Convergence of *Igf2* expression and adhesion signalling via RhoA and p38 MAPK enhances myogenic differentiation

Fiona A. Lovett[1], Ivelisse Gonzalez[1], Dervis A. M. Salih[1], Laura J. Cobb[1], Gyanendra Tripathi[1], Ruth A. Cosgrove[1], Adele Murrell[2], Peter J. Kilshaw[1] and Jennifer M. Pell[1,*]

[1]The Babraham Institute, Babraham Research Campus, Cambridge, CB2 4AT, UK
[2]Department of Oncology, Cambridge University, MRC-Hutchison Centre, Cambridge, CB2 2XZ, UK
*Author for correspondence (e-mail: jenny.pell@bbsrc.ac.uk)

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Summary

Cell-cell contact is essential for appropriate co-ordination of development and it initiates significant signalling events. During myogenesis, committed myoblasts migrate to sites of muscle formation, align and form adhesive contacts that instigate cell-cycle exit and terminal differentiation into multinucleated myotubes; thus myogenesis is an excellent paradigm for the investigation of signals derived from cell-cell contact. PI3-K and p38 MAPK are both essential for successful myogenesis. Pro-myogenic growth factors such as IGF-II activate PI3-K via receptor tyrosine kinases but the extracellular cues and upstream intermediates required for activation of the p38 MAPK pathway in myoblast differentiation are not known. Initial observations suggested a correlation between p38 MAPK phosphorylation and cell density, which was also related to N-cadherin levels and *Igf2* expression. Subsequent studies using N-cadherin ligand, dominant-negative N-cadherin, constitutively active and dominant-negative forms of RhoA, and MKK6 and p38 constructs, reveal a novel pathway in differentiating myoblasts that links cell-cell adhesion via N-cadherin to *Igf2* expression (assessed using northern and promoter-reporter analyses) via RhoA and p38α and/or β but not γ. We thus define a regulatory mechanism for p38 activation that relates cell-cell-derived adhesion signalling to the synthesis of the major fetal growth factor, IGF-II.

Key words: Cadherin, Cell-cell contact, IGF-II, Myogenic differentiation, p38 MAPK, RhoA

Introduction

Cell-cell contact and membrane fusion are crucial events in the development of multicellular organisms, being necessary for a wide range of processes. The developmental potential of cell lineages is often only realised when a certain cell density is exceeded – the 'community effect' (Gurdon et al., 1993) – and it is likely that key signals arise from such cell-cell contact that are essential for the regulation subsequent cell fate and function (Braga, 2002).

During myogenesis, committed but proliferating mononuclear myoblasts form cell-cell contacts, exit from the cell cycle, and fuse to become multinucleated myotubes, accompanied by upregulation of muscle-specific genes (Tapscott, 2005). Myogenesis thus provides an excellent paradigm for the investigation of signalling pathways stimulated by intercellular contacts, and the adhesive properties of cells are in large part regulated by the cadherin superfamily of glycoproteins (Derycke and Bracke, 2004). In developing muscle, at least four cadherin family members are present (N-cadherin, M-cadherin, R-cadherin and cadherin 11) (Marthiens et al., 2002); however, knockout studies of both N-cadherin (Charlton et al., 1997) and M-cadherin (Hollnagel et al., 2002) suggest that they exhibit considerable functional redundancy. Activation of cadherin-mediated cell-cell adhesion accelerates the myogenic differentiation program (Goichberg and Geiger, 1998; Redfield et al., 1997). The use of exogenous N-cadherin ligand to mimic intercellular contacts has demonstrated that N-cadherin signalling can substitute for cell-contact-mediated cell-cycle arrest and myogenin expression in myoblasts (Gavard et al., 2004).

Two key signalling pathways are required for myogenesis: PI3-K (phosphatidylinositol 3-kinase) and p38 MAP kinase, which regulate the activation of MEF2 transcription factors and MyoD. PI3-Ks are important mediators of tyrosine kinase receptor signal transduction; inhibition of PI3-K blocks myogenesis (Jiang et al., 1998; Kaliman et al., 1996) and Akt is an essential downstream target of PI3-K in myoblast differentiation (Fujio et al., 1999; Jiang et al., 1999). p38 MAPK is also essential for myoblast differentiation (Zetser et al., 1999; Zhao et al., 1999), and recently novel functions have been identified in the activation of a temporally regulated subset of myogenesis genes (Penn et al., 2004) and in targeted chromatin remodelling during myogenesis (Simone et al., 2004). p38 MAPK consists of four isoforms, three of which – α, β (Li et al., 2000) and γ (Lechner et al., 1996) – are present in myoblasts.

Autocrine IGF-II is thought to be the major upstream activator of the PI3-K/Akt pathway in myogenesis and acts via the IGF type 1 receptor (IGF1R) to regulate both PI3-K and Akt, as well as activating myogenic transcription factors (Coolocan et al., 1997; Wilson et al., 2003). The absolute importance of IGF1R signalling in muscle development is

illustrated by the poor muscle development and dystrophic phenotype of *Igf1r* null mice, which usually die at birth as a result of weakness of respiratory muscle (Liu et al., 1993).

In marked contrast to the PI3-K/Akt pathway, little is known about the upstream activator(s) of the p38 MAPK pathway in myogenesis, other than probable MAPKKs, even though these have been sought (reviewed by Forcales and Puri, 2005). In preliminary studies, we observed a correlation between initial cell density, p38 MAPK phosphorylation and subsequent myogenesis. Therefore the first aim of this study was to investigate whether cell-cell contact, via cadherin-mediated activation, could provide an extracellular signal for p38 MAPK activation and, if so, to identify key signalling intermediates. We further observed that expression of the key myogenic growth factor, IGF-II, also correlated with initial cell density, and therefore our second aim was to examine whether the putative adherens-activated p38 pathway had a role in the regulation of growth factor synthesis. We reveal a pathway from cadherin activation via RhoA and p38 MAPK to *Igf2* transcription and accelerated myoblast differentiation. More detailed analysis identified that, despite its preferential abundance in skeletal muscle, p38γ is not activated by N-cadherin signalling, but rather that p38α and/or β are important in regulating *Igf2* expression. We thus identify novel links between cell-cell-contact-stimulated signalling events and growth factor synthesis and action.

Results

Increasing cell density enhances myogenic differentiation, *Igf2* expression and N-cadherin protein levels in C2 cells

Preliminary experiments were performed in which C2 myoblasts were plated at subconfluent (1×10^5 cells/60 mm plate) or confluent (4×10^5 cells/60-mm plate) densities and induced to differentiate as described in the Materials and Methods. In subconfluent cultures, differentiation was

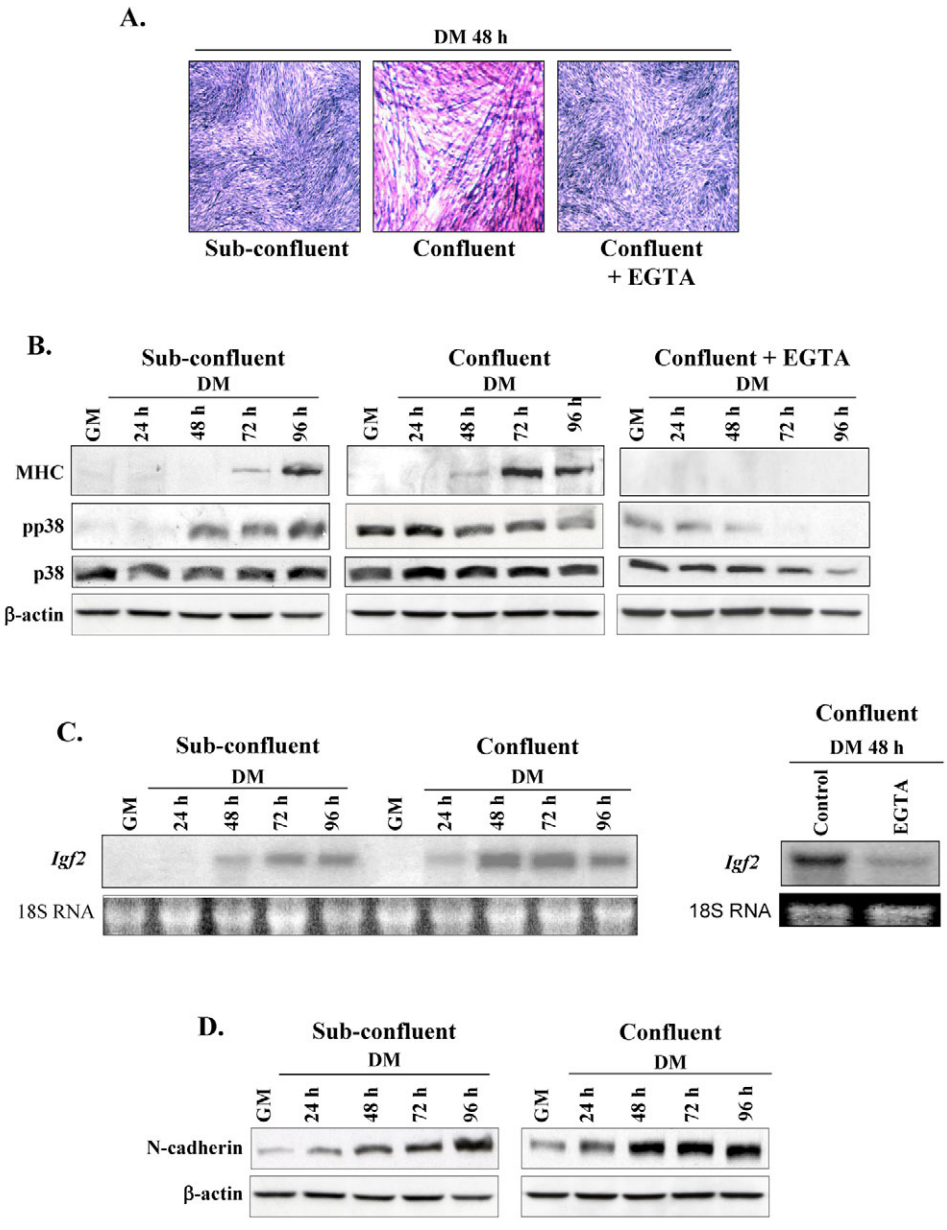

Fig. 1. Increased cell density enhances myogenic differentiation of C2 cells. (A) H and E staining of C2 myoblasts seeded at the different cell densities of subconfluency (1×10^5 cells/60-mm-diameter plate) and confluency (4×10^5 cells/60-mm-diameter plate), and also at confluency in the presence of 1.75 mM EGTA; cells were stained 48 hours after the initiation of differentiation induced by transfer of cells from growth medium (GM) to differentiation medium (DM), as described in the Materials and Methods. Magnification, ×50. (B) Western blot detection of MHC, phospho-p38 MAPK (pp38), total p38 MAPK and β-actin in differentiating C2 cells at subconfluency, confluency, and confluency in the presence of 1.75 mM EGTA, sampled at the times indicated after transfer from GM to DM. (C) Northern blot of *Igf2* mRNA levels in differentiating myoblasts sampled as in B; 18S rRNA ethidium bromide staining is shown as a loading control. (D) Western blot detection of N-cadherin during myogenesis sampled as in B; β-actin is shown as a loading control.

