Ex vivo treatment with nitric oxide increases mesoangioblast therapeutic efficacy in muscular dystrophy

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Summary

Muscular dystrophies are characterized by primary wasting of skeletal muscle for which no satisfactory therapy is available. Studies in animal models have shown that stem cell-based therapies may improve the outcome of the disease, and that mesoangioblasts are promising stem cells in this respect. The efficacy of mesoangioblasts in yielding extensive muscle repair is, however, still limited. We found that mesoangioblasts treated with nitric oxide (NO) donors and injected intra-arterially in α-sarcoglycan-null dystrophic mice have a significantly enhanced ability to migrate to dystrophic muscles, to resist their apoptogenic environment and engraft into them, yielding a significant recovery of α-sarcolgycan expression. In vitro NO-treated mesoangioblasts displayed an enhanced chemotactic response to myotubes, cytokines and growth factors generated by the dystrophic muscle. In addition, they displayed an increased ability to fuse with myotubes and differentiating myoblasts and to survive when exposed to cytotoxic stimuli similar to those present in the dystrophic muscle. All the effects of NO were cyclic GMP-dependent since they were mimicked by treatment with the membrane permeant cyclic-GMP analogue 8-bromo-cGMP and prevented by inhibiting guanylate cyclase. We conclude that NO donors exert multiple beneficial effects on mesoangioblasts that may be used to increase their efficacy in cell therapy of muscular dystrophies.

Key words: Muscular dystrophy, Stem cells, Nitric oxide, Cyclic GMP, Apoptosis, Differentiation

Introduction

Muscular dystrophies are clinically and molecularly heterogeneous genetic diseases characterised by a primary wasting of skeletal muscle that compromises patient mobility. This is caused, in the majority of the cases, by the lack of one of several proteins located either at the plasma membrane or, less frequently, within internal membranes. Such deficiency increases the probability of damage during contraction, and eventually leads to fibre degeneration, accompanied by severe local inflammation with infiltration of immune-competent cells (Emery, 2002). Inflammation is followed by sclerosis, thus starting a vicious circle that reduces oxygen supply and increases the likelihood of degeneration for surviving and regenerated fibres (Emery, 2002). In the most severe forms, such as Duchenne muscular dystrophy, regeneration is progressively exhausted, and skeletal muscle is replaced by fat and fibrous tissue. This condition leads to progressive muscle weakness and eventually paralysis, ultimately causing death by respiratory and/or cardiac failure (Emery, 2002). Although the underlying molecular defects are now known, there is still no available satisfactory therapy.

The current therapies, based on corticosteroid administration, provide only temporary improvements and are associated with severe side-effects (Manzur et al., 2004). The other pharmacological strategies attempted so far, including administration of protease inhibitors, and drugs that regulate calcium homeostasis or act on protein and lipid metabolism, have yielded no favourable outcomes in clinical trials and did not enter the clinical practice (Skuk et al., 2002).

In the past few years experimental approaches offering an alternative to classical pharmacological treatments have been developed, among which the injection of myogenic stem cells and gene therapy with adeno-associated viral vectors appear to hold particular promise (Cossu and Sampaolesi, 2004). Significant restoration of muscle structure and function in the α-sarcoglycan (αSG)-null mouse model of muscular dystrophy have been obtained using mesoangioblasts, a population of vessel-associated, mesoderm stem cells that express angioblast and mesoderm markers (Minasi et al., 2002; Sampaolesi et al., 2003). Although promising, this cell therapy is still far from yielding a complete reconstitution of the skeletal muscle structure. The main reasons for the only partial effect of

mesoangioblasts are not yet known, but it is conceivable that it may arise from a limited homing of these cells to muscle, as well as a reduced ability of these cells to fuse and to resist the cytotoxic environment existing in the damaged muscle, where several pro-inflammatory and pro-apoptotic stimuli may be present (Tews and Goebel, 1996; Rando et al., 1998; Engvall and Wewer, 2003). To date, no studies have reported specific pharmacological approaches to enhance the therapeutic efficacy of cell therapy, even though we showed that exposure to cytokines such as high mobility group box 1 (HMGB1) and stromal cell-derived factor 1 (SDF-1) increases mesoangioblast colonisation of dystrophic muscle (Palumbo et al., 2004; Galvez et al., 2006).

A good candidate molecule to increase the efficiency of muscle repair by mesoangioblasts is nitric oxide (NO), a short-lived messenger generated by skeletal muscle to play important roles in regulating its own physiological functions (Wolosker et al., 1997; Bredt, 1998; Balon and Nadler, 1997; Stamler and Meissner, 2001; Eu et al., 2003; Shen et al., 1994; Wang et al., 1995; Clementi and Meldolesi, 1997; Nisoli et al., 2004). Several pieces of evidence support this idea. NO actively participates in processes important to muscle regeneration, such as the myogenic programme activated by insulin-like growth factor (IGF)-II (Carrasco et al., 2002; Kaliman et al., 1999) or by stimulating myoblast fusion (Pisconti et al., 2006). In addition, NO contributes to activation of satellite cells by mediating the production of hepatocyte growth factor (HGF) and follistatin (Anderson, 2000; Tatsumi et al., 2002; Pisconti et al., 2006).

Here, we report that a brief ex-vivo treatment of mesoangioblasts with NO donors enhances their ability to migrate, resist death-inducing stimuli of the type known to be present in dystrophic muscles and fuse with regenerating myofibres. The action of NO is cyclic GMP (cGMP)-dependent and appears to be mediated by classical pathways involved in myogenesis and muscle repair. Our results indicate that the treatment with NO exerts multiple actions that enable mesoangioblasts to limit muscle damage in vivo more efficiently, thus opening an opportunity for improving the efficacy of cell therapy.

Results

NO enhances homing of mesoangioblasts delivered intra-arterially in αSG-null mice

Mesoangioblasts isolated from the dorsal aorta of wild-type E9.5 C57BL/6 mouse embryos (clone D16) (Minasi et al., 2002) and injected into the femoral artery are able to engraft into muscle and yield both morphological and functional repair in αSG-null mice (Duclos et al., 1998; Sampaolesi et al., 2003).

We investigated whether NO enhanced the therapeutic function of these cells. To this end, mesoangioblasts were cultured for 12 hours in growth medium in the presence or absence of either (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate] (DETA-NO) (20 μM) or isosorbide mononitrate (IMN) (50 μM), two widely employed, structurally unrelated NO-releasing compounds (Yamamoto and Bing, 2000). At the concentrations used these NO-donors release concentrations of NO in the physiological range (Clementi et al., 1998). Mesoangioblasts (5×10^5 per animal) were then washed free of the drugs and injected into the right femoral artery of αSG-null mice. Mice

