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RESEARCH ARTICLE

Mesenchymal stem cell mechanotransduction is cAMP dependent and regulated by adenylyl cyclase 6 and the primary cilium

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ABSTRACT

Mechanical loading is a potent stimulus of bone adaptation, requiring the replenishment of the osteoblast from a progenitor population. One such progenitor is the mesenchymal stem cell (MSC), which undergoes osteogenic differentiation in response to oscillatory fluid shear. Yet, the mechanism mediating stem cell mechanotransduction, and thus the potential to target this therapeutically, is poorly understood. In this study, we demonstrate that MSCs utilise cAMP as a second messenger in mechanotransduction, which is required for flow-mediated increases in osteogenic gene expression. Furthermore, we demonstrate that this mechanosignalling is dependent on the primary cilium and the ciliary localised adenylyl cyclase 6. Finally, we also demonstrate that this mechanotransduction mechanism can be targeted therapeutically to enhance cAMP signalling and early osteogenic signalling, mimicking the beneficial effect of physical loading. Our findings therefore demonstrate a novel mechanism of MSC mechanotransduction that can be targeted therapeutically, demonstrating a potential mechanotherapeutic for bone-loss diseases such as osteoporosis.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Bone, Oscillatory fluid shear, Mechanotherapeutic, Osteoporosis

INTRODUCTION

Bone has long been established as a mechanosensitive organ that can adapt its structure to meet the demands of its mechanical environment, maintaining an optimal strength-to-weight ratio (Robling et al., 2007; Robling and Turner, 2002; Frost, 1963). This mechanically driven adaptation requires a continued replenishment of bone-forming osteoblasts, achieved through the differentiation of mesenchymal stem cells (MSCs) (Knight and Hankenson, 2013). This loading-induced MSC osteogenic lineage commitment is thought to occur in part via direct mechanosensing of physical stimuli, leading to MSC osteogenic differentiation and bone formation (Govey et al., 2013; Hoey et al., 2012b; Curtis et al., 2018). Loading-induced deformation of bone creates a complex mechanical microenvironment within the stem cell niche consisting of strain, pressure and fluid flow (Gurkan and Akkus, 2008). A large body of work has demonstrated that physiological oscillatory fluid shear (OFS) is a potent regulator of bone homeostasis, triggering an osteogenic response in osteoblasts, osteocytes and stem cells (Yourek et al., 2010; Thompson et al., 2012). In particular, a systematic study investigating the effect of OFS on MSCs demonstrated that OFS triggers an increase in cyclo-oxygenase 2 (*Cox2*, also known as *PTGS2*), osteopontin (*Opn*, also known as *SPP1*) and runt-related transcription factor 2 (*Runx2*) expression at early time points (1, 2 and 4 hrs) that resulted in enhanced collagen and mineral deposition over 21 days (Stavenschi et al., 2017). Despite the known contribution of mechanical loading to MSC osteogenic differentiation and bone formation, the mechanism by which these cells transduce mechanical stimuli into cellular activity, mechanotransduction, remains poorly understood. Deciphering these mechanisms would greatly aid in the development of mechanotherapies to prevent bone loss and promote regeneration in skeletal diseases such as osteoporosis (Rando and Ambrosio, 2018).

Second messengers are one of the initiating biochemical components of intracellular signalling cascades triggered by a biophysical stimulus. Intracellular calcium (Ca^{2+}) has been the predominant second messenger studied in response to fluid shear, where rapid fluxes in intracellular Ca^{2+} following OFS result in downstream transcriptional activity in bone cells (Hung et al., 1995; Lewis et al., 2017; Lyons et al., 2017). Moreover, human MSCs have demonstrated intracellular Ca^{2+} increases following fluid shear that are required for downstream osteogenic gene expression (Liu et al., 2015; Corrigan et al., 2018). Alternatively, cyclic adenosine 3′,5′-monophosphate (cAMP) is another second messenger activated in response to fluid shear but its role in the osteogenic lineage is less well studied (Kamenetsky et al., 2006). Fluid shear has been shown to induce an increase in cAMP production in a time- and magnitude-dependent manner in rat calvarial osteoblasts (Reich et al., 1990), indicating that cAMP is mechanoresponsive in bone cells. Moreover, cAMP signalling is biphasic in osteocytes undergoing an initial decrease followed by an increase after 30 min of OFS (Kwon et al., 2010). A recent study in human MSCs demonstrated that activation of cAMP signalling augmented MSC osteogenesis in an ectopic bone formation model in immune-deficient mice (Siddappa et al., 2008), indicating that MSCs may too utilise cAMP as a potential pre-osteogenic signal; however, the role of cAMP as a second messenger in MSC mechanotransduction is poorly understood.

cAMP is universally generated by adenylyl cyclases, a family of enzymes that catalyse the cyclisation of adenosine triphosphate (ATP)

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into cAMP (Kamenetsky et al., 2006). The adenylyl cyclase family comprises nine distinct transmembrane isoforms (AC1–AC9, also known as ADCY1–ADCY9), where each of the transmembrane adenylyl cyclases have individual regulatory properties, and the nine subtypes are expressed in only a limited number of tissues (Hanoune and Defer, 2001; Defer et al., 2000). Specifically, adenylyl cyclase 6 (AC6, also known as ADCY6) has been shown to be expressed in mature bone cells and is required for loading-induced bone formation *in vivo* (Lee et al., 2014). Interestingly, skeletally mature mice with a global deletion of AC6 did not present with a skeletal phenotype but formed significantly less bone than control mice following ulnar loading, demonstrating that AC6 is involved specifically in mechanotransduction leading to functional bone mechanoadaptation (Lee et al., 2014). Additional *in vitro* studies have demonstrated a role for AC6 in osteocyte mechanotransduction (Kwon et al., 2010), but the potential role of AC6 in MSCs remains unknown.