observed morphologically by the alignment and elongation of cells by 48 hours (Fig. 1A) but fusion of myoblasts into multinucleated myotubes was not observed until 72-96 hours after initiation of differentiation. However, myogenesis was accelerated in confluent cultures, with myotubes observed after just 48 hours (evidenced by haematoxylin and eosin staining of structures composed of several cell lengths accumulating pink-stained protein; Fig. 1A) and MHC expression occurring at least 24 hours earlier than in subconfluent seeded cultures (Fig. 1B). Since cadherins require Ca^{2+} to form effective cell-cell contacts with neighbouring cells (Pokutta et al., 1994), myoblasts were induced to differentiate in the presence of the Ca^{2+} chelator, EGTA. Differentiation was abolished with cells unable to fuse and failing to express MHC (Fig. 1A,B), suggesting a key role for cadherins. Phosphorylation of the essential myogenic signalling protein, p38 MAPK, was accelerated in confluent cultures compared with subconfluent cultures, whereas the abolition of cell adhesion by EGTA abrogated p38 phosphorylation after 48 hours (Fig. 1B).

IGF-II, via IGF1R signalling, is important for efficient myogenesis, and transfer of myoblasts to differentiation medium stimulates essential autocrine *Igf2* expression (Florini et al., 1991). C2 cells seeded at higher density exhibited accelerated and increased *Igf2* expression compared with subconfluent cultures (Fig. 1C); when cell adhesion was impeded by addition of EGTA, *Igf2* expression was significantly reduced (Fig. 1C). Furthermore, N-cadherin protein levels were upregulated during myoblast differentiation, in a cell-density-dependent manner (Fig. 1D). These initial observations confirm a pro-myogenic role for cell-cell contact in myoblasts and suggest that key adherens-mediated cell signalling may be involved in the regulation of *Igf2* expression.

Modulation of N-cadherin-mediated adhesion regulates p38 MAPK activity and *Igf2* expression

The construct cN390Δ encodes a form of N-cadherin lacking a large portion of the extracellular domain, thus inhibiting its ability to form trans dimers with cadherins on neighbouring cells (Fig. 2A) (Fujimori and Takeichi, 1993). Stable expression of this construct (demonstrated by its HA tag, Fig. 2D) delayed myogenesis; initial elongation and alignment of myoblasts appeared normal but subsequent fusion to form myotubes was not observed (Fig. 2B). Expression of the late myogenic marker, MHC, was also reduced in the cN390Δ-expressing cells (Fig. 2C). As

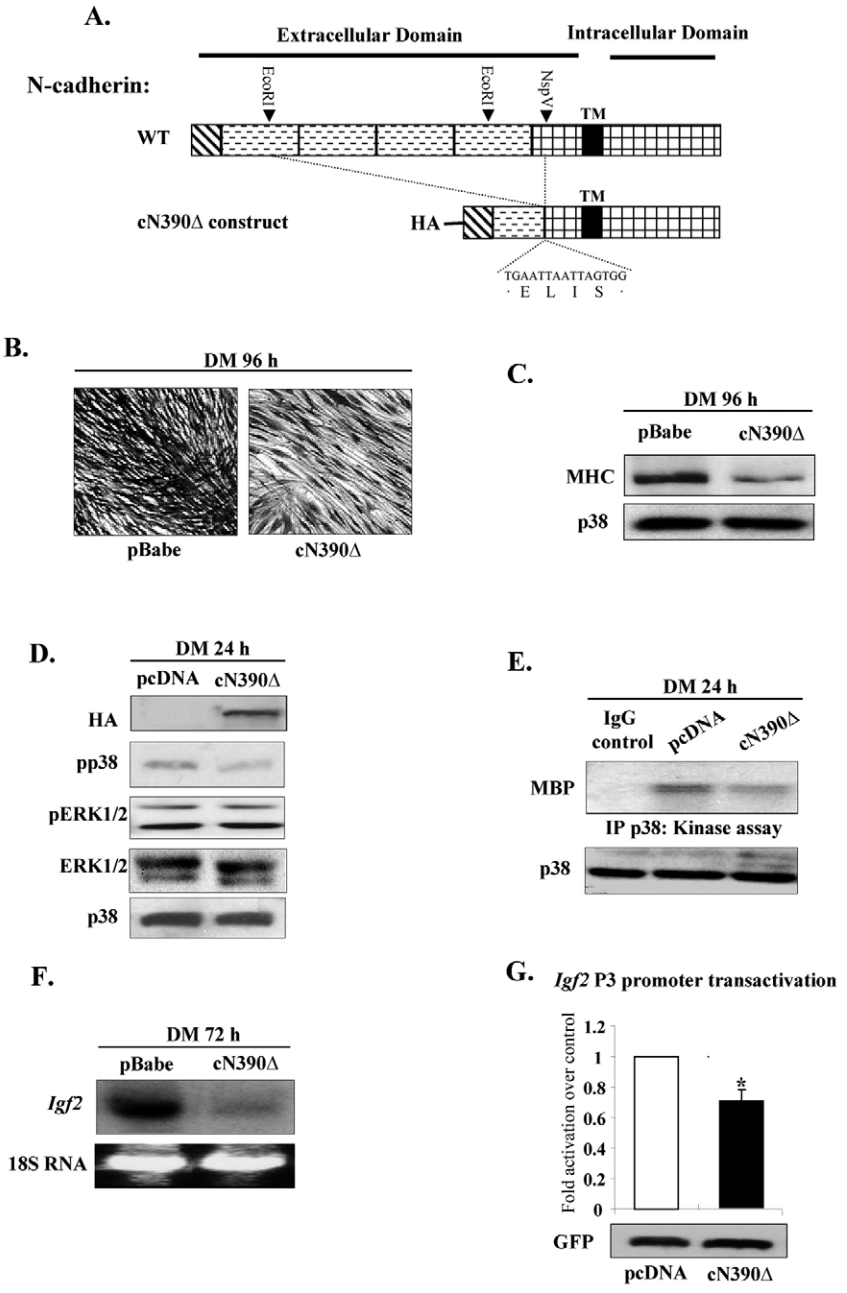

Fig. 2. Dominant-negative N-cadherin delays myogenesis and decreases p38 MAP kinase activation and *Igf2* expression. (A) Schematic of the dominant-negative cN390Δ N-cadherin construct. (B) H and E staining of differentiating C2 cells retrovirally transduced with cN390Δ or pBabe only. Magnification, ×50. (C) Western blot of MHC in cN390Δ or pBabe stably transfected cells; total p38 is shown as a loading control. (D) Western blot to detect the HA tag (cN390Δ cells only), phospho-p38 (pp38), total p38, phospho-ERK1/2 and total ERK1/2 in vector alone (pcDNA) and cN390Δ transiently transfected cells. (E) p38 MAPK kinase activity and total p38 levels by western blot analysis in vector alone (pcDNA) and cN390Δ transiently transfected cells; MBP, myelin basic protein. (F) Northern blot analysis of *Igf2* expression in C2 cells stably transfected with either cN390Δ or pBabe alone; 18S rRNA is shown as a loading control. (G) *Igf2* P3 promoter luciferase reporter activity in C2 cells transiently transfected with either cN390Δ or pcDNA alone. Fold activation levels are expressed as the ratio of luciferase activity of cN390Δ transfected cells to that of cells transfected with vector alone: values were normalised to the relative GFP expression levels per unit of protein; **P*<0.05 compared with an activation value of 1.0.

our initial observations suggested that p38 MAPK phosphorylation was cell-density dependent (Fig. 1B), we examined the effects of the dominant-negative N-cadherin on p38 activity at 24 hours after the initiation of myogenesis, a key stage of p38 activation (Gonzalez et al., 2004). Both p38 phosphorylation (Fig. 2D) and kinase activity (Fig. 2E) were significantly reduced after transfection with cN390Δ cadherin. To ensure that the effects on p38 were not due to a generalised reduction in cell function, we also examined the abundance and phosphorylation of ERK1/2; no changes in total or phospho-ERK were observed (Fig. 2D).

Since *Igf2* mRNA levels and N-cadherin protein abundance broadly correlated during myogenesis, we examined the effects of cN390Δ cadherin on *Igf2* expression. *Igf2* mRNA levels were dramatically reduced 72 hours after the initiation of myogenesis in cells stably transfected with cN390Δ, a time when *Igf2* expression is usually high (Fig. 2F). We also examined *Igf2* transcription using a luciferase P3 promoter-reporter for the *Igf2* P3 transcript; the dominant-negative N-cadherin significantly reduced P3 promoter activity (Fig. 2G) but to a lesser extent than the decrease observed for mature mRNA levels. This could indicate multiple levels of regulation of *Igf2* expression (i.e. nascent mRNA synthesis versus mRNA stability) but is also probably due to differences in method of transfection and time of sampling for each of the observations.

