding to 7 and 14 days post-injury). A subset of the *dko* mice received Notch treatments but no myotoxic injury; these mice received the treatments as described above and were allowed to recover for 4 days before assessment of muscle function. This was the equivalent of the 7 day post-injury time point in the other groups.

Muscle function. Mice were anaesthetised by intraperitoneal injection of sodium pentobarbital (60 mg/kg) and TA muscle function was assessed *in situ* as described previously (Gehrig *et al.*, 2010). Briefly, TA muscles were stimulated by supramaximal 0.2 ms square wave pulses of 350 ms duration, delivered via two wire electrodes adjacent to the deep peroneal branch of the sciatic nerve. Optimal muscle length for contraction (L_o) was defined as the muscle length at which maximal isometric twitch force (P_t) was attained, and the absolute maximal isometric tetanic force (P_o) was determined from the plateau of the frequency-force relationship. Measurements of P_o were normalised to the cross-sectional area of the muscle (CSA; calculated using L_o and muscle mass) in order to determine

maximum specific force (sP_o), that allows for relative comparisons of force production between muscles of different sizes. Immediately following the functional assessments, the muscles were carefully excised, trimmed of any adherent non-muscle tissue and tendons and weighed. Muscles were then either snap-frozen in liquid N_2 for extraction of RNA and protein, or mounted in Tissue-Tek OCT (Sakura Finetek Europe B.V. The Netherlands) embedding medium and frozen rapidly in thawing isopentane for histological analyses. Muscles were stored at -80°C until analysed. The mice were killed by surgical excision of the heart while still anaesthetised deeply.

Immunohistology. Transverse sections (5 μm) were cut from the midbelly of each muscle on a cryostat microtome and serial sections were placed onto glass slides (Superfrost® Plus, Menzel-Gläser, Kensington, VIC, Australia). General muscle histology was determined by staining sections with haematoxylin and eosin (H & E) to visualise muscle fibres, and digital images of stained sections were obtained using a microscope and camera (Carl Zeiss, Wrek, Göttingen, Germany) with associated imaging software (Axiovision V4.7.1.0).

RNA extraction and qPCR. RNA was extracted from ~5-10 mg of muscle using TRI-reagent (Ambion, Austin, TX) as per manufacturer's instructions. Approximately 500 ng of RNA was reverse transcribed to cDNA using the Affinity Script reverse transcription kit (Stratagene, Cedar Creek, TX) followed by treatment with RNAse H (Invitrogen, Mulgrave, Vic). The cDNA was quantified using the Quant-iT OliGreen ssDNA Reagent and Kit (Molecular Probes, Eugene, OR) with fluorescence measured at 428 nm. The qPCR was performed using a MX3000p thermal cycler system with Multiplex Brilliant QPCR Master Mix (Stratagene, Cedar Creek, TX) using conditions published previously (Lamon *et al.*, 2009). The primer sequences are provided in Table 1. C_t values were converted to arbitrary

units (AU) using the log power calculation. All qPCR results were normalised against cDNA quantification values.

Protein extraction and western blotting. Total protein was extracted using RIPA buffer (Millipore, North Ryde, Australia) with 1 μL/mL protease inhibitor cocktail (Sigma, Castle Hill, Australia) and 10 μL/mL Halt Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific, Rockford, USA). Following overnight agitation at 4°C, the homogenate was centrifuged (13,000 RPM, 15 min, 4°C), supernatant was removed and total protein content was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA) according to the manufacturer's instructions. Electrophoresis was performed using a 4-12% NuPAGE® Novex Bis-Tris Gel (Invitrogen, Carlsbad, CA) in NuPAGE® SDS MOPS Running Buffer (Invitrogen, Carlsbad, CA) or a 15% SDS–PAGE in a buffer containing 12 mM Tris-HCl pH 8.8, 200 mM glycine and 0.1% SDS. Protein transfer was performed in a Bjerrum buffer containing 50 mM Tris, 17 mM glycine and 10% methanol using PVDF membranes. The membranes were blocked with 5% BSA in PBS and incubated overnight at 4°C with the primary antibody. The HES-1 primary antibody used for human samples (NBP1-19029, Novus Biological, Littleton, USA) was diluted 1:500 in 2.5% BSA. The HES-1 primary antibody used for mouse samples (2922-1, Epitomics, Burlingame, USA) was diluted 1:1000 in PBS. Following washing, the membranes were incubated for 1 hour with a goat anti-rabbit IgG antibody labeled with an infrared-fluorescent 800 nm dye (Alexa Fluor® 800, Invitrogen, Carlsbad, CA) diluted 1:5000 in PBS containing 50% Odyssey® blocking buffer (LI-COR Biosciences, Lincoln, USA) and 0.01% SDS. After washing, the proteins were exposed on an Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, USA) and individual protein band optical densities were determined using ImageJ Software (National Institutes of Health, Bethesda, USA). The blots were normalised against

the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (G8795, Sigma-Aldrich, Sydney, Australia).