Primary cilia are solitary, immotile microtubule-based organelles that protrude from the membrane in various cell types, including bone (Hoey et al., 2012a), and have recently emerged as a nexus of intra- and extracellular signalling (Singla and Reiter, 2006; Lee et al., 2015). Extending into the extracellular milieu, the primary cilium is ideally positioned to relay both biochemical [HH (Singla and Reiter, 2006), TGFβ (Clement et al., 2013; Labour et al., 2016) and BMP (Monnich et al., 2018)] and biophysical [fluid shear (Hoey et al., 2012b), compression (Wann et al., 2012) and pressure (Luo et al., 2014)] cues and are required to maintain tissue homeostasis, with defects in the cilium leading to a number of diseases known as ciliopathies (Hildebrandt et al., 2011). Specifically, in bone, the depletion of primary cilia in mature bone cells or their progenitors results in an inhibition of the osteogenic response to fluid shear (Malone et al., 2007; Hoey et al., 2011, 2012b), with these findings verified using *in vivo* models (Temiyasathit et al., 2012; Chen et al., 2016), demonstrating the important role of the cilium in bone and stem cell mechanobiology. To identify new anabolic therapeutics, recent efforts have attempted to decipher the molecular mechanism of cilia-mediated mechanotransduction with contrasting results. Cilia-mediated mechanotransduction is predominately believed to be propagated by Ca^{2+} signalling with initial studies by Praetorius and Spring (2001) being verified by advanced cilia-localised genetically encoded Ca^{2+} indicators (Jin et al., 2014; Lee et al., 2015). Interestingly, work has recently emerged suggesting that primary cilia are not Ca^{2+}-responsive mechanosensors, as previously thought (Delling et al., 2016). Although these discrepancies may be associated with differences in the Ca^{2+} sensors utilised, it has raised the possibility of an alternative second messenger in cilia mechanotransduction, namely cAMP. Previous work has demonstrated that ciliary cAMP levels are fivefold higher than cytosolic cAMP in mouse embryonic fibroblasts and murine inner medullary collecting duct cells (IMCD3), and that this compartmentalised cAMP is regulated by adenylyl cyclases that localise to the ciliary microdomain (Moore et al., 2016). Moreover, AC6 was shown to localise to the primary cilium of cholangiocytes and osteocytes and was required for fluid shear-induced cAMP signalling (Masyuk et al., 2006; Mick et al., 2015; Kwon et al., 2010), indicating that cilium may also be a cAMP-responsive mechanosensor and that molecular mechanisms may be cell type-dependent. However, despite the progress in research into cilia-mediated mechanotransduction in mature bone cells, its mechanism in stem cells remains unknown.

Therefore, the aim of this study was to determine the molecular mechanism of cilia-mediated mechanotransduction in MSCs, which would facilitate the development of novel mechanotherapies to promote early osteogenic signalling in these cells. Here, with the use of custom-built bioreactors, we demonstrate that MSCs utilised cAMP signalling as a second messenger in mechanotransduction, and that this cAMP response is dependent on the primary cilium and the localisation of AC6 to this organelle. Moreover, we demonstrate mechanotherapeutic potential by biochemically targeting cAMP signalling, mimicking the beneficial effect of physical loading, which demonstrates a potential novel anabolic treatment to enhance MSC osteogenesis.

RESULTS

MSCs utilised cAMP signalling as a second messenger in mechanotransduction

To determine whether MSCs utilise cAMP signalling as a second messenger in fluid shear mechanotransduction, we quantified cAMP levels immediately following exposure for 2, 15 and 30 min of OFS. We found that in cells subjected to 15 min of OFS, cellular cAMP levels significantly increased by 2.9-fold compared to no-flow controls ($P<0.05$; Fig. 1A), indicating cAMP as a second messenger in MSC mechanotransduction. This response was temporal, in that no changes were seen following 2 min of OFS and returned to basal levels after 30 min of OFS (Fig. 1A), demonstrating similar profiles to that seen with Ca^{2+} signalling. As no change was seen following either 2 or 30 min of OFS, our remaining experiments focused on investigating the mechanisms involved in flow-induced increases of cAMP after 15 min of OFS.

