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RESEARCH ARTICLE

Cadherin-11 regulates both mesenchymal stem cell differentiation into smooth muscle cells and the development of contractile function *in vivo*

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ABSTRACT

Although soluble factors, such as transforming growth factor β1 (TGF-β1), induce mesenchymal stem cell (MSC) differentiation towards the smooth muscle cell (SMC) lineage, the role of adherens junctions in this process is not well understood. In this study, we found that cadherin-11 but not cadherin-2 was necessary for MSC differentiation into SMCs. Cadherin-11 regulated the expression of TGF-β1 and affected SMC differentiation through a pathway that was dependent on TGF-β receptor II (TGFβRII) but independent of SMAD2 or SMAD3. In addition, cadherin-11 activated the expression of serum response factor (SRF) and SMC proteins through the Rho-associated protein kinase (ROCK) pathway. Engagement of cadherin-11 increased its own expression through SRF, indicative of the presence of an autoregulatory feedback loop that committed MSCs to the SMC fate. Notably, SMC-containing tissues (such as aorta and bladder) from cadherin-11-null ($Cdh11^{-/-}$) mice showed significantly reduced levels of SMC proteins and exhibited diminished contractility compared with controls. This is the first report implicating cadherin-11 in SMC differentiation and contractile function *in vitro* as well as *in vivo*.

KEY WORDS: Mesenchymal stem cells, Cell–cell adhesion, Adherens junctions, Smooth muscle cells, Differentiation, Vascular contractility

INTRODUCTION

Cell–cell adhesion is mediated through the interaction of cell adhesion molecules (CAMs), including cadherins, integrins, selectins and immunoglobulin-like CAMs. These CAMs regulate multiple aspects of cellular behaviour, including proliferation, differentiation, apoptosis and embryonic stem cell self-renewal and differentiation (Cavallaro and Dejana, 2011; Li et al., 2012; Niessen et al., 2011). One class of CAMs includes cadherins – transmembrane glycoproteins that mediate Ca^{2+}-dependent homophilic interactions between cells. These intercellular connections (or adherens junctions) are composed of a supramolecular complex, which, among other molecules, contains various catenins (including β-catenin, α-catenin and p120-catenin), linking it to the actin cytoskeleton (Stepniak et al., 2009). Different types of cadherins are expressed in different types of cells. For example, E-cadherin is mostly found in epithelial cells, whereas cadherin-2 (N-cadherin) is highly expressed in neurons and in mesenchymal cells. Cadherin-11 – a type II cadherin – was first identified in mouse osteoblasts, but is also expressed in mesenchymal cells. Interestingly, cadherin-11 and cadherin-2 are considered as indicators of the epithelial to mesenchymal transition (EMT) (Zeisberg and Neilson, 2009). During the process of EMT, the expression of E-cadherin is repressed, whereas cadherin-11 and cadherin-2 are upregulated, indicating a possible role of cadherins in diverse biological processes such as fibrosis or carcinogenesis (Tomita et al., 2000).

More recently, cadherins have been found to regulate stem cell maintenance and differentiation. E-cadherin is necessary for maintaining the pluripotency of embryonic stem cells, as well as for cellular reprogramming. During the latter, ectopic expression of E-cadherin could substitute for one of the pluripotency factors, namely Oct4 (Redmer et al., 2011). Interestingly, cadherin-2 was found to be necessary for the long-term engraftment of hematopoietic stem cells and the establishment of hematopoiesis after bone marrow transplantation (Hosokawa et al., 2010). Cadherin-11 and cadherin-2 also play an important role in the transition of fibroblasts to myofibroblasts during the granulation tissue phase of wound healing. In this capacity, they transmit the force of intercellular adhesion to the network of actin stress fibers, increasing contraction and facilitating wound closure (Hinz and Gabbiani, 2003; Hinz et al., 2004). In addition, mesenchymal cadherins play a crucial role during MSC differentiation, as both cadherin-2 and especially cadherin-11 are required for osteogenesis but prevent adipogenesis. Conversely, lack of cadherin-11 induced the expression of key adipogenic regulators [e.g. peroxisome proliferator-activated receptor γ (PPARγ)] and adipogenic differentiation (Di Benedetto et al., 2010; Kawaguchi et al., 2001a; Kawaguchi et al., 2001b; Shin et al., 2000). Recently, cadherin-11 was implicated in pulmonary fibrosis (Schneider et al., 2012) and the inflammatory process of rheumatoid arthritis (Lee et al., 2007). However, the potential role of cadherin-11 in smooth muscle cell (SMC) differentiation has not been studied, and little is known about the mechanism through which adherens junctions control stem cell lineage commitment.

In the present study, we investigated the role of adherens junction formation in cell–cell-adhesion-induced MSC differentiation into SMCs. We found that cell–cell adhesion induced the differentiation of MSCs into contractile SMCs and that this process was mediated through cadherin-11. Cadherin-11 was a regulator of myogenesis, ultimately regulating the

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expression of serum response factor (SRF). Cadherin-11 was also necessary for contractile function in organs of diverse origin, such as blood vessels and bladder. Our results shed light on the mechanisms of cell–cell-adhesion-induced MSC differentiation and have potential implications for the development of strategies to control stem cell fate.

RESULTS

High cell density promoted MSC differentiation into SMCs

We examined whether cell density affected the differentiation of MSCs towards the SMC lineage. To test this hypothesis, we employed MSCs derived from human bone marrow (BM-MSCs) or human hair follicle (HF-MSCs). The latter were derived in our laboratory and were shown to be clonally multipotent, as individual cells could be induced to differentiate into fat, bone, cartilage and SMCs (Bajpai et al., 2012; Liu et al., 2008; Liu et al., 2010). BM-MSCs or HF-MSCs were plated at low density (3×10^3 cells/cm^2) or high density (30×10^3 cells/cm^2) in growth medium [DMEM supplemented with 10% MSC-FBS and 1 ng/ml basic fibroblast growth factor (bFGF)] and, 5 days later, the cells were immunostained for the myogenic proteins, α-smooth muscle actin (αSMA, officially known as ACTA2) and calponin-1 (CNN1). Expression of these proteins increased in high-density cultures of HF-MSC (Fig. 1A) or BM-MSC (supplementary material Fig. S1A), indicative of differentiation along the SMC lineage. The protein levels of αSMA, CNN1 and smooth muscle myosin heavy chain (MYH11) were also increased in HF-MSCs by 3-fold (±0.45), 670-fold (±6.5) and 16-fold (±1.2), respectively (Fig. 1B). Similarly to HF-MSCs, the protein levels of αSMA, CNN1 and MYH11 in BM-MSCs were increased by 23-fold (±3.9), 223-fold (±137) and 2.8-fold (±1.6), respectively (supplementary material Fig. S1B). Finally, quantitative real-time (qRT)-PCR showed that, at high density, the mRNA level of the early markers αSMA, caldesmon (CALD1) and SM22 (officially known as TAGLN) in HF-MSCs increased by 8.2-fold (±0.2), 4.5-fold (±0.4) and 23.4-fold (±4.4), respectively (Fig. 1C). However, as shown in supplementary material Fig. S2, culture at high density did not affect the expression of RUNX2, SPARC or ACAN, and it decreased the expression of SOX9, suggesting that high density alone was not sufficient to induce osteogenic or chondrogenic differentiation in the absence of differentiation-inducing factors.

Expression of αSMA has been previously correlated with the ability to generate force – the defining property of SMCs (Chen et al., 2007; Hinz et al., 2001). Therefore, we examined whether high cell density increased MSC contractility, by measuring hydrogel contraction. To this end, HF-MSC were plated at 3×10^3 cells/cm^2 or 30×10^3 cells/cm^2 in growth medium and cultured for 5 days before they were embedded in fibrin hydrogels (at 10^6 cells/ml). At 1 h after gelation, the hydrogels were overlaid with growth medium and released from the walls to allow for gel compaction, which was monitored by measuring the area of each gel at the indicated times. In contrast to cells from low-density cultures, HF-MSCs originating from high-density cultures were contractile even in the absence of myogenic differentiation medium (i.e. in growth medium) (Fig. 1D). These data demonstrate that high-density culture might promote HF-MSC differentiation into contractile SMCs.