We next examined the effects of stimulating N-cadherin. Myoblasts were treated with a chicken N-cadherin dimer fusion protein (NcadFc), that consisted of the extracellular domain of N-cadherin fused to an Fc domain, linked by disulphide bonds at the Fc hinge (Fig. 3A); this protein is a well-established means of recapitulating cadherin-mediated cell-cell contact, forming complexes with and activating endogenous N-cadherins (Gavard et al., 2004). Myoblasts were seeded at subconfluency and 0.5 μg/ml N-cadherin ligand (previously determined as an optimal dose) was added to the cells upon switching to differentiation medium. Control myoblasts were treated with an equivalent volume of extraction from mock-transfected Cos7 cells. Levels of p38 phosphorylation were increased in NcadFc-stimulated cells compared with that in controls by 24 hours (Fig. 3B), whereas there was little change in phosphorylation of ERK1/2. This increase in p38 phosphorylation was supported by an equivalent increase in kinase activity (Fig. 3C). Stimulation of N-cadherin-mediated adhesion by NcadFc also induced an enhancement of *Igf2* expression (Fig. 3D) and activation of the *Igf2* P3 promoter (Fig. 3E) compared with cells treated with the Cos7 extract.

Taken together, these results demonstrate that manipulation of N-cadherin-dependent adhesion correlates with the phosphorylation of the key signalling protein, p38 MAP kinase and the expression of the pro-myogenic growth factor *Igf2*, leading to the hypothesis that N-cadherin signalling could be an important regulator of subsequent p38 activity and *Igf2* expression.

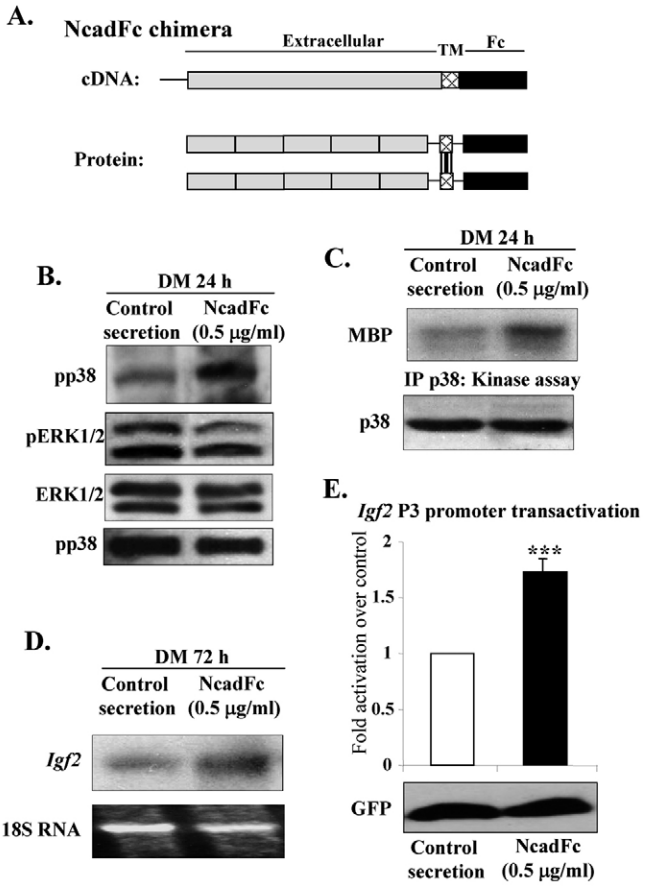

Fig. 3. Stimulation of cadherin with the NcadFc ligand activates p38 and induces *Igf2* expression. (A) Schematic of the N-cadherin/Fc fusion protein (termed NcadFc ligand). (B) Western blot detection of phospho-p38 (pp38), phospho ERK1/2 (pERK1/2), total p38 and total ERK1/2 in myoblasts treated with extract from control Cos7 cells or NcadFc derived from transfected Cos7 cells. (C) p38 MAP kinase activity of myoblasts treated as in B; total p38 levels were determined by western blotting. (D) Northern blot analysis of *Igf2* expression in myoblasts treated as in B; ethidium bromide staining of the 18S rRNA bands is shown as a loading control. (E) *Igf2* P3 promoter luciferase reporter activity in C2 cells treated as in B. *Igf2* promoter activity was expressed as a ratio of luciferase activity of the control secretion treated cells compared with NcadFc-treated cells; ***$P<0.001$ compared with levels in the control. Western blot analysis of the lysates shows the GFP expression levels.

RhoA activation reflects N-cadherin-mediated adhesion

RhoA has been suggested as a downstream target for N-cadherin in myoblasts (Charrasse et al., 2002). We therefore initially investigated RhoA activation during myogenesis; a biphasic response was observed in subconfluent cells (Fig. 4A, left panel), with an initial transient decrease in GTP-bound RhoA 12 hours after the initiation of differentiation, which switched to a modest activation at 24 hours. In confluent cells, RhoA activation occurred within 12 hours and to a greater extent than in subconfluent myoblasts (Fig. 4A, right panel), thus increasing the magnitude of RhoA activity as well as accelerating its kinetics of activation; however, in common with the subconfluent cells, RhoA activation was transient. RhoA has multiple downstream targets, of which it has been suggested that excessive Rho kinase (ROCK) activity is detrimental to myogenic differentiation (Nishiyama et al., 2004). We therefore examined expression of the late-myogenic marker, MHC, in the presence and absence of the established ROCK inhibitor, Y-27632. After 48 hours (when MHC is usually just detectable in our culture system) inhibition of

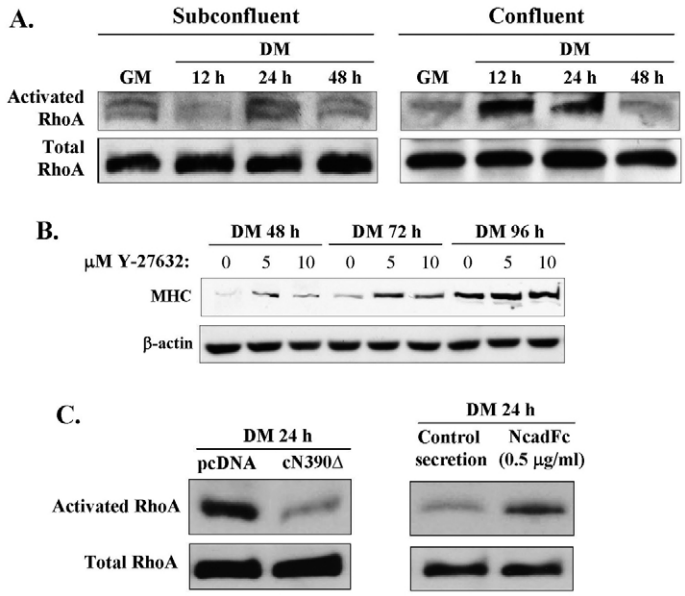

Fig. 4. RhoA activation during myoblast differentiation and in response to cadherin signalling. (A) Western blot determination of activated (GTP-bound) RhoA and total RhoA during myoblast differentiation. (B) Western blot analysis of MHC and β-actin in differentiating C2 myoblasts treated with the ROCK inhibitor Y-27632. (C) Western blot analysis of total and activated RhoA in lysates from C2 cells transfected with cN390Δ or vector alone (left panels) or with NcadFc and control secretion (right panels).

ROCK enhanced the abundance of MHC protein, and this acceleration was sustained to at least 72 hours (Fig. 4B). These findings therefore confirm an anti-myogenic role for ROCK.

The effect of N-cadherin signalling on RhoA activity in C2 myoblasts was determined using the dominant-negative and stimulatory forms of N-cadherin described above. Disruption of N-cadherin adhesion by transfection with cN390Δ caused a dramatic decrease in RhoA activation compared with the pcDNA empty vector (Fig. 4C, left panels). Conversely, N-cadherin ligand stimulated a significant increase in RhoA activity compared with appropriate controls (Fig. 4C, right panels). Thus, RhoA activation is regulated by N-cadherin-mediated adhesion in differentiating myoblasts.

RhoA activates p38 MAP kinase

To investigate whether RhoA has a role in p38 activation in myogenesis, we transfected myoblasts with constitutively active (L63 RhoA) or dominant-negative (T19N RhoA) RhoA mutants. The presence of both mutants in C2 cells was evidenced by a band shift in total RhoA, owing to their increased molecular weight because of Myc and HA tags (Fig. 5A) and by the presence of immunoreactive Myc and HA in western blots (Fig. 5B). Constitutively active L63 RhoA exhibited a high RhoA activity (in addition to that resulting from endogenous RhoA), whereas dominant-negative RhoA reduced endogenous RhoA activity (Fig. 5A, upper panel).

The phosphorylation status of p38 MAP kinase in C2 myoblasts was significantly enhanced after transfection with constitutively active L63 RhoA compared with pcDNA-transfected cells, whereas dominant-negative T19N RhoA caused a reduction in p38 phosphorylation (Fig. 5B); the mean results of all experiments are presented via semi-quantitative densitometry in the histogram, with L63 inducing a twofold increase and T19N RhoA a 50% decrease in pp38. In marked contrast, ERK1/2 phosphorylation was independent of RhoA status. The changes in p38 phosphorylation were supported by p38 kinase assays demonstrating significant increases in response to L63 RhoA and decreases due to T19 RhoA (Fig. 5C).

As SRF is a key positive downstream target of RhoA in myogenesis (Wei et al., 1998), SRF promoter activity was investigated in myoblasts with changed N-cadherin and RhoA signalling. N-cadherin fusion protein stimulated whereas dominant-negative N-cadherin inhibited SRF promoter-reporter activity (Fig. 5D). Similarly, both constitutively active RhoA and wild-type RhoA enhanced but dominant-negative RhoA decreased SRF promoter-reporter activity.

