Disease Models & Mechanisms 6, 197-205 (2013) doi:10.1242/dmm.009993

Osteopontin deficiency delays inflammatory infiltration and the onset of muscle regeneration in a mouse model of muscle injury

Kitipong Uaesoontrachoon[1,*], Dimuthu K. Wasgewatte Wijesinghe[1], Eleanor J. Mackie[1,‡] and Charles N. Pagel[1,‡,§]

SUMMARY

Osteopontin is secreted by skeletal muscle myoblasts and stimulates their proliferation. Expression of osteopontin in skeletal muscle is upregulated in pathological conditions including Duchenne muscular dystrophy, and recent evidence suggests that osteopontin might influence the course of this disease. The current study was undertaken to determine whether osteopontin regulates skeletal muscle regeneration. A whole muscle autografting model of regeneration in osteopontin-null and wild-type mice was used. Osteopontin expression was found to be strongly upregulated in wild-type grafts during the initial degeneration and subsequent early regeneration phases that are observed in this model. Grafted muscle from osteopontin-null mice degenerated more slowly than that of wild-type mice, as determined by histological assessment, fibre diameter and fibre number. The delayed degeneration in osteopontin-null grafts was associated with a delay in neutrophil and macrophage infiltration. Centrally nucleated (regenerating) muscle fibres also appeared more slowly in osteopontin-null grafts than in wild-type grafts. These results demonstrate that osteopontin plays a non-redundant role in muscle remodelling following injury.

INTRODUCTION

Efficient regeneration of injured or dystrophic skeletal muscle relies upon the coordinated action of invading inflammatory cells that induce muscle fibre necrosis and phagocytosis (Lescaudron et al., 1999; Tidball and Wehling-Henricks, 2007), and an endogenous pool of quiescent myogenic stem cells known as satellite cells that form new muscle fibres (Zammit et al., 2006). The failure of efficient muscle regeneration, such as occurs in dystrophic muscle, results in fibrosis of the muscle, which reduces its functional capacity and its ability to regenerate in response to further injury (Huard et al., 2002). The factors that control and coordinate inflammation and myogenesis during muscle regeneration are both complex and poorly understood. This study aims to examine the role of the multifunctional glycoprotein osteopontin in these processes.

Osteopontin, also known as early T-lymphocyte activation 1 protein (ETA1), secreted phosphoprotein 1 (SPP1) and bone sialoprotein 1 (BSP1), is a member of the small integrin-binding N-linked glycoprotein family of proteins, which interacts with certain variants of the hyaluronan receptor CD44 and with a variety of integrins by both RGD-dependent and independent mechanisms (Denhardt and Guo, 1993). Osteopontin exists both as an adhesive component of the extracellular matrix and as a soluble molecule with cytokine-like functions (O'Regan and Berman, 2000). It is expressed by a wide range of cells and promotes attachment, proliferation, migration, chemotaxis and apoptosis of macrophages, lymphocytes, osteoblasts and a range of tumour cells (Giachelli et al., 1998; O'Regan et al., 1999; Standal et al., 2004), regulating many pathological and physiological processes, including tissue repair, inflammation and fibrosis (Mori et al., 2008; O'Regan and Berman, 2000; Pardo et al., 2005). Although no developmental abnormalities have been reported in mice in which the osteopontin gene has been targeted by homologous recombination (Rittling et al., 1998), altered responses to injury such as skin incision, spinal cord injury, bone healing and ventricular and lung fibrosis have been described (Berman et al., 2004; Duvall et al., 2007; Hashimoto et al., 2007; Liaw et al., 1998; Sam et al., 2004).

Osteopontin has been described as a component of the inflammatory milieu of dystrophic and injured skeletal muscles (Haslett et al., 2002; Hirata et al., 2003; Porter et al., 2002). We have recently demonstrated that osteopontin is expressed by myoblasts in areas of focal muscle necrosis in the muscles of dystrophic *mdx* mice (dystrophin-deficient mice used as a model for muscular dystrophy), and that recombinant mouse osteopontin is able to influence adhesion, proliferation and differentiation of skeletal muscle myoblasts (Uaesoontrachoon et al., 2008). However, the role of osteopontin in muscle injury and regeneration remains unclear. For example, Vetrone and colleagues found that osteopontin promotes fibrosis of aged dystrophic mouse skeletal diaphragm muscles (Vetrone et al., 2009), whereas Pegoraro and colleagues reported that a polymorphism (rs28357094) in the osteopontin gene promoter, which reduces osteopontin mRNA expression in transfected HeLa cells (Giacopelli et al., 2004), is correlated with decreased muscle strength and a younger age at loss of ambulation in patients with Duchenne muscular dystrophy (Pegoraro et al., 2011). These findings suggest that the role that osteopontin plays in response to injury is complex, contributing to both muscle repair

[1]Faculty of Veterinary Science, University of Melbourne, Parkville, Victoria 3010, Australia
*Present address: Research Center for Genetic Medicine, Children's National Medical Center, 111 Michigan Avenue N.W., Washington, DC 20010, USA
[‡]These authors contributed equally to this work
[§]Author for correspondence (cpagel@unimelb.edu.au)

Received 5 April 2012; Accepted 7 August 2012

TRANSLATIONAL IMPACT

Clinical issue

Muscle injury resulting from trauma, or as a result of myopathic diseases such as Duchenne muscular dystrophy, causes activation of endogenous myogenic cells and infiltration of the muscle by inflammatory cells. Infiltrating inflammatory cells induce necrosis of damaged sections of muscle fibres and phagocytose the necrotic tissue, allowing muscle fibres to regenerate through the terminal differentiation of activated myogenic cells. Although osteopontin has been described as a component of the inflammatory milieu of dystrophic and injured skeletal muscles, its role remains unclear because experimental evidence from dystrophic muscles suggests that osteopontin expression can contribute to both increased muscle strength and fibrosis. One possible explanation is that chronic osteopontin overexpression (such as occurs in dystrophic muscles) drives muscle fibrosis, whereas acute osteopontin overexpression promotes efficient muscle regeneration. This study investigated the effect of acute osteopontin expression on the early stages of muscle regeneration, using whole muscle autografting of *extensor digitorum longus* muscles from either wild-type or osteopontin-null mice.

Results

The results of this study demonstrate that an increase in osteopontin expression is rapidly induced following a single severe injury and is then progressively lost over 14 days. This period of time coincides with infiltration of the muscles by neutrophils and macrophages, and with muscle fibre necrosis and phagocytosis. There was also evidence of muscle fibre regeneration at the later time points. In muscle grafts from osteopontin-null mice, muscle fibre necrosis and infiltration of muscles by neutrophils and macrophages were significantly delayed compared with grafted muscles from wild-type mice. Furthermore, the onset of muscle regeneration was delayed in grafted osteopontin-null muscles compared with grafted wild-type muscles.