were sacrificed 21 days after injection, and the quadriceps, gastrocnemius, soleus and tibialis anterior muscles collected. The expression of αSG, an index of wild-type mesoangioblast integration, was then evaluated, by real-time PCR, in quadriceps, gastrocnemius and soleus muscles. Expression of αSG in the tibialis anterior was evaluated by western blot analyses and immunohistochemistry. Using this single-injection protocol the restoration of αSG expression by untreated mesoangioblasts was clearly detectable, although lower than that obtained with repeated cell injections (Sampaolesi et al., 2003). Mesoangioblasts pretreated with DETA-NO or IMN yielded a recovery of αSG mRNA levels in the various muscles that was significantly higher than that induced by untreated mesoangioblasts (Fig. 1A). Accordingly, αSG expression analysed by western blotting and immunohistochemistry was also increased (Fig. 1B,C, and data not shown), and the areas in which αSG expression was increased by NO-treated mesoangioblasts were larger in size than those observed with untreated mesoangioblasts and contained more αSG-positive fibres. In the tibialis anterior the increase in area size was of 26.3±1.3% and 22.7±2.0% with DETA–NO- and IMN-treated mesoangioblasts, respectively (n=15, P<0.01 vs. control). In the same muscle, of a total fibre number of 418±28, the numbers of αSG-positive fibres were 109±12, 184±15 and 142±9 after injection with control mesoangioblasts, DETA–NO- and IMN-treated mesoangioblasts, respectively (n=20, IMN- or DETA–NO-treated mesoangioblasts: P<0.01 vs. control). In all these experiments the effect of pretreatment with DETA-NO and IMN was dependent on NO generation because the treatments with the amine DETA or isosorbide did not yield significant effects (data not shown).

The NO-dependent enhancement of mesoangioblast homing in vivo is mediated through cGMP generation

A key downstream effector of NO is cGMP, which is generated following activation of the 'soluble', NO-dependent guanylate cyclase (Moncada et al., 1991). Although mesoangioblasts express none of the NO-synthesizing enzymes, in preliminary experiments they were found to be endowed with the NO-dependent guanylate cyclase (data not shown). To examine the cGMP-dependency of the effect of NO, mesoangioblasts were incubated for 12 hours with the cell-permeable analogue of cGMP, 8-bromo-cGMP (8-Br-cGMP) (1 mM), or DETA-NO combined with 1H-(1,2,4) oxadiazolo [4,3-α]quinoxalin-1-one (ODQ) (1 μM), a selective guanylate cyclase inhibitor that prevents NO-dependent cGMP generation (Garthwaite et al., 1995). Pretreatment of mesoangioblasts with 8-Br-cGMP enhanced αSG expression in a manner similar to the pretreatment with the NO donors (Fig. 1A-C). The combined pretreatment with DETA-NO and ODQ consistently abolished this effect. Pretreatment with ODQ alone did not have significant effects (data not shown). These results indicate that the effect of NO on mesoangioblast homing is cGMP-dependent.

Mechanisms through which NO/cGMP increases homing of mesoangioblasts in vivo

Various mechanisms may conceivably explain the enhanced therapeutic efficacy of the NO-treated mesoangioblasts, including an increased ability to migrate through vessels and

into the site of damage, resist the apoptogenic stimuli present in the dystrophic muscle and fuse with, and repair, existing myofibres. We decided to investigate these mechanisms in vivo separately from each other. To assess chemoattraction and cell movement, green fluorescent protein (GFP)-expressing mesoangioblasts pretreated or not with IMN, DETA-NO, 8-Br-cGMP or DETA-NO plus ODQ were injected into the right femoral artery. Mice were sacrificed 6 hours later, and the quadriceps, gastrocnemius and tibialis anterior muscles collected. Also collected were the liver, spleen and lungs, i.e. the filter organs in which mesoangioblasts tend to accumulate when injected intra-arterially (Sampaolesi et al., 2003). The percentage of cells found in the various muscles and organs was evaluated measuring GFP expression by real-time PCR. Pretreatment with DETA-NO or IMN significantly increased the ability of mesoangioblasts to reach the muscles (Fig. 2A) and reduced the percentage of mesoangioblasts retained in the filter organs (Fig. 2B).

To distinguish between the effect of NO on apoptosis and on the ability to reach target muscles, the pretreated mesoangioblasts were injected directly into the right tibialis anterior muscle. Twelve hours later, treated and controlateral (control) muscles were removed and cell death assessed by staining with the TUNEL technique. As shown in Fig. 2C, the dystrophic muscle showed signs of apoptosis and induced apoptosis of injected mesoangioblasts, recognised by their GFP positivity. This apoptosis was clearly reduced if the cells were pretreated with either NO donor before their injection. Treatment with DETA-NO reduced apoptosis by $58\pm4.9\%$ [$n=3$, $P<0.01$ vs not treated (NT)].

To analyse the effect of NO on muscle repair, the pretreated mesoangioblasts were injected directly into the right tibialis anterior muscle as above, but muscles were removed and analysed after 21 days. The pretreatment with either NO donor resulted in a significantly increased expression of αSG, measured by both quantitative real-time PCR (Fig. 2D) and western blot analyses (Fig. 2E). The fact that restoration of αSG levels by untreated mesoangioblasts was reduced compared with that observed with intra-arterially delivered cells (compare NT in Fig. 1B and Fig. 2E) may reflect an increased cell death due to this specific route of administration (Beauchamp et al., 1999) (B. G. Galvez, unpublished observation).

Of importance, the pretreatment with 8-Br-cGMP mimicked the effects of NO on all the mechanisms investigated, while co-incubation with DETA-NO and ODQ abolished it (Fig. 2A-E). Pretreatment with ODQ alone did not have significant effects on either parameter investigated (data not shown). These results indicate that NO has independent effects that help to enhance homing of mesoangioblasts in a cGMP-dependent manner.

NO/cGMP enhances migration of mesoangioblasts in vitro in response to chemoattractants

We performed different sets of in vitro experiments to investigate in detail the mechanisms of the NO/cGMP effects on mesoangioblast functions observed in vivo. We evaluated the effect of NO/cGMP pretreatment on migration of mesoangioblasts using a transwell system in which the upper and lower chambers were separated by a gelatin-coated cell-permeable membrane. Mesoangioblasts pretreated or not with
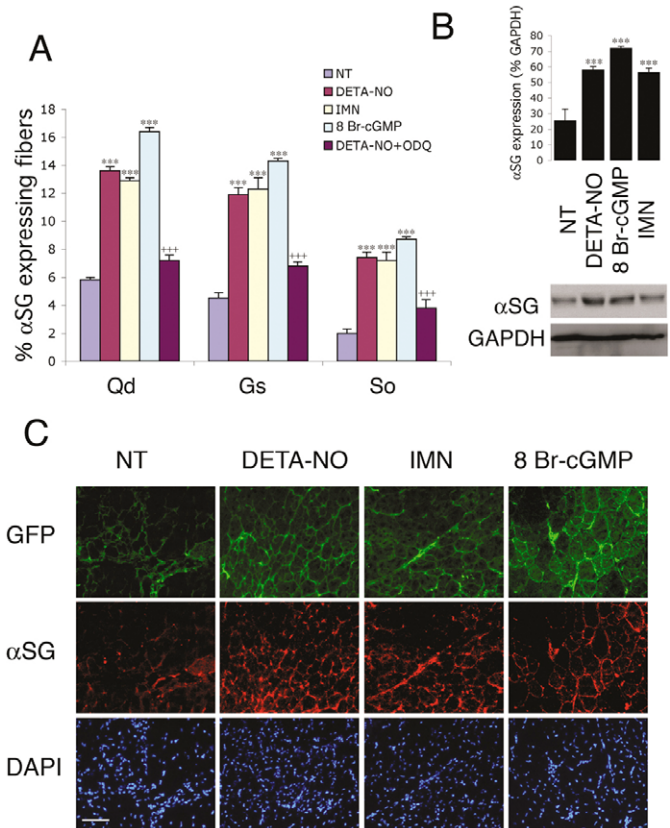