As cAMP signalling is predominately regulated via adenylyl cyclases, we utilised a general adenylyl cyclase inhibitor, MDL-12,330A hydrochloride (MDL), to investigate whether this OFS-induced increase in cAMP was adenylyl cyclase-dependent. Firstly, we performed a dose response study for 15 min to determine the optimum concentration of MDL that would inhibit adenylyl cyclase activity, but that did not affect basal cAMP levels. MDL at concentrations of 100 and 200 µM resulted in significant decreases in basal cAMP levels (Fig. 1B). A concentration of 10 µM was chosen as it was the lowest concentration of MDL and did not elicit any significant changes in cAMP levels (Fig. 1B). We found that treating MSCs with 10 µM MDL blocked the OFS-induced increases in cAMP ($P<0.05$; Fig. 1C), demonstrating that the OFS-mediated cAMP response was dependent on adenylyl cyclase activity. Thereafter, to investigate whether cAMP second messenger signalling is required for fluid shear-induced MSC osteogenesis, adenylyl cyclase activity was inhibited as above and osteogenic gene expression was analysed following 2 h of OFS. We found that MSCs displayed a positive early osteogenic response when subjected to a regime that has previously been shown to elicit osteogenic responses after 2 h of OFS at 1 Pa, 1 Hz (Stavenschi et al., 2017), where there was a significant increase in the expression of the osteogenic genes *Cox2* and *Opn* ($P<0.05$) and approached significance in *Runx2* ($P=0.072$) when compared to static no-flow conditions (Fig. 1D–F). However, when we blocked cAMP signalling with MDL treatment, this significant increase was lost in all genes analysed (Fig. 1D–F), demonstrating that MSCs utilise cAMP as a second messenger in loading-induced osteogenesis.

MSCs express seven of the nine transmembrane adenylyl cyclase isoforms, including AC6

Adenylyl cyclases are GTP-dependent enzymes that are responsible for the conversion of ATP to cAMP (Hanoune and Defer, 2001). Nine membrane-bound isoforms have been identified, with seven of these, AC2–AC7 and AC9, expressed in mature bone cells (Kwon et al., 2010). To determine what adenylyl cyclase isoforms are expressed in progenitor cells, utilising quantitative real-time PCR

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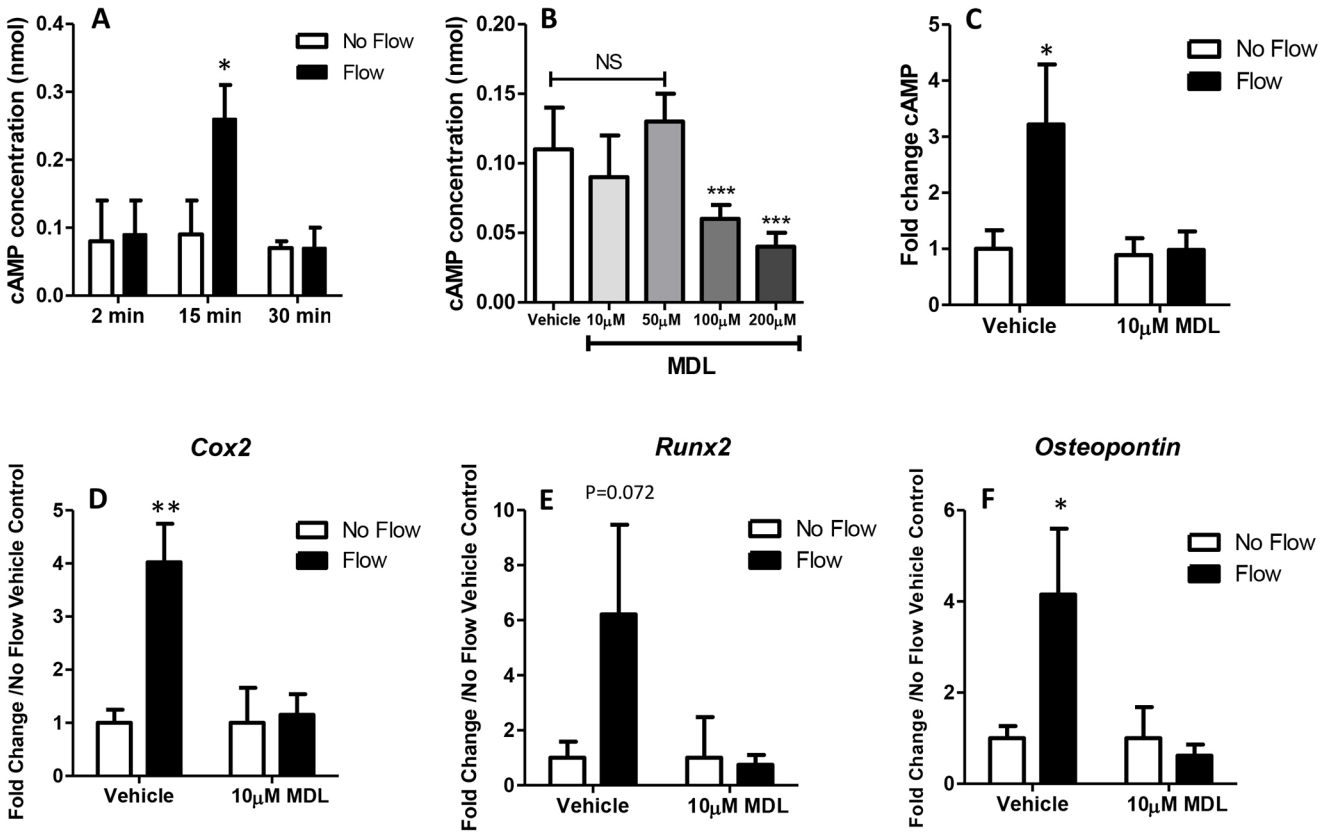