High cell density increased cadherin-11 expression

Immunostaining of HF-MSCs after 3 days in culture showed that β-catenin was localized at the cell–cell contact sites and that its levels increased over time in culture (Fig. 2A), concomitant with increases in cell density, suggesting that the density of

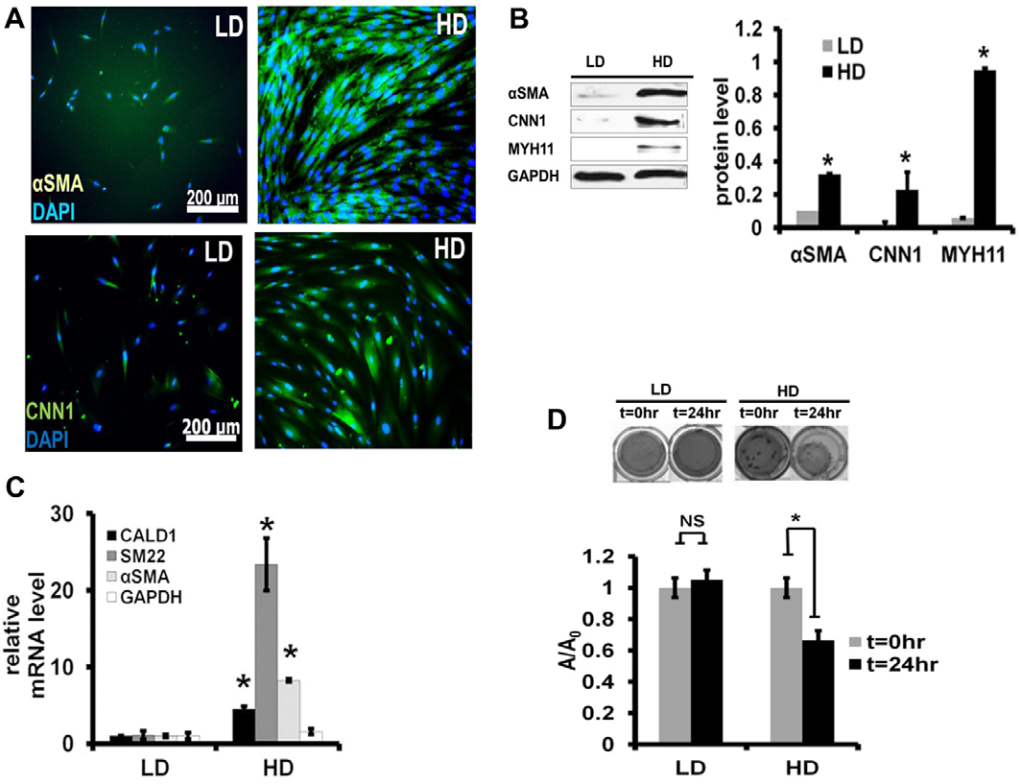

Fig. 1. Cell–cell contact promotes myogenic differentiation of HF-MSCs. HF-MSCs were seeded at 3×10^3 (low density, LD) or 30×10^3 cells/cm^2 (high density, HD) and, 5 days later, they were evaluated. (A) Immunostaining for αSMA (green, upper panels) or CNN1 (green, lower panels). Nuclei were counterstained with DAPI (blue). Scale bars: 200 μm. (B) Western blots for αSMA, CNN1 and MYH11 (left panel) were quantified to show relative protein levels (right panel). (C) qRT-PCR analysis of αSMA, CALD1 and SM22. Data show the mean±s.d.; *P<0.05 between high-density and low-density cells. (D) Fibrin hydrogel compaction. Cells were embedded in fibrin hydrogels (10^6 cells/ml) in the presence of growth medium. After polymerization, the gels were released from the walls and 24 h later the gels were photographed. The area of each gel ('A') was measured using ImageJ software and normalized to the initial area (A_0). Data show the mean±s.d.; *P<0.05; NS, not significant.

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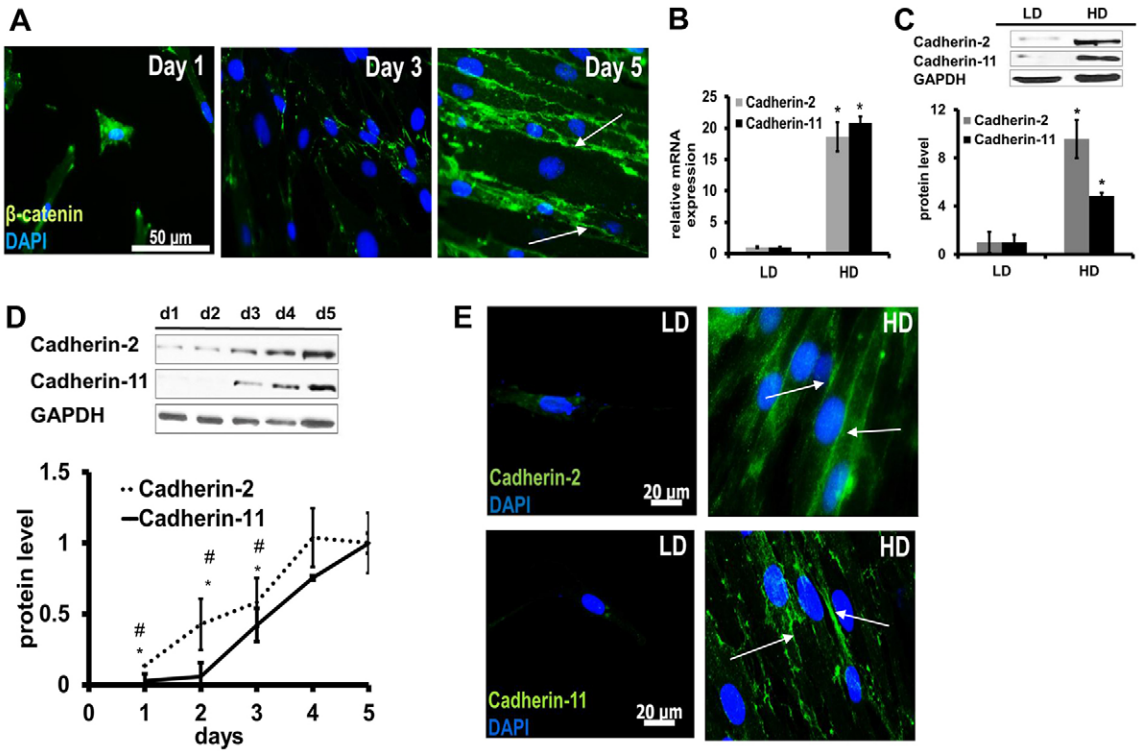

Fig. 2. Cell–cell contact increases cadherin-11 expression. (A) Kinetics of adherens junction formation. HF-MSCs were seeded at 2×10^4 cells/cm^2 and on day 1, 3 or 5 they were immunostained for β-catenin (green). Arrows indicate adherens junctions. Scale bar: 50 μm. (B,C) HF-MSCs were seeded at 3×10^3 (low density, LD) or 30×10^3 cells/cm^2 (high density, HD) and, 5 days later, they were evaluated. (B) qRT-PCR and (C) western blotting for cadherin-2 and cadherin-11. Data show the mean±s.d.; *$P<0.05$ between the high-density and low-density samples. (D) Kinetic profile of cadherin-2 or cadherin-11 proteins over a period of 5 days (seeding density of 2×10^4 cells/cm^2). Data show the mean±s.d.; *$P<0.05$ between the corresponding time-point and day 5 for cadherin-2; $^{\#}P<0.05$ between the corresponding time-point and day 5 for cadherin-11. (E) Immunostaining for cadherin-11 (green, lower panels) and cadherin-2 (green, upper panels) in high-density and low-density cultures. In A and E, nuclei were counterstained with DAPI (blue). Arrows indicate adherens junctions. Scale bars: 20 μm.

adherens junctions increased with increasing MSC density. This result prompted us to hypothesize that the level of mesenchymal cell cadherins, cadherin-2 and/or cadherin-11 might be increased with increased cell density. Indeed, qRT-PCR showed that the mRNA level of cadherin-2 and cadherin-11 increased by 18-fold and 20-fold in cells cultured at high density compared with those cultured at low density (Fig. 2B). In agreement, western blotting showed that cadherin-2 and cadherin-11 protein levels also increased by about 5-fold and 10-fold, respectively (Fig. 2C). Also, a kinetic experiment showed that the levels of both proteins increased as cell density increased over time in culture, reaching a maximum by day 4 or 5 (Fig. 2D). Finally, immunostaining showed that, similar to β-catenin, cadherin-11 and cadherin-2 were localized at the cell–cell contact sites in a confluent cell monolayer (Fig. 2E).