DMD muscle samples. Human muscle specimens were sourced from the Telethon Network of Genetic Biobanks (Neuromuscular Tissue Bank, Padova, Italy). Biopsies were taken from the vastus lateralis muscles of patients with DMD (aged 1-9 years) or healthy controls (aged 18-25 years) using either a Bergstrom or open biopsy technique. For the healthy control subjects informed consent was obtained and the procedures were approved by the Deakin University Human Ethics Committee. All biopsies were frozen immediately in liquid nitrogen and stored at -80°C until analysis. It should be noted that in all studies utilising muscle samples from DMD patients that the disease progression will vary widely even among similarly aged individuals and this variability in the pathology needs to be recognised. In 18-25 year old healthy controls we would expect normal (healthy) muscle fibre histopathology. In contrast, even in young (1-9 year old) DMD patients we would expect muscle fibre integrity to be compromised with normal signalling within the muscle to be perturbed – reflecting ongoing cycles of muscle fibre degeneration, inflammation, and regenerative mechanisms. Therefore, even though the ages of the patients and controls are different, it should not compromise the main objective to compare differences in the muscle pathology and these should be evident between the two populations examined.

Statistical analyses. All values are expressed as mean ± SEM. Groups were compared using either an unpaired Student's t-test, or a one- or two-way ANOVA with a post hoc multiple comparison procedure where appropriate. All statistical analyses were performed using Prism version 3 software (Graphpad Software Inc.). In all cases significance was defined as $P<0.05$.

Results

Members of the Notch signalling pathway are dysregulated in various models of muscular dystrophy. Compared with C57BL/10 mice, *mdx* mice had lower levels of Jagged2 and Numb mRNA and higher levels of Hes6 mRNA in the TA muscle. In TA muscles from the severely dystrophic *dko* mice lower levels of Jagged2, Notch1, Notch3, Numb and Hes1 mRNA and significantly higher expression of Hes6 mRNA were observed. Additionally, *dko* mice had significantly lower levels of Jagged2, Notch1, Notch3, Hes1 and Hes6 mRNA compared with *mdx* mice (Figure 1A). At the protein level, Hes1 was increased in the TA muscle of *mdx* mice compared with both the C57BL/10 and *dko* mice (Figure 1B). The diaphragm muscle from *mdx* mice also had lower Notch1 mRNA levels compared with C57BL/10 mice (Figure 1C). The Hes1 protein was highly expressed in the diaphragm of C57BL/10 mice, but not detectable in the majority of diaphragm samples from *mdx* and *dko* mice (Figure 1D). In human DMD patients, Notch1 and Hes1 mRNA expression was decreased, while Numb and Notch3 expression were increased compared with healthy controls (Figure 1E). Hes1 protein levels were increased in the muscles of DMD patients compared with healthy control subjects (Figure 1F).

Notch manipulation has little effect on muscle regeneration in C57BL/10 mice. Three days after inducing complete degeneration of the TA muscle with Notexin, Notch signalling was manipulated *in vivo* via injection of a Notch activator or inhibitor, as described previously (Conboy *et al.*, 2003). TA muscle function was examined after a further 4 days or 11 days of recovery (i.e. 7 or 14 days after myotoxic injury). As evidence that Notch signalling was activated a 38% increase in the mRNA levels of the Notch transcriptional target Hes1 was observed (Figure 2A). Treatment of C57BL/10 mice with the Notch activator did not influence absolute (Figure 2B) or relative (Figure 2C) maximal force,

muscle mass, twitch characteristics (time-to-peak tension, ½ relaxation time, and dP/dt) (Table 2) or the frequency-force relationship (Figure 2D) of regenerating muscles. However, treatment with the Notch inhibitor reduced specific force of the regenerating muscles at 7 days post-injury (Figure 2C), decreased force production at each stimulation frequency (Figure 2E), and increased time-to-peak tension and decreased dP/dt at 7 days post-injury (Table 2). Notch activation (α-Notch) or inhibition (Jagged Fc) did not influence muscle regeneration at 7 days post-injury (Figure 2F).

Notch manipulation has little effect on muscle regeneration in mdx *mice.* Activation or inhibition of the Notch pathway in *mdx* mice after myotoxic injury did not affect maximal (Figure 3A) or specific (Figure 3B) force, muscle mass, twitch characteristics (Table 2) or the frequency-force relationship (Figure 3C). However, Notch inhibition decreased force production at each stimulation frequency (Figure 3D). Notch activation (α-Notch) or inhibition (Jagged Fc) did not influence muscle regeneration at 7 days post-injury as shown in H&E stained (Figure 3E).

Notch manipulation has little effect on muscle regeneration in dko *mice.* To assess the effect of Notch manipulation in a more clinically relevant model, Notch signalling was manipulated in the TA muscles of severely dystrophic *dko* mice that were not injured myotoxically. Activation or inhibition of the Notch pathway did not affect maximum absolute (Figure 4A) or specific (Figure 4B) force, muscle mass, twitch characteristics (Table 3) or the frequency-force relationship (Figure 4C). However, Notch inhibition increased force production at each stimulation frequency (Figure 4D). Finally, it was of interest to examine whether Notch activation may have beneficial effects on regeneration of muscles from *dko* mice with the added insult of myotoxic injury. In these highly damaged muscles (dystrophic damage + myotoxic damage) Notch activation produced a significant reduction in the mass of the regenerating muscles (Table 3), but this did not affect twitch characteristics

(Table 3), maximal (Figure 4E) or specific force (Figure 4F) or the frequency-force relationship (Figure 4G).