Fig. 1. MSCs utilise cAMP signalling as a second messenger during mechanotransduction. (A) Effect of oscillatory fluid flow over MSCs at 1 Pa, 1 Hz for 2, 15 and 30 min on cAMP concentration (N=3, n=6). (B) Dose-response test of the adenylyl cyclase inhibitor MDL on cAMP concentration in MSCs (N=3, n=6). (C) Effect of 10 µM MDL on cAMP concentration following oscillatory fluid flow at 1 Pa, 1 Hz for 15 min (N=3, n=9). (D–F) Effect of MDL on *Cox2* (D), *Runx2* (E) and *Opn* (F) gene expression following oscillatory fluid flow at 1 Pa, 1 Hz for two hours (N=2, n=6), compared to no-flow vehicle control. Statistical tests: unpaired two-tailed Student's *t*-test with Wilcoxon correction (A), one-way ANVOA with Bonferroni post-test (B), and two-way ANOVA with Bonferroni post-tests (C–F). Values are means±s.e.m. for three independent replicates. *P<0.05, **P<0.01, ***P<0.001; NS, not significant.

(qRT-PCR), we found that there are also seven isoforms present in MSCs, but interestingly, the expression pattern is different to that seen in osteocytes. Here, we found that the genes *Ac1*, *Ac3*, *Ac4* and *Ac6–Ac9* are expressed, whereas *Ac2* and *Ac5* were not detected (Fig. 2A). Interestingly, of the seven isoforms present, *Ac6* was one of the more abundantly expressed and has previously be shown to be critical for loading-induced bone formation (Lee et al., 2014). To investigate AC6 further, we examined AC6 protein expression and spatial organisation using immunocytochemistry and found that AC6 is expressed across the MSC cell membrane (Fig. 2B). No staining was found when AC6 primary antibody was withheld (Fig. S1).

Adenylyl cyclase 6 is required for fluid shear-induced cAMP signalling in MSCs

To investigate the role of AC6 in flow-mediated changes in cAMP signalling, we depleted AC6 in MSCs using small-interfering RNA (siRNA). *Ac6* gene expression levels, as quantified by qRT-PCR,

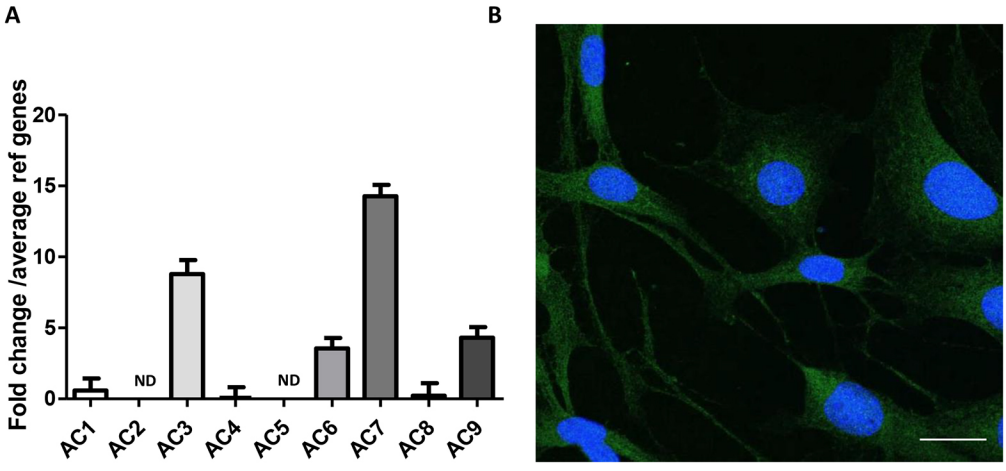

Fig. 2. MSCs express seven of the nine transmembrane adenylyl cyclase isoforms, including AC6. (A) Mean±s.e.m. relative gene expression of adenylyl cyclases *Ac1–Ac9* in MSCs, quantified by qRT-PCR. *Ac3*, *Ac6*, *Ac7* and *Ac9* show the highest expression of the nine adenylyl cyclases analysed. *Ac2* and *Ac5* were not detected (n=3). (B) Verification of AC6 protein expression in MSC with the use of immunocytochemistry. Cells were stained for AC6 (green) and nuclei counterstained with DAPI (blue). Scale bar: 20 µm. ND, not detected.