Implications and future directions

These results indicate that acute osteopontin expression promotes muscle inflammation, necrosis and regeneration after a single severe injury, supporting the hypothesis that acute osteopontin expression is important for tissue remodelling in skeletal muscle. This study increases understanding of the molecular regulation of early muscle regeneration and helps to elucidate the role of osteopontin in injured and diseased muscles.

and muscle fibrosis. On the basis of this evidence, we hypothesised that, although chronic overexpression of osteopontin promotes muscle fibrosis, acute osteopontin overexpression is important in coordinating muscle inflammation and regeneration and, therefore, in promoting efficient muscle repair.

The current study was performed to study the effect of acute osteopontin expression on the early stages of muscle regeneration and used whole muscle autografting of extensor digitorum longus (EDL) muscles from wild-type and osteopontin-null littermate mice. In this study, a number of histological and morphometric parameters were examined in grafted and sham-operated muscles in order to determine whether muscle inflammation, necrosis, phagocytosis and myogenesis were altered in osteopontin-deficient muscles. The results of the study contribute to our understanding of the roles of osteopontin in the responses of skeletal muscle to injury.

RESULTS

Osteopontin deficiency delays muscle fibre degeneration and regeneration in response to a single severe injury

Whole muscle autografting of EDL muscles from wild-type and osteopontin-null mice was used to determine the role of osteopontin following a single severe muscle injury. Histological analysis of grafted muscles indicated that, initially, loss of muscle fibres and infiltration of the grafted muscles by mononucleate cells were delayed in osteopontin-null muscles compared with wild-type muscles. Thus, 5 days after grafting, fewer persisting muscle fibres and more mononucleate cells were observed in grafted wild-type muscle (Fig. 1A,C) than in osteopontin-null muscles (Fig. 1B,D). Furthermore, 2 days later, histological evidence suggested that muscle fibre regeneration was more advanced in wild-type muscles than in osteopontin-null muscles. Regenerated muscle fibres, identified by their basophilia and central nucleation, were a greater feature of grafted wild-type muscles, particularly in the region of the graft adjacent to the adherent tibialis anterior (TA) muscle (Fig. 1E,G), than of grafted osteopontin-null muscles (Fig. 1F,H). At 14 and 20 days after grafting, no histological differences between the two genotypes were observed (data not shown).

Morphometric analysis of grafted osteopontin-null and wild-type muscles

The mean minimal diameter of muscle fibres from grafted muscles was measured in histological sections and expressed as a percentage of the minimal diameter of muscle fibres from contralateral sham-operated muscle (Fig. 2A). At 3 days post-surgery, the mean fibre diameter of wild-type muscles was significantly smaller than that of osteopontin-null muscles. At later time points (5, 7 and 14 days), fibre diameter continued to decline but no significant difference was observed between the two genotypes (Fig. 2A).

The total number of muscle fibres per section was also counted at each time point for grafted and sham-operated muscles of both genotypes. Grafted wild-type muscles contained a significantly lower total fibre number (relative to sham) than did grafted osteopontin-null muscles 3 days after surgery. At this time point, approximately 35% of the number of muscle fibres present in sham-operated muscles remained in grafted wild-type muscles, whereas approximately 77% of fibres remained in grafted osteopontin-null muscles. Between 5 and 7 days post-surgery, the number of muscle fibres (relative to sham) in wild-type muscles increased, whereas in osteopontin-null muscles the value continued to decline (Fig. 2B). Thus, by 7 days post-surgery, grafted wild-type muscles contained a significantly greater proportion of muscle fibres (78% of sham value) than did grafted osteopontin-null muscles (34% of sham value; Fig. 2B). In the grafted muscles 14 days after surgery, there was no significant difference in the number of fibres (relative to sham) between the two genotypes (Fig. 2B).

Muscle fibre regeneration was evaluated by determining the proportion of the total fibre number that contained centrally located nuclei. There was no evidence of centrally nucleated fibres in sham-operated muscles at any of the time points or in grafted muscles of either genotype at 3 days after grafting (Fig. 2C). Five days after surgery, wild-type grafted muscles contained a significantly higher proportion of centrally nucleated fibres (53%) than osteopontin-null (14%) grafted muscles (Fig. 2C). At 7 and 14 days post grafting there was no significant difference between the two genotypes in the proportion of muscle fibres containing central nuclei (Fig. 2C).

Immunolocalization of osteopontin in grafted and sham-operated muscles

In sham-operated wild-type muscles, no osteopontin staining was observed in the muscle fibres or interstitial cells at any of the time