Constitutively active RhoA can rescue dominant-negative N-cadherin-mediated inhibition of p38 activation

Since both N-cadherin and RhoA signalling modulated p38 activation, we next investigated the hierarchy of this regulation. Co-transfection of myoblasts with cN390Δ and L63 RhoA restored the reduced p38 MAP kinase activity that occurred after transfection with cN390Δ alone, to levels above those for vector-only transfected cells (Fig. 6A,B). Thus, constitutively active RhoA is able to rescue the inhibitory effects of dominant-negative N-cadherin on p38 MAP kinase activity, supporting the notion that RhoA is downstream of N-cadherin and upstream of p38 in myoblast differentiation.

N-cadherin promotes *Igf2* expression via RhoA in myogenesis

As *Igf2* expression and N-cadherin signalling were correlated, we examined the role of RhoA in a putative pathway from formation of adherens junctions to *Igf2*. Constitutively active L63 RhoA induced a significant increase in *Igf2* expression at 72 hours DM, compared with pcDNA transfected control C2 myoblasts, whereas dominant-negative T19N RhoA caused a significant reduction in expression levels (Fig. 7A, upper panel); *Igf2* P3 promoter transactivation studies supported these findings (Fig. 7A, lower panel).

We investigated whether L63 RhoA could rescue the inhibitory effects of cN390Δ on *Igf2* expression. Constitutively active L63 RhoA rescued the actions of the dominant-negative N-cadherin on *Igf2* mRNA levels (Fig. 7B, upper panel) and these findings were corroborated by *Igf2* P3 promoter-reporter activity (Fig. 7B, lower panel). Wild-type RhoA increased both *Igf2* mRNA levels and P3 promoter activity; Y-27632 had no effect on either *Igf2* message levels or P3 promoter assays implying that they are independent of ROCK activation (data not shown). Overall, these findings suggest that N-cadherin signalling regulates *Igf2* expression via RhoA.

p38 MAP kinase mediates N-cadherin- and RhoA-dependent *Igf2* expression in myogenesis

We next investigated whether p38 had a role in the modulation of *Igf2* expression. MKK6EE, the constitutively active phosphomimetic mutant of the p38 upstream MAP kinase kinase, MKK6, was used to activate p38, and dominant-negative p38 MAP kinase was used to suppress p38 activity

(p38dn) in C2 myoblasts. Fig. 8A demonstrates that MKK6EE increased and p38dn decreased the abundance of the p38 target caveolin-3 and phospho-ATF2. *Igf2* expression was significantly enhanced in cells transfected with MKK6EE while expression was decreased in p38dn transfected cells (Fig. 8B). *Igf2* P3 promoter transactivation studies supported these findings, with MKK6EE causing over a threefold increase in P3 promoter activity whereas p38dn significantly reduced promoter activity (Fig. 8C).

We next investigated the possibility that constitutive activation of p38 MAP kinase, using MKK6EE, could rescue the inhibition of *Igf2* expression after transfection with dominant-negative N-cadherin cN390Δ or T19N RhoA. Co-transfection of MKK6EE with either cN390Δ or T19N RhoA rescued *Igf2* mRNA levels significantly above those for dominant-negative cN390Δ and T19N RhoA constructs alone (Fig. 8D). This rescue was confirmed with *Igf2* P3 promoter transactivation studies (Fig. 8E).

Overall therefore, we suggest that p38 MAP kinase lies downstream of the RhoA signal, which in turn, is downstream of N-cadherin; together, this pathway has an important role in determining *Igf2* expression in myoblasts.

p38 isoforms in myogenesis

We next investigated which p38 isoform was important in signalling from N-cadherin to *Igf2*. Unfortunately, no phosphospecific antibodies selective for p38γ are currently commercially available, but p38γ phosphorylation status has been inferred from crossreactivity using a p38α antibody (Kuma et al., 2005) that yields a band co-migrating with that for total p38γ in immunoprecipitates. Using this antibody, positive regulation of p38α and/or p38β phosphorylation by L63 RhoA and MKK6EE, and negative regulation by cN390Δ and T19N RhoA was observed (Fig. 9A). Small increases in p38γ phosphorylation were observed in response to L63 RhoA and MKK6EE; surprisingly however, p38γ phosphorylation was not decreased by cN390Δ and T19N RhoA (Fig. 9A). We therefore examined whether differential phosphorylation of p38α/β and p38γ occurred in response to specific upstream stimuli. Phosphorylation of p38α/β but not p38γ increased in response to treatment of myoblasts with N-cadherin ligand (Fig. 9B). As the level of p38γ phosphorylation seemed very low [probably

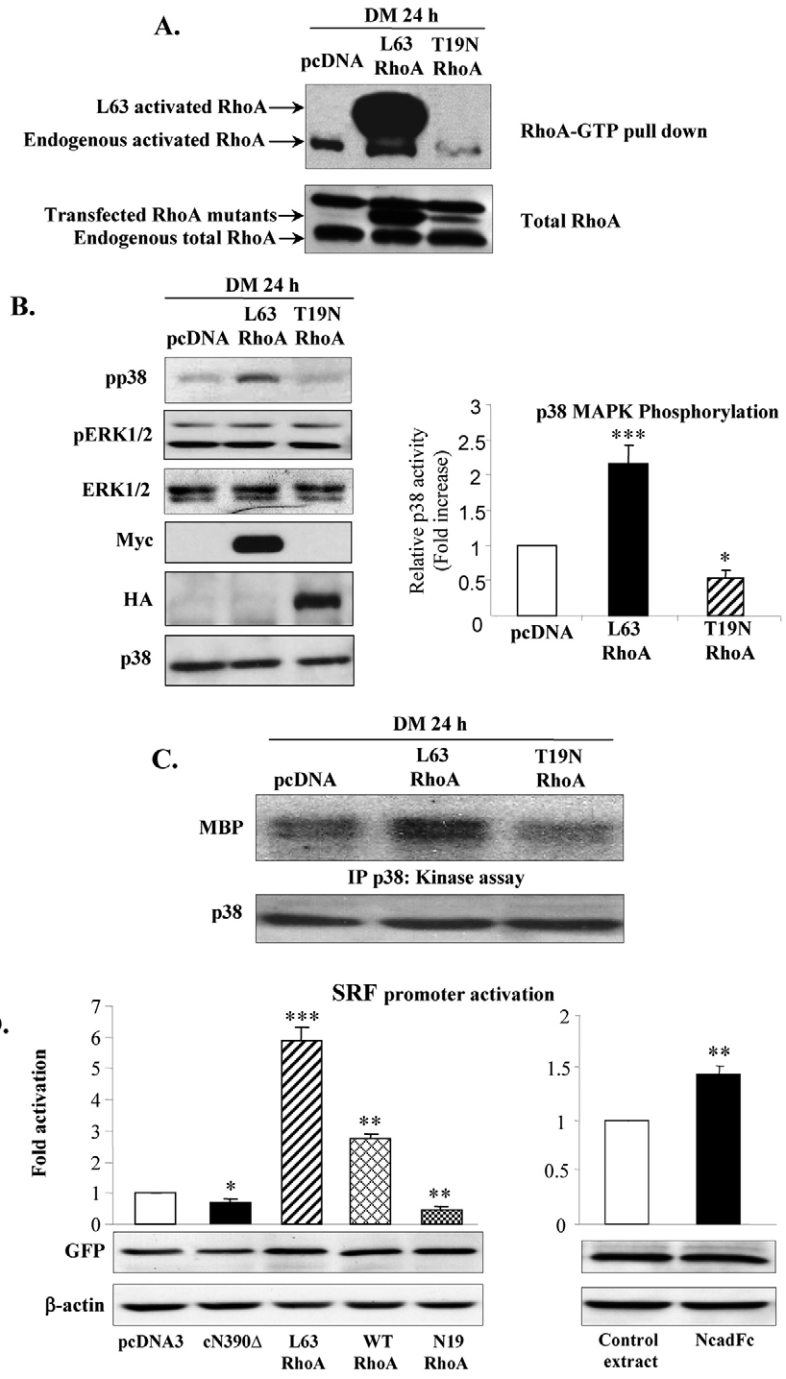

Fig. 5. RhoA activity regulates p38 MAPK phosphorylation and kinase activity. (A) Western blot determination of active and total RhoA in lysates from C2 cells transiently transfected with empty vector (pcDNA), constitutively active RhoA (L63 RhoA) and dominant-negative RhoA (T19N RhoA). The band shifts observed for L63 and T19N RhoA are due to their Myc and HA tags (shown in B); thus the lower molecular weight bands show endogenous levels of RhoA. (B) Western blot determination of phospho-p38 (pp38), phospho-ERK1/2 (pERK1/2), total ERK1/2, Myc and HA tags and total p38 in whole cell lysates of C2 cells transfected and sampled as described in A. Phospho- and total p38 were semi-quantified by scanning densitometry and p38 phosphorylation normalised for total p38 calculated (lower panel); data are expressed as fold increases of p38 phosphorylation above pcDNA control levels; *P<0.05, ***P<0.001 compared with levels in the controls. (C) p38 MAP kinase activity in myoblasts transfected with pcDNA, L63 RhoA or T19N RhoA; total p38 levels were determined by Western blotting. (D) SRF promoter reporter activity in lysates extracted from C2 cells transiently transfected with pcDNA3, cN390Δ, L63 RhoA, WT RhoA or N19 RhoA (left panel) or treated with control extract or NcadFc (right panel); Western blot analysis of lysates shows transfected GFP and β-actin levels.

because of a relatively low affinity of the antibody (Kuma et al., 2005)], we transfected C2 myoblasts with HA-tagged p38γ (Marinissen et al., 2001); despite clear expression of the construct, shown by the presence of the HA tag, no phosphorylation of p38γHA was observed in response to N-cadherin ligand (Fig. 9C). Since it has been reported that lysophosphatidic acid (LPA) can stimulate p38γ phosphorylation via activation of RhoA (Marinissen et al., 2001), we treated C2 myoblasts with LPA (as a positive control) and confirmed a modest increase in RhoA activation (Fig. 9D). C2 myoblasts were treated with the higher dose of LPA (5 μM); phosphorylation of transfected p38γHA was substantially increased, whereas that for p38α/β was not (Fig. 9E). We conclude that p38 isoforms might be differentially regulated by RhoA in a manner that depends upon the mechanism by which RhoA itself is activated.