Cadherin-11 but not cadherin-2 was necessary for SMC differentiation

Because the expression of cadherin-2 and cadherin-11 increased significantly during the establishment of cell–cell contact, we examined whether either one or both of these proteins were necessary for αSMA expression and myogenic differentiation. To this end, we first employed the shRNA-encoding LentiViral Dual Promoter (shLVDP) vector (Alimperti et al., 2012) to knock down cadherin-2 in HF-MSCs (siCDH2) by >90% (Fig. 3A), which did not affect the total level of β-catenin, as shown by western blotting (Fig. 3A), but decreased the presence of β-catenin at the adherens junctions (Fig. 3B). Additionally, western blotting and immunostaining showed that αSMA protein levels were decreased by ~50% in siCDH2 cells (Fig. 3A,C). However, knocking down cadherin-2 had no significant effect on the levels of cadherin-11, CNN1 or MYH11 (Fig. 3A). The expression of αSMA, CNN1 and MYH11 correlated with the ability of siCDH2 cells to compact fibrin hydrogels. Specifically, gel compaction by siCDH2 cells was delayed but eventually reached similar levels as compared with control cells (Fig. 3D).

On the other hand, knocking down cadherin-11 in HF-MSCs (siCDH11) (Fig. 3E) had profound effects on MSC differentiation. First, knocking down cadherin-11 decreased the total level of β-catenin (Fig. 3E) and eliminated adherens junction formation even in high-density cultures, as shown by the loss of β-catenin from the cell–cell contact sites (Fig. 3F). In addition, the protein levels of αSMA, CNN1, MYH11 and cadherin-2 were diminished in siCDH11 cells (Fig. 3E). Attenuation of αSMA expression in siCDH11 cells was also confirmed by immunostaining (Fig. 3G). Finally, siCDH11 cells failed to contract fibrin hydrogels over a time period of 72 h (Fig. 3H). Taken together, these data indicate that cadherin-11 is necessary for MSC differentiation into SMCs. Because loss of cadherin-11 had more profound effects on high-density-induced expression of SMC genes as compared with loss of cadherin-2, in the rest of the study we explored the role of cadherin-11 in more detail.

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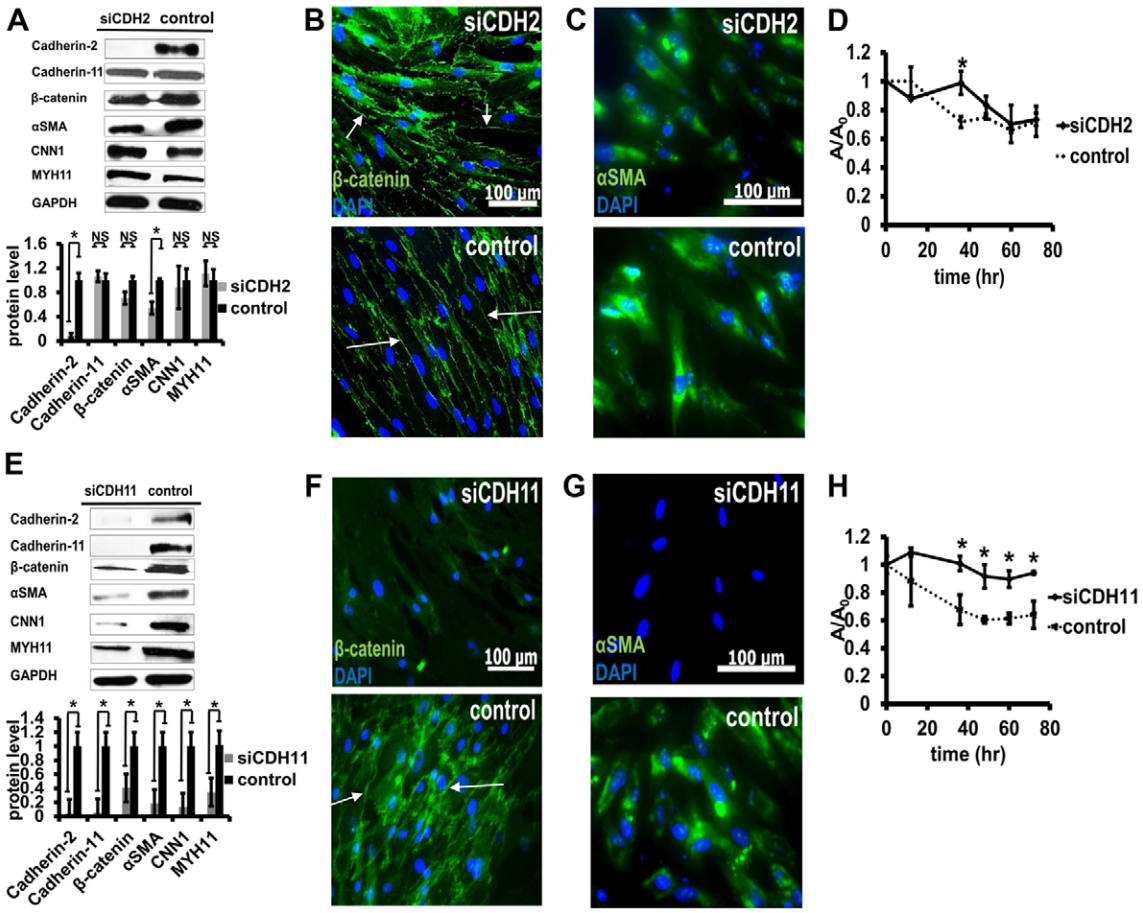

Fig. 3. Cadherin-11 but not cadherin-2 is required for cell-density-induced myogenic differentiation. (A) Western blots of cadherin-2, cadherin-11, β-catenin, αSMA, CNN1 and MYH11 in siCDH2 and control HF-MSCs (upper panel) were quantified to show relative protein levels (lower panel). (B) Immunostaining for β-catenin (green) in siCDH2 and control HF-MSCs. Arrows indicate adherens junctions. (C) Immunostaining for αSMA (green) in siCDH2 and control HF-MSCs. (D) Kinetics of hydrogel compaction by siCDH2 or control HF-MSCs embedded in fibrin hydrogels (10^6 cells/ml). After polymerization, the gels were released from the walls and photographed at the indicated times. At each time-point, the area of each gel ('A') was measured using ImageJ software and normalized to the initial area (A_0). (E) Western blots of cadherin-2, cadherin-11, β-catenin, αSMA, CNN1 and MYH11 in siCDH11 and control cells (upper panel) were quantified to show relative protein levels (lower panel). For A and E, data show the mean±s.d.; *P<0.05; NS, not significant. (F) Immunostaining for β-catenin (green) in siCDH11 and control cells. Arrows indicate adherens junctions. (G) Immunostaining for αSMA (green) in siCDH11 and control cells. In B, C, F and G, nuclei were counterstained with DAPI (blue). All scale bars: 100 μm. (H) Kinetics of fibrin hydrogel compaction by siCDH11 or control HF-MSC. For D and H, data show the mean±s.d.; *P<0.05 between the indicated sample and the corresponding control.

Cadherin-11-mediated intercellular adhesion increased the expression of TGF-β1

Because TGF-β1 is a well-known inducer of myogenic differentiation of MSCs, we hypothesized that cadherin-11-mediated intercellular junctions might promote myogenic differentiation by increasing the expression of TGF-β1. Indeed, we found that the mRNA expression of TGF-β1 increased by ~50-fold (Fig. 4A) and the TGF-β1 protein level increased by ~2-fold in high-density cultures over time (Fig. 4B). Immunostaining showed that, in low-density cultures, phosphorylation of SMAD2 and SMAD3 (pSMAD2/3) was weak but cells from high-density cultures showed high levels of pSMAD2/3 that was localized in the nucleus, similar to that observed in TGF-β1-treated cells (Fig. 4C). Quantification of nuclear fluorescence intensity showed that cells from high-density culture contained ten times the amount of pSMAD2/3 per cell when compared with cells from low-density culture, and this was further increased by the addition of exogenous TGF-β1, as expected (Fig. 4D). Similarly, western blotting showed that the level of SMAD2 phosphorylation (pSMAD2) increased in high-density cultures (Fig. 4E). As expected, treatment with exogenous TGF-β1 (10 ng/ml) induced SMAD2 phosphorylation (Fig. 4E) and a significant increase in αSMA expression (supplementary material Fig. S3A) in low-density and high-density cultures, indicating synergistic effects of cell–cell contact and TGF-β1 on SMC differentiation.

Notably, the amount of TGF-β1 mRNA was reduced in siCDH11 but not in siCDH2 cells (Fig. 4F), suggesting that cadherin-11 is necessary for the expression of TGF-β1. Accordingly, immunostaining showed that pSMAD2/3 was present in the nuclei of siCDH2 cells but was absent from siCDH11 cells (Fig. 4G,H).