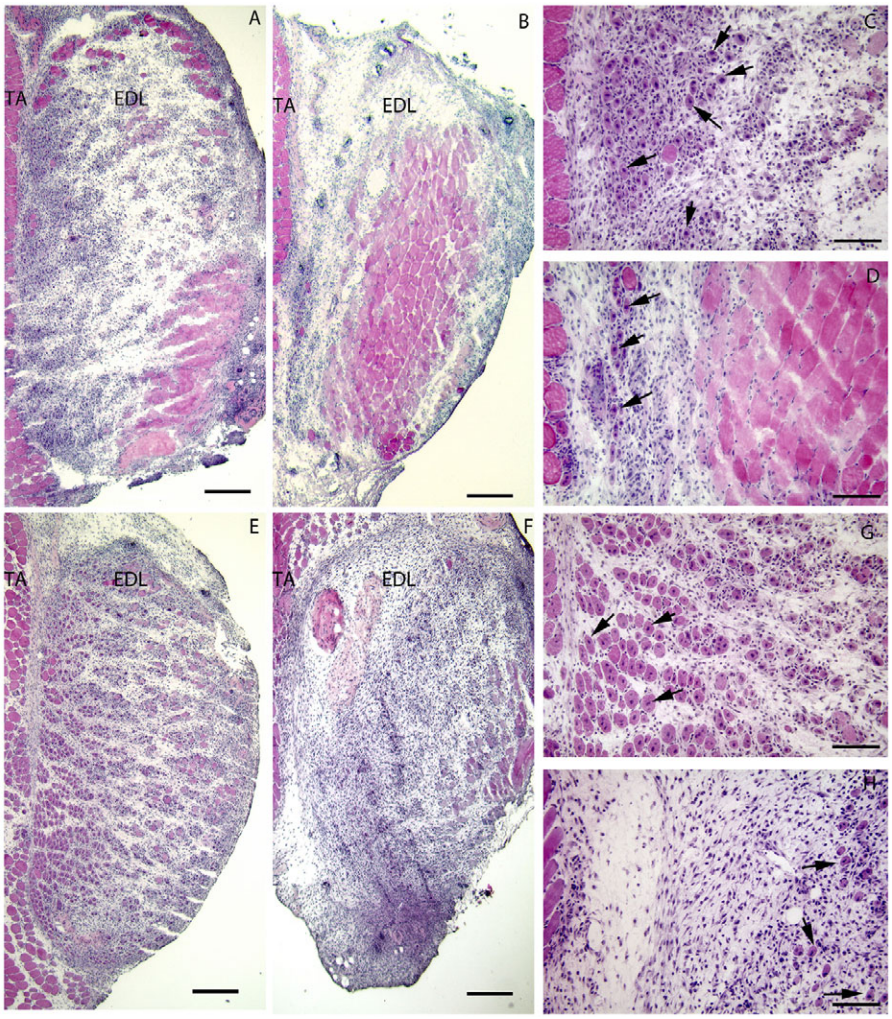

Fig. 1. Histological analysis of grafted and sham-grafted muscles. (A-H) Representative H & E stained sections of wild-type and osteopontin-null EDL grafts at 5 days (wild-type: A,C; osteopontin-null: B,D) and 7 days (wild-type: E,G; osteopontin-null: F,H) after surgery. Scale bars: 250 μm (A,B,E,F) and 100 μm (C,D,G,H). Arrows indicate basophilic centrally nucleated fibres.

points. In these muscles, osteopontin immunoreactivity was limited to capillary-like structures within the muscle (Fig. 3A). Three days after surgery in grafted wild-type muscles, strong perinuclear osteopontin immunoreactivity was detected in interstitial cells and intense cytoplasmic osteopontin staining was observed in necrotic muscle fibres, which were identified at this stage as fibres containing internal nuclei (Fig. 3B). By contrast, intact muscle fibres (lacking internal nuclei) at this time point showed only light cytoplasmic immunoreactivity (Fig. 3B).

At 5 and 7 days after surgery, osteopontin immunoreactivity was weaker and more variable, and was again observed in the cytoplasm of mononucleate interstitial cells. Osteopontin staining was now observed in regenerated (small diameter, centrally nucleated) muscle fibres, which constituted the bulk of the muscle; some of these fibres showed intense cytoplasmic staining, but most showed weak immunoreactivity only at their periphery (Fig. 3C). At the last two time points, 14 and 20 days post-surgery, only faint osteopontin staining was observed between muscle fibres (data not shown). No osteopontin immunostaining was observed in wild-type muscles incubated with a non-immune primary antibody (data not shown), or in osteopontin-null muscles at any of the time points post-surgery (Fig. 3D).

Inflammatory cell infiltration into grafted osteopontin-null and wild-type muscles

In order to study potential mechanisms for the observed delay in skeletal muscle degeneration and regeneration in osteopontin-deficient muscles, macrophages and neutrophils were localized in sections of grafted muscle by immunohistochemistry using antibodies raised against the macrophage marker F4/80 and the neutrophil marker Ly6G (Fig. 4). In grafted muscles, F4/80- or Ly6G-positive cells were observed at each of the time points studied, appearing first in the region of the graft adjacent to the TA muscle and persisting longest in the region of the graft furthest from the TA. No F4/80- or Ly6G-positive cells were observed in sham-grafted muscles at any of the time points studied. The average number of macrophages was significantly higher in wild-type than in osteopontin-null grafted muscles at 3 days after surgery but not at later time points (Fig. 5A). Three days after surgery there were significantly fewer neutrophils in sections of grafted muscles from the osteopontin-null mice than in those from wild-type mice (Fig. 5B). By 7 days post-grafting, significantly more neutrophils were observed in osteopontin-null than wild-type grafted muscles. No significant differences between genotypes in the number of neutrophils present in muscle sections were observed at 1, 5 and 14 days after surgery.

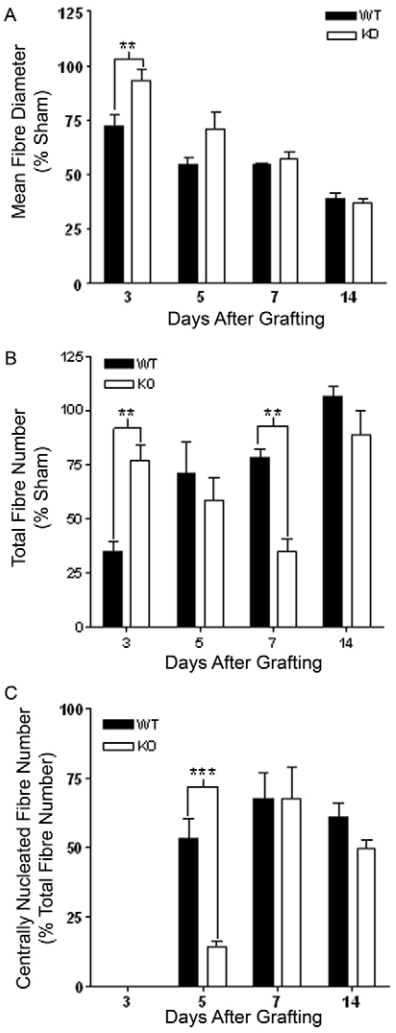

Fig. 2. Histomorphometric analysis of grafted and sham-grafted muscles. (A) Mean minimal Feret's diameter of the muscle fibres of wild-type (WT) and osteopontin-null (KO) EDL muscles at 3, 5, 7 and 14 days after surgery, expressed as percentage of mean minimal Feret's diameter of contralateral sham grafted muscle. (B) Mean total fibre number of grafted EDL muscles from wild-type and osteopontin-null mice at 3, 5, 7 and 14 days after surgery, expressed as a percentage of the value for mean contralateral sham grafted muscles. (C) Centrally nucleated muscle fibres as a percentage of the total fibre number of grafted wild-type and osteopontin-null EDL muscles. Data are expressed as means ± s.e.m. (n=5 animals from each genotype and time point). Two-way ANOVA, Bonferroni post-hoc test, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ for comparisons between wild-type and osteopontin muscles.

Osteopontin expression is not associated with increased fibrosis in grafted muscles

The possibility of fibrosis being present in the grafted muscles of either genotype was investigated using Picrosirius Red staining of collagen in sections of grafted and sham-grafted muscles at 20 days post-surgery. No difference in the pattern of Picrosirius Red staining was observed between wild-type and osteopontin-null muscles, either within the EDL muscle or at the interface between the TA and the EDL, in grafted or sham-grafted muscles (Fig. 6).

DISCUSSION

In this study, we have demonstrated that in response to a single severe injury, muscle fibre necrosis and regeneration are delayed in muscles from mice that lack osteopontin. Due to the role of osteopontin in inflammation, fibrosis and tissue remodelling in other tissues and its elevated expression in dystrophic muscles (Haslett et al., 2002; Porter et al., 2002), interest has recently been focused on the role of osteopontin in the pathogenesis of dystrophinopathies (Pegoraro et al., 2011; Vetrone et al., 2009). In dystrophin-deficient muscles, contraction-mediated injury causes persistent asynchronous cycles of segmental necrosis and regeneration in small foci of muscle fibres (Bridges, 1986; Petrof et al., 1993). These cycles of muscle fibre injury and regeneration are thought to underlie the chronic inflammatory signature and osteopontin overexpression that are characteristic of dystrophin-deficient muscles (Haslett et al., 2002; Porter et al., 2002; Uaesoontrachoon et al., 2008).