Fig. 1. NO/cGMP increase sarcoglycan production by intra-arterially delivered mesoangioblasts. Mesoangioblasts (5×10^5) pretreated for 12 hours without (NT) or with DETA-NO (20 μM), IMN (50 μM), 8-Br-cGMP (1 mM) or DETA-NO plus ODQ (1 μM) were injected into the right femoral artery of 4-month-old αSG-null mice. Mice were sacrificed after 21 days and quadriceps (Qd), gastrocnemius (Gs) and soleus (So) muscles collected. (A) αSG mRNA expression, an index of mesoangioblast homing, was measured by real-time PCR. Values ± s.e.m. are expressed as the percentage of the total injected cells ($n=3$). (B,C) αSG expression, evaluated by western blot analysis (B, showing both a representative western blot image and densitometric values, $n=3$) and immunohistological detection (C, representative of three consistent experiments). The graph in B reports the ratio of densitometric values ± s.e.m. of αSG vs. those of glyceraldehyde-3 phosphate dehydrogenase (GAPDH) used as an internal loading control. Triple asterisks and crosses, $P<0.001$ vs NT and DETA-NO-treated cells, respectively. Bar in C, 400 μm.

IMN, DETA-NO, 8-Br-cGMP or DETA-NO plus ODQ were plated into the upper chamber and serum-starved overnight. Migration was triggered by the addition of vascular endothelial growth factor (VEGF) (10 ng/ml), a known chemoattractant of these cells, in the lower chamber (Palumbo et al., 2004), transforming growth factor-β (TGF-β) (100 ng/ml), tumour necrosis factor-α (TNF-α) (10 ng/ml), hepatocyte growth factor (HGF) (10 ng/ml) or basic fibroblast growth factor (bFGF) (10 ng/ml), i.e. chemokines and growth factors known to be present in the environment of the regenerating dystrophic muscle (Charge and Rudnicki, 2004). Migration was measured after 6 hours and compared with that triggered by bovine serum albumin (BSA) (0.1%), used as a negative control. As shown in Fig. 3A, migration of mesoangioblasts was dependent on the
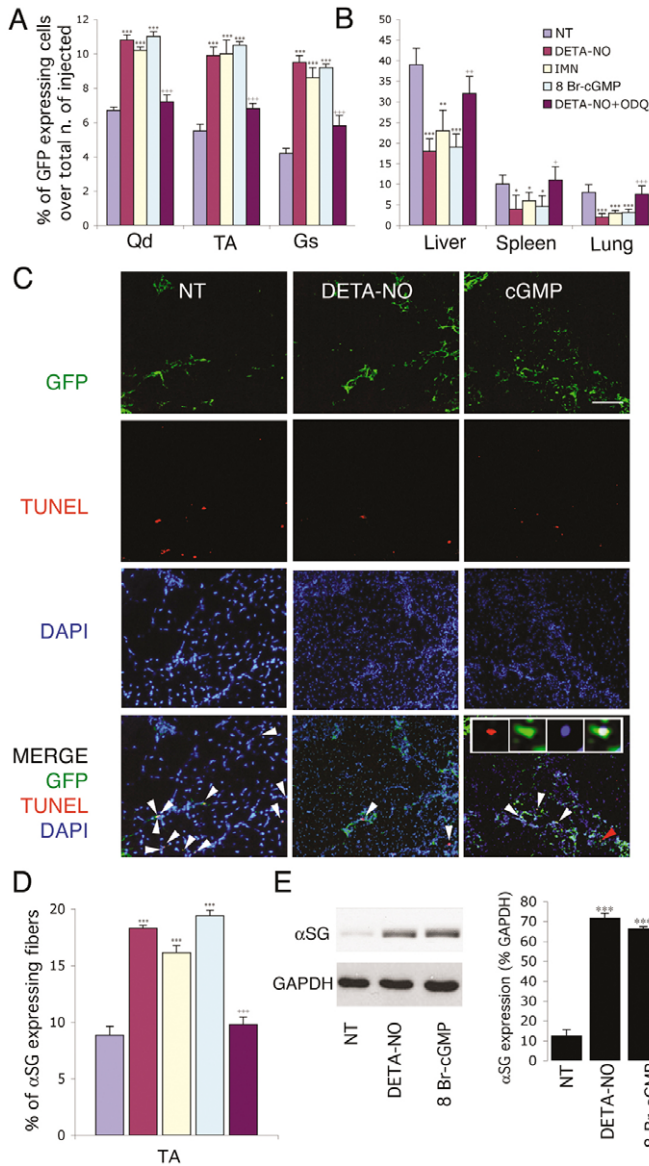
Fig. 2. NO/cGMP increase mesoangioblast migration and engrafting in vivo. (A,B) GFP-expressing mesoangioblasts (5×10^5) pretreated for 12 hours without (NT) or with DETA-NO (20 μM), IMN (50 μM), 8-Br-cGMP (1 mM) or DETA-NO plus ODQ (1 μM) were injected into the right femoral artery of 4-month-old αSG-null mice. After 6 hours quadriceps (Qd), tibialis anterior (TA) and gastrocnemius (Gs) muscles, as well as liver, spleen and lung were collected and the number of mesoangioblasts migrated into them calculated by quantitative real-time PCR for GFP. Values are expressed as the percentage of the total injected cells (*n*=3). (C) GFP-expressing mesoangioblasts (5×10^5) pretreated as indicated above were injected into the tibialis anterior muscles of 4-month-old αSG-null mice. After 12 hours muscles were recovered. Apoptosis of GFP-expressing mesoangioblasts was assessed by the TUNEL technique (images from one out of three reproducible experiments). Also shown is the DAPI staining of the nuclei and its overlay with the GFP and TUNEL staining. Arrows in the merge panels show selected GFP, TUNEL and DAPI-positive cells. The red arrow indicates the specific cell for which the GFP, TUNEL, DAPI and merge stainings are magnified in the insets. (D,E) Same conditions as in C, except that mice were sacrificed three weeks later, tibialis muscles were recovered and αSG expression was analysed by real-time PCR (D, *n*=3) and western blot analysis (E, showing both a representative western blot image and densitometric values, *n*=3). The graph in E reports the ratio of densitometric values of αSG vs. those of GAPDH used as an internal loading control. Triple asterisks and crosses, *P*<0.001 vs. NT and DETA-NO-treated cells, respectively; error bars in A, B, D and E, s.e.m.). Bar in C, 400 μm. The inset in the panel is at 6× magnification.

conditions, VEGF, HGF and TNFα triggered significant migration of control, untreated mesoangioblasts (Fig. 3D). Pretreatment with DETA-NO, IMN or 8-Br-cGMP increased migration further. The effect of DETA-NO was abolished when it was administered together with ODQ.

