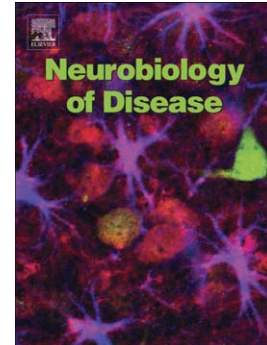


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BMP antagonists enhance myogenic differentiation and ameliorate the dystrophic phenotype in a DMD mouse model

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Summary

Duchenne Muscular Dystrophy (DMD) is an X-linked lethal muscle wasting disease characterized by muscle fiber degeneration and necrosis. The progressive pathology of DMD can be explained by an insufficient regenerative response resulting in fibrosis and adipose tissue formation. BMPs are known to inhibit myogenic differentiation and in a previous study we found increased expression of BMP family member BMP4 in DMD myoblasts. The aim of the current study was therefore to investigate whether inhibition of BMP signaling could be beneficial for myoblast differentiation and muscle regeneration processes in a DMD context. All tested BMP inhibitors, Noggin, dorsomorphin and LDN-193189, were able to accelerate and enhance myogenic differentiation. However, dorsomorphin repressed both BMP and TGF β signaling and was found to be toxic to primary myoblast cell cultures. In contrast, Noggin was found to be a potent and selective BMP inhibitor and was therefore tested *in vivo* in a DMD mouse model. Local adenoviral-mediated overexpression of Noggin in muscle resulted in increased expression of the myogenic regulatory genes *Myog* and *Myod1* and improved muscle histology. In conclusion, our results suggest that repression of BMP signaling may constitute an attractive adjunctive therapy for DMD patients.

Keywords: DMD, BMP signaling, myogenic differentiation

Introduction

Duchenne Muscular Dystrophy (DMD) is a lethal X-linked muscle wasting disorder caused by large deletions, insertions or point mutations in the *DMD* gene, which encodes the dystrophin protein. DMD muscle pathology has a progressive nature. The absence of functional dystrophin protein induces muscle fiber degeneration and necrosis. Subsequent local inflammation triggers fibrosis and fatty tissue infiltration, which results in replacement of muscle fibers with fibrotic and fatty tissue and loss of muscle function (reviewed in (Blake et al., 2002)). Although no treatment exists to date that can reverse the progressive muscle pathology of DMD, substantial effort and progress has been made in the development of novel therapies for DMD, which can roughly be divided into two groups; therapies aiming for restoration of dystrophin expression and therapies aiming for improvement of the overall condition of the muscle by repressing the molecular pathways that aggravate DMD pathology. The complexity of molecular pathways involved in the progressive pathophysiology of the disease makes it difficult to identify all the molecular players involved in DMD pathology, but several key players have been identified by expression profiling (Chen et al., 2000; Haslett et al., 2002; Pescatori et al., 2007; Sterrenburg et al., 2006). Importantly, signaling cascades that are known to be pro-inflammatory and pro-fibrotic, such as the nuclear Factor- κ B (NF- κ B) and Transforming Growth Factor- β 1 (TGF β 1) pathways, were reported to be induced in DMD patients and in the *mdx* mouse model for DMD (Acharyya et al., 2007; Bernasconi et al., 1995; Chen et al., 2005; Cohn et al., 2007). In addition, TGF β 1 and the related family member myostatin have been described to act as direct negative regulators of muscle mass and muscle regeneration by repressing proliferation and differentiation of muscle stem cells (also known as satellite cells) and may therefore play a role in the further impairment of muscle regeneration in DMD. Several studies showed that blocking the myostatin- and TGF β -induced signaling cascades improved the dystrophic phenotype and muscle function of *mdx* mice by counteracting fibrosis and/or stimulating muscle regeneration (Bogdanovich et al., 2002; Cohn et al., 2007; Haidet et al., 2008). The results of these studies provide insight in the molecular mechanism of DMD pathology and hold promise that specific pathways can be targeted in the future to improve DMD. However, the

complete spectrum of molecular players involved in pathological processes such as fibrosis, inflammation and regeneration and their spatiotemporal interplay during the progression of the disease remains to be elucidated.

BMPs are secreted proteins that form a large subfamily within the TGF β superfamily and which fulfill essential roles during embryonic development and in adult life. The specificity of downstream signaling cascades depends on the specific interaction of BMP proteins with different type I and type II receptor kinases, which subsequently activate intracellular Smad1/5/8 proteins as well as other protein kinases such as p38 MAP kinase (Miyazono et al., 2010). By genome wide expression profiling, we previously identified BMPs as potential novel players in DMD pathology. In muscles of *mdx* mice the expression of several BMP signaling components was found to be increased (Turk et al., 2005). In addition, BMP4 levels were found to be consistently elevated in myoblast cultures derived from DMD patients compared to myoblasts isolated from healthy individuals, and finally the BMP antagonist, gremlin 2, was found to be downregulated in DMD muscle (Pescatori et al., 2007; Sterrenburg et al., 2006). These findings suggest that increased BMP signaling maybe directly involved in DMD pathology.

The exact role and potential impact of deregulated BMP signaling on DMD pathology is not known, however several studies suggest that BMPs are involved in myoblast proliferation and/or myogenic differentiation. In myoblast culture both BMP2 and BMP4 repress myogenic differentiation and stimulate differentiation towards the osteoblast lineage (Dahlqvist et al., 2003; Katagiri et al., 1997; Yamamoto et al., 1997). During embryonic muscle differentiation repression of local BMP signaling by secretion of BMP inhibitors such as Noggin and Gremlin is crucial for proper differentiation of muscle progenitors cells (Linker et al., 2003; Reshef et al., 1998; Tzahor et al., 2003). Noggin loss-of-function results in perinatal death in mice and a range of developmental defects, including severe reduction of skeletal muscle size, presumably as a consequent of defective terminal muscle differentiation (Tylzanowski et al., 2006). These studies suggest that BMPs repress myogenic differentiation during embryonic development. In contrast, a recent study showed that BMP4 overexpression in chicken limbs increases the number of fetal muscle progenitors and muscle fibers, suggesting BMP signaling