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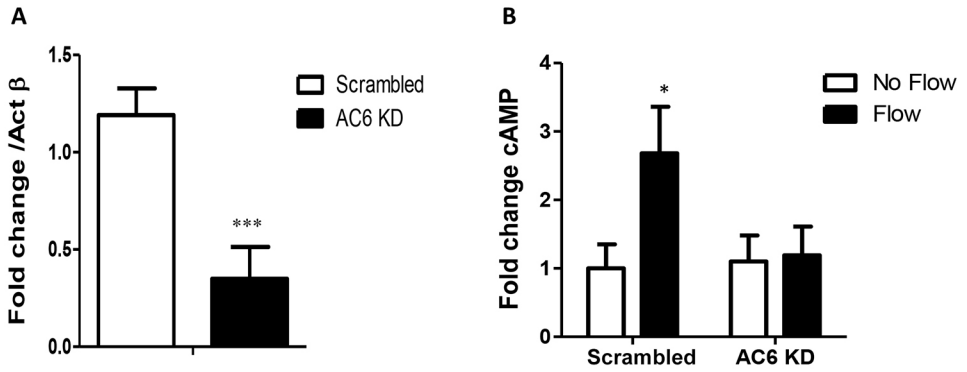

Fig. 3. AC6 is required for fluid shear-induced cAMP signalling in MSCs. (A) *Ac6* expression was successfully knocked down in MSCs using siRNA technology, as verified by qRT-PCR, normalised to *Actβ* expression and compared to scrambled siRNA control (N=3, n=13). (B) Effect of oscillatory fluid flow over MSCs treated with scrambled and *Ac6* siRNA at 1 Pa, 1 Hz for 15 min on cAMP concentration (N=3, n=9), compared to scrambled no-flow control. Statistical tests: unpaired two-tailed Student's *t*-test (A) and two-way ANOVA with Bonferroni post-test (B). Values are means±s.e.m. for a minimum of three independent replicates. *P<0.05, ***P<0.001.

were 75% lower in MSCs treated with *Ac6* siRNA when compared to scrambled siRNA controls (P<0.001; Fig. 3A). MSCs treated with either off-target scrambled or *Ac6* siRNA were subjected to 15 min of OFS to assess the role of AC6 in OFS activation of cAMP signalling. When exposed to flow, MSCs treated with scrambled siRNA exhibited a 2.6-fold increase in cAMP signalling (P<0.05; Fig. 3B), demonstrating no effect of the transfection treatment. However, upon depletion of AC6 no change in cAMP activity was found in MSCs following shear (Fig. 3B), demonstrating the specific role of AC6 in regulating mechanically activated cAMP signalling.

AC6 is required for fluid shear-induced osteogenic gene expression in MSCs

Given the demonstrated role of AC6 in fluid shear-induced increases in cAMP signalling, we next investigated whether AC6 also plays a role in downstream osteogenesis. MSCs treated with either scrambled or *Ac6* siRNA as above were subjected to OFS at 1 Pa, 1 Hz as previously described (Stavenschi et al., 2017). Expression of *Cox2*, *Runx2* and *Opn* in the no flow control group were unaffected by the *Ac6* siRNA treatment, demonstrating that AC6 activity does not significantly influence basal osteogenic gene expression (Fig. 4). Furthermore, analysis of osteogenic gene expression in MSCs treated with scrambled siRNA and exposed to flow, resulted in significant 4-, 3.16- and 3.60-fold increases in *Cox2*, *Runx2* and *Opn* mRNA levels, respectively, compared to scrambled siRNA no-flow controls (P<0.05; Fig. 4). However, similar to that seen with cAMP signalling, depletion of AC6 resulted in a complete loss of the fluid shear-induced increases in osteogenic gene expression (Fig. 4), demonstrating that AC6 is a critical component of MSC mechanotransduction through regulation of cAMP second messenger signalling.

AC6 is localised to the microdomain of the primary cilium in MSCs

Given the previously demonstrated role of the primary cilium in MSC mechanotransduction and spatial organisation of adenylyl cyclases at the primary cilium in other cell types, we next examined whether AC6, responsible for the flow-mediated changes in cAMP and gene expression, is localised to the primary cilium in MSCs. Utilising immunocytochemistry, MSCs were co-immunostained for AC6 and acetylated α-tubulin, which is enriched within the primary cilium. Primary cilia were identified in the perinuclear region of the cell, extending as rod-like structures on the apical surface. As previously demonstrated (Kwon et al., 2010), AC6 was found throughout the cell membrane but upon co-staining for acetylated α-tubulin, a distinct spatial organisation was revealed within the primary cilium, with intense staining found along the ciliary