In this study we have used whole muscle grafting as a model of muscle regeneration that lacks the chronic inflammation found in dystrophic muscle and looked at the effect of acute osteopontin expression on the response of skeletal muscle to injury. Whole muscle autografting was chosen as a model of muscle injury over other established models of muscle injury such as ischemia reperfusion injury (Vignaud et al., 2010), crush injury (McGeachie and Grounds, 1987) and injection of myolytic compounds such as the snake venom notexin (Harris et al., 2003). Whole muscle grafting involves removing the entire EDL muscle from its bed, severing the vascular and neural connections of the grafted muscles, thus causing severe ischaemia and subsequent necrosis of all the fibres of the muscles (Hansen-Smith and Carlson, 1979). This model was chosen for the current study because it involves a single injury that results in a severe, reproducible, synchronous and predictable process of muscle degeneration and regeneration (Hansen-Smith and Carlson, 1979). In addition, it is a well characterised model, having been used to study skeletal muscle regeneration in a number of contexts including pathogenesis of the dystrophinopathies, angiogenesis, exercise and pharmacodynamics (Grounds et al., 2005; Morgan et al., 1989; Roberts and McGeachie, 1990; Roberts and McGeachie, 1992a; Roberts and McGeachie, 1992b; Roberts and McGeachie, 1994).

Although osteopontin is not a constituent of normal skeletal muscle, it is rapidly upregulated in response to injury (Barbosa-Souza et al., 2011; Hirata et al., 2003) and has been shown to be expressed by myoblasts in vitro and in pathological lesions in vivo (Uaesoontrachoon et al., 2008). The lack of cellular staining in uninjured wild-type muscles in the current study suggests that quiescent satellite cells do not express detectable levels of osteopontin. Despite this, by 3 days post-surgery, strong osteopontin immunoreactivity was observed in grafted wild-type muscles associated with single interstitial cells as well as in muscle fibres containing multiple internal nuclei, which are considered to be necrotic (Hirata et al., 2003). Whether the source of osteopontin in these fibres are muscle fibres themselves or the invading inflammatory cells remains unclear. Although osteopontin staining declined over the time course, strong osteopontin immunostaining was also observed in regenerated (small diameter, centrally nucleated) muscle fibres between 5 and 7 days post-grafting and faint osteopontin staining was still detectable even at the later time points studied.

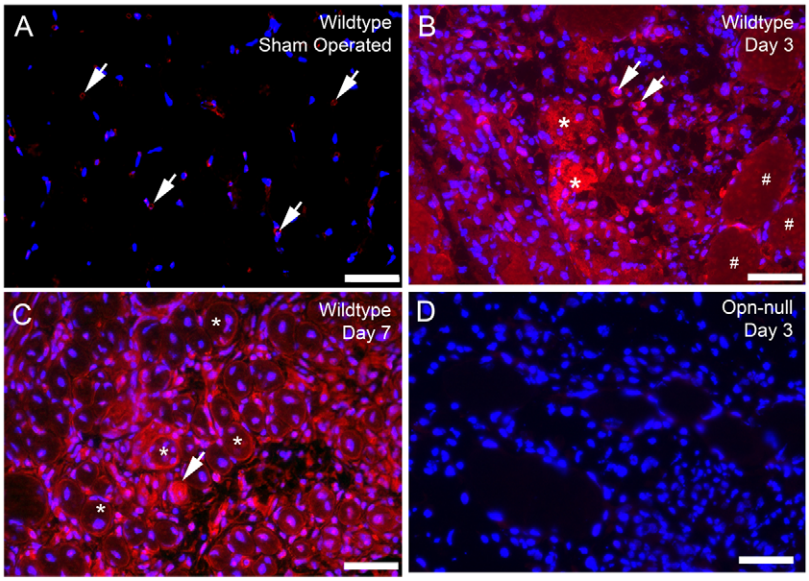

Fig. 3. Osteopontin immunostaining of grafted and sham-grafted muscles. (A-D) Representative transverse sections from EDL whole muscle grafts immunostained with anti-osteopontin antibody (LF-175: red) and counter stained with DAPI (blue). (A) Sham-operated wild-type control. Arrows indicate osteopontin-positive capillary-like structures. (B) Wild-type EDL muscle 3 days after grafting. Arrows, interstitial cells; asterisks, muscle fibres with internal nuclei; #, muscle fibres lacking internal nuclei. (C) Wild-type EDL muscle 7 days after grafting. Arrow, small diameter centrally nucleated fibre with intense osteopontin staining; asterisks, small diameter centrally nucleated fibres with weak, peripheral osteopontin staining. (D) Osteopontin-null EDL muscle at 3 days after grafting. Scale bars: 50 μm.

As well as increased expression of osteopontin, by 3 days post-surgery, clear signs of degenerative changes, including decreased fibre diameter and fibre number, were evident in injured wild-type muscles. By contrast, the relatively normal histology and morphometric parameters of injured osteopontin-deficient muscles at 3 days post-surgery indicated that muscle fibre necrosis was delayed in the absence of osteopontin. Evidence of the onset of muscle fibre necrosis in grafted osteopontin-null mice did not occur until 5-7 days post-surgery. In addition to the observed delay in muscle fibre degeneration in grafted osteopontin-null muscles, muscle regeneration also appeared delayed. Thus, at 5 days post-surgery grafted muscles from wild-type mice contained a significantly greater proportion of centrally nucleated muscle fibres than grafted osteopontin-null muscles. At 7 days post-grafting, regeneration still appeared to be more advanced in wild-type than in osteopontin-null muscles. However, by 14 days post-surgery, regeneration in osteopontin-null muscles appeared to have caught up with that of wild-type muscles because no significant differences were observed at this or subsequent time points. The ability of osteopontin-null muscles to eventually undergo muscle fibre necrosis and regeneration, albeit delayed, suggests that osteopontin is not essential for muscle repair, possibly due to compensation for the loss of osteopontin by other unidentified proteins (Boudiffa et al., 2010; Monfoulet et al., 2010).