regulates the number of myogenic precursor cells in fetal muscle (Wang et al., 2010). Interestingly, in adult muscle BMP signaling has been implied to play a role in satellite cell activation, presumably through Id1 and Id3, suggesting that BMP signaling is important for muscle regeneration (Clever et al., 2010; Frank et al., 2006; Fukada et al., 2007; Ono et al., 2010; Wang et al., 2010). In summary, these studies suggest a yet unidentified role for BMP signaling in adult muscle regeneration which might be perturbed in DMD as a consequence of continuously elevated BMP signaling. We hypothesize that the use of BMP antagonists may be useful to inhibit BMP signaling in DMD muscle and may ameliorate the progressive DMD pathology. The objective of this study was therefore to determine the effect of different BMP inhibitors on myogenic differentiation in vitro and the effect of selective BMP repression in a DMD mouse model.

Materials & methods

Cell culture and transfections

Mouse myoblast cell line C2C12 was maintained in DMEM supplemented with 10% FBS, Pen/strep and Glutamax (Gibco). Primary human myoblasts were maintained in F-10 HAM medium supplemented with 20% FBS and pen/strep and grown at 37 °C at 5% CO₂. For the differentiation assay, both the C2C12 cells and primary human myoblasts were cultured in differentiation medium after cells reach 80%-90% confluency, which is composed of DMEM (Gibco) supplemented with 2% FBS. C2C12 cells were grown at 37°C in a humidified incubator 10% CO₂. Transient transfections and reporter assays were performed in triplicates as previously described with BRE-luc and CAGA₁₂-luc reporter constructs (Dennler et al., 1998; Korchynski and ten Dijke, 2002). In all reporter assays, a β -galactosidase expression plasmid was co-transfected and served as a control to correct for transfection efficiency. The experiments were performed in triplicates. For CAGA-Luc, the luciferase activity was analyzed 8 hours after TGF- β ₃ (5 ng/ml) addition. Recombinant human BMP4 was a gift from Yongping Cai and Anne-Marie Cleton-Jansen, Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands. Dorsomorphin was purchased from Biomol (BML-275). Noggin protein used in cell culture was a gift from Regeneron, USA.

Mice

Male *mdx/utrn*^{+/-} mice were obtained through crossing of *mdx* and *utrn*^{-/-}. At 4 weeks of age, mice were anesthetized with isoflurane and injected intramuscular in the gastrocnemius and triceps muscles with ad-Noggin (1×10^9 pfu/injection), ad-lacZ (1×10^9 pfu/injection) or saline. The adeno containing Noggin cDNA was obtained from Regeneron, the adeno containing the LacZ cDNA was in the same backbone (serotype 5). Four weeks after injection muscles were isolated and analyzed. The animal experiments were carried out with approval of the Animal Experimental Commission of the Leiden University Medical Center, Leiden and according to the Dutch Government guidelines.

Histology

Muscle tissue for histological analysis and RNA isolation was snap frozen in liquid nitrogen-cooled 2-methylbutane (Sigma Aldrich, The Netherlands) and sectioned at 8 μm with a Shandon cryotome (Thermo Fisher Scientific Co., Pittsburgh, PA, USA). Sections were fixed using ice cold acetone for 5 minutes and stained with hematoxylin and eosin (Sigma Aldrich, The Netherlands). For the X-gal staining, the sections were fixed for 4 minutes using cold fixative solution (0.2% glutaraldehyde, 1.5% paraformaldehyde, 2mM MgCl_2 , 5mM EGTA and 100mM Sodium-phosphate-buffer pH 7.3) and washed twice in PBS, first wash 1 minute and second wash 10 minutes. Sections are stained overnight in the dark at room temperature with staining solution (5mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5mM $\text{K}_4\text{Fe}(\text{CN})_6$, 1mg/ml X-gal, 2mM MgCl_2 and 100mM Sodium-phosphate-buffer pH 7.3). Then the sections were washed with PBS, dehydrated and embedded in Pertex (Histolab, Göteborg). H&E staining and Masson's Trichrome staining were performed on frozen sections (8 μm). Pictures were taken using a Leica microscope of a representative section and analyzed using ImageJ software as described previously by our group (van Putten et al., 2010). Briefly, Image J was used to determine total tissue area of the original picture, after which the area of healthy cells corresponding with the eosin component was obtained after colour deconvolution using a H&E colour deconvolution plugin. Subsequently, the percentage of fibrosis/necrosis was determined by subtracting the eosin area from the total area. To determine the percentage of fibrosis on the Masson's

Trichrome stained sections a similar colour deconvolution protocol was used. Average fiber size was determined by measuring 300 fibers per section (random fields) using Adobe Photoshop (with ruler tool after setting measurement scale). The greatest distance between the opposite sides of the narrowest aspect of the fiber was measured. The percentage of centrally nucleated fibers was averaged over determined in three random fields per section (500-1000 fibers total/muscle).

Western blotting and Immunofluorescence

Antibody used for immunoblotting were as following: actin (Sigma), Myogenin (F5D; BD Pharmingen), Smad1/5/8 phosphorylation was detected using a previously described antibody recognizing phosphorylated SMAD1/5/8 (Persson et al., 1998). Western blotting was performed as previously described using standard techniques (Persson et al., 1998). Antibodies used for immunofluorescence were as following: Desmin (H-76, Santa Cruz Biotech), Myosin heavy chain (MF20; Developmental Studies Hybridoma Bank), embryonic Myosin heavy chain (eMyHC; F1.652, DSHB). Immunofluorescence was performed as previously described (Sterrenburg et al., 2006). For determining the average percentage of eMyHC+ fibers ten random fields per section were counted for each muscle (2000-3000 fibers total per muscle).

qRT-PCR

Total RNA was isolated from muscle tissue using the RNeasy isolation kit (Machery Nagel) according to the manufacturers protocol. Subsequently, cDNA was reverse transcribed from 1ug total RNA using the Revert Aid protocol (Fermentas) with random hexamer primers. Quantitative Real-Time PCR analysis was performed using the Roche 480 lightcycler and the relative expression level of the genes of interest was determined in triplicate for each sample using the $2^{-\Delta\Delta CT}$ method using Genex Analysis software (Biorad). Values were normalized to *Gapdh* expression. Measurements were performed in triplo and statistical analysis between groups was performed using a two-tailed Students *t*-test. The sequences of the different primers used in this study can be provided upon request.