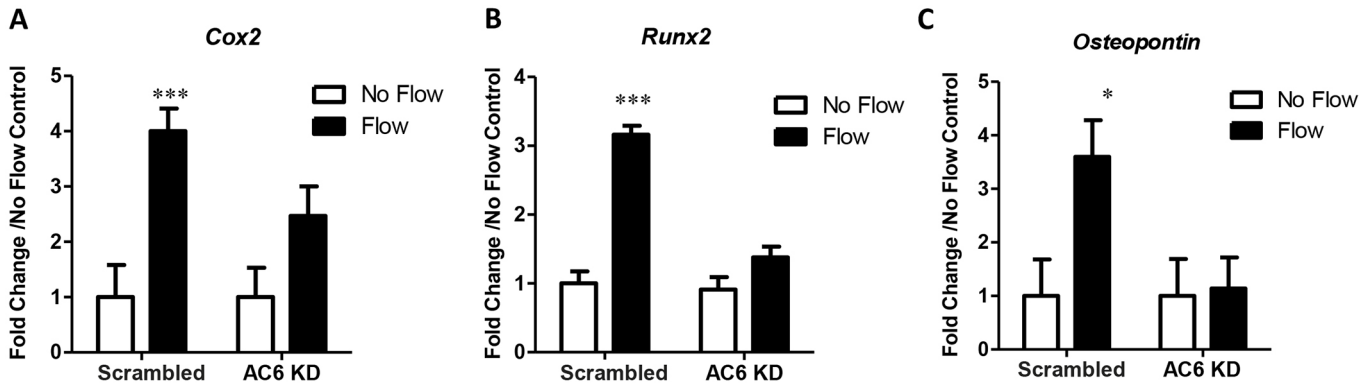

Fig. 4. Adenylyl cyclase 6 is required for fluid shear-induced osteogenic gene expression in MSCs. (A–C) Effect of oscillatory fluid flow at 1 Pa, 1 Hz on expression of osteogenic genes *Cox2* (A), *Runx2* (B) and *Opn* (C) after 2 h (N=4, n=9–12), as determined by qRT-PCR. All groups are compared to no-flow scrambled control. Statistical test: two-way ANOVA with Bonferroni post-hoc. Values are means±s.e.m. for a minimum of three independent replicates. *P<0.05, ***P<0.001.

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microdomain (Fig. 5). This therefore indicates that cilia-mediated MSC mechanotransduction may be dependent on AC6 and its regulation of cAMP signalling. No staining was detected when AC6 primary antibody was withheld (Fig. S2).

Primary cilium-mediated mechanotransduction in MSCs is cAMP dependent

The primary cilium has previously been shown to be required for stem cell mechanotransduction, specifically with regards to fluid shear-induced increases in osteogenic gene expression (Hoey et al., 2012b). Therefore, given the localisation of AC6 to the primary cilium compartment and the demonstrated role of cAMP in MSC mechanotransduction, the role of the primary cilium in fluid shear-induced cAMP signalling was then investigated. The formation of cilia was inhibited through the utilisation of siRNA targeting *Ift88*, which is a principal motor protein required for ciliogenesis. The transfection resulted in significantly diminished *Ift88* mRNA expression, which in turn significantly reduced the incidence of primary cilia by 74% as demonstrated by immunocytochemistry (P<0.05; Fig. 6A–D). MSCs transfected with siRNA targeting *Ift88* or scrambled siRNA were subjected to OFS, and cAMP signalling was analysed as above. The scrambled siRNA treatment did not affect basal or fluid shear-induced increases in cAMP in MSCs following fluid shear (3.74-fold, P<0.05; Fig. 6E). However, upon inhibition of primary cilia, the fluid shear-induced increase in cAMP was lost (Fig. 6E), mirroring that with AC6 depletion. This therefore demonstrates that the cilium is a cAMP-responsive mechanosensor in MSCs, which potentially utilises AC6 to mediate this second messenger and downstream osteogenic response, demonstrating a novel molecular mechanism of cilia-mediated mechanotransduction in MSCs.

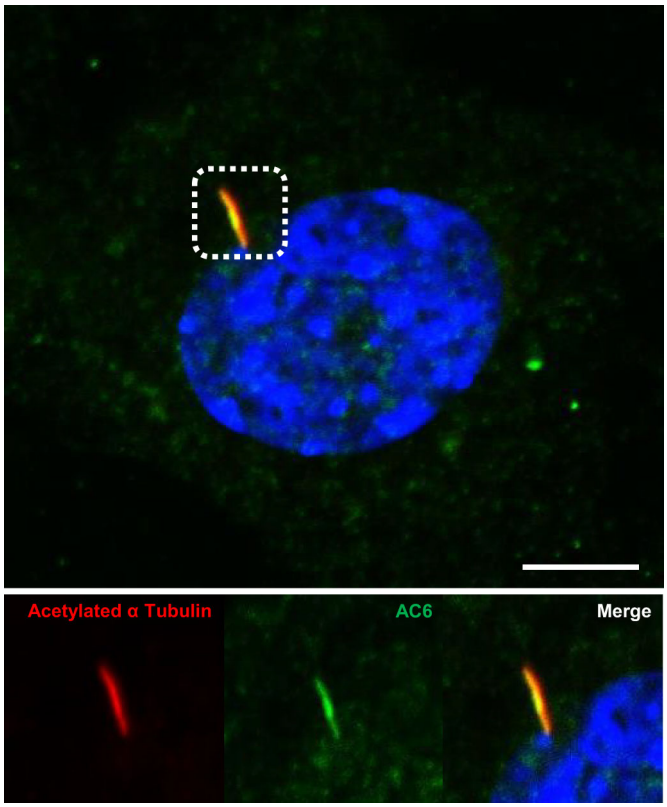

Fig. 5. AC6 is localised to the primary cilium in MSCs. AC6 (green) and acetylated α-tubulin (red) co-localisation to the primary cilia (identified as linear structures enriched in acetylated α-tubulin). Nuclei are counterstained with DAPI (blue). N=3, n=77. Scale bar: 5 µm.

cAMP signalling can be biochemically activated with an adenylyl cyclase activator, demonstrating artificial mechanotransduction and mechanotherapeutic potential