As whole muscle grafting involves severing of the vascular connections of the grafted muscles to induce ischemia, one possible explanation for the observed delay in muscle fibre necrosis and regeneration in osteopontin-null muscles could be a delay in revascularisation of the muscle in the absence of osteopontin. Revascularisation of whole muscle grafts has previously been shown to begin approximately 72 hours after surgery (Roberts and McGeachie, 1990). However, in a mouse model of hind limb ischemia reduced perfusion was found at 7 and 14 days, but not 3 days post-ischemia in osteopontin-null mice compared with wild-type controls (Duvall et al., 2008). Thus, the timing of the delay in necrosis of osteopontin-null muscles, which was apparent as early

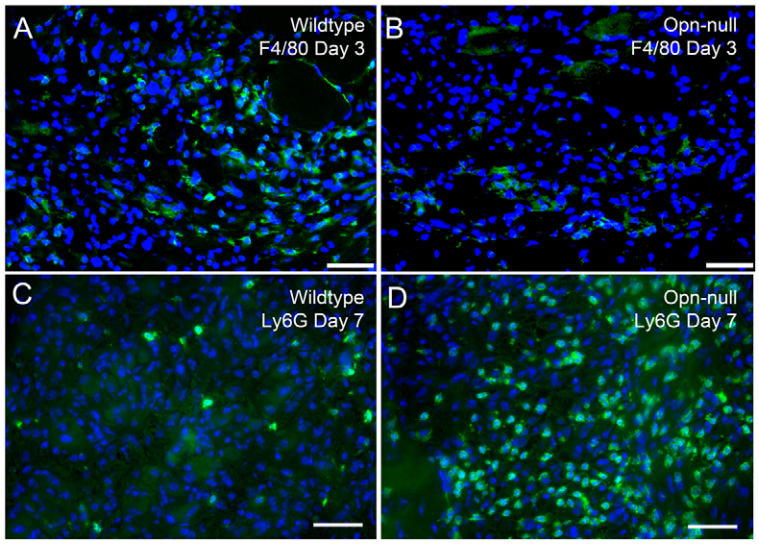

Fig. 4. Localization of macrophages and neutrophils in grafted and sham-grafted muscles. (A-D) Representative transverse sections of wild-type (A,C) and osteopontin-null (B,D) EDL whole muscle grafts at 3 (A,B) or 7 (C,D) days post-grafting, immunostained with the antibody F4/80 (A,B, green) or the antibody Ly6G (C,D, green), and counter-stained with DAPI (blue). Scale bars: 50 μm.

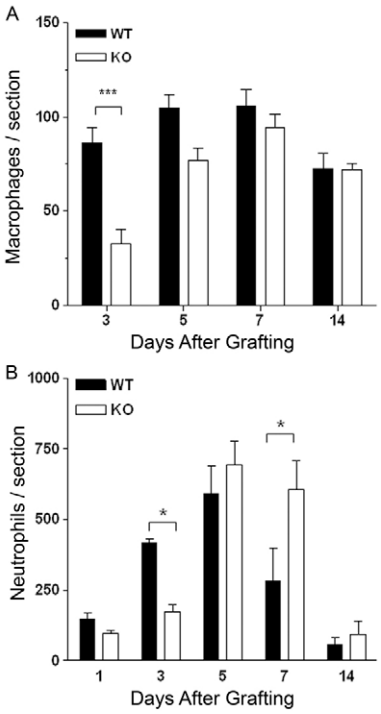

Fig. 5. Number of macrophages and neutrophils in grafted and sham-grafted muscles. (A,B) Macrophages (A) and neutrophils (B) were counted in histological sections of grafted muscles from wild-type (WT) and osteopontin-null (KO) mice at different times after grafting. Data are expressed as means ± s.e.m. (n=5 animals from each genotype and time point). Two-way ANOVA, Bonferroni post-hoc test, *P≤0.05, ***P≤0.001 for comparisons between wild-type and osteopontin-null muscles.

as 3 days post grafting, suggests that delayed revascularisation in osteopontin-null muscles is unlikely to be the mechanism by which the observed effect occurs.

Osteopontin has been shown to promote migration of macrophages (Giachelli et al., 1998; Vetrone et al., 2009) and neutrophils (Banerjee et al., 2006) in tissues in response to injury. Furthermore, as both macrophages and neutrophils have been shown to promote muscle fibre necrosis (Hodgetts et al., 2006; Nguyen and Tidball, 2003; Pizza et al., 1998), it is likely that these cells are responsible for initiating muscle fibre necrosis following initial trauma in the autograft model used in this study. The lower macrophage and neutrophil numbers in the grafted muscles of osteopontin-null mice at 3 days after surgery and the significantly higher numbers of neutrophils present in osteopontin-null muscles at 7 days post-surgery suggest that infiltration of the injured muscles by neutrophils and macrophages (and the subsequent loss of neutrophils) occurs more slowly in the absence of osteopontin. Thus, a delay in inflammatory infiltration and recruitment of macrophages and neutrophils to sites of skeletal muscle injury is likely to be the reason for the observed delay in muscle fibre necrosis in grafted osteopontin-null muscles. Further evidence in support of a delayed inflammatory response as the mechanism for this delay comes from a study in which whole muscle EDL grafts were conducted in mice treated with Remicade or Enbrel to block TNFα-mediated inflammation (Grounds et al., 2005). The authors reported a similar delay in muscle fibre necrosis and myogenesis at 5 days post-surgery to that observed in the current study.

Osteopontin exerts significant effects on myoblast adhesion, proliferation and differentiation in vitro (Uaesoontrachoon et al., 2008). It is, therefore, possible that the delay in muscle regeneration observed in osteopontin-null mice in the current study relates to a requirement for a direct interaction of osteopontin with muscle cells. However, the inflammatory response of muscle to injury is known to be important in stimulating muscle fibre regeneration (Lescaudron et al., 1999; Tidball, 2005). Thus, it is not possible to determine whether the delay in regeneration in osteopontin-null mice is a secondary consequence of the delay in the inflammatory response or a direct effect of osteopontin deficiency on myogenesis, or a combination of the two.

Excessive collagen deposition and fibrosis is a major pathological feature of the muscles of patients with Duchenne muscular dystrophy and the diaphragm muscle of *mdx* mice (Stedman et al., 1991) and osteopontin has been shown to drive fibrosis of aged, chronically injured *mdx* muscles (Vetrone et al., 2009). In the current study, no evidence of excessive muscle collagen deposition was found in wild-type grafted muscles compared with osteopontin-null grafted muscles, suggesting that in response to a single severe injury acute osteopontin overexpression by itself does not induce collagen deposition and fibrosis.

In conclusion, the results of this study indicate that acute osteopontin expression promotes muscle inflammation, necrosis and regeneration after a single severe injury. Thus, this study supports the hypothesis that acute osteopontin expression is important for tissue remodelling (Pegoraro et al., 2011), whereas chronic osteopontin overexpression drives chronic inflammation and therefore muscle fibrosis (Vetrone et al., 2009). More generally, the results suggest that early osteopontin expression is likely to support a rapid return to normal muscle function following injury. These observations contribute to our understanding of the molecular regulation of early muscle regeneration, and might assist in the development of therapeutic approaches to conditions involving inadequate regeneration, such as the muscular dystrophies.