Discussion

The Notch signalling pathway is a key regulator of cellular proliferation and differentiation (Artavanis-Tsakonas *et al.*, 1999) and plays an important role in skeletal muscle regeneration after many types of muscle injury (Conboy & Rando, 2002; Sun *et al.*, 2007; Sun *et al.*, 2008; Kitamoto & Hanaoka, 2010; Wen *et al.*, 2012). Dystrophic muscles are susceptible to damage and have impaired regeneration that leads to progressive deterioration of muscle structure and function. We examined the regulation of several members of the Notch signalling pathway in different dystrophic models, and then determined whether activation or inhibition of Notch improved muscle regeneration in *mdx* and *dko* dystrophic mice, revealing several novel findings. In the TA muscle of *mdx* and *dko* mice, as well as in quadriceps muscle of DMD patients, there was little similarity in the regulation of Notch pathway genes, including Delta, Jagged1, Jagged2, Notch1, Notch3, Numb, Hes1 and Hes6, compared with control mice and otherwise healthy humans. However, there was commonality within the three dystrophic models for a reduction in Notch1 and Hes1 mRNA in TA muscles of *dko* mice and in quadriceps muscles of DMD patients, as well as a reduction in Hes1 mRNA in diaphragm muscles of *mdx* mice. Next, we manipulated Notch signalling in TA muscles of C57BL/10, *mdx* and *dko* mice after injury and assessed the effect on functional regeneration of the muscle. Stimulation of Notch signalling had no effect on functional regeneration of injured muscles in any of the mouse models. Furthermore, while inhibition of Notch signalling produced modest deficits in the frequency-force relationship of regenerating TA muscles from C57BL/10 and *mdx* mice, it paradoxically produced modest enhancement of the frequency-force relationship in *dko* mice.

Changes in the frequency-force relationship reflect possible alterations in the kinetics Ca^{2+} release from the sarcoplasmic reticulum and subsequent binding to regulatory proteins, or possibly to changes in fibre type composition – a left-shift indicating a muscle with a 'slower' phenotype and a right-shift indicating a 'faster' muscle phenotype. However, given that the specific force of the regenerating muscles was changed by roughly the same percentage at all stimulation frequencies, it is more likely that the changes in force may simply reflect changes in the amount of contractile proteins. While there were modest changes in the frequency-force relationship with perturbed Notch signalling, our overall conclusion was that manipulation of Notch signalling did not significantly influence functional regeneration of muscles from any of the three murine models examined.

The observation of different levels of regulation of various members of the Notch pathway across the three models of DMD is an important but unsurprising finding largely because of the variation in dystrophic pathology between the different models employed. The *mdx* mouse lacks dystrophin due to a point mutation on the dystrophin gene and is the most widely used murine model of DMD (Sicinski *et al.*, 1989). Although *mdx* mice share the same genetic deficit as in DMD patients, they generally exhibit a more benign phenotype, attributed largely to an enhanced regenerative capacity (Dupont-Versteegden & McCarter, 1992; Petrof *et al.*, 1993). A more phenotypically accurate murine model of DMD is the *dko* mouse which exhibits severe muscle wasting and weakness, significant spinal deformities (i.e. kyphosis) from an early age, and premature death (Deconinck *et al.*, 1997; Grady *et al.*, 1997). Both the *mdx* and *dko* mice had lower levels of Jagged2 mRNA in TA muscles compared with C57BL/10 mice. Jagged 2 was unchanged in the quadriceps muscles of DMD patients compared with healthy controls. Jagged2 is a Notch1 ligand in muscle (Luo *et al.*, 1997) and therefore a reduction in Jagged2 expression may contribute to reduced Notch signalling. Numb expression was also reduced significantly in both mouse models of

muscular dystrophy, but the expression of Numb in DMD patients was increased. This is in contrast to Turk *et al.* (2005), who found that Numb expression was increased in the limb muscles of *mdx* mice. Numb is a negative regulator of Notch1 signalling (Guo *et al.*, 1996; Beres *et al.*, 2011) and is also involved in muscle regeneration, particularly in the determination of satellite cell fate (Shinin *et al.*, 2006). Its upregulation in DMD suggests that it may inhibit Notch1.