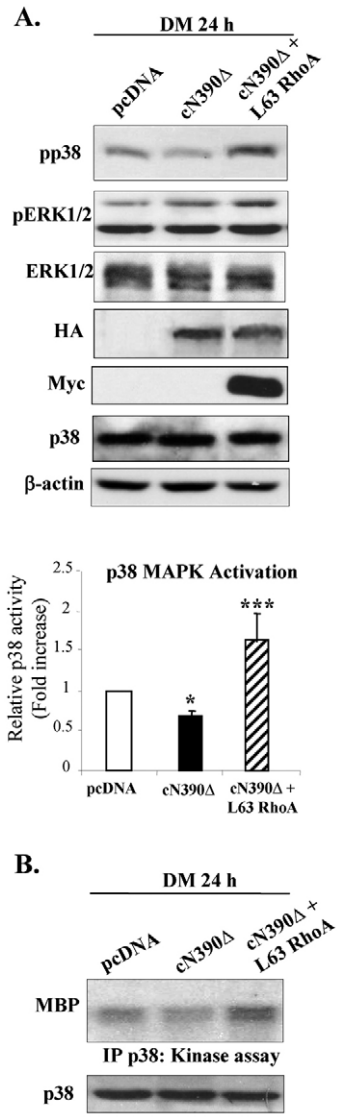

Fig. 6. Constitutively active RhoA rescues p38 activity in dominant-negative N-cadherin transfected myoblasts. (A) Western blot detection of phospho-p38 (pp38), phospho-ERK1/2 (pERK1/2), total ERK1/2, HA and Myc tags, total p38 and β-actin in lysates from C2 cells that were transfected with pcDNA, cN390Δ, or co-transfected with cN390Δ and L63 RhoA. Phospho- and total p38 were semi-quantified by scanning densitometry, and p38 phosphorylation normalised for total p38 (lower panel); data are expressed as fold increases of p38 phosphorylation above pcDNA control levels; **P*<0.05; ****P*<0.001 compared with levels in the control. (B) p38 MAP kinase activity (MBP phosphorylation) in lysates from C2 cells treated as in A; total p38 levels were determined by western blotting.

Discussion

The transmission and integration of signals deriving from cell-cell interactions and subsequent cell-specific gene expression are essential for successful embryogenesis. Here, we have used a myogenic paradigm to investigate such signals because myoblasts undergo many of the cellular events essential for tissue development: proliferation, cell-contact-mediated cell-cycle exit, formation of intercellular adherens junctions, extensive chromatin remodelling and upregulation of lineage-specific genes. We describe a novel pathway, linking signals

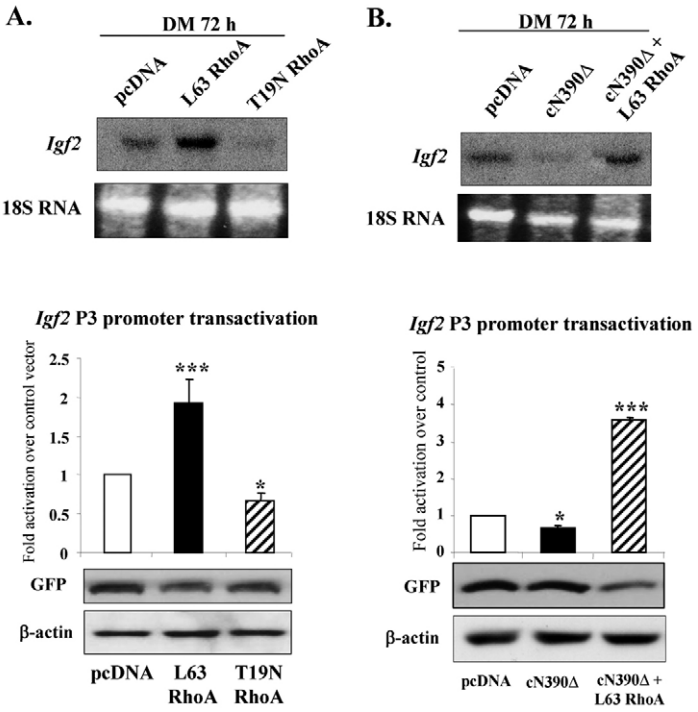

Fig. 7. RhoA lies downstream of N-cadherin in the regulation *Igf2* expression. (A) Northern blot analysis (upper panels) of *Igf2* mRNA levels in C2 cells transfected with either empty vector (pcDNA), L63 RhoA or T19N RhoA; total RNA is shown by ethidium bromide staining of 18S rRNA. *Igf2* P3 promoter luciferase reporter activity (lower panels), in cells co-transfected as above with L63 RhoA, T19N RhoA or control vector (pcDNA), and the *Igf2* P3 promoter-reporter construct. Fold activation levels are expressed as the ratio of luciferase activity of L63 and T19N RhoA transfected cells to that in cells transfected with pcDNA: values were normalised to the relative GFP expression levels per unit of protein; **P*<0.05, ****P*<0.001 (compared with an activation value of 1.0). Western immunoblotting to detect β-actin is shown as a loading control. (B) Northern blot analysis of *Igf2* mRNA levels (upper panels) and *Igf2* P3 promoter-reporter activity (lower panels) in C2 cells transfected with cN390Δ, pcDNA or co-transfected with cN390Δ and L63 RhoA, and examined exactly as for A.

arising from N-cadherin intercellular adherens junction formation with activation of downstream RhoA and specific p38 MAPK isoforms to regulate and activate *Igf2* transcription, a key autocrine pro-myogenic regulatory peptide. The significance of this is twofold. First, we defined a novel link between extracellular cell-cell contact with the synthesis of a major embryonic growth factor, providing an example of an essential signalling link and 'community effect' (Holt et al., 1994) necessary for coordinated development; such links between the extracellular environment and epigenetic modification to enhance gene expression are important (e.g. Kim et al., 2005). Second, we identified a mechanism, beyond immediate upstream kinases, that regulates p38 MAPK activity in myogenesis. Further, we characterised that, despite the high abundance of p38γ in muscle, p38α and/or β are important in the regulation of *Igf2* expression in differentiating myoblasts.

In this study, we used an N-cadherin ligand to recapitulate specific events associated with the formation of adherens junctions. This soluble and dimeric N-cadherin Fc fusion protein has been imaged using atomic force technology (Harrison et al., 2005) and substitutes for events associated with cell-contact-mediated cell-cycle arrest and subsequent myogenic differentiation (Gavard et al., 2004). It also distinguishes between primary versus secondary signalling associated with the formation of adherens junctions, thus avoiding those signals that first require, but are not initiated by, cell-cell contact (Yap and Kovacs, 2003). To complement these stimulatory studies, we also used a dominant-negative N-cadherin lacking the intracellular domain. We further observed a significant several-fold increase of endogenous N-cadherin expression as myogenesis proceeded, which was cell-density dependent. Recent findings suggest that cadherins form further multiprotein complexes with immunoglobulin superfamily members and activate pro-myogenic signals (reviewed by Krauss et al., 2005). Taken together, these findings suggest that cadherins act in a potentially complex manner both upstream and downstream of myogenic regulators, providing a feed-forward mechanism often used in myogenesis (e.g. Penn et al., 2004).

The downstream response to cadherin signalling is cell-type and/or cadherin specific. For example, increasing cell density decreased RhoA activity in MDCK and HEK293 cells (Noren et al., 2001), and it has been proposed that cadherin