Next, we wanted to investigate whether the biochemical activation of this novel MSC mechanotransduction mechanism via adenylyl cyclases could mimic the observed cAMP signalling responses to OFS. A dose-response study was carried out to determine whether the adenylyl cyclase activator, forskolin (FSK), could biochemically mimic the effects of OFS on cAMP signalling. We found that 15 min of treatment with 0.01 mM and 0.001 mM of FSK induced 25- and 12-fold increases in cAMP levels, respectively (P<0.001; Fig. 7A), which were considerably larger increases than that seen following OFS. However, cAMP levels increased 2.5-fold compared to the vehicle control in cells treated with 0.1 µM FSK (P<0.05; Fig. 7B). As the aim of this study was to biochemically induce similar increases in cAMP to those seen with fluid shear, cells were treated with 0.1 µM FSK for further studies. Importantly, 0.1 µM FSK treatment was not only able to mimic cAMP signalling following OFS, but also that of the osteogenic response, where treatment with 0.1 µM FSK resulted in significant increases in *Cox2*, *Runx2* and *Opn* expression (P<0.05; Fig. 7C–E), to a similar degree to that seen with fluid shear. This result demonstrated artificial mechanotransduction and highlighted FSK as a potential anabolic mechanotherapeutic to enhance MSC osteogenesis. Moreover, given the demonstrated role of AC6 in fluid shear-induced increases in cAMP signalling and osteogenic gene expression, we next investigated whether AC6 also plays a role in FSK-induced increases in osteogenesis. MSCs treated with either scrambled or *Ac6* siRNA, as above, were subjected to 0.1 µM FSK treatment as previously described. The expression of *Cox2* and *Opn* in the vehicle control group showed a trend towards an increase in expression; however, this was not significant (P>0.05; Fig. 7F–H). Furthermore, analysis of osteogenic gene expression in MSCs treated with scrambled siRNA and exposed to FSK resulted in significant 3.19-, 3.43- and 2.59-fold increases in *Cox2*, *Runx2* and *Opn* mRNA levels, respectively, compared to no-treatment controls (P<0.05; Fig. 7F–H). However, similar to the changes seen with cAMP signalling and OFS, depletion of AC6 resulted in a complete loss of the increase in osteogenic gene expression following FSK treatment (Fig. 7F–H), demonstrating that AC6 is a critical component of MSC osteogenesis.

Primary cilia have been shown previously to be required for fluid flow-induced increases in osteogenic gene expression (Hoey et al., 2012b) and this study has demonstrated the role of primary cilia in cAMP signalling. Therefore, we wanted to investigate the role of the primary cilium in FSK-induced osteogenesis. As previously described, the formation of cilia was inhibited with the treatment of siRNA targeting *Ift88*. MSCs transfected with siRNA targeting *Ift88* or scrambled siRNA were subjected to treatment with 0.1 µM FSK, and osteogenic gene expression was analysed as above. The scrambled siRNA treatment did not affect basal or FSK-induced increases in *Cox2*, *Runx2* or *Opn* in MSCs (3.14-, 3.41- and 2.59-fold, P<0.05; Fig. 7I–K). However, upon inhibition of primary cilium formation, the FSK-induced increase in osteogenic gene expression was lost (Fig. 7I–K), mirroring that with AC6 depletion. This therefore demonstrates that activating osteogenic genes via FSK treatment requires a functional cilium in MSCs.

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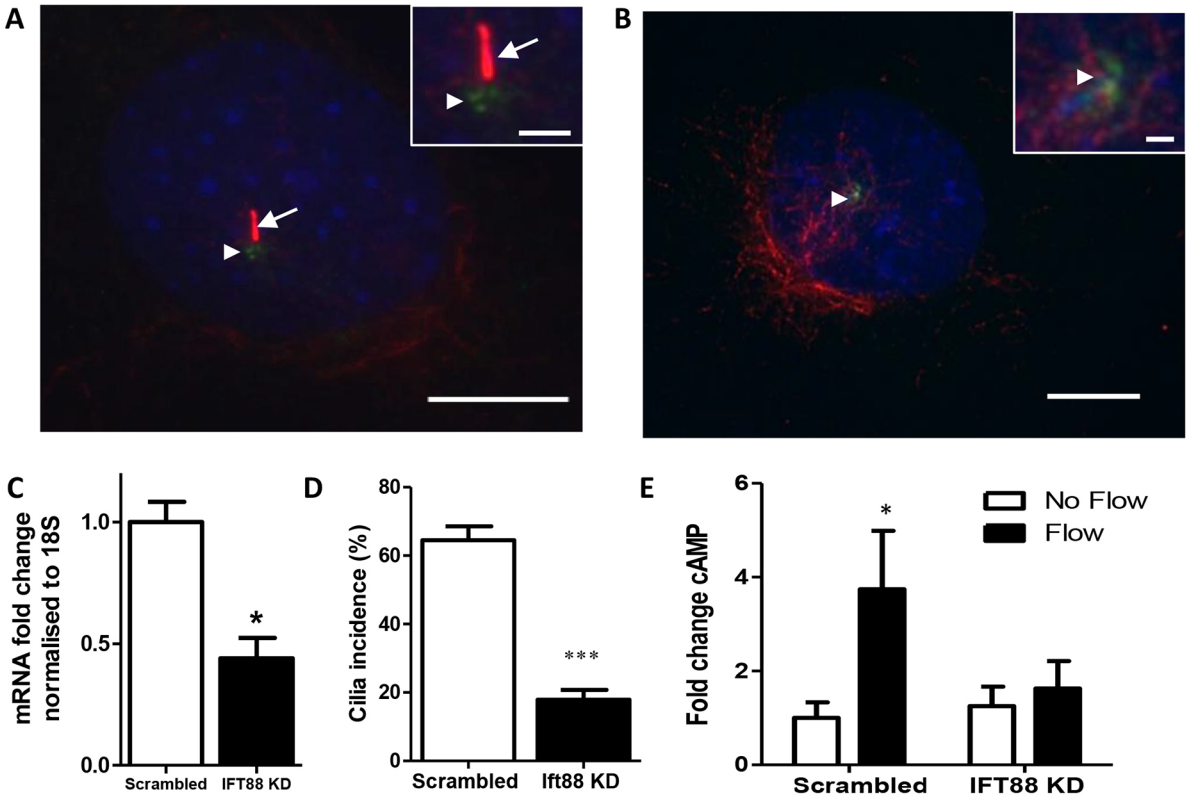