We studied whether differentiated L6E9 myotubes and non-differentiating L6E9 myoblasts attracted mesoangioblasts and whether NO affected this event. We found that a significant number of mesoangioblasts migrated through the H5V cell monolayer in the presence of myotubes. By contrast, myoblasts failed to trigger migration (Fig. 3E). Pretreatment with either DETA-NO or IMN further increased mesoangioblast migration triggered by myotubes. The effect of NO was mimicked by 8-Br-cGMP and inhibited by ODQ. Conversely, none of the pretreatments triggered migration towards undifferentiated myoblasts (Fig. 3E). Of importance, mesoangioblast pretreatment with ODQ alone had no significant effects in any of the migration experiments (data not shown).

Differentiation of mesoangioblasts into skeletal muscle cells is enhanced by NO/cGMP treatment in vitro

When co-cultured with myogenic cells, mesoangioblasts fuse with them and differentiate into skeletal muscle (Minasi et al., 2002). To investigate the effect of NO on this event we performed two different sets of experiments: in the first mesoangioblasts and L6E9 myoblasts were co-cultured and induced to differentiate together for 5 days (co-fusion experiments), whereas in the second mesoangioblasts were co-cultured with preformed L6E9 myotubes (post-fusion experiments). Cells were fixed and stained with the anti-myosin heavy-chain monoclonal antibody (mAb) MF20 to identify myotubes and differentiated myocytes and with 4′,6-

stimulus used, with TGFβ and TNFα being the most powerful chemoattractants, whereas smaller but still significant migration was induced by VEGF, HGF and bFGF. As shown in the graph and representative images of Fig. 3B,C, pretreatment with either DETA-NO or IMN increased mesoangioblast migration in response to all stimuli used. The effect of NO was mimicked by 8-Br-cGMP and inhibited by ODQ, indicating the cGMP-dependence of the effect of NO. Interestingly, neither NO nor 8-Br-cGMP enhanced migration significantly in the absence of chemoattractants (Fig. 3B; data not shown).

We then investigated whether the stimulatory effect of NO/cGMP was still maintained in a transwell migration assay through an endothelial cell layer. To this end mouse microendothelial H5V cells were plated onto the upper membrane of the transwell insert and cultured until they reached confluence. In this assay we used LacZ-expressing mesoangioblasts so that migrated cells were distinguished from endothelial cells by β-galactosidase staining. Under these

diamidino-2-phenylindole (DAPI). The DAPI staining allowed us to distinguish between rat nuclei (L6E9 cells) and mouse nuclei (mesoangioblasts) on the basis of the different heterochromatin distribution (Blau et al., 1983). Pretreatment of mesoangioblasts with DETA-NO, IMN or 8-Br-cGMP increased their ability to fuse to both myoblasts and myotubes. The effect of DETA-NO was no longer observed in the presence of ODQ (Fig. 3F,G). ODQ had no effects on the fusogenic ability of mesoangioblasts when administered alone (data not shown).

NO/cGMP treatment inhibits death of mesoangioblasts induced by different stimuli in vitro

NO, when generated at low concentrations often increases cell resistance to apoptosis, an effect that may persist even after its removal (Liu and Stamler, 1999; Falcone et al., 2004; Perrotta et al., 2004). We investigated whether NO/cGMP had pro-survival effects on mesoangioblasts. To this end, mesoangioblasts pretreated or not with DETA-NO, IMN, 8-Br-cGMP or DETA-NO plus ODQ were exposed for a further 24 hours to cytotoxic stimuli known to be present in the microenvironment of the dystrophic muscle, i.e. TNFα (100 ng/ml) and the reactive oxygen species generating agents H_2O_2 (100 μM) and As_2O_3 (20 μM) (Tews and Goebel, 1996; Rando et al., 1998; Emery, 2002; Engvall and Wewer, 2003; Hodgetts et al., 2003). Cell death was determined 24 hours later by measuring both Annexin V staining of phosphatidylserine exposed on the outer leaflet of the plasma membrane and propidium iodide (PI) incorporation. Results obtained are summarised in Fig. 4A, and representative dot-plot analyses showing the results using As_2O_3 as the death-inducing stimulus are shown in Fig. 4B. Cell death-induction by all stimuli used was prevented by pretreatment with either NO donor or 8-Br-cGMP and restored by co-incubation of DETA-NO with ODQ (Fig. 4A,B). We then assessed whether NO protected against cell-mediated cytotoxicity, an event known to occur in the damaged, dystrophic muscle (Hohlfeld and Engel, 1990). To this end, mesoangioblasts, after the various pretreatments indicated above, were stained with the green fluorescent dye 5-chloromethylfluorescein (CFMDA) and then co-incubated for 16 hours with cytotoxic T lymphocytes. The results obtained show that NO conferred protection against cytotoxicity, evaluated by measuring PI incorporation (Fischer et al., 2002) (Fig. 4C). Also in this case, the effect of NO depended on cGMP generation.

Expression-profile analysis shows that NO increases expression of specific genes in mesoangioblasts

We investigated whether the effects of NO on mesoangioblast function described in this study involved a change in the expression of specific genes. Mesoangioblasts were pretreated with or without DETA-NO, as above, and their gene-expression profile analysed by microarray analysis. Fluorescent-labelled cRNA was hybridized to the Affymetrix Murine Genome MOE430 GeneChip arrays. From this first analysis and from a reverse transcription (RT)-PCR validation assay we found that NO did not cause profound changes in the overall profile of gene expression, but the expression of specific genes that may explain its effects on mesoangioblasts was affected (Table 1). In particular, NO increased the expression of follistatin, insulin-like growth factor I (IGF-I) and
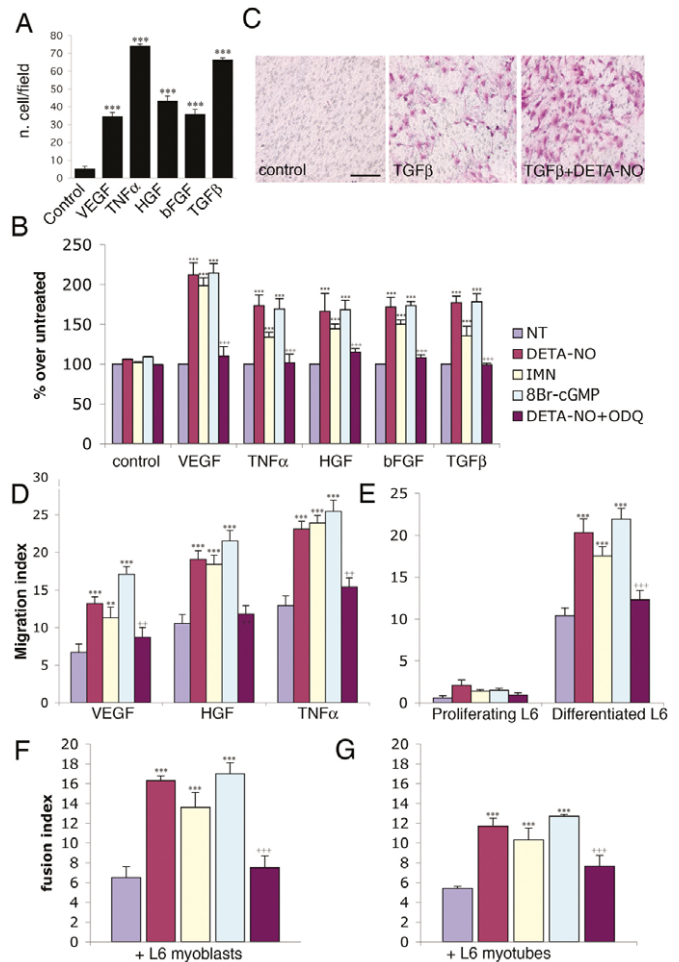