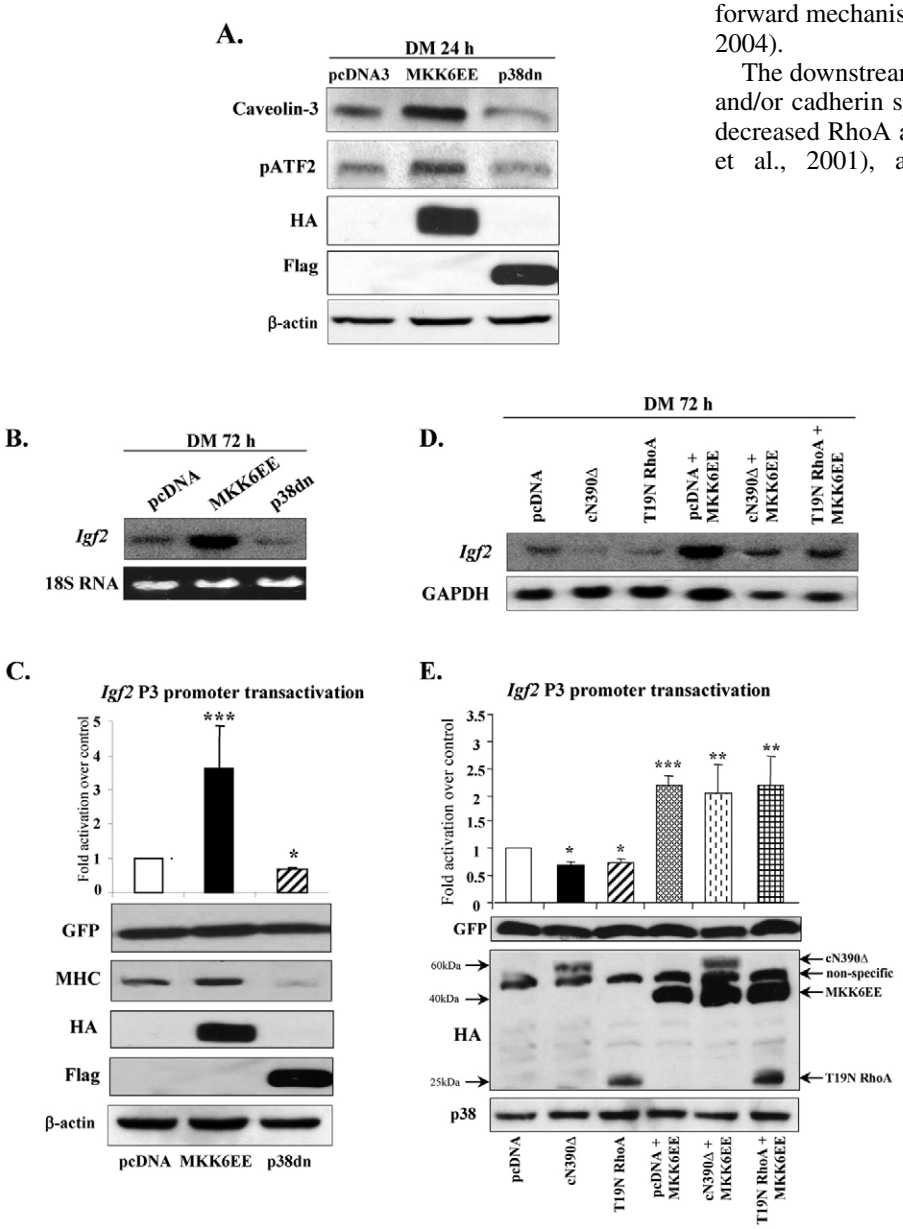

Fig. 8. p38 MAP kinase lies downstream of RhoA in the regulation of *Igf2* expression. (A) Western blot detection of caveolin-3, phospho-ATF2, HA tag (MKK6EE expression), Flag tag (p38dn expression) and β-actin in lysates from C2 cells that were transfected with control vector (pcDNA), constitutively active upstream activator of p38 MAPK, MKK6 (MKK6EE) and p38 dominant-negative (p38dn) constructs. (B) Northern blot analysis of *Igf2* mRNA levels in C2 cells transiently transfected as in A; ethidium bromide staining of 18S rRNA is shown as a loading control. (C) *Igf2* P3 promoter transactivation in C2 cells treated as in A but co-transfected with *Igf2* P3 promoter and GFP. The lower panels additionally show western blot detection of MHC, HA tag (MKK6EE expression), flag tag (p38dn expression) and β-actin. **P*<0.05, ****P*<0.001 (compared with an activation value of 1.0). (D) Northern blot analysis of *Igf2* mRNA levels in C2 cells transiently transfected with control vector (pcDNA), cN390Δ, T19N RhoA alone or co-transfected with MKK6EE; GAPDH mRNA levels are shown as a loading control. (E) *Igf2* P3 promoter-reporter activity in C2 cells treated as in C but co-transfected with *Igf2* P3 promoter and GFP. Western immunoblot determination (lower panel) of HA tag (detects cN390Δ, T19N RhoA and MKK6EE expression), and total p38 (loading control). There is non-specific binding at ~50 kDa in the HA immunoblot which is present in all lanes. **P*<0.05; ***P*<0.01; ****P*<0.001 compared with an activation value of 1.0.

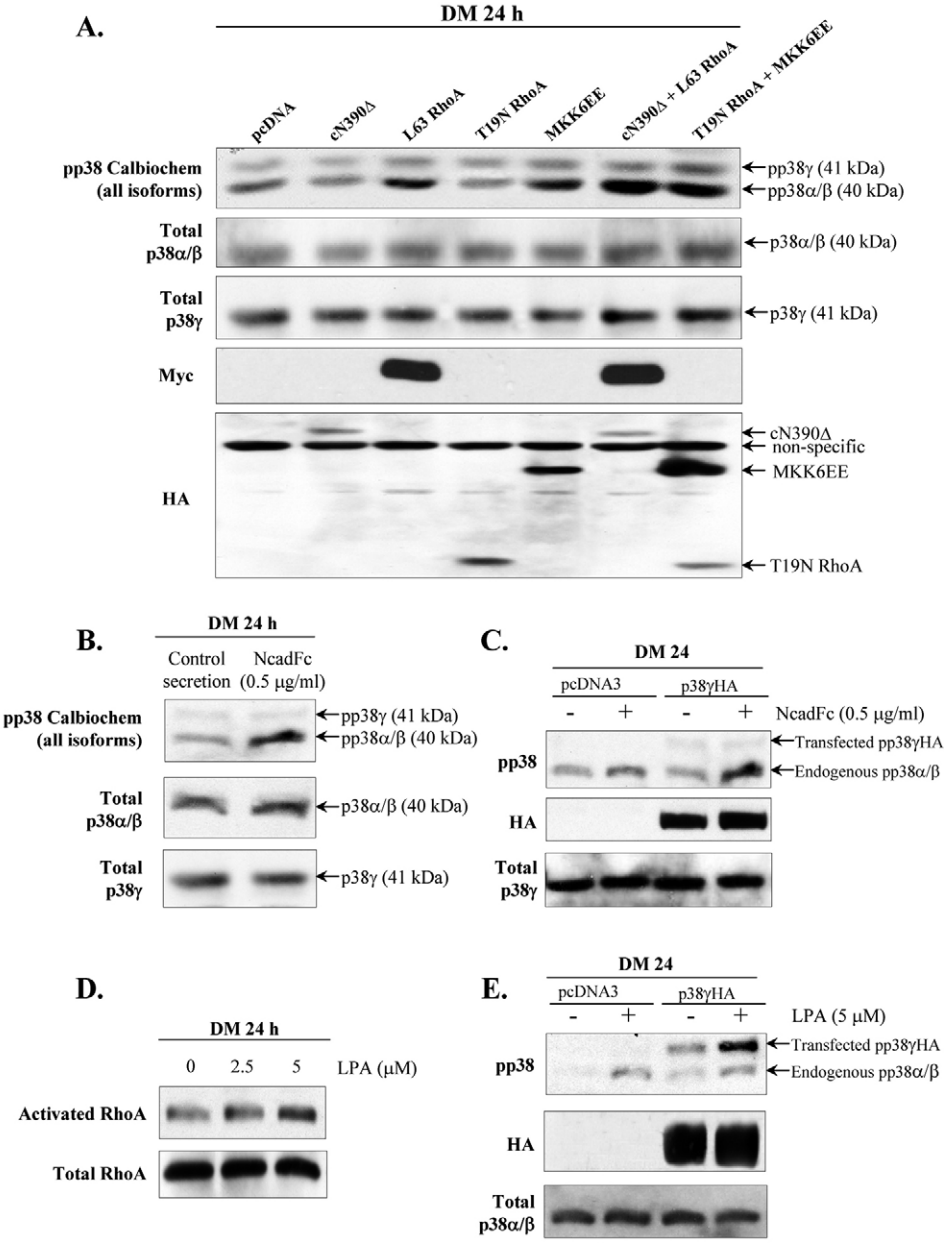

Fig. 9. Differential phosphorylation of p38 isoforms by N-cadherin, LPA and activated RhoA. (A) Western blot analysis of pp38 using antibodies that recognise pp38α/β and pp38γ, total p38α/β and total p38γ in C2 cells transiently transfected with pcDNA, cN390Δ, L63 RhoA, T19N RhoA, MKK6EE or combinations of these. (B) Western blot analysis of pp38 using antibodies that recognise pp38α/β and pp38γ, total p38α/β and total p38γ in C2 cells treated with either control extract or NcadFc ligand. (C) As B, except that C2 cells were transfected with pcDNA or p38γHA. (D) Western blot of total and activated RhoA in response to LPA treatment. (E) Western blot analysis of pp38 using antibodies that recognise pp38α/β, total p38α/β and HA in C2 cells transfected with pcDNA or p38γHA and treated with LPA.

engagement decreased RhoA signalling and the formation of focal adhesions in epithelial cells. We observed increased RhoA activity in response to N-cadherin ligand and decreased RhoA activity in myoblasts transfected with dominant-negative N-cadherin, in agreement with conclusions derived from earlier observations (Charrasse et al., 2002). However, conflicting opinions exist concerning the positive and/or negative effects of RhoA in myogenesis. Even though it is established that the activities of Rac and Cdc42 decrease during myoblast differentiation (Charasse et al., 2002; Heller et al., 2001), surprisingly few studies have attempted to assess changes in activated RhoA, its importance in myogenesis instead being inferred following transfection of cells with RhoA mutants. A rapid decrease in RhoA activity was observed in C2C12 myoblasts after induction of differentiation that declined further after 2 days (Nishiyama et al., 2004). We demonstrate a transient but substantial and cell-density-dependent increase in RhoA activity 12-24 hours after initiation of differentiation – a period not investigated by Nishiyama et al. (Nishiyama et al., 2004). Charrasse et al. (Charrasse et al., 2006) suggest that RhoA is only upregulated after the initiation of myoblast fusion, which is not necessarily compatible with the observation that RhoA is required for upregulation of specific pro-myogenic genes (Carnac et al., 1998; Takano et al., 1998; Wei et al., 1998). Activation of RhoA, by inhibition of p190RhoGAP, enhances myogenesis and directs adipogenesis or myogenesis cell fate decisions towards the myogenic lineage (Sordella et al., 2003). In marked contrast, several studies report the inhibition of myoblast differentiation by RhoA (Beqaj et al., 2003; Meriane et al., 2000; Nishiyama et al., 2004). Taken together, these conflicting observations illustrate the complex nature of RhoA action.