METHODS

Animals

Osteopontin-null mice on a C57Bl/6J genetic background (Rittling et al., 1998), generously provided by David Denhardt (Rutgers University, New Brunswick, NJ), were maintained as heterozygous breeding stock at the University of Melbourne, School of Veterinary Science. All work with animals was approved by the Veterinary Science Animal Experimentation Ethics Committee of the University of Melbourne (AEC ID 811049.1) and all procedures involving animals were conducted in accordance with the conditions of this approval.

Whole muscle autografting

Whole muscle autografting was performed on anesthetized, genotyped 12-week-old male littermate wild-type and osteopontin-null mice, as previously described (Roberts and McGeachie, 1990). Briefly, the whole EDL muscle of the right hind-limb was removed free of adherent tissues, repositioned over the TA muscle and secured by suturing the distal EDL tendon to the distal tendon of

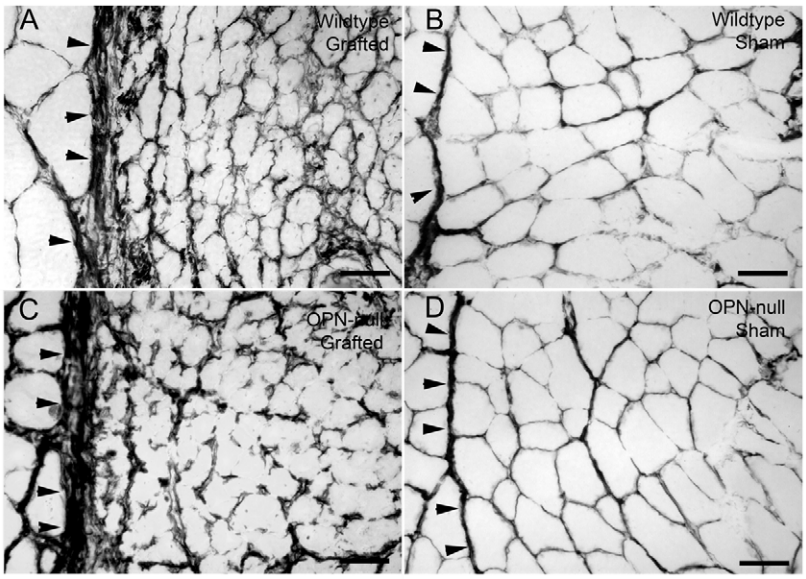

Fig. 6. Fibrosis in grafted and sham-grafted muscles. (A-D) Representative transverse sections stained with Picrosirius Red from grafted (A,C) and sham-grafted (B,D) wild-type (A,B) and osteopontin-null (C,D) EDL muscles at 20 days after grafting. Arrows indicate the interface between the TA and EDL in each section. Scale bars: 50 μm.

the TA and the proximal tendon of the EDL onto the proximal end of TA muscle with 5-0 braided silk (Johnson and Johnson Medical, New Brunswick, NJ) (Fig. 7). The skin was then sutured closed. For sham-operated controls, the skin of the left hind-limb was incised and reflected from the muscle, then closed and sutured without disturbing the muscle. Mice were randomly assigned into different groups (n=5) and grafts were left for 1, 3, 5, 7, 14 and 20 days before mice were euthanized by cervical dislocation.

Histology and morphometry

The EDL and TA muscles were removed intact from both limbs and sectioned transversally in the mid-belly region (Fig. 7) prior to embedding in OCT compound and freezing in isopentane chilled in liquid nitrogen. Muscles were sectioned (10 μm) using a cryostat and post-fixed in 4% (w/v) paraformaldehyde prior to staining with hematoxylin and eosin (H & E), or Picrosirius Red using a proprietary kit (Polyscience, Warrington, PA) following the manufacturer's instructions. Staining was followed by dehydration, clearing and mounting.

Histological and morphometric analyses were performed using Image Pro Plus 4.0 (MediaCybernetics, Bethesda, MD) on representative sections from the mid-region of the grafted and sham-operated muscles. At each time point, the Feret's minimal diameter of the individual muscle fibres was measured by determining the minimum distance between two parallel tangents at the opposite border of the muscle fibres, as previously described (Briguet et al., 2004). The mean minimal fibre diameter of grafted muscles was expressed as a percentage of the mean minimal fibre diameter of contralateral control muscles. The total number of fibres per section and the number of centrally nucleated fibres per section were also counted. The total fibre number of grafted muscles was expressed as a percentage of the total fibre number of contralateral control muscles, and centrally nucleated fibres were expressed as a percentage of the total fibre number of each muscle.

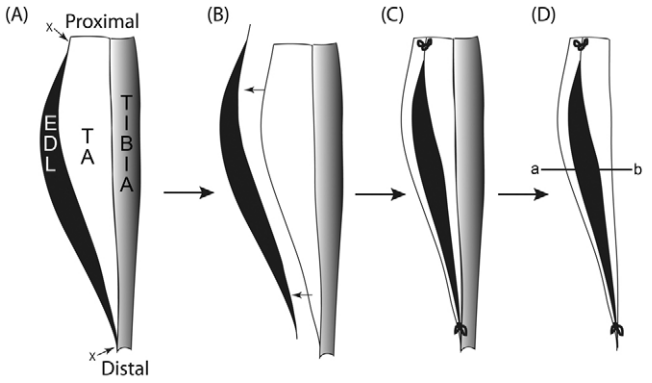

Fig. 7. Illustration of whole muscle autograft method. Anterior view of mouse right hind limb, showing extensor digitorum longus (EDL, black) and tibialis anterior (TA, white) muscles together with the tibia (grey). The proximal and distal tendons (×) of the EDL muscle were cut (A). The whole EDL muscle was then removed from its original muscle bed, severing vascular and neural connections (B). The EDL was then repositioned over the TA muscle and secured by suturing the distal tendon of the EDL to the distal tendon of the TA and the proximal tendon of the EDL onto the proximal end of TA muscle (C). At each time point, mice were euthanized and the EDL and the attached TA muscles were removed together and sectioned transversally in the mid-belly region (a-b) prior to embedding and freezing (D).

Immunohistochemistry

Tissue sections were blocked for 1 hour in phosphate-buffered saline (PBS) containing 10% serum, then incubated overnight at 4°C with rabbit anti-mouse osteopontin (1:300, LF-175; kindly provided by Larry Fisher, NIDCR, NIH, Bethesda, MD), rat anti-mouse F4/80 for the detection of macrophages (Austyn and Gordon, 1981; Hirsch et al., 1981) (2 μg/ml; Serotec, Oxford, UK), rat anti-mouse Ly6G for the detection of neutrophils (Fleming et al., 1993) (5 μg/ml; BD Biosciences, San Jose, CA) or the relevant non-immune IgG diluted in PBS. The following day, sections were washed three times in PBS and incubated with either swine anti-rabbit IgG conjugated to tetramethyl rhodamine isothiocyanate (1:250; DakoCytomation, Glostrup, Denmark) or donkey anti-rat IgG conjugated to Alexa Fluor 488 (1:100; Life Technologies, Grand Island, NY) for 30 minutes at room temperature. Sections

were then washed three times in PBS and were mounted in aqueous mountant containing 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and viewed under fluorescent illumination. F4/80-positive cells were counted and expressed as macrophages per section, and Ly6G-positive cells were counted and expressed as neutrophils per section.