Fig. 3. NO/cGMP increase mesoangioblast migration and myogenic differentiation in vitro. (A-C) Mesoangioblast migration on gelatin-coated membranes. (A) Comparison of the chemoattractant properties of VEGF (10 ng/ml), TGFβ (100 ng/ml), TNFα (10 ng/ml), HGF (10 ng/ml) or bFGF (10 ng/ml) measured in a 6-hour migration assay (n=5). (B) Effect of pretreatment of mesoangioblasts for 12 hours without (NT) or with DETA-NO (20 μM), IMN (50 μM), 8-Br-cGMP (1 mM) or DETA-NO plus ODQ (1 μM). Migration is expressed as the percentage of that observed in untreated controls (NT) (n=5). (C) Representative images of the transmigrated mesoangioblasts after staining with crystal violet. (D,E) Mesoangioblast transmigration through an H5V endothelial cell monolayer. In these experiments, LacZ-expressing mesoangioblasts, pretreated with or without DETA-NO, IMN, 8-Br-cGMP and ODQ as above were used. Transmigration induced by TNFα, VEGF, HGF (D) or L6E9 myoblasts and myotubes (E) was measured after 6 hours. The migration index was calculated as described in the Materials and Methods (n=4). (F,G) Mesoangioblasts pretreated for 12 hours without (NT) or with DETA-NO (20 μM), IMN (50 μM), 8-Br-cGMP (1 mM) or DETA-NO plus ODQ (1 μM) were co-cultured for 5 days in differentiating conditions together with rat L6E9 myoblasts (co-fusion conditions, F) or preformed L6E9 myotubes (post-fusion conditions, G). The graphs show the fusion index, i.e. the number of murine mesoangioblast-derived nuclei in myosin-expressing cells with more than two nuclei vs. the total number of rat and murine nuclei measured (n=5). Double and triple asterisks and crosses, P<0.01 and P<0.001, respectively vs. NT (asterisks) and DETA-NO-treated cells (crosses). Error bars, s.e.m. Bar in C, 200 μm.
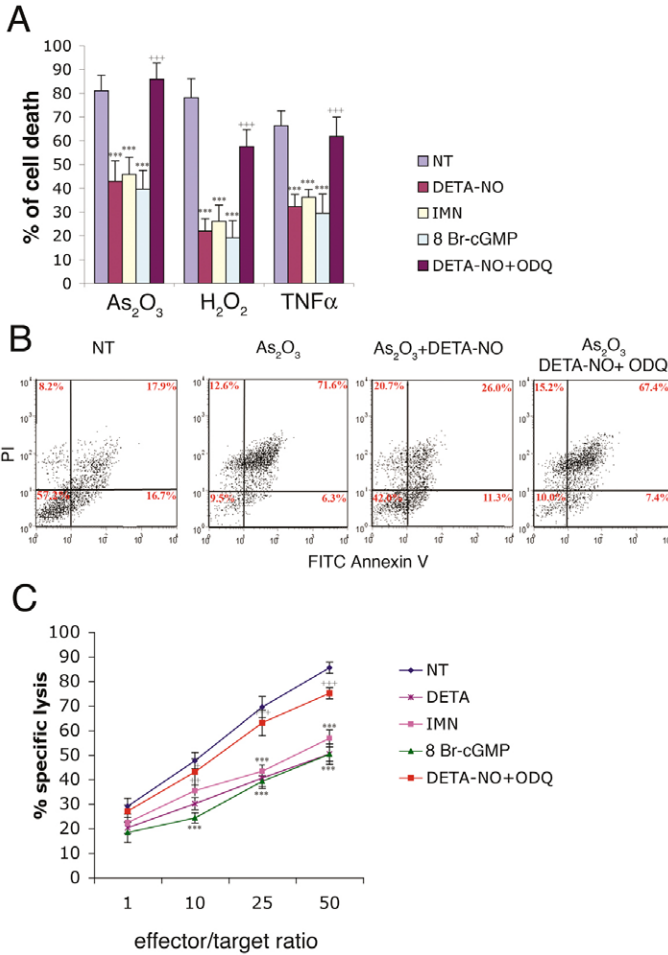
Fig. 4. NO/cGMP protect mesoangioblasts from cell death-inducing stimuli. (A,B) Mesoangioblasts were pretreated for 12 hours without (NT) or with DETA-NO (20 μM), IMN (50 μM), 8-Br-cGMP (1 mM) or DETA-NO plus ODQ (1 μM) and incubated for 24 hours in the absence (NT) or presence of TNFα (100 ng/ml), H_2O_2 (1 μM) or As_2O_3 (20 μM). (A) Cell death was determined by flow cytometry measuring Annexin V staining of phosphatidylserine exposed on the outer leaflet of the plasma membrane and PI incorporation, and expressed as the percentage of that observed in controls (i.e. mesoangioblasts pretreated without drugs and incubated in the absence of apoptogens) (n=4). (B) Representative dot-plot analyses showing the results obtained in one of four consistent experiments using As_2O_3 as the cell death-triggering stimulus. (C) Protection by NO/cGMP of mesoangioblasts (target) from cell death (specific lysis) induced by incubation for 16 hours with cytotoxic T lymphocytes (effector) at the indicated effector/target ratios. Single, double and triple asterisks and crosses, $P<0.05$, $P<0.01$ and $P<0.001$, respectively vs. NT (asterisks) or DETA–NO-treated cells (crosses). Error bars, s.e.m.

metalloproteases, all involved in the network of events governing myoblast fusion and differentiation (Tatsumi et al., 2002; Horsley and Pavlath, 2004; Iezzi et al., 2004). In addition, the pretreatment with NO increased the expression of receptors for HGF, VEGF and TNFα, as well as members of families of adhesion molecules known to play a role in migration, such as cadherins and integrins (Carmeli et al., 2004; Horsley and Pavlath, 2004; Krauss et al., 2005). Finally, NO increased the expression of two anti-apoptotic members of

Table 1. Changes in expression of mouse genes induced by mesoangioblast treatment with DETA-NO

Gene	Fold increase or decrease
Mmp11	+2.83±0.13
Mmp19	+1.91±0.21
Adam12	+1.16±0.09
Adam15	+1.74±0.08
Cdh13	+2.97±0.13
Cdh15	+1.71±0.10
Itgb1	+2.27±0.12
Fst	+2.13±0.09
Igf1	+0.80±0.05
Hgf	+1.21±0.07
Met	+1.92±0.07
Flt1	+1.01±0.91
TnfrI	+2.43±0.11
TnfrII	+1.71±0.10
Bcl-xL	+2.34±0.13
Bcl2	+1.12±0.06
Bax	−1.87±0.09

Mesoangioblasts were treated with or without DETA-NO (20 μM) for 12 hours. RT-PCR analyses using specific primers were performed and densitometric values, normalized for the respective GAPDH values, are expressed as fold increase over NT (*n*=4). *Mmp*, metalloprotease; *Met*, *Flt1*, *TnfrI* and *TnfrII* are receptors for HGF, VEGF and TNFα, respectively.

the Bcl protein family, Bcl-2 and Bcl-xL, and reduced the expression of pro-apoptotic Bax (Adams et al., 2001).