Cadherin-11 regulated SMC differentiation in part through the TGF-β pathway

These results suggested that cell–cell contact through cadherin-11 might have promoted MSC differentiation through the autocrine action of TGF-β1. To address this hypothesis, we knocked down TGF-β receptor II (TGFβRII) by using shRNA in HF-MSCs

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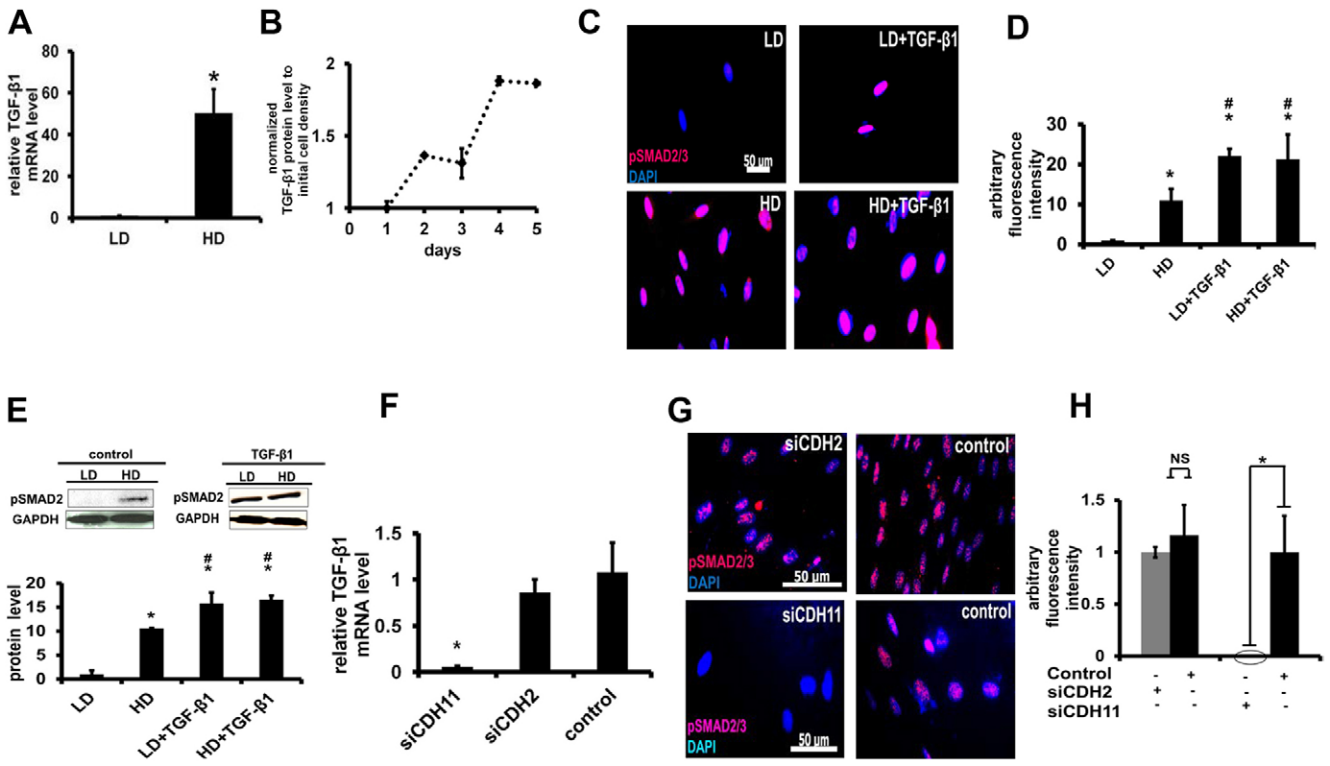

Fig. 4. High cell density increases the expression of TGF-β1. HF-MSCs were seeded at 3×10^3 (low density, LD) or 30×10^3 cells/cm^2 (high density, HD). (A) qRT-PCR for TGF-β1 on day 5 post-seeding. *$P<0.05$ between high-density and low-density cells. (B) HF-MSCs were seeded at 2×10^4 cells/cm^2, and the concentration of TGF-β1 protein in the medium was measured by enzyme-linked immunosorbent assay (ELISA) over a period of 5 days. (C) Immunostaining on day 3 for pSMAD2/3 (pink). (D) Quantification of nuclear pSMAD2/3 fluorescence intensity per cell. (E) Western blots for pSMAD2 (upper panels) were quantified to show relative protein levels (lower panel). For D and E, *$P<0.05$ between the indicated samples and the low-density sample; $^{\#}P<0.05$ between the high-density sample and HD+TGF-β1 or LD+TGF-β1. (F) qRT-PCR for TGF-β1 in siCDH11, siCDH2 or control cells on day 5 post-seeding. *$P<0.05$ between siCDH11 and control cells. (G) Immunostaining for pSMAD2/3 (pink) in siCDH2 or siCDH11 cells. In C and G, nuclei were counterstained with DAPI (blue). Scale bars: 50 μm. (H) Quantification of the fluorescence intensity per cell using ImageJ. *$P<0.05$; NS, not significant. All quantitative data are shown as the mean±s.d.

(siTGFβRII) (Fig. 5A). Knocking down TGFβRII significantly reduced the expression of αSMA, CNN1, MYH11 and cadherin-2 but had no effect on cadherin-11 (Fig. 5B). However, blocking the SMAD2/3 pathway using SB431452 (SB4, 10 μM) for 5 days had no significant effect on the levels of cadherin-2, cadherin-11 or any of the myogenic proteins αSMA, CNN1 and MYH11 (Fig. 5C). Also, cells in high-density cultures formed adherens junctions even in the presence of SB4 (Fig. 5D).

Interestingly, exogenous TGF-β1 failed to promote the expression of myogenic genes (Fig. 5E) or contraction of hydrogels (Fig. 5F) by siCDH11 cells, even though it increased the levels of phosphorylated SMAD2 (Fig. 5E). This result suggests that exogenous TGF-β1 could not compensate for the loss of cadherin-11. Overall, our results show that cadherin-11 regulates myogenic differentiation – at least in part – through a TGFβRII-dependent but SMAD2/3-independent pathway.

Cadherin-11-mediated ROCK activation was necessary for myogenic differentiation induced by high-density culture

Because blocking the SMAD2/3 pathway did not eliminate the expression of myogenic proteins, we hypothesized that additional pathway(s) might be mediating the effects of cadherin-11 on SMC differentiation. It has been shown previously that the Rho-associated protein kinase (ROCK) pathway is involved in myogenic differentiation and affects the levels of αSMA and other myogenic genes through MYOCD or SRF (Mack et al., 2001; Wang et al., 2002; Wang and Olson, 2004; Yoshida and Owens, 2005). A recent study also showed that Rho-associated, coiled-coil-containing protein kinase 1 (ROCK1) binds to E-cadherin through p120-catenin (Smith et al., 2012). Collectively, these results prompted us to examine whether any of the ROCK isoforms bound to cadherin-11.

Indeed, immunoprecipitation experiments showed that cadherin-11 immunoprecipitated with ROCK2 (Fig. 6A). This result suggested that cadherin-11 and ROCK2 might bind to one another directly or indirectly, and it prompted us to hypothesize that the ROCK pathway might be activated by cadherin-11 engagement through cell–cell adhesion when cells reach high density. Indeed, we found that the protein myosin-binding subunit of myosin phosphatase 1 (MYPT-1, officially known as PPP1R12A) – a ROCK substrate – was phosphorylated in high-density but not in low-density MSC cultures (Fig. 6B). This result was confirmed by using immunostaining, which also showed that phosphorylated (p)MYPT-1 localized to the cell nuclei (Fig. 6C). Interestingly, phosphorylation of MYPT-1 was significantly reduced in siCDH11 cells, as shown by western blotting (Fig. 6D) and immunostaining (Fig. 6E), suggesting that high-density-induced ROCK activation was dependent on cadherin-11. In agreement, treatment with TGF-β1 failed to phosphorylate MYPT-1 in siCDH11 cells (Fig. 6F), suggesting