Notch1 mRNA was decreased significantly in muscles from both *dko* mice and DMD patients. Notch3 mRNA was also similarly decreased in the muscles of *dko* mice but increased in DMD patients. Notch3 is suggested to repress Notch1 signalling in regenerating muscle (Kitamoto & Hanaoka, 2010) and so the decrease in Notch3 in *dko* mice may represent an attempt to enhance Notch1 signalling to promote muscle regeneration. The large (~five-fold) increase in Notch3 in muscles from DMD patients may contribute to the overall decreased efficiency of the Notch1 pathway. Hes1, a downstream target of Notch1, also was similarly downregulated (as for Notch1) in muscles from both the *dko* mice and DMD patients. Surprisingly Hes1 protein levels were elevated in *mdx* and DMD patients. The reason for this is unknown but may reflect the negative autoregulation of Hes1 mRNA by the Hes1 protein. Sustained increase in Hes1 protein represses *hes1* mRNA synthesis (Hirata *et al.*, 2002). Our results confirm the Notch pathway is compromised during muscular dystrophy, the degree of which varies with the dystrophic phenotype in the model being investigated. The variability in the regulation of certain genes in the Notch signalling pathway in these different models of muscular dystrophy highlights the importance of these comparisons when trying to understand the clinical relevance of regulatory pathways in the dystrophic pathology. The findings also warrant caution when translating findings between species.

We found that while acute activation of Notch signalling produced an increase in Hes1 expression in uninjured TA muscles, it had no significant effect on the functional characteristics of regenerating TA muscles of C57BL/10 mice. It has been observed that activation of the Notch pathway in young, healthy mice did not enhance regeneration after injury, presumably because the pathway is already at maximal efficiency in healthy animals (Conboy *et al.*, 2003). In contrast, we found that inhibition of Notch signalling produced functional deficits in regenerating muscles, supporting previous observations that inhibiting Notch impaired regeneration in healthy animals and confirming the importance of the Notch pathway for normal muscle repair (Conboy *et al.*, 2003). In *mdx* mice, activation of Notch signalling in regenerating TA muscles had no effect on functional parameters. Similarly, inhibition of Notch signalling did not affect muscle mass or maximal force, except for small decreases in the frequency-force relationship, which we believe may reflect the superior muscle regenerative capacity in *mdx* mice (Dupont-Versteegden & McCarter, 1992; Petrof *et al.*, 1993).

Since the muscles of *dko* mice are in constant cycles of degeneration and regeneration, the effects of Notch manipulation were first investigated without inducing myotoxic damage. This approach also more accurately resembles how treatment might be administered for DMD patients. Notch activation did not affect muscle mass, maximum force or the frequency-force relationship as observed in the C57BL/10 and *mdx* mice. As was seen in C57BL/10 mice, inhibition of the Notch pathway in *dko* mice had no effect on muscle mass or maximal force. However, unlike the other two mouse models, Notch inhibition significantly increased the frequency-force relationship in *dko* mice; an unexpected finding likely attributed to the more complex and disrupted muscle fibre regeneration in *dko* mice. Increased Notch signalling is vital for stimulating satellite cell activation and proliferation in the early stages of fibre regeneration after injury (Luo *et al.*, 2005). However, it is generally

accepted that successful muscle fibre regeneration cannot be achieved without a subsequent decrease in Notch signalling for myogenic differentiation (Conboy & Rando, 2002; Sun *et al.*, 2007; Sun *et al.*, 2008). As the muscles of *dko* mice (much like the muscles of DMD patients) will, at any given time, be undergoing either degeneration or regeneration, it is likely that acute manipulation (by either activation or inhibition) of the Notch pathway will affect muscle fibres either positively or negatively, depending on whether they are in the early or late stages of regeneration. To truly measure whether Notch activation can assist muscle regeneration in *dko* mice, it was necessary for all muscle fibres to be regenerating contemporaneously. We therefore injured muscles of *dko* mice with an intramuscular injection of Notexin to cause complete destruction of the muscle and to instigate a common 'time zero' for regeneration of all muscle fibres. Although Notch activation caused regenerating muscles of *dko* mice to be significantly smaller than controls and to have a slightly higher twitch force, there was no significant difference in maximal force or in the frequency-force relationship, confirming that activation of the Notch pathway does not enhance functional muscle regeneration in these mouse models of DMD.

The Notch pathway is important in skeletal muscle regeneration and acute activation of the pathway has previously been demonstrated to enhance muscle regeneration in aged mice (Conboy *et al.*, 2003). Our findings suggest that while the Notch signalling pathway is involved in muscle regeneration in dystrophic mice, acute activation of Notch does not provide any beneficial effect on muscle regeneration based on multiple functional assessments. Although Notch activation did not enhance regeneration in either of our mouse models of dystrophy, it must be noted that the expression of Notch proteins in the *mdx* and *dko* mice was not consistent with that in DMD, and caution is therefore warranted when translating our functional data from mouse models to DMD patients. Nonetheless, our data suggest that manipulation of Notch signalling may not be an effective intervention for DMD

patients, and that other pathways may play more important roles in regeneration during muscular dystrophy.

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Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

All animal experiments, functional testing and histology were performed in the lab of GSL. All PCR and western blotting analyses were performed in the lab or APR. JEC, SMG, APR and GSL conceived and designed the experiments. JEC, JT, AC, TN, SG, SL, CA, APR and GSL contributed to collection and analysis of data. JEC, APR and GSL wrote the draft manuscript, and all authors checked for scientific content and contributed to the final drafting.