Discussion

A previous study using mesoangioblasts showed that these stem cells have therapeutic efficacy in the αSG-null mouse (Sampaolesi et al., 2003), a model of Duchenne muscular dystrophy (Duclos et al., 1998). Here we show that a brief ex-vivo treatment of mesoangioblasts with NO significantly increases various independent parameters important for their therapeutic activity, i.e. the ability to reach dystrophic muscles, to resist their apoptogenic environment and to engraft into them. The combination of these effects resulted in increased efficiency in homing of intra-arterially delivered mesoangioblasts that yielded an enhanced structural recovery of the damaged muscle. Of importance, the increased homing of mesoangioblasts to the muscle was accompanied by a concomitant reduction in their number in the relevant filter organs. This number is large and may cause damage to these organs (Cossu and Sampaolesi, 2004). Thus, increasing homing to the target tissue has two positive outcomes, the delivery of more cells to repair the target tissue and a reduction in the number of cells that are trapped in filter organs.

The treatment with NO shows two characteristics. First, it is persistent, since a brief exposure of mesoangioblasts yielded a long-lasting increase in their function; second, it depends on the generation of cGMP following activation of guanylate cyclase, an enzyme known to be stimulated by nanomolar concentrations of NO and to mediate physiological actions of the gas (Moncada et al., 1991). Both these characteristics are crucial from a therapeutic standpoint because the amelioration of mesoangioblast function was obtained with a short-term drug exposure, compatible with minimal manipulation of cells ex vivo, and with concentrations of NO in the physiological range.

Using in vitro experiments, we studied in further detail the mechanisms responsible for the beneficial effects of NO

observed in vivo. We investigated the effect of NO on migration of mesoangioblasts towards multinucleated myotubes. These experiments were performed in the presence of an endothelial cell layer in a transwell system, an assay commonly used to examine the chemotactic properties of specific molecules, and proven as a reliable indicator of the mechanisms that govern cellular trafficking in vivo (Puri et al., 2004). Moreover, this in vitro setting mimicks the microenvironment encountered by mesoangioblasts after intra-arterial injection, since myotubes correspond to newly regenerated, immature fibres (Charge and Rudnicki, 2004). We found that pretreatment with NO significantly increased migration of mesoangioblasts towards myotubes. Interestingly, in control experiments using proliferating myoblasts, migration of mesoangioblasts was almost undetectable and not significantly modified by NO, suggesting that some factors secreted by newly-formed muscle fibres stimulate mesoangioblast migration and synergise with the action of NO.

We also investigated the ability of NO to ameliorate mesoangioblast migration triggered by cytokines and growth factors known to be produced in the pro-inflammatory environment of the regenerating dystrophic muscle, such as TNFα, HGF, bFGF and TGFβ (Charge and Rudnicki, 2004; Wagers and Conboy, 2005) or VEGF, a chemoattractant for mesoangioblasts (Palumbo et al., 2004). NO significantly increased mesoangioblast migration triggered by all of these chemoattractants, both in the presence and absence of an endothelial cell layer. Of importance, all the effects of NO in these in vitro assays were found to be cGMP-dependent, consistent with the in vivo migration results. The fact that NO increases migration triggered by myotubes and a variety of chemoattractants is of relevance for the therapy of muscular dystrophy, since it indicates that the beneficial actions of NO apply to the various pro-inflammatory conditions that may arise during the course of the disease.

An important aspect of damaged muscle is that it originates a pro-apoptotic microenvironment in which cytokines such as TNFα, oxidative stress and immune-competent cells play a role, and that may contribute to the poor outcome of transplantation of myogenic cells (Tews and Goebel, 1996; Rando et al., 1998; Emery, 2002; Engvall and Wewer, 2003; Hohlfeld and Engel, 1990; Hodgetts et al., 2003). In vivo, we found that NO/cGMP reduced the number of apoptotic mesoangioblasts observed in muscle sections; in vitro, the exposure to NO/cGMP increases the ability of mesoangioblasts to resist the apoptogenic effects of TNFα, reactive oxygen species and cytotoxic T lymphocytes. This evidence supports the fact that NO protects mesoangioblasts from apoptosis, consistent with its cGMP-dependent anti-apoptotic role described for other cell types (Liu and Stamler, 1999).

The observation that injection of NO-pretreated mesoangioblasts led to a recovery of αSG expression by muscle fibres suggests an additional mechanism that may contribute to the in vivo effect of NO is an increased mesoangioblast ability to fuse to pre-existing myofibres. A role for NO in myoblast fusion has indeed been shown by some studies (Kaliman et al., 1999; Pisconti et al., 2006), although whether this also applies to mesoangioblasts is not known. To investigate this possibility we used an in vitro fusion assay in which mesoangioblasts were co-incubated with either preformed myotubes or differentiating myoblasts. Pretreatment

with NO led to increased fusion of mesoangioblasts in both types of assays. Also in this case the effect was cGMP-dependent, and therefore consistent with the in vivo results.

Signalling events activated by the NO/cGMP pathway are multiple and comprise both short-term modifications of protein function and activity and long-term effects on gene transcription (Pilz and Casteel, 2003). The persistency of the effect of NO/cGMP on mesoangioblasts observed here is consistent with the latter mechanism of action. Moreover, induction and repression of specific genes is also crucial in the process of myogenesis, myoblast fusion and muscle repair (Buckingham, 2001; Charge and Rudnicki, 2004; Cossu and Biressi, 2005). We have therefore investigated whether regulation of gene transcription explained the effects of NO/cGMP on mesoangioblasts. We found that NO increased expression by mesoangioblasts of several plasma membrane receptors known to play key roles in myogenesis, such as members of metalloproteases and disintegrin families, adhesions molecules, integrins and cadherins (Carmeli et al., 2004; Horsley and Pavlath, 2004; Krauss et al., 2005). NO also increased the expression of HGF, which participates in NO-induced satellite cell activation (Anderson, 2000; Tatsumi et al., 2002), and of IGF-I and follistatin, which contribute to muscle hypertrophy (Glass, 2003; Iezzi et al., 2004), the latter recently shown to mediate NO-induced satellite cell fusion (Pisconti et al., 2006). Interestingly, NO also increased the expression of receptors for chemoattractants, and this, together with the effect on adhesion molecules, may explain why NO-treated mesoangioblasts are endowed with and increased migratory ability. NO also inhibited the mesoangioblast expression of proapoptotic Bax, while increasing the expression of anti-apoptotic Bcl-2 and Bcl-xL (Adams et al., 2001). This, together with the observation that the anti-apoptotic action of NO was not restricted to a specific stimulus, strongly suggests that NO switches off the apoptotic programme elicited by the damaged muscle environment in mesoangioblasts, rather than acting as a simple negative modulator of selected apoptotic signals. Although the changes in the levels of expression of the various genes investigated were not striking, the pattern of modifications induced in mesoangioblasts by NO/cGMP is consistent with a general switch on of signalling pathways involved in myogenesis and muscle repair. How and to what extent the changes in gene expression are integrated in shaping the enhanced function of NO-treated mesoangioblasts remains to be investigated.