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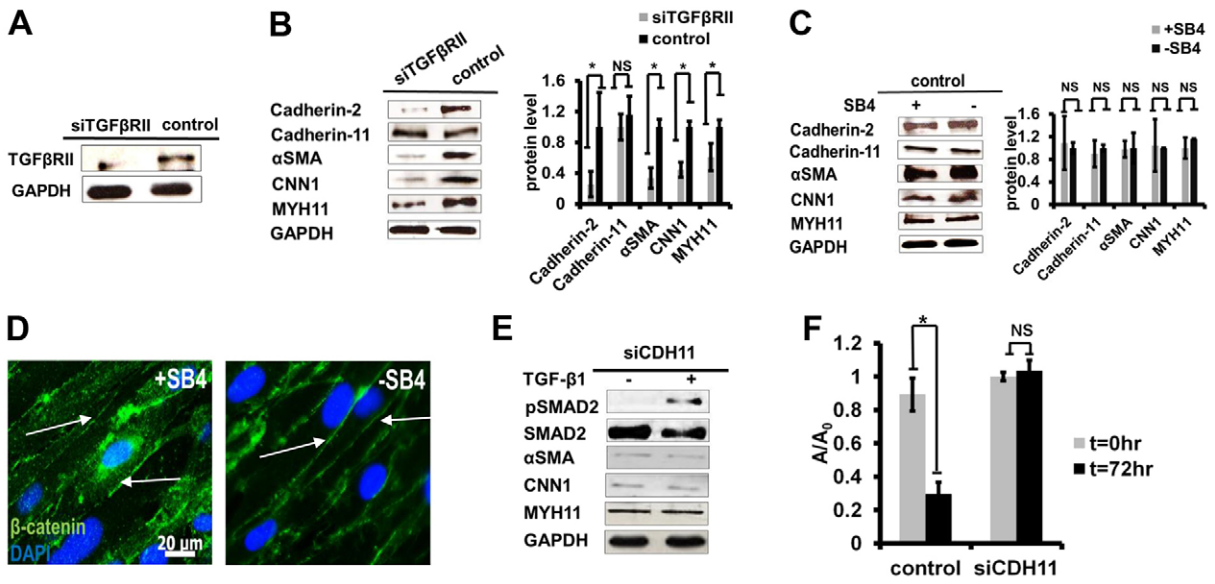

Fig. 5. High-density-induced SMC differentiation is dependent on TGFβRII signaling. (A,B) siTGFβRII cells were seeded at high-density and, 5 days later, they were lysed. (A) Western blot for TGFβRII. (B) Western blots for cadherin-2, cadherin-11, αSMA, CNN1 and MYH11 (left panel) were quantified to show relative protein levels (right panel). (C) HF-MSCs were seeded at 3×10^4 cells/cm^2 and were treated with SB4 (10 μM) for 5 days. Western blots for cadherin-2, cadherin-11, αSMA, CNN1 and MYH11 (left panel) were quantified to show relative protein levels (right panel). (D) Immunostaining for β-catenin (green). Nuclei were counterstained with DAPI (blue). Arrows indicate adherens junctions. Scale bar: 20 μm. (E) siCDH11 cells were treated with TGF-β1 for 3 days or remained untreated, and the indicated proteins were measured by western blotting. (F) Fibrin hydrogel compaction with siCDH11 or control cells in the presence of exogenous TGF-β1 (10 ng/ml). At each time-point, the area of each gel ('A') was measured using ImageJ software and normalized to the initial area (A_0). All quantitative data are shown as the mean±s.d.; *$P<0.05$; NS, not significant.

that exogenous TGF-β1 could not activate ROCK in the absence of cadherin-11.

Next we examined whether ROCK activation was necessary for high-density-induced adherens junction formation and SMC differentiation. Indeed, blocking ROCK with the chemical inhibitor Y27632 (Y27) diminished adherens junction formation (Fig. 6G), inhibited high-density-induced phosphorylation of MYPT-1 (Fig. 6H), significantly reduced high-density-induced expression of cadherin-11 and diminished cadherin-2, αSMA, CNN1 and MYH11 expression (Fig. 6I). Finally, knocking down both ROCK isoforms ROCK1 and ROCK2 by using small interference RNA (siRNA) siROCK reduced αSMA protein levels (Fig. 6J). These results suggested that activation of ROCK1 or ROCK2 through cadherin-11 are necessary for high-density-induced SMC differentiation.

A positive-feedback loop increases cadherin-11 expression through SRF

Interestingly, knocking down cadherin-11 significantly decreased the expression of both ROCK1 and ROCK2 (Fig. 6K). Because ROCK1 and ROCK2 are known to affect myogenic gene expression (Castellani et al., 2006; Olson and Nordheim, 2010; Pagiatakis et al., 2012), we examined whether cadherin-11-mediated intercellular adhesion affected the expression of the key myogenic regulator, SRF. Indeed, the expression of SRF was significantly reduced both by treatment with Y27 and in siCDH11 cells (Fig. 6L), suggesting that SRF is downstream of ROCK and cadherin-11. By contrast, SRF was not affected by knocking down cadherin-2 (Fig. 6L). However, the expression of cadherin-11 (as well as that of cadherin-2 and αSMA) was strongly reduced in HF-MSCs expressing shRNA for SRF (siSRF) (Fig. 6M), suggesting that cadherin-11 might be regulated by SRF. Taken together, our results indicate the presence of a feedback loop, whereby cadherin-11 regulates SRF through ROCK and, in turn, SRF regulates the expression of cadherin-11.

Cdh11$^{-/-}$ mice lack SMC proteins and contractile function in vascular and urinary tissues

Cadherin-11 is present in all mesenchymal cells throughout the embryo, including the cells of the stomach, intestine, pharynx, lungs, rib shafts, bladder and blood vessels (Halbleib and Nelson, 2006; Kuijpers et al., 2007; Monahan et al., 2007; Simonneau et al., 1995). To assess the physiological implications of our findings, we examined the effects of cadherin-11 in blood vessels and two tissues that contain SMCs originating from the mesoderm, similar to mesenchymal stem cells (Bohnenpoll et al., 2013; Eichmann et al., 2005; Satoskar et al., 2005; Wasteson et al., 2008). To this end, we measured SMC protein expression and contractility of aortas and bladders from cadherin-11-null (Cdh11$^{-/-}$) and wild-type mice. Interestingly, the SMC layers in the bladder of Cdh11$^{-/-}$ mice exhibited significantly reduced levels of the SMC-specific proteins αSMA and MYH11. The expression levels of these proteins were found to be ~25% and 20% of the levels present in wild-type tissues, respectively (Fig. 7A,B).

Next, we examined whether the lack of cadherin-11 affected the contractile function of these tissues. Specifically, we measured the isometric tension per gram of tissue generated by cylindrical rings of aorta or bladder tissues in response to several agonists, by using an isolated tissue bath as we reported previously (Liu et al., 2007a; Swartz et al., 2005; Yao et al., 2005). Bladders from Cdh11$^{-/-}$ mice ($n=5$) exhibited significantly lower contractility in response to the thromboxane A_2 receptor agonist U46619 (U4, 10^{-6} M) and KCl (118 mM) as compared with wild-type bladders ($n=5$, $P<0.05$). In response

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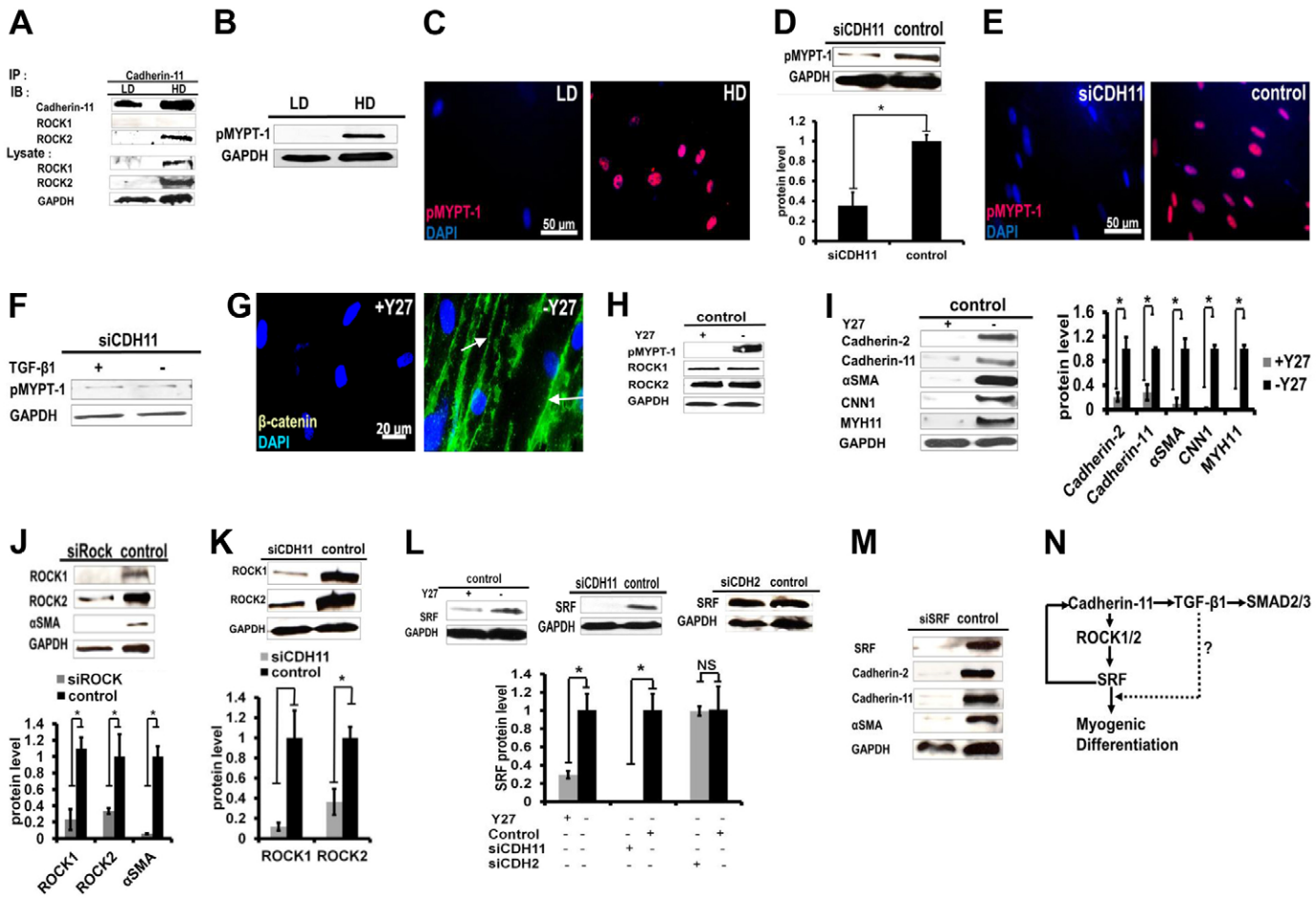