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Figure 1: mRNA and protein levels of Notch signalling components in various models of dystrophy. Comparison of mRNA expression of Notch signalling proteins in uninjured tibialis anterior (TA) muscles of C57BL/10, *mdx* or *dko* mice **(A)** (**P* < 0.05 vs. C57BL/10, #*P* < 0.05 vs. *mdx*, ANOVA, n=9-14). Hes1 protein levels in the TA muscle of C57BL/10, *mdx* and *dko* mice **(B)** (**P* < 0.05 vs. C57BL/10, ANOVA, n=6-8). Comparison of mRNA expression of Notch signalling proteins in the diaphragm muscle of C57BL/10 and *mdx* mice **(C)** (**P* < 0.05 vs. C57BL/10, ANOVA, n=9-10). Hes1 protein levels in the diaphragm muscle of C57BL/10, *mdx* and *dko* mice **(D)** (**P* < 0.05 vs. C57BL/10, ANOVA, n=5-6). Comparison of mRNA expression of Notch signalling proteins in quadriceps muscles of DMD patients and healthy controls **(E)** (**P* < 0.05, Student's unpaired *t*-test, n=10). Hes1 protein levels in quadriceps muscles of DMD patients and healthy controls **(F)** (**P* < 0.05, Student's unpaired *t*-test, n=10).

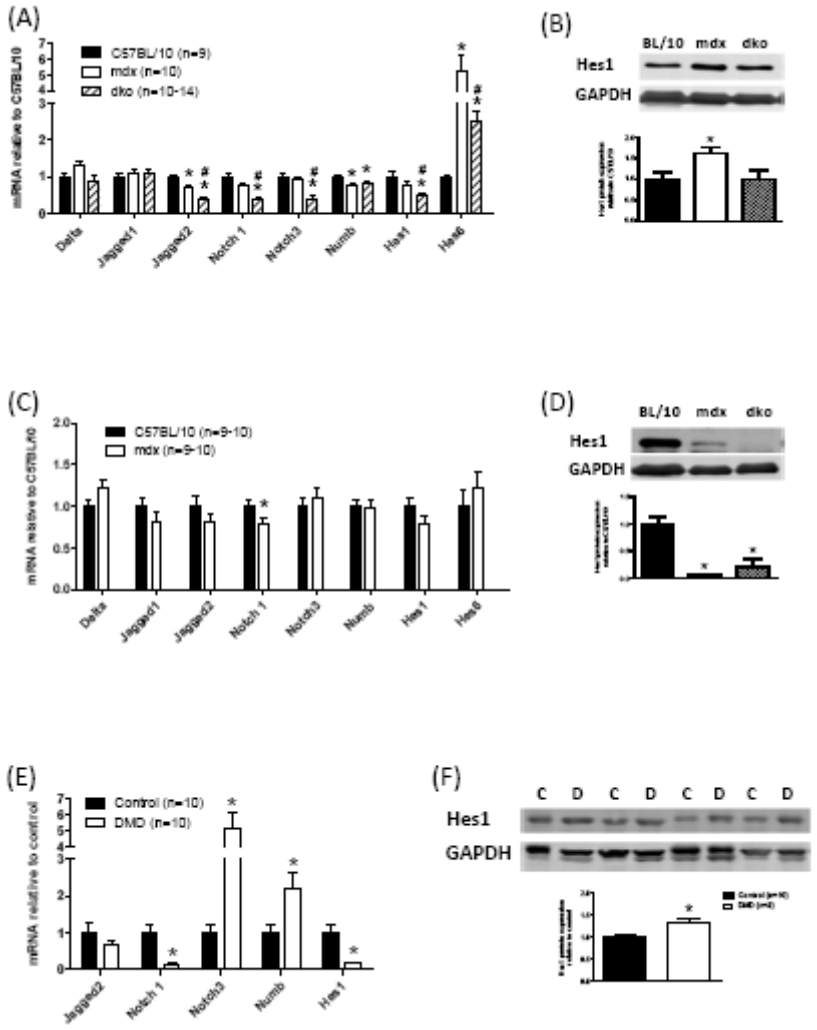

Figure 2: Effect of Notch manipulation on functional regeneration in C57BL/10 mice. Activation of Notch signalling with α-Notch increased the mRNA levels of the Notch target gene Hes1**(A)** (**P* < 0.05 vs. vehicle control, Student's unpaired *t*-test). In TA muscles following myotoxic injury, neither activation of Notch signalling with α-Notch nor inhibition of Notch signalling with Jagged-Fc had any significant effect on absolute maximum force at 7 or 14 days post-injury **(B)**, but Jagged-Fc did significantly inhibit specific force at 7 days post-injury **(C)** (**P* < 0.05 vs. vehicle control, Student's unpaired *t*-test). Activation of the Notch pathway did not alter the frequency-force relationship **(D).** Inhibition of the Notch pathway decreased force produced at each stimulation frequency **(E)** (#*P* < 0.05, treatment main effect, 2-way ANOVA)**.** H&E stained sections of regenerating muscles (7 days post-injury) following acute Notch activation or inhibition **(F)**. IgG = vehicle control for α-Notch, Fc = vehicle control for Jagged-Fc.