Which among the various effects of NO on mesoangioblasts described here is the most relevant in in vivo conditions is difficult to predict, and enhanced migration, resistance to apoptosis and ability to fuse may conceivably all play a role. However, the anti-apoptotic effect of NO is the most dramatic in terms of fold changes and has already been shown to play a key role with other cell types in vivo (Perrotta et al., 2004). This strongly suggests that protection against apoptosis plays a crucial role in determining the NO-dependent enhanced homing of mesoangioblasts.

In conclusion, we propose that the treatment of mesoangioblasts with NO significantly enhances their homing to dystrophic muscle, yielding a recovery of its function. Since this treatment does not require extensive cell manipulation and is non-toxic it appears worth pursuing, especially in view of the new NO donors and compounds able to increase cGMP

concentration that are currently undergoing validation for clinical use (Burgaud et al., 2002; Nakane, 2003). In addition, because of its simplicity, the treatment might be easily combined with other strategies to yield an enhanced therapeutic effect combining increased mesoangioblast survival and ability to reach damaged muscles and engraft into them. Finally, this therapeutic approach may be helpful not only in muscular dystrophies but also in other pathological states in which stem cell therapy has been proposed, including congenital myopathies and muscle atrophy (Wagers and Conboy, 2005).

Materials and Methods

Reagents
Cell culture reagents were purchased from Cambrex (Verviers, Belgium); the anti-sarcomeric myosin MF20 and anti-αSG mAbs from DSHB (University of Iowa, Iowa City, IA, USA) and NovoCastra (Newcastle, UK), respectively. In immunoblot and immunohistochemistry analyses primary Abs were detected by appropriate secondary Abs conjugated with horseradish peroxidase (BioRad, Hercules, CA, USA) or Alexa-Fluor 488 and 594, respectively (Molecular Probes, Eugene, OR, USA). Polycarbonate membrane transwell inserts used for the in vitro chemotaxis assays were purchased from Costar (Milan, Italy); mouse VEGF, TNF-α, HGF, bFGF and TGF-β from R&D Systems (Abingdon, UK); and fluorescein isothiocyanate (FITC)-Annexin V and PI from Bender MedSystem (Vienna, Austria). The reagents for RNA extraction and cDNA preparation were obtained by Invitrogen Life Technologies (Carlsbad, CA, USA). ODQ and DETA-NO were from Alexis Italia (Florence, Italy), and all the other reagents were purchased from Sigma (Milan, Italy).

Animals
αSG-null C57BL/6 mice were a kind gift of K. Campbell (Iowa University, Iowa City, IA, USA). Animals were housed in the pathogen-free facility at our institution and treated in accordance with the European Community guidelines, and with the approval of the Institutional Ethical Committee. Animals were used when 4 months old, since at this stage αSG-null mice are characterized by the development of histopathological features of muscle dystrophy with ongoing fibre degeneration and modest spontaneous regeneration (Duclos et al., 1998).

Cell culture
Wild-type, GFP- or LacZ-expressing D16 mesoangioblasts were generated and cultured as described (Minasi et al., 2002; Sampaolesi et al., 2003; Palumbo et al., 2004). In all experiments the pretreatment of mesoangioblasts with DETA-NO (20 μM), IMN (50 μM), 8-Br-cGMP (1 mM), ODQ (1 μM) or DETA-NO plus ODQ was for 12 hours, after which cells were washed free of the drugs and suspended in culture medium. Delivery of mesoangioblasts was either by intra-arterial injection through the right femoral artery with 5×10^5 mesoangioblasts per animal, performed exactly as described (Sampaolesi et al., 2003), or by injection of 5×10^5 cells directly into tibialis anterior muscles.

The rat skeletal muscle cell line L6E9 was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15% foetal bovine serum (FBS), 200 μM glutamine, 1000 U/ml penicillin and streptomycin growth medium (GM). Differentiation was obtained by shift to a differentiation medium (DM) containing 2% horse serum. The microendothelium cell line H5V (a kind gift of Elisabetta Dejana, University of Milano, Milan, Italy) was cultured in DMEM containing 10% FBS, 200 μM glutamine, 1000 U/ml penicillin and streptomycin.

Quantitative real-time PCR analysis
Tissue samples were homogenized and RNA extracted using TRIzol reagent (Invitrogen) and reverse transcribed into complementary DNA by using a Taqman kit (Platinum Taq DNA polymerase; Invitrogen). Real-time quantitative PCR analyses were performed using a Mx3000P real-time PCR detection system (Stratagene, La Jolla, CA, USA). Each cDNA sample was amplified in duplicate by using the SYBR Green Supermix (BioRad) for GFP (GFP primers: forward (FW), 5′-AAGTTCATCTGCACCACCG-3′; reverse (Rev), 5′-TCCTTGAAGAAGATG-GTGCG-3′). Data are expressed as the percentage of migrated cells, which is calculated by comparing the level of GFP messenger in our sample with that corresponding to the total input of injected cells. Real-time PCR analyses of αSG expression were performed using the Taqman Universal PCR master mixture containing AmpliTaq Gold DNA with commercial primers (Applied Biosystems, Foster City, CA, USA) for αSG detection. For Taqman assays, the level of αSG measured represents the specific signal detected in the sample from mesoangioblast-injected αSG-null mouse muscles vs. that of a positive control for αSG expression (corresponding wild-type mouse muscles).

Protein extraction and immunoblot analysis
Muscle tissues were dissected and homogenized in 100 mM NaHCO$_3$, 1 mM EDTA, 2% sodium dodecyl sulphate and protease inhibitor cocktail (Complete, Roche Diagnostics) and centrifuged (1000 *g*) for 10 minutes at 4°C to discard cellular debris. Sample preparation and western blot analyses were performed as described (Bulotta et al., 2001).

Immunohistochemistry
Tibialis muscles recovered from the mesoangioblast-injected animals were dissected and frozen in liquid N$_2$-cooled isopentane. Serial muscle sections were immunostained as previously described (Sampaolesi et al., 2003) with the anti-GFP, anti-laminin or anti-αSG Abs. Primary Abs were detected using appropriate secondary Abs and nuclei were visualized with the DNA dye DAPI. Quantification of αSG-positive areas and fibre number was performed in five randomly selected fields of at least three non-adjacent transverse αSG-laminin double-stained sections along the longitudinal axis of the muscle.

Differentiation experiments
L6E9 myoblasts were plated in growth medium at a density of 1.5×10^5 and shifted in DM medium 24 hours later. D16 mesoangioblasts suspended in DM were added to L6E9 cultures either at the moment of medium shift (5×10^4 cells/dish, co-fusion experiments) or after 3 days (2×10^4 cells/dish, post-fusion experiments), i.e. when L6E9 myotubes were already formed.