The serine/threonine kinase RhoA target ROCK has a principal function in mediating RhoA action on cell morphology via the actin cytoskeleton and in cell movement (Jaffe and Hall, 2005); specifically, ROCK activates contractility via phosphorylation of myosin light chain (reviewed by Riento and Ridley, 2003). Constitutively active ROCK inhibited myogenesis by blocking myoblast fusion and subsequent upregulation of MHC; blocking ROCK activity restored fusion (Nishiyama et al., 2004). In our initial studies, wild-type or constitutively active RhoA did not accelerate myogenesis as assessed by MHC protein levels; therefore we used the established ROCK inhibitor Y-27632 at doses low enough to specifically inhibit ROCK (Ishizaki et al., 2000). This induced increased MHC expression in both the presence

and absence of transfected RhoA, consistent with accelerated myogenesis. However, it did not affect p38 MAPK activation and SRE or *Igf2* promoter-reporter activities, importantly indicating that activation of p38 and subsequent upregulation of *Igf2* expression are not regulated by ROCK, and also suggesting that removal of negative cytoskeletal constraints induced by ROCK facilitated myogenesis.

RhoA has a pivotal role in the upregulation of early immediate genes, and when RhoA-mediated upregulation of Fos and Jun are considered, parallel pathways involving MAPKs are revealed: (1) RhoA upregulates Fos via MAPK phosphorylation of TCF (Janknecht and Hunter, 1997); (2) p38γ is activated by RhoA to upregulate Jun expression in 3T3 fibroblasts (Marinissen et al., 2001) and (3) RhoA also upregulates Jun via ROCK and JNK (Marinissen et al., 2005). Thus, MAPKs appear to be an intermediate in RhoA signalling to regulate gene expression. Here, we identify a specific pathway in myoblasts, in which RhoA signals to p38α or p38β and activates autocrine *Igf2* expression.

As p38γ is more abundant in skeletal muscle than in other tissues, we initially predicted that this isoform would be key in mediating RhoA activity; to our surprise, however, p38α/β and not p38γ were activated in myoblasts in which RhoA activity was stimulated by cell-cell contact or N-cadherin signalling. We did however, stimulate preferential p38γ phosphorylation by LPA, recapitulating the observations of Marinissen et al. (Marinissen et al., 2001) and, as further positive controls, constitutively active RhoA and MKK6 were able to phosphorylate all p38 isoforms investigated. Thus, we demonstrate that RhoA GTPase signalling is context dependent and suggest that the mechanism by which RhoA is activated dictates the isoform of p38 that is subsequently phosphorylated. Our findings also reveal novel myoblast-specific regulation during myogenesis; for example, in NIH 3T3 cells, constitutively active RhoA activated p38γ but had minimal effects on p38α phosphorylation (Marinissen et al., 2001), contrasting with the RhoA-activated p38 targets in myoblasts. Furthermore, our demonstration of the involvement of p38 as an N-cadherin-activated RhoA target in myoblasts markedly contrasts with that of Charrasse et al. (Charrasse et al., 2002), who did not observe cell-contact activation of p38 during myogenesis, instead suggesting that Rac1 and Cdc42 maintained 'sufficient' p38 activity for myogenesis to proceed; we have observed increased p38 phosphorylation as myoblasts become confluent, even in high-serum growth medium (data not shown).

The necessity for a functional p38 MAPK pathway in myoblast differentiation has been established for a number of years (e.g. Cuenda and Cohen, 1999; Zetser et al., 1999). In addition to its role in the phosphorylation of the myogenic transcription factor MEF2 (Zhao et al., 1999), p38 also phosphorylates E47 protein, promoting the formation of MyoD-E protein complexes that are essential for myogenic progression (Lluis et al., 2005). p38 initiates chromatin remodelling by phosphorylating a subunit of the SW1-SNF complex, and targeting the complex to myogenic loci (Simone et al., 2004). Furthermore, p38 facilitates myogenic signalling, e.g. via transactivation of Akt2 (Gonzalez et al., 2004) and has a key role in temporal regulation of myogenesis (Penn et al., 2004). Thus, by multiple mechanisms, p38 transmits external stimuli to directly increase the transcriptional efficiency of myogenic genes.

Even though primary stimuli responsible for activation of other key myogenic signalling pathways e.g. PI3-K/Akt, are characterized, those responsible for p38 MAPK activation have not been identified to date. Here, we demonstrate one possible mechanism that is primarily regulated by formation of adherens junctions. We thus expand the role of p38 MAPKs to include mediation of cell-cell contact and RhoA activity to target upregulation of the myogenic growth factor IGF-II, in a p38 isoform-specific manner. Direct interactions of RhoA and MAPKs have not been demonstrated to date; however, alternative mechanisms have been suggested, e.g. direct via interaction with upstream MAP kinase kinases (Gallagher et al., 2004), or via specific scaffold proteins (Morrison and Davis, 2003). It is possible that p38α/β are activated by an intermediate RhoA target in myoblasts. The Rho kinase, ROCK, is essential for JNK activation (Marinissen et al., 2005) but, as we demonstrated that the ROCK inhibitor Y-27632 did not inhibit p38 phosphorylation in differentiating myoblasts, ROCK is unlikely to be a candidate. The closely related serine/threonine kinases, PKN (PRK1) and PRK2 (Amano et al., 1996; Deaton et al., 2005) are ubiquitously expressed RhoA effectors; PKN mediates RhoA activation of p38γ in NIH 3T3 cells (Marinissen et al., 2001) and can interact with MKK, MLTK (Takahashi et al., 2003) and therefore it cannot be ruled out at present as a putative RhoA intermediate in myoblasts. RhoA also activates, via complex mechanisms that may include PKN, the transcription factor SRF (Arai et al., 2002; Hill et al., 1995; Miralles et al., 2003). SRF is a member of an ancient DNA-binding protein family, which contains a highly conserved DNA binding/dimerisation domain (MADS box), as do the important pro-myogenic MEF2 transcription factors; it binds to and is required for expression of myogenic determination factors (Soulez et al., 1996). It is interesting, therefore, that we determined regulation of SRF promoter-reporter activity by p38.

The upregulation of *Igf2* by p38-dependent events has not been described before and the mechanism(s) by which this occurs remain to be defined. Examination of the mouse *Igf2* P3 promoter (Murrell et al., 2001) reveals the presence of multiple putative Sp1 sites and functional relevance of Sp1 for the *Igf2* P3 promoter has been demonstrated (Evans et al., 1988). Recently, association of p38α/β with Sp1 and subsequent Sp1 phosphorylation and increased transactivation activity were demonstrated in connective tissue cells (D'Addario et al., 2002). Further transactivation of *Igf2* P3 by the Ap-1 complex has also been reported (Caricasole and Ward, 1995). It is unlikely that SRF is directly involved in the regulation of *Igf2* transcription as the P3 promoter has no putative SRE. Further, even though SRF-null mice develop muscle myopathy, this occurs after the formation of muscle fibers and is due to defects in subsequent hypertrophy (Li et al., 2005).

In summary, we have identified a novel pathway that provides a mechanism to regulate p38 MAPK upregulation during myoblast differentiation, characterising a link between N-cadherin-mediated cell-cell contact to the synthesis of a key myogenic growth factor, IGF-II. A crucial effector of this pathway is RhoA, and we show that activation of p38 isoforms by RhoA is context-dependent in myogenesis, with p38α/β being the cell-cell-contact-regulated isoforms. The function of p38γ, which is expressed more abundantly in muscle than any

other tissue (Lechner et al., 1996), thus remains elusive. Given that it has PDZ-binding motifs it is possible that it has a scaffold rather than a kinase role, as has recently been suggested in K-Ras-mediated transformation (Tang et al., 2005).

Materials and Methods

Reagents, plasmids and antibodies

Reagents were purchased as follows: myelin basic protein (MBP), Sigma (Poole, Dorset, UK); cell culture reagents, Invitrogen (Paisley, Scotland); and [γ-^{32}P]ATP and [γ-^{32}P]CTP, Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK).

Constructs and expression vectors were generous gifts or acquired as follows: cN390Δ dominant-negative cadherin (Y. Takai, Osaka University, Japan); N-cadherin-Fc (Harrison et al., 2005); pBabe and BOSC23 viral helper cell line (S. Cook, Babraham Institute, Cambridge, UK); pGex GST-RBD (Rho-binding domain of Rhotekin, K. Burridge, University of North Carolina, USA); constitutively active L63 RhoA and wild-type RhoA (S. Vermeren, Babraham Institute); dominant-negative T19N RhoA (G. Bokoch, The Scripps Research Institute, USA); dominant-negative p38 (p38dn, R. Davis, University of Massachusetts Medical Center, USA); constitutively active MKK6 (MKK6EE, Z. Wu, Hong Kong University of Science and Technology, China); *Igf2* P3 reporter (Murrell et al., 2001); green fluorescent protein expression vector (pEGFP, Clontech Laboratories, Oxford, UK); pcDNA3 vector (Invitrogen).

Sources of antibodies were as follows: anti-MHC (MF20, University of Iowa, IA); anti-phospho-p38 (raised against Thr180/Tyr182 and surrounding residues of p38α; Promega); anti-phospho-p38 (raised against Thr180/Tyr182 and surrounding residues of p38α and stated to additionally recognise p38β and γ; Calbiochem, Nottingham, UK); anti-total p38, anti-Myc, and anti-phospho-ATF2 (Cell Signalling Technologies, Hitchin, UK); anti-total p38γ (monoclonal antibody, R&D Systems, Abingdon, UK); anti-N-cadherin and anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho ERK1/2 and anti-total ERK1/2 (BioSource International, Nivelles, Belgium); anti-haemagglutinin (anti-HA) and anti-Myc (Babraham Technix Monoclonal Antibody Service, Cambridge, UK); anti-GFP (Molecular Probes, Cambridge, UK); anti-Flag (Eastman Kodak Company, New Haven, Connecticut, USA); anti-caveolin-3 from BD Bioscience/Clontech (Cowley, UK); anti-β-actin (Abcam, Cambridge, UK); horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G and goat anti-mouse IgG both from Jackson Immunoresearch (Luton, UK).