Results

Endogenous BMP signaling is repressed during myoblast differentiation

To determine BMP activity during myoblast differentiation, we induced myogenic differentiation of C2C12 mouse myoblasts and subsequently determined pSmad1/5/8 levels at different time points. After the switch to low serum containing differentiation medium, C2C12 myoblasts fused and differentiated into multinucleated myotubes (Fig. 1A). As expected, the level of the myogenic transcription factor Myog increased from day1 onwards (Fig. 1B). Interestingly, *Bmp4* expression and pSmad1/5/8 levels peaked two days after initiation of myogenic differentiation and at this stage myogenin was already present (Fig.1B, C). The subsequent decrease of pSmad1/5/8 levels after two days correlated with a decrease in endogenous *Bmp4* expression (Fig. 1B, C). This was consistent with CAGE/SAGE expression analysis of undifferentiated versus differentiated C2C12 myoblasts, where *Bmp4* was found to be the only Bmp ligand whose expression decreased upon myogenic differentiation (M. Hestand, unpublished). Importantly, the expression of *Noggin*, a known endogenous BMP antagonist, increased during myogenic differentiation, suggesting that this may be an important mechanism for the downregulation of BMP signaling (Fig. 1C). Together these results show that BMP signaling is actively repressed upon myogenic differentiation.

Inhibition of BMP signaling by three BMP inhibitors

Several BMP antagonists have been used to inhibit BMP signaling in vitro and in vivo. To determine which BMP inhibitors can specifically inhibit BMP signaling in C2C12 myoblasts, we subsequently performed a luciferase reporter assay using three established BMP inhibitors: Noggin, dorsomorphin and LDN-193189 (LDN). Noggin can bind to specific BMPs, thereby inhibiting BMP binding to BMP receptors and blocking activation of downstream signaling (Li et al., 2008). The compounds dorsomorphin and the structurally related LDN are small molecule kinase inhibitors that bind and inhibit the kinase domain of BMP type I receptors (Cuny et al., 2008; Yu et al., 2008b). Luciferase reporter assays employing a BMP-responsive reporter construct (BRE-luc) and a TGF β /Smad3-responsive reporter construct (CAGA-luc) were used to measure the

potency and specificity of the inhibitors. Dorsomorphin repressed both basal and BMP4-induced BRE-luc activity (2-fold and 5-fold respectively), but only at a relatively high concentration of 4 μ M and was therefore the least potent antagonist (Fig. 2A,B). In addition, dorsomorphin inhibited CAGA-luc activity at a comparable dose (Fig. 2C), showing that this compound cannot solely be regarded as a BMP antagonist. LDN repressed both basal and BMP4-induced BRE-activity at a lower dose of 120nM (Fig. 2A, B), whereas CAGA-luc activity was only inhibited at a ten-fold higher dose (Fig. 2C), suggesting that specific BMP inhibition with this compound can be achieved at lower concentrations. Noggin also repressed both basal and BMP4-induced BRE-activity (5-fold and 40-fold respectively; Fig. 2A, B), but had no effect on CAGA-luc activity at low or high dose (Fig. 2C). Together these results suggest that Noggin is the most selective BMP inhibitor of the three compounds tested and can be used to specifically inhibit BMP signaling.

Specific inhibition of BMP signaling potentiates myoblast differentiation

We next determined the effect of Noggin, dorsomorphin and LDN on myogenic differentiation of C2C12 myoblasts. C2C12 cells were switched to differentiation medium with or without BMP antagonists and after three days of differentiation the cells were stained for desmin and myosin heavy chain (MyHC) to determine the differentiation index (percentage of MyHC⁺ cells) and fusion index (average number of nuclei/MyHC⁺ cell). All three BMP inhibitors were able to increase the differentiation index and fusion index in C2C12 myoblasts compared to the control, showing their ability to enhance myogenic differentiation (Fig. 3A-C). In addition, BMP4-induced repression of myogenic differentiation was antagonized by Noggin and LDN (Fig. S1A). Next, we determined the effect of the BMP inhibitors on the levels of pSmad1/5/8 and Myog at different time points of differentiation (Fig. 3C). All three inhibitors decreased pSmad1/5/8 levels after 24h and 48h treatment (Fig. 3C). After 24 hours of differentiation, the protein level of Myog was significantly increased after treatment with the BMP inhibitors compared to the control, whereas the difference was less apparent after two days, suggesting that treatment resulted in precocious myogenic differentiation (Fig. 3C). The apparent acceleration of myogenic differentiation was confirmed in Noggin-treated cells by

immunofluorescent staining of Myog, where the difference in percentage Myog⁺ nuclei compared to control cells was more apparent at the first three days of differentiation compared to day 4 and 6 (Fig. 3D and Fig. S1B). The difference in timing observed between the Myog immunofluorescent staining and Myog western blot is because cells for immunofluorescent staining have to be grown on glass rather than plastic, which slows down C2C12 myoblasts differentiation. Next, we determined the effect of the BMP inhibitors on the myogenic differentiation of human primary myoblast cultures obtained from muscle biopsies of healthy volunteers (Aartsma-Rus et al., 2003). Consistent with the results obtained in C2C12 cells, treatment with LDN and Noggin enhanced differentiation in primary myoblast cultures (Fig. 4). Surprisingly, dorsomorphin was found to be toxic in primary myoblast cell cultures and the cells demonstrated a reduced capacity to differentiate and fuse (Fig. 4). Together these results suggest that Noggin and LDN, but not dorsomorphin, are suitable compounds to enhance myogenic differentiation.