Fig. 6. Rock and SRF are activated downstream of cadherin-11 and are necessary for high-density-induced myogenic differentiation. HF-MSCs were seeded at 3×10^3 (low density, LD) or 30×10^3 cells/cm^2 (high density, HD). (A) Lysates were immunoprecipitated with antibody against cadherin-11. Immunoprecipitates (IP) and corresponding lysates were probed by western blotting (IB). (B) Western blot for pMYPT-1 in cells cultured at low or high density. (C) Immunostaining for pMYPT-1 (pink). Scale bar: 50 μm. (D) Western blot for pMYPT-1 in siCDH11 or control HF-MSCs (upper panel) was quantified to show relative protein levels (lower panel). (E) Immunostaining of siCDH11 or control cells for pMYPT-1 (pink). Scale bar: 50 μm. (F) siCDH11 cells were treated with TGF-β1 (10 ng/ml) and pMYPT-1 was detected by western blotting 3 days later. (G–I) HF-MSCs were seeded at 20×10^3 cells/cm^2, and on day 3 they were treated with Y27 (20 μM) for 1 day. (G) Immunostaining for β-catenin (green). Arrows indicate adherens junctions. Scale bar: 50 μm. In C, E and G, nuclei were counterstained with DAPI (blue). (H) Western blots for pMYPT1, ROCK1 and ROCK2 in Y27-treated and untreated cells. (I) Western blots for cadherin-2, cadherin-11, αSMA, CNN1 and MYH11 (left panel) were quantified to show relative protein levels (right panel). (J) Western blots for the indicated proteins in siROCK cells (upper panel) were quantified to show relative protein levels (lower panel). (K) The level of ROCK1 and ROCK2 in control and siCDH11 cells (lower panel) was determined from western blots (upper panel). (L) HF-MSCs were seeded at 20×10^3 cells/cm^2 and, on day 3, they were treated with Y27 (20 μM) for 1 day. Western blots for SRF in Y27-treated and non-treated cells, siCDH11 or siCDH2 cells (upper panel) were quantified to show relative protein levels (lower panel). All quantitative data in D and I–L show the mean±s.d.; *P<0.05; NS, not significant. (M) SRF was knocked down using shLVDP. Western blots for the indicated proteins in control and siSRF cells are shown. (N) Schematic illustrating the proposed mechanism of cadherin-11-mediated myogenic differentiation.

to U4, the force generated by wild-type bladder tissue was 128.7±62.3 mN/g versus a force of only 21.07±12.1 mN/g generated by Cdh11$^{-/-}$ tissues (P<0.05). Similarly, the forces generated by wild-type and Cdh11$^{-/-}$ bladder tissue in response to KCl were 469.65±271.6 mN/g and 23.4±16.2 mN/g, respectively (P<0.05) (Fig. 7C).

Similar to bladder, Cdh11$^{-/-}$ aortas expressed lower levels of αSMA and MYH11 as compared with wild-type aortas (Fig. 7D,E). Also, Cdh11$^{-/-}$ aortas (n=7) displayed significantly lower contractile forces compared with wild-type vessels (n=7) (Fig. 7F). Specifically, the forces generated by wild-type and Cdh11$^{-/-}$ aortas in response to U4 were 4821.5±377.8 mN/g and 377.8±226.2 mN/g, respectively (P<0.05). The forces generated by wild-type versus Cdh11$^{-/-}$ aortas in response to endothelin-1 (ET-1) were 1972.8±292.9 mN/g versus 578.5±323.7 mN/g (P<0.05); finally, the forces generated by wild-type versus Cdh11$^{-/-}$ aortas in response to KCl were 4252.1±1143 mN/g versus 1534.4±909.7 mN/g (P<0.05), respectively. Taken together, these measurements indicate that cadherin-11 is necessary for the expression of SMC proteins as well as for the development of contractility in SMC-containing tissues *in vivo*.

DISCUSSION

In this study, we investigated the role of cadherin-11 in MSC differentiation into SMCs and in the development of contractile function *in vivo*. So far, most studies have implicated cadherin-11 in osteogenic differentiation. In particular, it has been shown that the expression of cadherin-11 increases during osteogenesis

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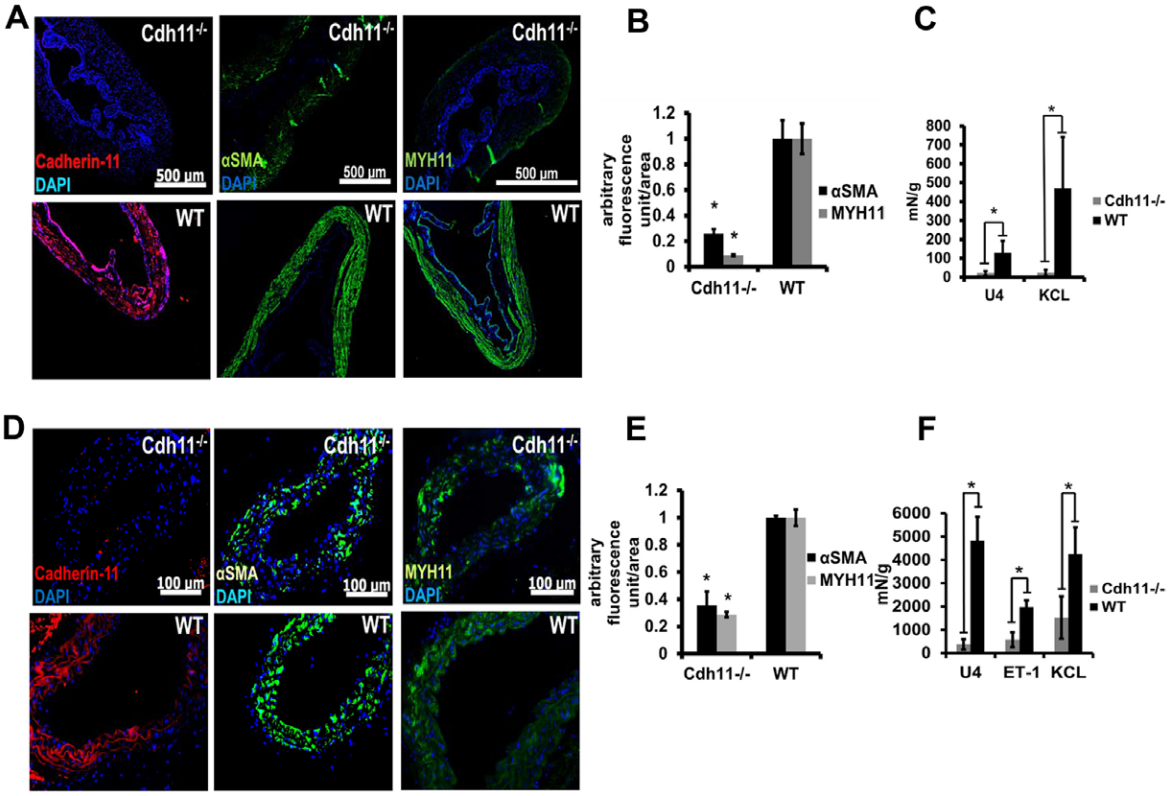

Fig. 7. Cadherin-11 regulates SMC protein expression and the contractility of SMC-containing organs *in vivo*. Bladders (A–C) or aortas (D–F) were obtained from 10-week-old Cdh11$^{-/-}$ or wild-type (WT) male mice. (A) Immunostaining for cadherin-11 (red, left panels), αSMA (green, middle panels) and MYH11 (green, right panels) in bladder tissue from Cdh11$^{-/-}$ and wild-type mice. Nuclei were counterstained with DAPI (blue). Scale bars: 500 μm. (B) Quantification of the fluorescence intensity of αSMA and MYH11 per area of tissue. (C) Contractile force generated in response to the indicated agonists in Cdh11$^{-/-}$ and wild-type bladders. (D–F) The data are as described for A–C for mouse aortas instead of bladders. Scale bars: 100 μm. All quantitative data show the mean±s.d.; *P<0.05 between the Cdh11$^{-/-}$ and wild-type tissues.