Statistical analysis

Data are presented as the mean ± s.e.m. Comparisons between wild-type and knockout mice were made using two-way ANOVA with, where appropriate, a Bonferroni post-hoc test. In all cases $P \leq 0.05$ was considered significant.

ACKNOWLEDGEMENTS

The authors thank Larry Fisher (NIH/NIDCR) for his generous gift of the osteopontin antibody LF-175, David Denhardt (Rutgers University) for making the osteopontin-null mice available and Su Toulson for care of the mice.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

K.U., E.J.M. and C.N.P. conceived and designed the experiments. K.U., D.W. and C.N.P. performed the experiments. K.U., E.J.M. and C.N.P. analyzed the data. K.U., E.J.M. and C.N.P. wrote the paper.

FUNDING

This work was supported by the National Health and Medical Research Council [grant number 284233].

REFERENCES

Austyn, J. M. and Gordon, S. (1981). F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* **11**, 805-815.

Banerjee, A., Apte, U. M., Smith, R. and Ramaiah, S. K. (2006). Higher neutrophil infiltration mediated by osteopontin is a likely contributing factor to the increased susceptibility of females to alcoholic liver disease. *J. Pathol.* **208**, 473-485.

Barbosa-Souza, V., Contin, D. K., Filho, W. B., de Araújo, A. L., Irazusta, S. P. and da Cruz-Höfling, M. A. (2011). Osteopontin, a chemotactic protein with cytokine-like properties, is up-regulated in muscle injury caused by Bothrops lanceolatus (fer-de-lance) snake venom. *Toxicon* **58**, 398-409.

Berman, J. S., Serlin, D., Li, X., Whitley, G., Hayes, J., Rishikof, D. C., Ricupero, D. A., Liaw, L., Goetschkes, M. and O'Regan, A. W. (2004). Altered bleomycin-induced lung fibrosis in osteopontin-deficient mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* **286**, L1311-L1318.

Boudiffa, M., Wade-Gueye, N. M., Guignandon, A., Vanden-Bossche, A., Sabido, O., Aubin, J. E., Jurdic, P., Vico, L., Lafage-Proust, M. H. and Malaval, L. (2010). Bone sialoprotein deficiency impairs osteoclastogenesis and mineral resorption in vitro. *J. Bone Miner. Res.* **25**, 2669-2679.

Bridges, L. R. (1986). The association of cardiac muscle necrosis and inflammation with the degenerative and persistent myopathy of MDX mice. *J. Neurol. Sci.* **72**, 147-157.

Briguet, A., Courdier-Fruh, I., Foster, M., Meier, T. and Magyar, J. P. (2004). Histological parameters for the quantitative assessment of muscular dystrophy in the mdx-mouse. *Neuromuscul. Disord.* **14**, 675-682.

Denhardt, D. T. and Guo, X. (1993). Osteopontin: a protein with diverse functions. *FASEB J.* **7**, 1475-1482.

Duvall, C. L., Taylor, W. R., Weiss, D., Wojtowicz, A. M. and Guldberg, R. E. (2007). Impaired angiogenesis, early callus formation, and late stage remodeling in fracture healing of osteopontin-deficient mice. *J. Bone Miner. Res.* **22**, 286-297.

Duvall, C. L., Weiss, D., Robinson, S. T., Alameddine, F. M., Guldberg, R. E. and Taylor, W. R. (2008). The role of osteopontin in recovery from hind limb ischemia. *Arterioscler. Thromb. Vasc. Biol.* **28**, 290-295.

Fleming, T. J., Fleming, M. L. and Malek, T. R. (1993). Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J. Immunol.* **151**, 2399-2408.

Giachelli, C. M., Lombardi, D., Johnson, R. J., Murry, C. E. and Almeida, M. (1998). Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo. *Am. J. Pathol.* **152**, 353-358.

Giacopelli, F., Marciano, R., Pistorio, A., Catarsi, P., Canini, S., Karsenty, G. and Ravazzolo, R. (2004). Polymorphisms in the osteopontin promoter affect its transcriptional activity. *Physiol. Genomics* **20**, 87-96.

Grounds, M. D., Davies, M., Torrisi, J., Shavlakadze, T., White, J. and Hodgetts, S. (2005). Silencing TNFalpha activity by using Remicade or Enbrel blocks inflammation in whole muscle grafts: an in vivo bioassay to assess the efficacy of anti-cytokine drugs in mice. *Cell Tissue Res.* **320**, 509-515.

Hansen-Smith, F. M. and Carlson, B. M. (1979). Cellular responses to free grafting of the extensor digitorum longus muscle of the rat. *J. Neurol. Sci.* **41**, 149-173.

Harris, J. B., Vater, R., Wilson, M. and Cullen, M. J. (2003). Muscle fibre breakdown in venom-induced muscle degeneration. *J. Anat.* **202**, 363-372.

Hashimoto, M., Sun, D., Rittling, S. R., Denhardt, D. T. and Young, W. (2007). Osteopontin-deficient mice exhibit less inflammation, greater tissue damage, and impaired locomotor recovery from spinal cord injury compared with wild-type controls. *J. Neurosci.* **27**, 3603-3611.

Haslett, J. N., Sanoudou, D., Kho, A. T., Bennett, R. R., Greenberg, S. A., Kohane, I. S., Beggs, A. H. and Kunkel, L. M. (2002). Gene expression comparison of biopsies from Duchenne muscular dystrophy (DMD) and normal skeletal muscle. *Proc. Natl. Acad. Sci. USA* **99**, 15000-15005.

Hirata, A., Masuda, S., Tamura, T., Kai, K., Ojima, K., Fukase, A., Motoyoshi, K., Kamakura, K., Miyagoe-Suzuki, Y. and Takeda, S. (2003). Expression profiling of cytokines and related genes in regenerating skeletal muscle after cardiotoxin injection: a role for osteopontin. *Am. J. Pathol.* **163**, 203-215.

Hirsch, S., Austyn, J. M. and Gordon, S. (1981). Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture. *J. Exp. Med.* **154**, 713-725.

Hodgetts, S., Radley, H., Davies, M. and Grounds, M. D. (2006). Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice. *Neuromuscul. Disord.* **16**, 591-602.

Huard, J., Li, Y. and Fu, F. H. (2002). Muscle injuries and repair: current trends in research. *J. Bone Joint Surg. Am.* **84**, 822-832.

Lescaudron, L., Peltékian, E., Fontaine-Pérus, J., Paulin, D., Zampieri, M., Garcia, L. and Parrish, E. (1999). Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant. *Neuromuscul. Disord.* **9**, 72-80.