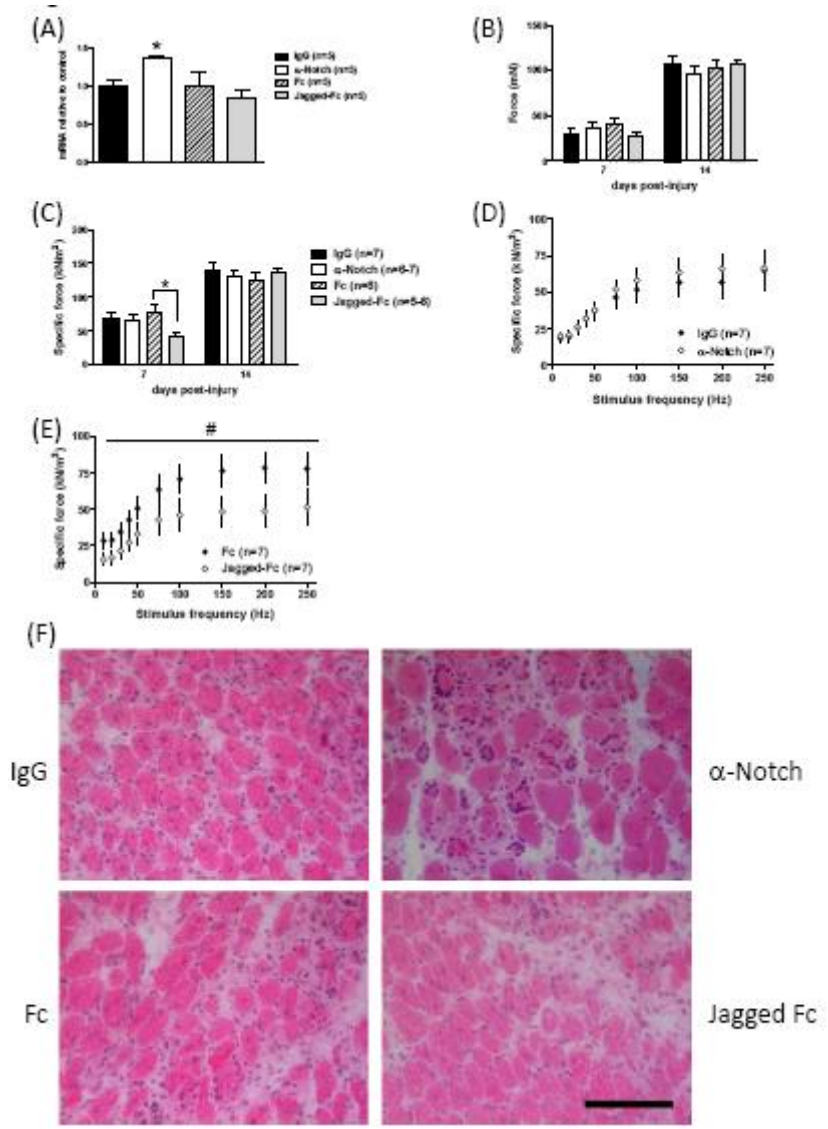

Figure 3: Effect of Notch manipulation on functional regeneration in muscles of *mdx* mice. Activation or inhibition of Notch signalling did not affect maximum absolute **(A)** or specific force **(B)**. Activation of Notch did not affect the frequency-force relationship at 7 or 14 days post-injury **(C)**. Inhibition of the Notch pathway decreased force produced at each stimulation frequency **(D)** (*#P* < 0.05, treatment main effect, 2-way ANOVA). H&E stained sections of regenerating muscles (7 days post-injury) following acute Notch activation or inhibition **(E)**. IgG = vehicle control for α-Notch, Fc = vehicle control for Jagged-Fc.