Adenoviral-mediated overexpression of BMP antagonist Noggin

To test the hypothesis that specific inhibition of BMP signaling may alleviate the dystrophic phenotype, we used Noggin to assess the effect of BMP inhibition in vivo in *mdx utrⁿ+/-* mice. In *mdx utrⁿ+/-* and *mdx utrⁿ-/-* mice the loss-of-function of the dystrophin-related protein utrophin results in a more severe dystrophic muscle phenotype than seen in the *mdx* mice (Deconinck et al., 1997; Zhou et al., 2008). We hypothesized that the effect of Noggin overexpression on the dystrophic phenotype would be more evident in *mdx utrⁿ+/-* mice compared to *mdx* mice, since the dystrophic phenotype is more prominent in these mice ((Zhou et al., 2008) and M. Putten, unpublished). For the in vivo overexpression, we used a Noggin adenoviral vector that was previously used in another study to repress BMP activity in vivo (Glaser et al., 2003). Five mice at the age of four weeks were injected intramuscular in the gastrocnemius muscle with the human Noggin cDNA containing adenovirus. As a control we injected the contralateral gastrocnemius muscle of each mouse with either *LacZ* adenoviral vector (n=3) or saline solution (n=2). After four weeks we isolated the muscles and we performed X-gal staining on sections to verify *LacZ* activity in the control injected gastrocnemius muscle.

High LacZ activity was seen in regenerating fibers (Fig. S2A and data not shown), although there was high variability between different injected muscles (data not shown). PCR and qRT-PCR analysis of the ad-Noggin injected muscles showed that human Noggin was detectable in all injected muscles, although the expression levels of Noggin in the different injected muscles showed a high level of variation (Fig. 5A and Fig. S2B). Isolation of the amplified product and subsequent sequence analysis verified the presence of human Noggin (data not shown).

We determined the expression of several genes involved in the process of regeneration, fibrosis and inflammation to determine the effect of Noggin overexpression on dystrophic muscle pathology. *Myog* and *Myod1* are involved in myogenesis and have been described as being negatively regulated by BMP signaling in myoblast cell culture (Dahlgvist et al., 2003, Vinals and Ventura, 2004). During adult muscle regeneration these genes are activated in the process of satellite cell proliferation and differentiation (Creuzet et al., 1998; Dahlgvist et al., 2003; Jin et al., 2000). qRT-PCR analysis showed that the expression of *Myog* and *Myod1* was significantly higher in the ad-Noggin injected muscles compared to control samples (on average 2-fold and 1.3-fold; Fig. 5B and Fig. S2B). The muscles with the highest expression of Noggin also showed a decrease in the expression of fibrosis-related genes *CTGF* and *Colla1* and of the BMP-target gene *Id1*, although on average the differences between control and ad-Noggin-injected muscles were not significant (Fig. 5B and Fig. S2B). The expression of genes involved in inflammation (*CD68* and *Lgals3*) showed no significant difference compared to the control groups (Fig. 5B). Next we determined the effect on muscle histology. The average in fibrosis/necrotic areas, as determined by H&E staining, was decreased by 20% in the ad-Noggin-treated group (Fig. 5C, D). No significant differences were found in the amount of collagen staining (determined with Masson's Trichrome staining) or the percentage of fibers containing centrally located nuclei (Fig S3B, C). Furthermore, the number of early regenerating fibers, as determined by embryonic myosin heavy chain (eMyHC) staining appeared to increase slightly in ad-Noggin injected samples, but this trend was not significant (Fig. 5C). Consistent with the increase in early regenerating fibers, the amount of small fibers was increased in the ad-Noggin group (Fig. S3A).

In summary, overexpression of Noggin appeared to enhance regeneration in *mdx utrn*^{+/-} muscles and alleviate the dystrophic phenotype.

Discussion

In this study we showed that different BMP antagonists can efficiently accelerate and enhance myogenic differentiation in vitro and provide the first evidence that BMP antagonists maybe beneficial in counteracting the dystrophic pathology of DMD. BMPs are established inhibitors of myogenesis: during embryogenesis repression of BMPs is necessary for proper initiation of myogenic differentiation of myoblasts. In this study we show that BMP signaling is downregulated during myogenic differentiation as evidenced by the decrease of pSmad1/5/8 levels. Although *Bmp4* expression was lower after 6 days of differentiation compared with 2 days, its expression was still pronounced, suggesting other regulatory mechanisms responsible for the low pSmad1/5/8 levels. Interestingly, we found that the expression of Noggin was increased upon myogenic differentiation, which may explain the lower pSmad1/5/8 levels in differentiated C2C12 cells (data not shown). Inhibition of endogenous BMP signaling as well as BMP4-induced signaling by treatment with BMP antagonists Noggin, dorsomorphin or LDN, accelerated and enhanced myogenic differentiation in C2C12 cells. The mechanism by which these BMP antagonists repress BMP signaling is different. Noggin is a secreted protein that selectively sequesters BMPs, including BMP4, and therefore inhibits both Smad-dependent BMP signaling and Smad-independent BMP signaling. In contrast, the small molecule compounds dorsomorphin and its structural derivative LDN bind to the kinase domains of the BMP type I receptors. Both Noggin and LDN were also able to induce myogenic differentiation in human primary myoblast cultures, showing that these BMP antagonists have a comparable effect on human myoblasts. Dorsomorphin, however, was found to repress myogenic differentiation of primary myoblasts, which is presumably a result of non-specific effects of the compound. Several other studies showed that dorsomorphin indeed also targets other kinases such as AMPK (Yu et al., 2008c) and VEGF type-2 receptor (Flk1/KDR) (Hao et al., 2010). In addition, we show in this study that dorsomorphin has a repressive effect on Smad3-dependent TGF β -activity and thus