Liaw, L., Birk, D. E., Ballas, C. B., Whitsitt, J. S., Davidson, J. M. and Hogan, B. L. (1998). Altered wound healing in mice lacking a functional osteopontin gene (spp1). *J. Clin. Invest.* **101**, 1468-1478.

McGeachie, J. K. and Grounds, M. D. (1987). Initiation and duration of muscle precursor replication after mild and severe injury to skeletal muscle of mice. An autoradiographic study. *Cell Tissue Res.* **248**, 125-130.

Monfoulet, L., Malaval, L., Aubin, J. E., Rittling, S. R., Gadeau, A. P., Fricain, J. C. and Chassande, O. (2010). Bone sialoprotein, but not osteopontin, deficiency impairs the mineralization of regenerating bone during cortical defect healing. *Bone* **46**, 447-452.

Morgan, J. E., Coulton, G. R. and Partridge, T. A. (1989). mdx muscle grafts retain the mdx phenotype in normal hosts. *Muscle Nerve* **12**, 401-409.

Mori, R., Shaw, T. J. and Martin, P. (2008). Molecular mechanisms linking wound inflammation and fibrosis: knockdown of osteopontin leads to rapid repair and reduced scarring. *J. Exp. Med.* **205**, 43-51.

Nguyen, H. X. and Tidball, J. G. (2003). Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells in vitro. *J. Physiol.* **547**, 125-132.

O'Regan, A. and Berman, J. S. (2000). Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. *Int. J. Exp. Pathol.* **81**, 373-390.

O'Regan, A. W., Chupp, G. L., Lowry, J. A., Goetschkes, M., Mulligan, N. and Berman, J. S. (1999). Osteopontin is associated with T cells in sarcoid granulomas and has T cell adhesive and cytokine-like properties in vitro. *J. Immunol.* **162**, 1024-1031.

Pardo, A., Gibson, K., Cisneros, J., Richards, T. J., Yang, Y., Becerril, C., Yousem, S., Herrera, I., Ruiz, V., Selman, M. et al. (2005). Up-regulation and profibrotic role of osteopontin in human idiopathic pulmonary fibrosis. *PLoS Med.* **2**, e251.

Pegoraro, E., Hoffman, E. P., Piva, L., Gavassini, B. F., Cagnin, S., Ermani, M., Bello, L., Soraru, G., Pacchioni, B., Bonifati, M. D. et al. (2011). SPP1 genotype is a determinant of disease severity in Duchenne muscular dystrophy. *Neurology* **76**, 219-226.

Petrof, B. J., Shrager, J. B., Stedman, H. H., Kelly, A. M. and Sweeney, H. L. (1993). Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. USA* **90**, 3710-3714.

Pizza, F. X., Hernandez, I. J. and Tidball, J. G. (1998). Nitric oxide synthase inhibition reduces muscle inflammation and necrosis in modified muscle use. *J. Leukoc. Biol.* **64**, 427-433.

Porter, J. D., Khanna, S., Kaminski, H. J., Rao, J. S., Merriam, A. P., Richmonds, C. R., Leahy, P., Li, J., Guo, W. and Andrade, F. H. (2002). A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. *Hum. Mol. Genet.* **11**, 263-272.

Rittling, S. R., Matsumoto, H. N., McKee, M. D., Nanci, A., An, X. R., Novick, K. E., Kowalski, A. J., Noda, M. and Denhardt, D. T. (1998). Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. *J. Bone Miner. Res.* **13**, 1101-1111.

Roberts, P. and McGeachie, J. K. (1990). Endothelial cell activation during angiogenesis in freely transplanted skeletal muscles in mice and its relationship to the onset of myogenesis. *J. Anat.* **169**, 197-207.

Roberts, P. and McGeachie, J. K. (1992a). The effects of pre- and posttransplantation exercise on satellite cell activation and the regeneration of skeletal muscle transplants: a morphometric and autoradiographic study in mice. *J. Anat.* **180**, 67-74.

Roberts, P. and McGeachie, J. K. (1992b). Propranolol retards revascularisation and impedes early myogenesis in regenerating skeletal muscle transplants: an autoradiographic and morphometric study in mice. *J. Anat.* **181**, 101-111.

Roberts, P. and McGeachie, J. K. (1994). The enhancement of revascularisation of skeletal muscle transplants using the beta 2-agonist isoprenaline. *J. Anat.* **184**, 309-318.

Sam, F., Xie, Z., Ooi, H., Kerstetter, D. L., Colucci, W. S., Singh, M. and Singh, K. (2004). Mice lacking osteopontin exhibit increased left ventricular dilation and reduced fibrosis after aldosterone infusion. *Am. J. Hypertens.* **17**, 188-193.

Standal, T., Hjorth-Hansen, H., Rasmussen, T., Dahl, I. M., Lenhoff, S., Brenne, A. T., Seidel, C., Baykov, V., Waage, A., Børset, M. et al. (2004). Osteopontin is an adhesive factor for myeloma cells and is found in increased levels in plasma from patients with multiple myeloma. *Haematologica* **89**, 174-182.

Stedman, H. H., Sweeney, H. L., Shrager, J. B., Maguire, H. C., Panettieri, R. A., Petrof, B., Narusawa, M., Leferovich, J. M., Sladky, J. T. and Kelly, A. M. (1991). The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* **352**, 536-539.

Tidball, J. G. (2005). Inflammatory processes in muscle injury and repair. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**, R345-R353.

Tidball, J. G. and Wehling-Henricks, M. (2007). Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. *J. Physiol.* **578**, 327-336.

Uaesoontrachoon, K., Yoo, H. J., Tudor, E. M., Pike, R. N., Mackie, E. J. and Pagel, C. N. (2008). Osteopontin and skeletal muscle myoblasts: association with muscle regeneration and regulation of myoblast function in vitro. *Int. J. Biochem. Cell Biol.* **40**, 2303-2314.

Vetrone, S. A., Montecino-Rodriguez, E., Kudryashova, E., Kramerova, I., Hoffman, E. P., Liu, S. D., Miceli, M. C. and Spencer, M. J. (2009). Osteopontin promotes fibrosis in dystrophic mouse muscle by modulating immune cell subsets and intramuscular TGF-beta. *J. Clin. Invest.* **119**, 1583-1594.

Vignaud, A., Hourde, C., Medja, F., Agbulut, O., Butler-Browne, G. and Ferry, A. (2010). Impaired skeletal muscle repair after ischemia-reperfusion injury in mice. *J. Biomed. Biotechnol.* **2010**, 724914.

Zammit, P. S., Partridge, T. A. and Yablonka-Reuveni, Z. (2006). The skeletal muscle satellite cell: the stem cell that came in from the cold. *J. Histochem. Cytochem.* **54**, 1177-1191.