After 5 days of co-culture, the medium was removed, the cells fixed and myotubes and nuclei revealed by immunofluorescence using the anti-myosin heavy-chain Ab (MF20, 1:3) and DAPI, respectively (Brunelli et al., 2004). The percentage of mesoangioblasts fused to rat myotubes was measured by counting the number of mouse nuclei in myosin heavy chain-stained rat fibres (5-8 random fields for each sample) on the basis of different staining with DAPI because of different heterochromatin distribution.

Migration assays
LacZ-expressing D16 mesoangioblasts (2×10^4) were plated in complete medium in the upper chamber of transwell inserts (membrane diameter: 6.5 mm; pore size: 8 μm) coated with type A gelatin from porcine skin. At the end of the pretreatments the cells were starved overnight in DMEM without serum. The following morning the lower chamber medium was substituted with DMEM containing differentiated L6E9 myotubes, undifferentiated L6E9 cells or one of the following chemotactic agents: TGFβ (100 ng/ml), VEGF (10 ng/ml), TNFα (10 ng/ml), HGF (10 ng/ml) or bFGF (10 ng/ml). In the transmigration experiments through endothelium, mouse microendothelial H5V cells were plated on the gelatin-coated transwell membrane (1×10^4 cells/dish) and grown in DMEM containing 10% FBS until they reached confluence, before the addition of mesoangioblasts. The tightness of the monolayers was checked by measuring the diffusion of BSA from the upper to the lower chamber. The number of mesoangioblasts that crossed the membrane pores was evaluated after 6 hours. To this end, non-migrated cells were mechanically removed from the upper side of the transwell system, whereas the cells in the lower side of the filter membrane were fixed in 4% paraformaldehyde for 10 minutes at 4°C and stained with crystal violet [0.1% in methanol-water (2:8) solution for 20 minutes at room temperature]. The number of migrated cells was measured using an inverted microscope and by counting 5-10 random fields of the lower face of the transwell membrane at 20× magnification or by X-Gal staining, performed according to standard protocols (Brunelli et al., 2003). Migration index was calculated by dividing the number of migrated cells in the presence of the chemotactic agents by the cells migrated in response to DMEM with 0.1% BSA (control).

Cell death experiments
After the various pretreatments, mesoangioblasts were incubated with or without TNFα (100 ng/ml), H$_2$O$_2$ (100 μM) or As$_2$O$_3$ (20 μM) for 24 hours. Cells were detached and stained with FITC-Annexin V and PI according to the manufacturer's instructions and analysed by flow cytometry as described (Sciorati et al., 1997). Cells showing single staining for Annexin V or double staining for Annexin V and PI were considered dead cells. To study the effect of cytotoxic T lymphocytes, 5×10^4 mesoangioblasts or control cells (splenocytes and RMA cells) were stained with the vital green fluorescent dye CFMDA (Perrotta et al., 2004) and incubated with increasing numbers of cytotoxic effector T cells (1:1, 1:5, 1:10, 1:25) (Ferrarini et al., 1999). Cell death was evaluated by flow cytometry measuring PI incorporation after 16 hours (Fischer et al., 2002).

Gene expression profiling
Biotin-labelled target synthesis as well as the Affymetrix Mouse Genome MOE430 GeneChip array hybridization and scanning were performed with Affymetrix instruments and by applying standard procedures and protocols on total RNA isolated from cultured cells using the RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA). The amount of a transcript mRNA (signal) was determined using the GCOS 1.2 absolute analysis algorithm as already described (Liu et al., 2002).

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RT-PCR

RNA (1 μg) collected from cells using an RNeasy Mini (or Micro) kit (Qiagen) was converted into double-stranded cDNA using the Thermoscript RT-PCR System cDNA synthesis kit (Invitrogen), according to the manufacturer's instructions. cDNA was then amplified using the following primers: Adam12 FW, 5′-ATCAGTGTCTTCGGCGTTC-3′; Adam12 Rev, 5′-GGCAATTCTTCCTGTT-GTTACATACC-3′; Adam15 Fw, 5′-GACCACTCCACAAGCATCTTAGG-3′; Adam15 Rev, 5′-GGGAGAATCATGGTCCAAACC-3′; Bax Fw, 5′-GGAATT-CGCCGTGATGGACGGGTCCGG-3′; Bax Rev, 5′-GGAATTCTCAGCCCAT-CTTCTTCCAGA-3′; Bcl-2 Fw, 5′-TTCGGTGTAACTAAAGACAC-3′; Bcl-2 Rev, 5′-CTCAAAGAAGGCCACAATCC-3′; Bcl-XL Fw, 5′-GTGAGTGGACGGTC-AGTG-3′; Bcl-XL Rev, 5′-TTGGACAATGGACTGGTTGA-3′; Cdh13 Fw, 5′-GACGCCTGACAAGCCATCTCCTAA-3′; Cdh 13 Rev, 5′-GACCCCCACAG-CTCCCTCCTC-3′; Cdh15 Fw, 5′-CTTGGGTGCCACGGAGA-3′; Cdh15 Rev, 5′-ATGCAGGCCCTCGGAGAC-3′; Hgf Fw, 5′-CTTGGCATCCACGATGTTC-AT-3′; Hgf Rev, 5′-TGGTGCTGACTGCATTTCTCA-3′; Igf1 Fw, 5′-GTG-GATGCTCTTCAGTTCGT-3′; Igf1 Rev, 5′-ACACTCCTAAAGACGATGTT-3′; Itgb1 Fw, 5′-TGTTCAGTGCAGAGCCTTCA-3′; Itgb1 Rev, 5′-CCTCATA-CTTCGGATTGACC-3′; Fst Fw, 5′-CTCTTCAAGTGGATGATTTTC-3′; Fst Rev, 5′-ACAGTAGGCATTATTGGTCTG-3′; Mmp11 Fw, 5′-ATTGATGCTGCCTT-CCAGGAT-3′; Mmp11 Rev, 5′-GGGCGAGGAAAGCCTTCTAG-3′; Mmp19 Fw, 5′-GCCCATTTCCGGTCAGATG-3′; Mmp19 Rev, 5′-AGGGATCCTCCAG-ACCACAC-3′; Tnfr1 Fw, 5′-CCACCATCTCGGTCATCAGGATTGCCT-3′; Tnfr1 Rev, 5′-TTCTCATGGAAGCTATGGGTATCACA-3′; Met Fw, 5′-AGA-AATTCATCAGGCTGTGAAGCGCG-3′; Met Rev, 5′-TTCCTCCGATCGCA-CACATTTGTCG-3′; Flt1 Fw, 5′-CAATGTGGAGAGCCGAGACA-3′; Flt1 Rev, 5′-GAGGTGTTGAAAGACTGGAACGA-3′; GAPDH Fw, 5′-TGAAGGTCG-GAGTCAACGGATTTGGT-3′; GAPDH Rev, 5′-CATGTGGGCCATGAGGTC-CACCAC-3′.

Statistical analysis

The results are expressed as means ± s.e.m.; n represents the number of individual experiments. Statistical analysis was performed using the Student's t-test for unpaired variables (two-tailed). Asterisks and crosses in the figure panels refer to statistical probabilities vs. untreated controls or DETA-NO-treated cells, respectively. Statistical probability values (P) of less than 0.05 were considered significant.

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