does not specifically target BMP signaling. This has been reported before and is due to the fact that dorsomorphin does not specifically inhibit kinase activity of BMP receptors *Acvr1* (ALK2), *Bmpr1a* (ALK3) and *Bmpr1b* (ALK6), but also targets the TGF β receptor *Tgfbr1* (ALK5), although with less efficiency. The reported inductive effect of dorsomorphin on myoblast apoptosis (Niesler et al., 2007) could explain the effects observed in primary myoblast cultures in our study, suggesting this compound is not suitable to enhance myogenic differentiation in vivo. LDN inhibited TGF β activity at higher doses and we observed no toxicity in vitro, suggesting a low dose of this compound can be used to specifically inhibit BMP signaling and enhance myogenic differentiation. In a previous study, our group showed that DMD patient myoblasts exhibit elevated BMP4 expression levels and in addition are more sensitive to BMP signaling. Therefore, we also determined the effect of the different BMP antagonists on the myogenic differentiation of three different DMD patient myoblast cultures. DMD myoblasts treated with Noggin or LDN showed a higher percentage of differentiated myoblasts, but the difference was not significant due to the low percentage of myogenic cells and the low percentage of myogenic differentiation (around 5%) in these cultures, which was most likely the result of high passage number of these cultures.

Based on our in vitro results we decided to overexpress Noggin using an adenoviral vector for selective in vivo repression of BMP signaling. Since our main objective was to determine the effect on muscle regeneration, we used *mdx utrⁿ+/-* mice that were 4 weeks old at the time of injection. This model shows more pronounced muscle regeneration in the limb muscle than *mdx* mice (data not shown). In a pilot experiment we already observed improved muscle histology after two weeks of the ad-Noggin injection (n=2, data not shown). In the experiment described here, we observed that Noggin can induce *Myog* and *Myod1* expression in vivo in dystrophic muscle of *mdx utrⁿ+/-* mice. This finding is suggestive of enhanced muscle regeneration, which was supported by the slight increase in number of eMyHC⁺ fibers, and the higher percentage of smaller fibers in the ad-Noggin group. In addition, significant improvement of muscle histology was seen for the percentage of fibrotic/necrotic areas by H&E staining, although no significant difference was found in the percentage of collagen levels (which detect only fibrosis) between control and ad-Noggin samples. Therefore, we presume that

enhanced or accelerated regeneration induced by Noggin results in a decrease in the necrosis and not fibrosis. To address the point whether or not BMP inhibition has an effect on fibrosis, which is an important contributor of DMD pathology, it may be better to repress BMP signaling in older *mdx* or *mdx utrⁿ+/-* mice (6 months), which have more pronounced fibrosis (Zhou et al., 2008).

Although our results suggest that local inhibition of BMP signaling affects the expression of genes involved in the muscle regeneration process resulting in improvement of the dystrophic phenotype, we observed considerable variance in response after ad-Noggin injection, which is most likely the result of high variation in the levels of Noggin expression when using an adenoviral expression vector. This is consistent with our observation that the most pronounced effect on gene expression was detected in muscle with the highest expression of Noggin. Moreover, it was previously shown that the full length protein is not very stable in vivo and overexpression of a modified Noggin protein, which lacks a heparin binding site was shown to have an increased half-life and to be more stable in vivo (Glaser et al., 2003). Further studies are therefore needed to investigate the effect of BMP inhibition in DMD context using a more stable and efficient method of expression.

BMP antagonists, such as recombinant Noggin, dorsomorphin and its structural derivative LDN, have been used to effectively antagonize BMP signaling in vivo (Cuny et al., 2008; Glaser et al., 2003; Lories et al., 2005; Wang et al., 2009; Yu et al., 2008b). This suggests that in the future BMP antagonists maybe of therapeutic use to treat disease where BMP signaling is aberrantly activated, such as Fibrodysplasia Ossificans Progressiva and, in our case, DMD (Sterrenburg et al., 2006; Yu et al., 2008a). However several important issues have to be addressed before BMP antagonists can be considered as a therapeutic option. First, it will be important to determine the potential role of BMP signaling in normal and dystrophic muscle. It has been described that BMP signaling is active in quiescent satellite cells and is downregulated upon activation of this cell population after muscle damage (Fukada et al., 2007). Recently, a novel role of BMP signaling in the regulation of satellite cell proliferation has been suggested (Clever et al., 2010; Ono et al., 2010), but the molecular mechanism and the potential role in dystrophic pathology is currently not known. Furthermore, overexpression of Noggin inhibits the

activity of several members of the BMP family (Balemans and Van, 2002) and therefore it is not exactly known which ligands and signaling components may contribute to the pathology of DMD. Second, the effect of long term treatment with BMP inhibitors is not known and may have serious side-effects. For example, it has been described that ectopic expression of Noggin in the mouse skeleton reduces bone density and increases the occurrence of spontaneous fractures (Devlin et al., 2003). Finally, additional signaling cascades, such as induced by TGF β and myostatin, also likely play an important role in DMD pathology and it will therefore be important to determine the additive or synergistic effect when targeting multiple pathways. Intriguingly, it is known that overexpression of follistatin or a soluble form of the type 2 receptor kinase ActrIIB, both proteins that inhibit the activity of BMPs, myostatin and activin, result in an excessive increase in muscle mass that cannot solely be explained by the neutralizing of myostatin (Gilson et al., 2009; Lee, 2007), showing that other ligands are involved in the regulation of muscle mass. Future experiments will therefore be focused on further dissecting the role the different signaling pathways in DMD pathology.