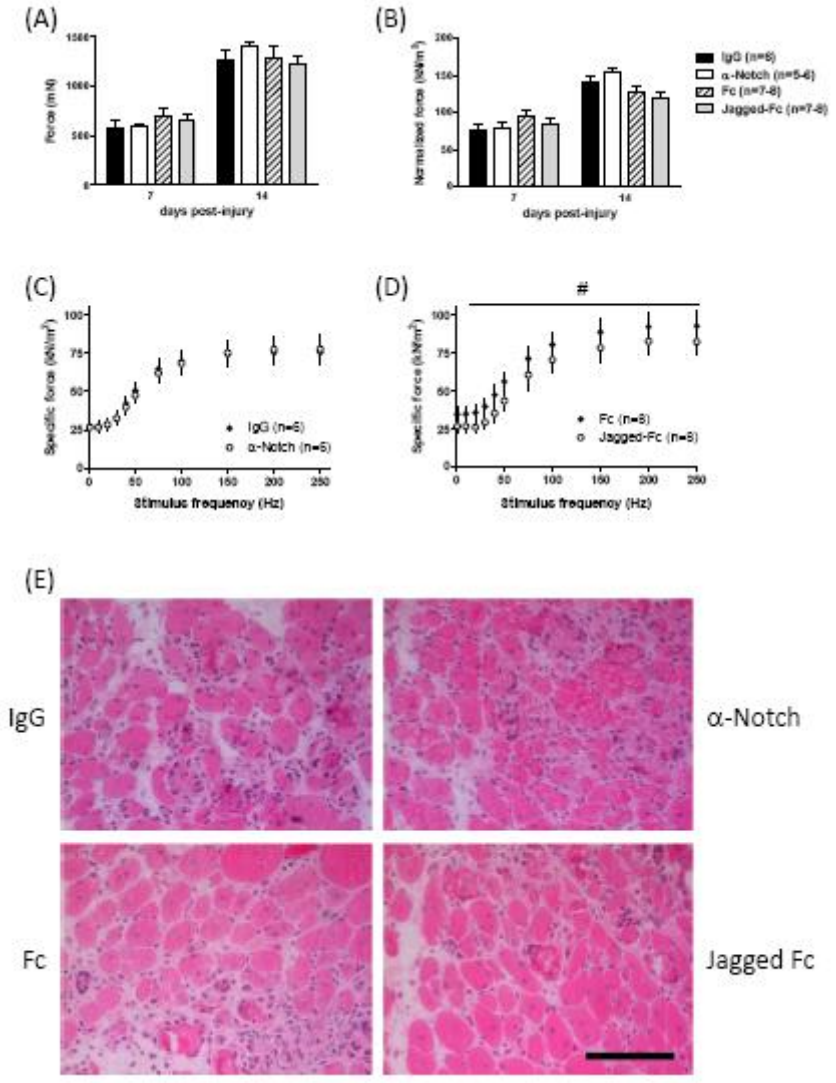

Figure 4: Effect of Notch manipulation on functional regeneration in muscles of *dko* mice. In uninjured TA muscles from *dko* mice, neither activation of Notch signalling with α-Notch nor inhibition of Notch signalling with Jagged-Fc had any effect on absolute force **(A)** or specific force **(B)** 4 days after treatment. Activation of the Notch pathway did not alter the frequency-force relationship in TA muscles at 4 days post-treatment, **(C),** while inhibition of the Notch pathway increased the force produced at each stimulation frequency **(D)** (*#P* < 0.05, treatment main effect, 2-way ANOVA)**.** In regenerating *dko* muscles after myotoxic injury, Notch activation did not significantly enhance absolute (**E**) or specific force **(F)** at 7 days post-injury nor did it have any effect on the frequency-force relationship **(G)**. IgG = vehicle control for α-Notch, Fc = vehicle control for Jagged-Fc.