(Greenbaum et al., 2012), and that inhibition of cadherin-2 and cadherin-11 prevents osteoblastic differentiation (Cheng et al., 1998; Kii et al., 2004). Interestingly, our work revealed a novel role for cadherin-11 in MSC differentiation into SMCs and in the development of contractile function, even in the absence of differentiation factors. Notably, the contractility of aortic vessels and bladder detrusor smooth muscle was diminished in Cdh11$^{-/-}$ mice, clearly implicating cadherin-11 in SMC function *in vivo*. This is the first study implicating cadherin-11 in MSC differentiation into SMCs and in the development of contractile function *in vitro* and *in vivo*.

Our findings show that cadherin-11 – but not cadherin-2 – is the key regulator of cell–cell-adhesion-induced myogenic differentiation of MSCs. We found that loss of cadherin-2 had a small effect on high-density-induced myogenic gene expression and contractile function. Knockdown of cadherin-2 had a moderate effect on the expression of αSMA (leading to a 50% decrease in αSMA expression) but had no effect on the expression of other late SMC markers. Because SMC-specific genes are regulated by transcription factors such as SRF binding to CaRG response elements (Owens, 1998; Owens, 2007; Wamhoff et al., 2006), this result suggests that cadherin-2 might affect αSMA through a CarG-independent (and perhaps also SRF-independent) pathway. Indeed, in contrast to siCDH11, siCDH2 cells expressed SRF at similar levels to control cells, suggesting that cadherin-2 did not affect SRF expression. However, ROCK1, ROCK2 or SRF knockdown reduced the level of cadherin-2, suggesting that the ROCK1/2–SRF pathway controlled cadherin-2 expression. By contrast, loss of cadherin-11 resulted in complete loss of cadherin-2, as well as all myogenic genes and contractile function. Cadherin-11 might regulate the expression of cadherin-2, possibly as a way of increasing the force of cell adhesion (Pittet et al., 2008), which, in turn, might serve to maintain cell–cell contact for longer periods of time, further promoting differentiation.

Interestingly, we identified two pathways through which cadherin-11 induced SMC differentiation. First, high-density culture increased the expression of TGF-β1 through cadherin-11, as knocking down cadherin-11 reduced the level of TGF-β1 as well as the phosphorylation of SMAD2/3. Although the mechanism remains unclear, cadherin-11 might regulate the secretion of TGF-β1 by activating SRF through the ROCK pathway. In turn, TGF-β1 at least partly mediated increased SMC differentiation, as knocking down TGFβRII decreased (but did not eliminate) the expression of SMC proteins as well as that of cadherin-2. Interestingly, the chemical inhibitor SB4, which blocks phosphorylation of SMAD2/3, had no effect on the expression of SMC proteins, suggesting that the effect of TGF-β1 on high-density MSC cultures might be mediated through a SMAD2/3-independent pathway. However, this result might also be a consequence of the presence of bFGF in the growth medium. bFGF is known to antagonize TGF-β1 and suppress the SMAD2/3 pathway (Cushing et al., 2008; Kawai-Kowase et al., 2004; Papetti et al., 2003; Schuliga et al., 2013; Shi et al., 2013),

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suggesting that chemical inhibition had no effect, perhaps because this pathway might have been already suppressed in our experiments. Interestingly, exogenous TGF-β1 failed to increase the expression of myogenic genes in siCDH11 cells, despite activating the SMAD2/3 pathway, suggesting that TGF-β1 could not compensate for the loss of cadherin-11. It is possible that TGF-β1 might affect an SRF co-factor that might not work in the absence of SRF in siCDH11 cells. However, currently we cannot exclude the possibility that TGF-β1 is not the main mediator of cadherin-11-induced myogenesis.

Although knocking down TGFβRII decreased the expression of SMC proteins, it did not eliminate them, suggesting that an additional TGF-β-independent pathway(s) might also be at work. Indeed, our experiments showed that, at high-density, cadherin-11 activated ROCK, which in turn activated SRF (the key transcriptional regulator of SMC genes) and the development of contractile function. Interestingly, cadherin-11 and ROCK2 formed a complex, suggesting that cadherin-11 engagement at the cell surface might induce the recruitment and activation of ROCK at the adherens junction sites. It will be interesting to understand how cadherin-11 recruits ROCK2 and whether other factors are involved in this process, as was recently shown for p120 and E-cadherin (Smith et al., 2012). As cell density increased, presumably more cadherin-11 molecules were engaged with their counterparts on the surface of neighboring cells, activating ROCK and SRF, which in turn further increased the expression of cadherin-11. Treatment with Y27 or shRNA against the ROCK isoforms ROCK1 and ROCK2 significantly decreased or eliminated the expression of SMC proteins as well as the expression of cadherin-2 and cadherin-11. Similarly, knocking down SRF decreased the expression of cadherin-11, indicating the presence of a positive-feedback loop that reinforces the expression of cadherin-11 and SRF. This positive-feedback mechanism might be necessary for inducing and maintaining MSC differentiation into SMCs at high density, even in the absence of exogenous differentiation factors such as TGF-β1. Our findings are summarized in a schematic (Fig. 6N) describing the mechanism of cadherin-11 action as suggested by our experiments.

Interestingly, the ROCK pathway was previously shown to affect the contractility of detrusor smooth muscle in urinary bladder or aortic smooth muscle cells (Peters et al., 2006), whereas knocking down ROCK decreased blood pressure in hypertensive rat and canine animal models (Takahara et al., 2003; Uehata et al., 1997) and reduced vascular inflammation and atherosclerosis (Noma et al., 2006). Taken together, these data suggest that intercellular adhesion through cadherin-11 might be important in diseases related to SMC function (e.g. bladder retention or hypotension), owing to the role for cadherin-11 in regulating the ROCK pathway. More studies are required to shed light on these interesting observations and to investigate potential broader implications of cadherin-11 loss in animal pathophysiology.

Previous studies have implicated mechanical forces in cell signaling, proliferation, migration and, more recently, in stem cell differentiation (Chen, 2008; Farge, 2003; Lee et al., 2006; Somogyi and Rørth, 2004). Mechanical forces promote stress fiber formation and might be transmitted between cells through surface proteins, such as those found at adherens junctions (Liu et al., 2007b; Tzima et al., 2005) or stretch-activated ion channels (Pellegrino et al., 1990; Sukharev and Corey, 2004; Tavernarakis and Driscoll, 2001). Interestingly, mechanical signals are integrated by distinct GTPases in a lineage-specific manner, with Rac1 regulating myogenesis (Gao et al., 2010), whereas RhoA regulates osteogenesis (McBeath et al., 2004). Blocking such cell-generated forces appears to alter many basic cellular functions such as proliferation, differentiation and migration (Huang et al., 1998; Lo et al., 2004; McBeath et al., 2004; Sordella et al., 2003). Recent experiments have also shown that mechanical forces through the surface substrate regulate MSC differentiation (Buxboim and Discher, 2010; Discher et al., 2005; Engler et al., 2006; Gao et al., 2010; Gilbert et al., 2010; McBeath et al., 2004). Our findings extend previous observations, as they implicate adherens junctions, and specifically cadherin-11, in the development of the contractile phenotype through ROCK. Therefore, they call attention to cell–cell adhesion force as a regulator of MSC differentiation and function.