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ACCEPTED MANUSCRIPT

Figure 1

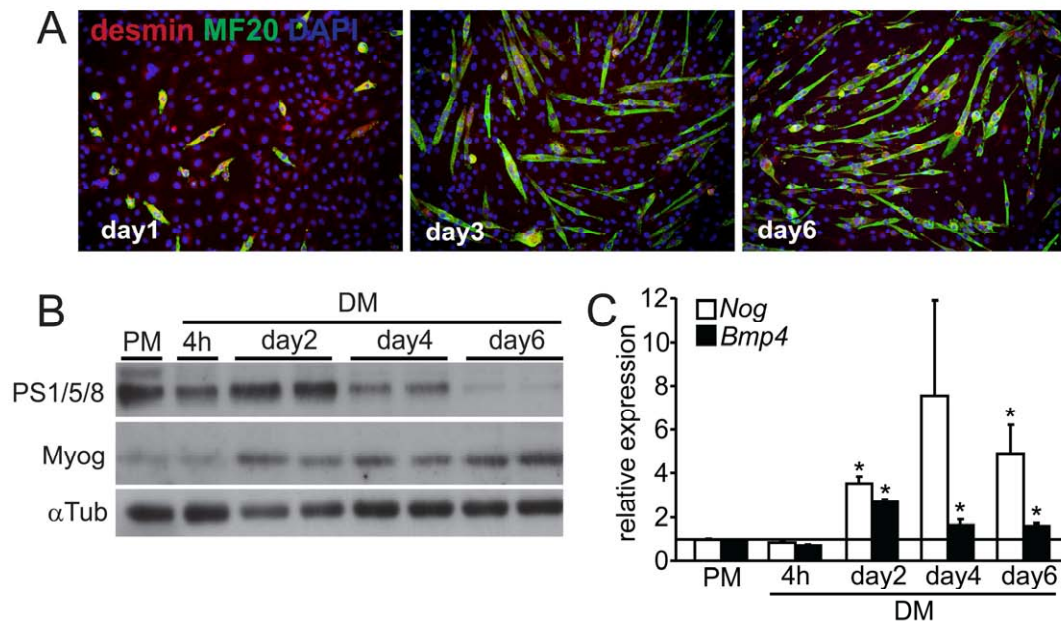


Fig. 1. Endogenous BMP signaling during myogenic differentiation. (A) Myogenic differentiation of C2C12 cells. Immunofluorescent staining of myogenic marker desmin in red and differentiation marker Myosin Heavy Chain (MyHC) in green. After 3 days of differentiation in low serum medium, formation of myotubes was observed. (B) Western blot analysis of pSmad1/5/8 and Myog levels on C2C12 protein lysates at different time points of differentiation. α Tubulin is shown as a loading control in all samples. (C) Quantitative Real-Time PCR analysis of *Bmp4* and *Noggin* expression on C2C12 cDNA at different time points of differentiation. Fold expression relative to PM is shown and error bars indicate s.d. of triplicate experiments. * $P < 0.01$ (compared with PM). PM, proliferation medium; DM, differentiation medium.

Figure 2

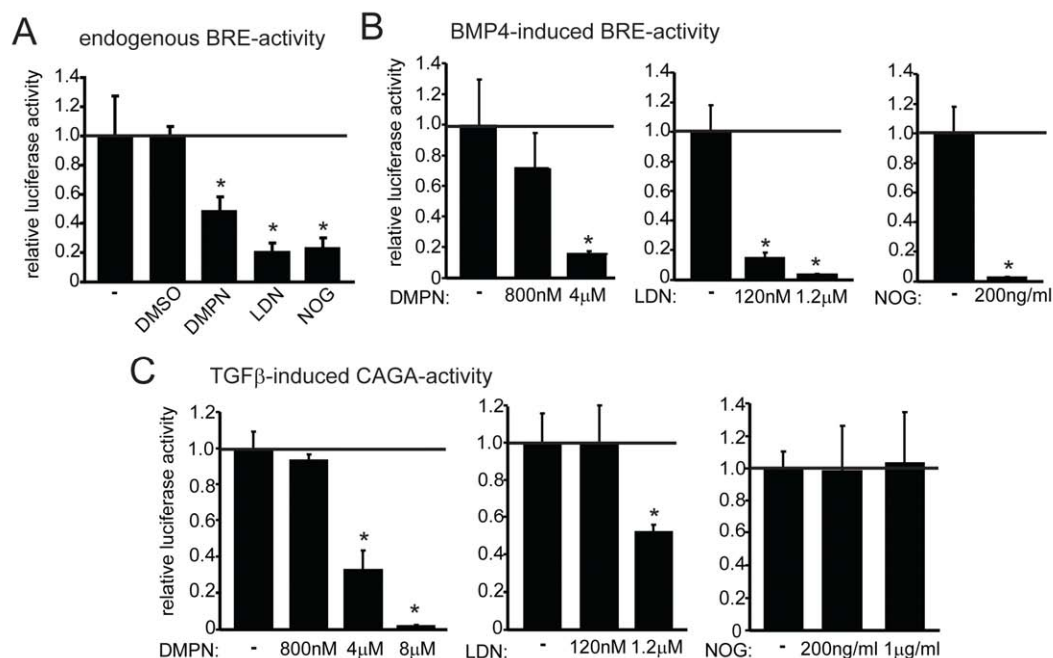


Fig. 2. Effect of BMP inhibitors on BMP and TGF β signaling. (A) Luciferase reporter assay with the BRE-luc reporter showing the activity of endogenous BMP signaling in transfected C2C12 cells treated with or without BMP antagonists Noggin (NOG; 200ng/ml), LDN-193189 (LDN; 120nM) and dorsomorphin (DMPN; 4 μ M). (B) Luciferase reporter assay with BRE-luc showing the activity of BMP4-induced signaling in transfected C2C12 cells treated with or without different concentrations of BMP antagonists (C) Luciferase reporter assay with the CAGA-luc reporter showing the activity of TGF β -induced signaling in transfected C2C12 cells treated with or without different concentrations of BMP antagonists. Error bars indicate s.d. of triplicate samples and the luciferase activity relative to the control is shown in each graph; * P <0.01.