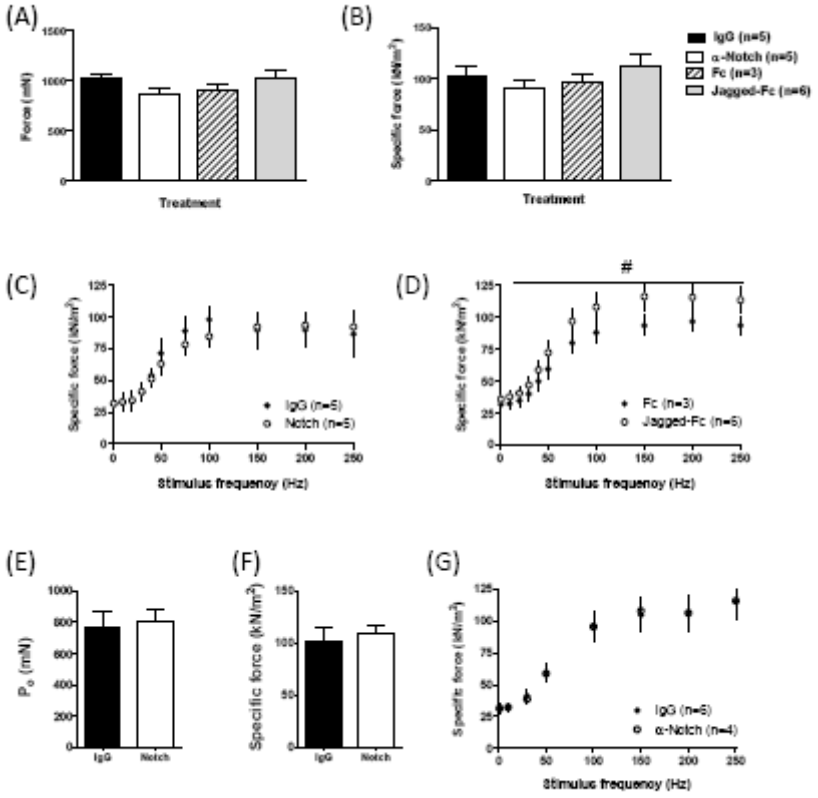

Table 1 – PCR primer sequences and annealing temperature

Primers for mouse targets			
Target	**Forward -5'**	**Reverse- 5'**	**Temp. °C**
Delta1	CTCCATACAGACTCTCCCGATGAC	ACAGAGCAACCTTCTCCGTAGTAG	60
Hes1	AGAGGCGAAGGGCAAGAATAAATG	CGGAGGTGCTTCACAGTCATTTC	60
Hes6	ACGGATCAACGAGAGTCTTCAGG	CCTCACGGTCAGCTCCAGAAC	60
Jagged1	GGAGGCGTGGGATTCCA	CCGAGTGAGAAGCCTTTCAATAAT	55
Jagged2	GCCTTAACGCTTTTTCTTGCA	TTGACGTTGATATGGCAGTTGAT	55
Notch1	CCTCTGACGCCCTTGCTCTG	TTGGAGTCCTGGCATCGTTGG	60
Notch3	TGATCGGCTCGGTAGTAATGC	GACAACGCTCCCAGGTAGTCA	55
Numb	CCGGCATGCTCCAATTG	TCTGGCTAAGAGCAGGAAAACC	55
Primers for human targets			
Target	**Forward -5'**	**Reverse- 5'**	**Temp. °C**
Hes1	GCTGAAAACACTGATTTTGGATGC	CCGGAGGTGCTTCACTGTC	60
Jagged2	TCAAGGTGGAGACGGTTGTTAC	TCCTCTCCCGCTCTTTCCTG	60
Notch1	GCAGGCAATCCGAGGACTATG	GTGGGTGTTCTGGCAGGATG	60
Notch3	CTGGCTACACGGGCACAC	GTTCACTTCGCAGTTCACACC	60
Numb	GACCTCATAGTTGACCAGACGATAG	TGACAGCCATGAAGCAGTGAC	60

Table 2 – Selected morphological and isometric twitch contractile properties of regenerating TA muscles (7 days post-injury) from C57BL/10 and *mdx* mice following acute Notch pathway manipulation at 3 days post-injury.

C57BL/10 **n**	**Hamster IgG** 7	**α-Notch** 7	**Fc** 7	**Jagged-Fc** 7
TA mass (injured/uninjured)	0.79 ± 0.06	0.87 ± 0.08	0.86 ± 0.04	0.94 ± 0.03
P_t (mN)	108.2 ± 24.4	128.8 ± 14.6	152.3 ± 22.1	105.0 ± 26.5
TPT (ms)	20.8 ± 1.3	18.4 ± 0.9	17.4 ± 1.0	22.0 ± 1.7**
½ RT (ms)	22.9 ± 0.7	18.8 ± 2.0	18.1 ± 1.9	25.6 ± 3.5
dP_t/dt (mN/ms)	22.7 ± 3.1	27.2 ± 2.1	29.2 ± 3.2	18.9 ± 2.3**
mdx **n**	**Hamster IgG** 6	**α-Notch** 6	**Fc** 8	**Jagged-Fc** 8
TA mass (injured/uninjured)	0.83 ± 0.06	0.85 ± 0.02	0.82 ± 0.04	0.80 ± 0.03
Pt (mN)	187.8 ± 21.1	215.2 ± 14.7	244.0 ± 38.8	215.3 ± 25.5
TPT (ms)	18.2 ± 1.0	17.1 ± 0.6	16.7 ± 0.6	17.3 ± 0.5
½ RT (ms)	18.2 ± 1.7	16.4 ±1.4	17.7 ± 2.2	16.6 ± 0.6
dPt/dt (mN/ms)	34.3 ± 5.2	38.5 ± 3.0	41.6 ± 6.7	37.6 ± 3.9

P_t - peak twitch tension; TPT - time to peak twitch tension; ½ RT - one-half relaxation time; dP_t/dt - maximum rate of force development during a twitch contraction. $^{**}P < 0.05$ vs vehicle control, Student's unpaired *t*-test.

Table 3 – Selected morphological and isometric twitch contractile properties of TA muscles from *dko* mice following acute Notch pathway manipulation.

Uninjured ***n***	**Hamster IgG** *5*	**α-Notch** *6*	**Fc** *3*	**Jagged-Fc** *6*
TA mass (injured/uninjured)	1.00 ± 0.02	0.98 ± 0.2	1.02 ± 0.02	1.02 ± 0.02
P_t (mN)	314.3 ± 16.0	285.4 ± 17.9	265.4 ± 13.6	313.1 ± 36.3
TPT (ms)	17.3 ± 0.9	16.4 ± 0.8	17.2 ± 0.4	17.5 ± 0.9
½ RT (ms)	16.2 ± 1.5	16.8 ± 1.2	17.4 ± 1.1	19.5 ± 3.2
dP_t/dt (mN/ms)	53.7 ± 4.4	49.6 ± 2.6	46.7 ± 1.1	51.2 ± 5.9
7-days post injury ***n***	**Hamster IgG** *7*	**α-Notch** *4*		
TA mass (injured/uninjured)	1.02 ± 0.02	0.90 ± 0.02**		
Pt (mN)	202.1 ± 21.0	223.4 ± 31.2		
TPT (ms)	17.0 ± 0.6	17.5 ± 1.3		
½ RT (ms)	17.9 ± 0.9	18.6 ± 0.6		
dPt/dt (mN/ms)	37.6 ± 2.6	41.5 ± 4.5		

P_t - peak twitch tension; TPT - time to peak twitch tension; ½ RT - one-half relaxation time; dP_t/dt - maximum rate of force development during a twitch contraction. $^{**}P < 0.05$ vs vehicle control, Student's unpaired *t*-test.