In general, the ability to constrict in response to physiological signals is an important property characterizing functional SMC and SMC-containing organs, such as blood vessels. The degree of tone in vascular or other smooth muscle cells (in a continuum from complete relaxation to complete contraction) is determined by the level of free intracellular Ca^{2+} $(Ca^{2+})_i$. SMC contractile agents increase the concentration of extracellular or intracellular calcium for contraction. Extracellular calcium can enter the cell through potential-dependent channels or potential-independent channels, often referred to as receptor-operated channels. An impressive variety of neurohumoral agents and second messenger systems act in concert to regulate the concentration of $(Ca^{2+})_i$ *in vivo*. It was not within the scope of this study to investigate each of these myriad systems independently. Rather, we focused on two major receptor-dependent (ET-1, U4) and one receptor-independent agonist (KCl) to evaluate the functional status of contraction of SMC-containing tissues such as aortic vessels and bladders.

Notably, our results with $Cdh11^{-/-}$ mice show that cadherin-11 is necessary for the expression of SMC proteins and for endowing SMC-containing organs with contractile ability. This is a novel finding, as previous studies have implicated cadherin-11 in modest osteopenia (Kawaguchi et al., 2001a; Kawaguchi et al., 2001b; Shin et al., 2000) and in pulmonary fibrosis (Schneider et al., 2012), but this is the first study implicating cadherin-11 in muscle contractile function. Indeed, we discovered that the SMC layer of the bladder and aorta of $Cdh11^{-/-}$ mice expressed significantly lower levels of αSMA and MYH11 that that of wild-type mice. In addition, contractility was diminished in both tissues, in agreement with our observations *in vitro*. Interestingly, although both bladder and aorta exhibited significantly impaired contractile function, the viability of $Cdh11^{-/-}$ animals was not compromised, suggesting that compensatory mechanisms might be at work. More work is required to determine how the cardiovascular system is affected by the loss of Cdh11 and what compensatory mechanisms – if any – might be at work.

In summary, the results presented here reveal a previously unknown role for cadherin-11-based adherens junctions in the process of MSC differentiation into SMCs as well as in the contractile function of SMC-containing tissues. These findings shed light on the mechanism of MSC differentiation, with potential implications for the development of strategies to understand and control stem cell fate decisions.

MATERIALS AND METHODS

Cell Culture

BM-MSCs (Stem Cell Technologies, Vancouver, Canada) and HF-MSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco,

Grand Island, NY) supplemented with 10% (v/v) MCS-FBS (Gibco), 1% (v/v) antibiotic-antimycotic (Gibco) and 1 ng/ml bFGF (BD Biosciences, San Jose, CA).

RNA interference, cloning and lentiviral vector preparation

The sh-LVDP vector has been described elsewhere (Alimperti et al., 2012). For shRNA cloning, the two complementary oligos were mixed at a 1:1 ratio to a final concentration of 50 µM and annealed as follows: 95℃ for 30 s, 72℃ for 2 min, 37℃ for 2 min and 25℃ for 2 min. We designed small interfering RNA (siRNA) oligonucleotides directed against specific sites of human cadherin-11 (siCDH11), cadherin-2 (siCDH2), TGFβRII (siTGFβRII), and the two ROCK isoforms ROCK1 and ROCK2 (siROCK) (BLAST confirmed). We also designed a scramble oligonucleotide (control) without homology to any region of the human genome as confirmed by BLAST. The siRNA sequences that were used here are listed in supplementary material Table S1. All cloning products were confirmed by sequencing with ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA).

For lentivirus production, 293T/17 cells were transfected with three plasmids; lentiviral vector (16.8 µg), psPAX2 (15 µg) and pMD.G (5 µg), using the standard calcium phosphate precipitation method. Virus was harvested at 24 h post-transfection, filtered through a 0.45-µm filter (Millipore, Bedford, MA), pelleted by ultracentrifugation (50,000 ***g*** at 4℃ for 2 h) and resuspended in fresh medium.

Immunostaining and western blotting

HF-MSCs and BM-MSCs were plated at high or low density or for the indicated times and were fixed and permeabilized. After incubation with blocking buffer [5% goat serum, 0.01% (v/v) Triton X-100 in PBS], samples were incubated with the following primary antibodies; mouse anti-human-αSMA (1:50 dilution, Sigma), mouse anti-human-CNN1 (1:100 dilution, Santa Cruz Biotechnology), rabbit anti-human-pSMAD2/3 (1:100, Cell Signaling Technology), mouse anti-human-β-catenin (1:200, BD Transduction Laboratories), rabbit anti-human-pMYPT-1 (1:50, Cell Signaling Technology), mouse anti-human-cadherin-2 (1:100, BD Transduction Laboratories) or rabbit anti-human-cadherin-11 (1:50, Cell Signaling Technology) in blocking buffer overnight at 4℃. After washing with PBS, samples were incubated with secondary antibodies (Alexa-Fluor-488-conjugated goat anti-mouse-IgG and Alexa-Fluor-488-conjugated goat anti-rabbit-IgG, 20 µg/ml; Molecular Probes), followed by staining with DAPI (25 µg/ml in TNE buffer: 10 mmol/l Tris-HCl, 2 mol/l NaCl, 1 mmol/l EDTA, pH 7.4; Molecular Probes). Fluorescent images were obtained using an inverted fluorescence microscope, the Zeiss AxioObserver (Zeiss, Thornwood, NY). Cells lysates were prepared for immunoblotting essentially as described previously (Lee et al., 2011).

qRT-PCR analysis

Total mRNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA was synthesized using the Superscript III cDNA Synthesis Kit (Invitrogen). The cDNA was subjected to RT-PCR using a SYBR Green Kit (Bio-Rad, Hercules, CA) and primers as listed in supplementary material Table S2. The expression level of each gene was quantified using the ΔC_T method and normalized to the expression level of the housekeeping gene RPL32.

Mice

Cadherin-11-null mice ($Cdh11^{-/-}$) and wild-type B6:129 F1 intercross mice (Schneider et al., 2012) were housed at Baylor College of Medicine with the approval of the Baylor College of Medicine Institutional Animal Care and Use Committee. Heart, lung and bladder tissues were removed from 10-week-old mice after the mice were euthanized. For histology, tissues were placed in 10% formalin. For vascular reactivity and mechanical testing, tissues were kept in culture medium on ice overnight prior to experimentation.

Histology and immunohistochemistry

The bladders and arteries of wild-type and $Cdh11^{-/-}$ male mice were fixed in 10% formalin and embedded in paraffin, using standard protocols (Liu et al., 2007a). For immunohistochemistry, tissue sections (5 µm) were deparaffinized in xylene and rehydrated in graded alcohol. Specimens were washed with PBS and permeabilized. After incubation in blocking buffer [5% goat serum, 0.01% (v/v) Triton X-100 in PBS], samples were incubated with the following primary antibodies; rabbit anti-human-cadherin-11 (1:100; Invitrogen), rabbit anti-human-αSMA (1:100, Abcam) or rabbit anti-human-MYH11 (1:100, Biomedical Technologies) in blocking buffer overnight at 4℃. After washing with PBS, samples were incubated with secondary antibody (Alexa-Fluor-488-conjugated goat anti-rabbit-IgG, 20 µg/ml; Molecular Probes), followed by staining with DAPI, as described above. Fluorescent images were obtained as described above.

Hydrogel compaction and vascular reactivity assays

Compaction of three-dimensional fibrin hydrogels and contractile force measurements were performed as described previously (Han et al., 2010; Liu et al., 2008; Liu et al., 2010). Briefly, contractile force measurements were performed using an isolated tissue bath system, and the isometric contraction was recorded using a PowerLab data acquisition unit and analyzed with Chart5 software (ADInstruments, Colorado Springs, CO). The following receptor-dependent and receptor-independent vascular agonists were used to test vasoconstriction: (1) KCl (118 mM), to open K^+-dependent channels; (2) ET-1 (10^{-8}–10^{-5} M), to activate endothelin receptors; and 3) U4 (10^{-9}–10^{-6} M), to activate thromboxane A_2 receptors.

Statistical analysis

Statistical analysis of the data was performed using a two-tailed Student's *t*-test ($a=0.05$) in Microsoft Excel (Microsoft, Redwood, CA). Each experiment was repeated three times, each time with at least triplicate samples.

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Competing interests

The authors declare no competing interests.

Author contributions

S.A.: conception, design, collection and/or assembly of data; data analysis and interpretation; manuscript writing. H.Y.: collection and/or assembly of data, data interpretation, manuscript writing. T.G. and S.K.A.: collection and/or assembly of data. S.T.A.: conception and design; data analysis and interpretation; manuscript writing; final approval of manuscript.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.134833/-/DC1

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