Figure 3

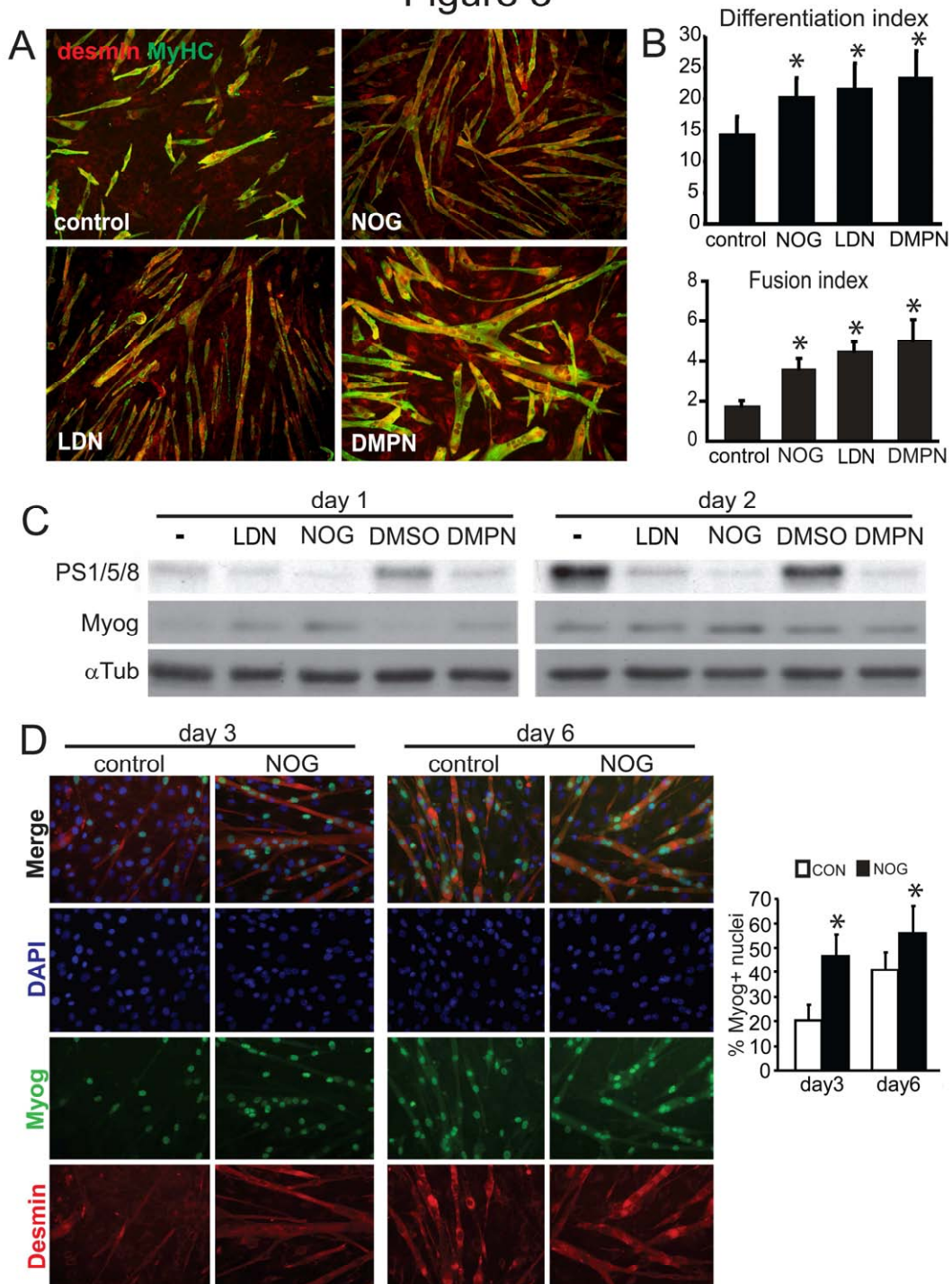


Fig. 3. Effect of BMP inhibitors on myogenic differentiation. (A) Immunofluorescent staining of desmin and MyHC showing the effect of Noggin (NOG; 200ng/ml), LDN-193189 (LDN; 120nM) and dorsomorphin (DMPN; 4 μ M) on myogenic differentiation of

C2C12 cells. Experiment was performed in duplo and representative pictures are shown. (B) Quantification of immunofluorescent staining in (A) showing the differentiation index and fusion index of C2C12 cells treated with or without the different BMP antagonists. Error bars indicate the s.d. of 10 areas measured in duplo. * $P < 0.01$ (C) Western blot analysis of C2C12 protein lysates showing the pSmad1/5/8 levels and Myogenin levels after treatment with the different compounds after 1 day and 2 days of differentiation. α Tubulin is shown as a loading control in all samples. (D) Immunofluorescent staining of Myog (in green) and desmin (myogenic cells; in red) of C2C12 cells differentiated for 3 days and 6 days with or without Noggin (200ng/ml). Top row shows merged pictures with DAPI (in blue) staining the total nuclei in the field. Representative pictures are shown from experiments performed in duplo. The graph on the right shows the quantification of the percentage of Myog⁺ nuclei compared to the total amount of nuclei. Error bars indicate the s.d. of 10 areas measured in duplo. * $P < 0.01$