Cloning

The cN390Δ coding region (in pBluescript SK) was PCR amplified using *PfuTurbo*® DNA polymerase (Stratagene) including insertion of a C-terminal HA tag (upstream primer: 5′-CCGGAATTCCGGCTCCTCGCCTCCATGTGCCGGA-TAG-3′; downstream primer: 5′-CCGGAATTCCGGTCAGGCATAATCGGGT-ACATCGTAAGGGTAGTCATCACCTCCACCGTAC-3′). The cN390ΔHA cDNA (termed cN390Δ) was cloned into pcDNA and pBabe expression vectors.

Production and quantification of NcadFc

The extracellular region of chick N-cadherin up to but not including the transmembrane domain was fused to human IgG1 Fc and the resultant N-cadherin-Fc fusion protein (termed NcadFc) expressed, extracted and quantified from Cos7 cells as described in Corps et al. (Corps et al., 2001). The NcadFc is secreted by the Cos7 cells and was quantified by ELISA. Briefly, 96-well plates were coated with 100 μl of 5 μg/ml goat anti-human Fc (Sigma) in PBS shaking overnight at 4°C. Wells were washed using PBS/0.05% Tween-20 before and after blocking with 200 μl of 10% FBS in PBS at room temperature for 1 hours. A 1:10 dilution of the N-cadherin-Fc fusion secretion in a volume of 500 μl was prepared in the diluents (PBS/0.05% Tween-20 and 1 mg/ml BSA). NcadFc standards of known concentration were prepared in the same way. 200 μl of the relevant diluted supernatant or standard was added to wells and doubling dilutions were made. After incubation with shaking for 1 hour at room temperature, wells were washed before addition of 100 μl biotinylated goat anti-human Fc at 1:500 dilution in PBS/0.05% Tween-20 +1 mg/ml BSA and incubation for 1 hour at room temperature. After washing, 100 μl avidin-HRP (Sigma) at 1:2000 dilution in PBS/0.05% Tween-20 +1 mg/ml BSA was added to wells and incubated for 1 hour at room temperature. Wells were washed and assayed for peroxide activity: the colour was developed with 100 μl per well of fresh 0.055% (w/v) 2,2′-azino-bis (3-ethyl), benzthiazoline-6-sulphonic acid (ABTS) and 0.0018% (v/v) hydrogen peroxide in citrate/phosphate buffer (pH 4.3). The colour was allowed to develop for 10 minutes at room temperature before measuring the absorbance at 405 nm. A standard curve was made using values from the standard supernatant and from this the concentration of the NcadFc fusion protein was estimated.

Initially, we confirmed that the NcadFc bound to N-cadherin produced by C2 cells using a homophilic adhesion assay. Briefly, 100 μl of 5 μg/ml goat anti-human Fc (Sigma), which binds to chick NcadFc (Harrison et al., 2005), was used to coat 96-well ELISA plates, and wells washed with PBS and 1 mg/ml BSA in PBS, followed by blocking with 10% FBS in PBS. Serial dilutions of NcadFc (in Hanks buffer containing 1 mg/ml BSA and starting at 1 μg/ml) were added to the wells, with a zero concentration control, and a mutant NcadFc which is unable to form homophilic adhesions but still binds to the anti-human Fc. Confluent C2 myoblasts were dissociated into a single cell suspension and added to the wells for 30 minutes at 37°C to allow adhesions to form between the bound NcadFc and N-cadherins on the surface of C2 myoblasts. Unbound cells were washed away. The acid phosphatase assay (addition of 50 μl of 50 mM sodium acetate pH 5, 0.5% Triton X-100, 40 μM FDP (fluorescein diphosphate) with subsequent fluorescence measured via excitation at 495 nm and emission at 530 nm) was used to quantify bound cells. A standard curve was constructed using 20-100% added cell suspension by brief centrifugation.

Cell culture

C2 myoblasts, derived from mouse muscle satellite cells, were seeded either at subconfluency (1×10^5 cells in 60-mm-diameter plates) or at confluency (4×10^5 cells/plate) on to 2% gelatin-coated plates and initially cultured in growth medium [GM: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 10% newborn calf serum]. Differentiation was induced 48 hours after seeding by replacing GM with differentiation medium (DM: DMEM with 2% horse serum). When indicated, exogenous IGF-II, EGTA, NcadFc (or equivalent control materials) were added at the doses indicated in figure legends to the medium immediately after cells were moved to DM; media (and additives) were changed every 24 hours. All reagents were added to DM. In initial studies, NcadFc was administered to C2 cells by coating the gelatin plates with goat anti-human Fc (Sigma) and NcadFc; however, as similar responses were observed when NcadFc was added directly to the DM, this form of administration was used for all studies described here.

Transfections

For stable transfections, BOSC23 viral helper cells were grown in DMEM supplemented with 10% fetal bovine serum and seeded on to 60-mm plates. At 50% confluency, cells were calcium phosphate transfected (Danos and Mulligan, 1998) with pBabe or pBabe-cN390ΔHA vectors. Viral titres were collected for 48 hours after transfection and used to infect C2 myoblasts in suspension. C2 myoblasts were infected with viral titres and seeded at 1×10^5 cells on 60-mm-diameter plates. Cells were cultured in GM and selected for the presence of pBabe vector with 2 μg/ml puromycin for 2 weeks.

For transient transfections, 1×10^5 C2 myoblasts were seeded in 60-mm gelatin-coated plates in GM; after 24 hours they were transfected with the indicated construct or pcDNA3 using Effectene™ (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions and were cultured in GM for a further 24 hours before transfer to DM. Cells were co-transfected with a GFP-expression plasmid (pEGFP) to determine transfection efficiency between samples (~35%) (Gonzalez et al., 2004).

Whole cell extract preparation

C2 cells were washed three times with phosphate buffered saline (PBS) and harvested in 250 μl of lysis buffer (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 1 mM EGTA pH 8, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$) containing protease and phosphatase inhibitors (10 mM NaF, 1 mM PMSF, 1 mM Na metavanadate, 5 μg/ml aprotinin, 10 μg/ml leupeptin). Cellular debris was removed by centrifugation at 13,000 ***g*** for 1 minute. Cell extracts were frozen in liquid nitrogen and stored at –80°C until use. Total protein in the extracts was determined by the Bradford assay (Bio-Rad, Hemel Hempstead, UK).

Cell morphology

C2 cells were analysed morphologically by Gill's haematoxylin and eosin (H and E) staining as described previously. H and E stains nuclei deep purple and protein pink, giving a good indication of overall myogenic progression (Langley et al., 2002). Myoblasts were washed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature.

Northern blotting

Total RNA from C2 cells was obtained by using RNeasy Mini protocol according to the manufacturer's instructions (Qiagen) and northern blotting was performed as described (Gonzalez et al., 2004), using cDNA probes for *Igf2* or GAPDH that were ^{32}P-labeled by random priming (High Prime protocol; Roche, Lewes, UK).

Western blot analysis

50 μg of protein from cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Watford, UK) by electroblotting. The membranes were blocked with 0.2% I-block™ (Applied Biosystems, Warrington, UK); 0.1% Tween20 in PBS overnight at 4°C and probed with corresponding primary and secondary antibodies. Blots were washed in PBS plus 0.1% Tween20 and antigen-antibody complexes were visualised using enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Where re-probing of the blots was performed,

membranes were stripped with 5% acetic acid (pH 2.8) for 5 minutes at room temperature before being washed and blocked as described. Scanned autoradiographs were semi-quantified using AIDA 2D densitometry software and normalised as a function of the expression/phosphorylation of the various proteins.

Luciferase promoter reporter assays

C2 cells were co-transfected with *Igf2* P3 luc and pEGFP (for determination of transfection efficiency) exactly as described previously (Gonzalez et al., 2004; Wu et al., 2000), and harvested to measure luciferase activity using a luciferase assay kit (Promega, UK). Where appropriate, the luciferase reporter was co-transfected with cN390Δ (in pcDNA), L63 RhoA, T19N RhoA, wild-type RhoA, p38dn, MKK6EE or control empty vector (pcDNA3).

p38 immune complex kinase assay

p38 kinase assays were performed as described (Gonzalez et al., 2004). Briefly, 200 μg whole cell extracts were incubated with 5 μl total p38 antibody for 12 hours at 4°C with continuous rotation. Extracts were then incubated with 50 μl protein-A-Sepharose bead suspension (Amersham Pharmacia Biotech, UK) for 1 hour. The immune complexes were washed three times with lysis buffer and once with kinase reaction buffer (30 mM Tris-HCl, pH 8, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 25 mM β-glycerol phosphate and 0.1 mM sodium vanadate). Beads were resuspended with 30 μl kinase reaction buffer containing 7 μg MBP, 10 μM/3 μCi [γ-^{32}P]ATP and incubated at 30°C for 30 minutes in a shaking incubator. Reactions were stopped by addition of 10 μl of 4× Laemmli SDS sample buffer. Samples were subjected to 12.5% SDS-PAGE and products were visualised by autoradiography. Results were quantified using a phosphorimager. To ensure equal protein loading, gels were stained with Coomassie Blue and lysates subjected to western blotting analysis.

Preparation of GST-RBD beads and RhoA activity assay

GST-RBD (i.e. GST-Rho-binding domain of Rhotekin) beads were prepared by the established method of Ren and Schwartz (Ren and Schwartz, 2000). C2 cells were lysed in 50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF and cocktail protease inhibitors. 200 μg of each sample were incubated with 25 μg GST-RBD beads for 1 hour at 4°C. Beads were then washed four times in Tris buffer (50 mM Tris-HCl pH 7.6, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM PMSF and cocktail inhibitors) before addition of SDS sample buffer. RhoA fractions were analysed by western blotting with RhoA antibody.

Statistical analyses

Statistical differences between treatments were analysed using analysis of variance and/or Student's *t*-test, as indicated. Values are presented as means ± s.e.m. All experiments were performed independently at least three times.

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