Figure 4

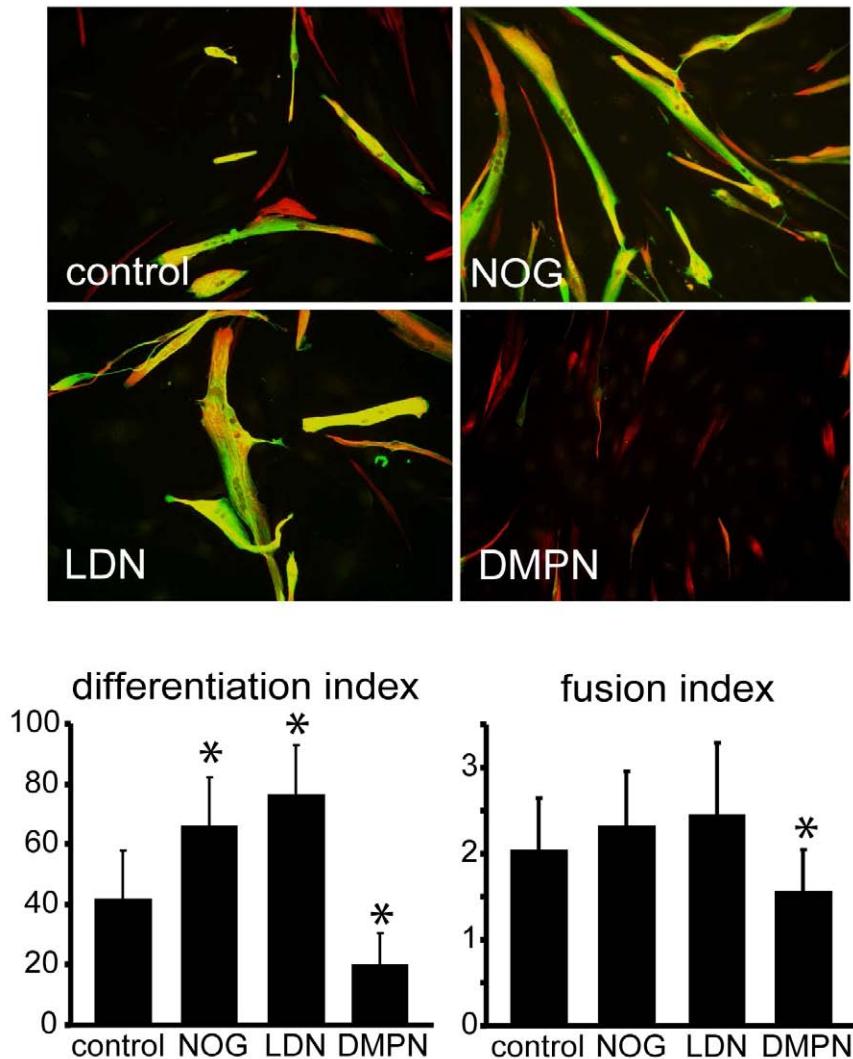


Fig. 4. Effect of BMP inhibitors on myogenic differentiation of primary human myoblasts. Immunofluorescent staining of human primary myoblasts differentiated for 7 days with or without Noggin (NOG; 200ng/ml), LDN-193189 (LDN; 120nM) and dorsomorphin (DMPN; 4 μM). Staining shows desmin (all myogenic cells; in red) and MyHC (differentiated myotubes; in green). Experiment was performed in duplo and representative pictures are shown. Error bars indicate s.d. of 10 measured areas in duplo; $*P < 0.01$

Figure 5

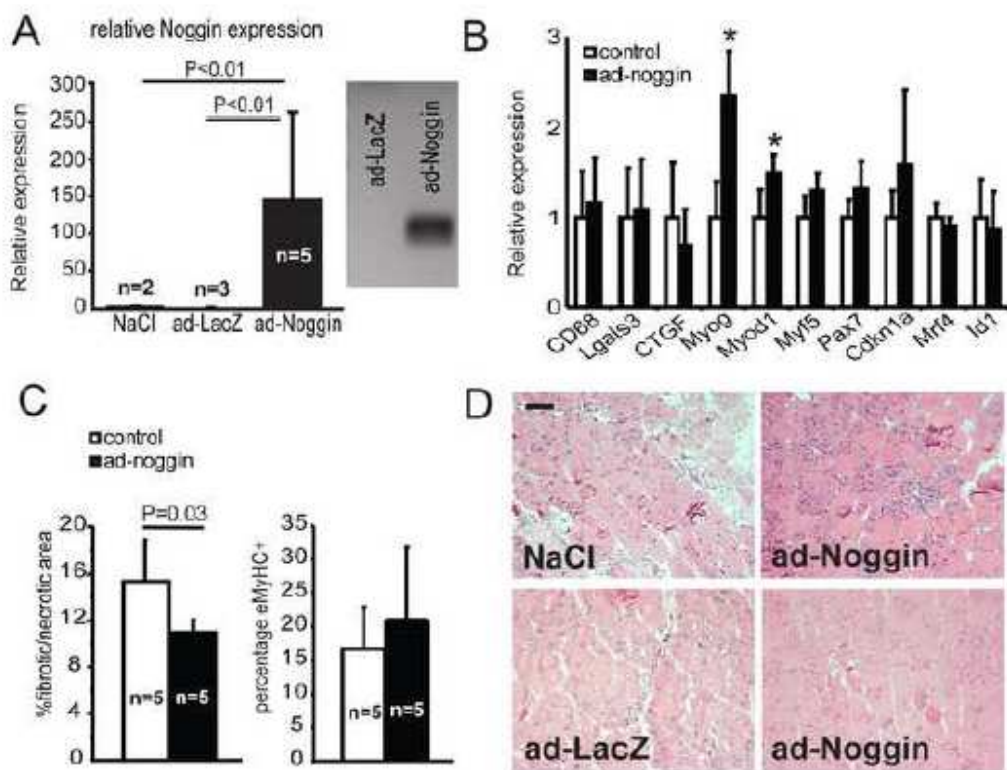


Fig. 5. Adenoviral-mediated overexpression of Noggin in dystrophic muscle. (A) The left panel shows quantitative Real-Time PCR analysis of control (n=5) and ad-Noggin (n=5) gastrocnemius cDNA with primers specific for human Noggin. The right panel shows a representative gel with RT-PCR product amplified with human Noggin specific primers. The amplified products of all ad-Noggin samples were isolated, sequenced and identified as human Noggin. (B) Quantitative Real-Time PCR analysis of myogenic, inflammation and fibrosis marker gene expression in gastrocnemius samples of control (n=5) and ad-Noggin (n=5). Error bars represents s.d. * $P < 0.01$ (C) Quantitative analysis of fibrotic/necrotic areas as determined with H&E staining and the percentage of eMyHC+ fibers of control (n=5) and ad-Noggin (n=5) muscle sections. Error bars represents s.d. (D) Representative pictures of H&E stainings of control muscle (NaCl and LacZ) and contralateral ad-Noggin injected muscle. Scale bar = 100 μ m