Fig. 6. Primary cilium-mediated mechanotransduction in MSCs is cAMP dependent. (A–C) Intraflagellar transport protein 88 (IFT88) expression was knocked down in MSCs using *Ift88* siRNA as verified by immunostaining (A,B) and qRT-PCR (C) (*n*=4). Cells were treated with either scrambled siRNA (A) or *Ift88* siRNA (B) and stained for primary cilia, identified as linear structures enriched in acetylated α-tubulin, (red; arrows) and centrioles (green; arrowhead). Nuclei are counterstained with DAPI (blue). Scale bars: 5 µm and 1 µm (insert). (D) Effect of scrambled and *Ift88* siRNA treatment on cilia incidence (*N*=4, *n*=143). (E) Effect of oscillatory fluid flow over MSCs treated with scrambled and *Ift88* siRNA at 1 Pa, 1 Hz for 15 min on cAMP concentration (*N*=3, *n*=9). Statistical tests: unpaired two-tailed Student's *t*-test with Wilcoxon correction (C,D) and two-way ANOVA with Bonferroni post-hoc (E). Values are means±s.e.m. for three independent replicates. **P*<0.05, ****P*<0.001.

DISCUSSION

Mechanical loading is a potent stimulus of bone adaptation, requiring the replenishment of the osteoblast from a progenitor population. One such progenitor is the MSC, which undergoes osteogenic differentiation in response to OFS (Stavenschi et al., 2017). Yet, the mechanism mediating stem cell mechanotransduction, and thus the potential to target this therapeutically, remains poorly understood. In this study, we demonstrate that MSCs utilise cAMP as a second messenger in mechanotransduction, required for flow-mediated increases in early osteogenic gene expression. Furthermore, we show that this mechanosignalling is dependent on the primary cilium and the ciliary localised adenylyl cyclase 6. Importantly, we also demonstrate that this mechanotransduction mechanism can be targeted therapeutically to enhance early osteogenic signalling in MSCs, mimicking the beneficial effect of physical loading. Our findings therefore demonstrate a novel mechanism of MSC mechanotransduction that can be targeted therapeutically, demonstrating a potential mechanotherapeutic for bone loss diseases such as osteoporosis.

MSCs utilise cAMP as a second messenger to transduce a biophysical stimulus into a biochemical osteogenic response. By mimicking the marrow mechanical environment via application of OFS, MSCs were shown to rapidly upregulate intracellular cAMP levels 2.6-fold over static controls. This rapid increase in cAMP is in agreement with work carried out in osteoblasts, where there was an increase in cAMP not only over time, but also over increasing shear magnitudes, demonstrating mechanosensitivity (Reich et al., 1990), and demonstrates the utilisation of cAMP signalling as an important second messenger in the skeleton. Interestingly, when this cAMP response was inhibited, MSCs exhibited a loss of flow-induced increases in expression of osteogenic genes, *Cox2*, *Runx2* and *Opn*, suggesting that the initial increase in cAMP is a critical component of early loading-induced osteogenic signalling. Mechanical loading-induced osteogenesis has previously been linked to both cAMP and Ca^{2+} signalling (Thompson et al., 2012; Liu et al., 2015; Reich et al., 1990). However, we recently demonstrated that a complete loss of Ca^{2+} signalling following TRPV4 inhibition only partially blunted the osteogenic response to fluid shear in MSC (Corrigan et al., 2018). Given the complete loss of early osteogenic gene responses following cAMP inhibition in this study, this may suggest a more dominant role for cAMP signalling as a second messenger in MSC mechanotransduction. This alternative mechanism of cAMP signalling agrees with previous work in bone MSCs (BMSCs), where phosphate promoted osteogenesis via a ATP-induced cAMP/PKA pathway (Wang et al., 2016). Further to this, work *in vivo* has shown that activation of the cAMP pathway results in robust increases in bone formation in transplanted hMSCs (Siddappa et al., 2008). Our findings therefore support a role for cyclic adenosine monophosphate as an important messenger in MSC mechanotransduction and osteogenesis.

Adenylyl cyclase 6 is required for OFS-induced increases in cAMP and osteogenic gene expression. There is a growing body of evidence detailing the expression patterns of adenylyl cyclases within lineage-committed cells, whereas the isoforms that are

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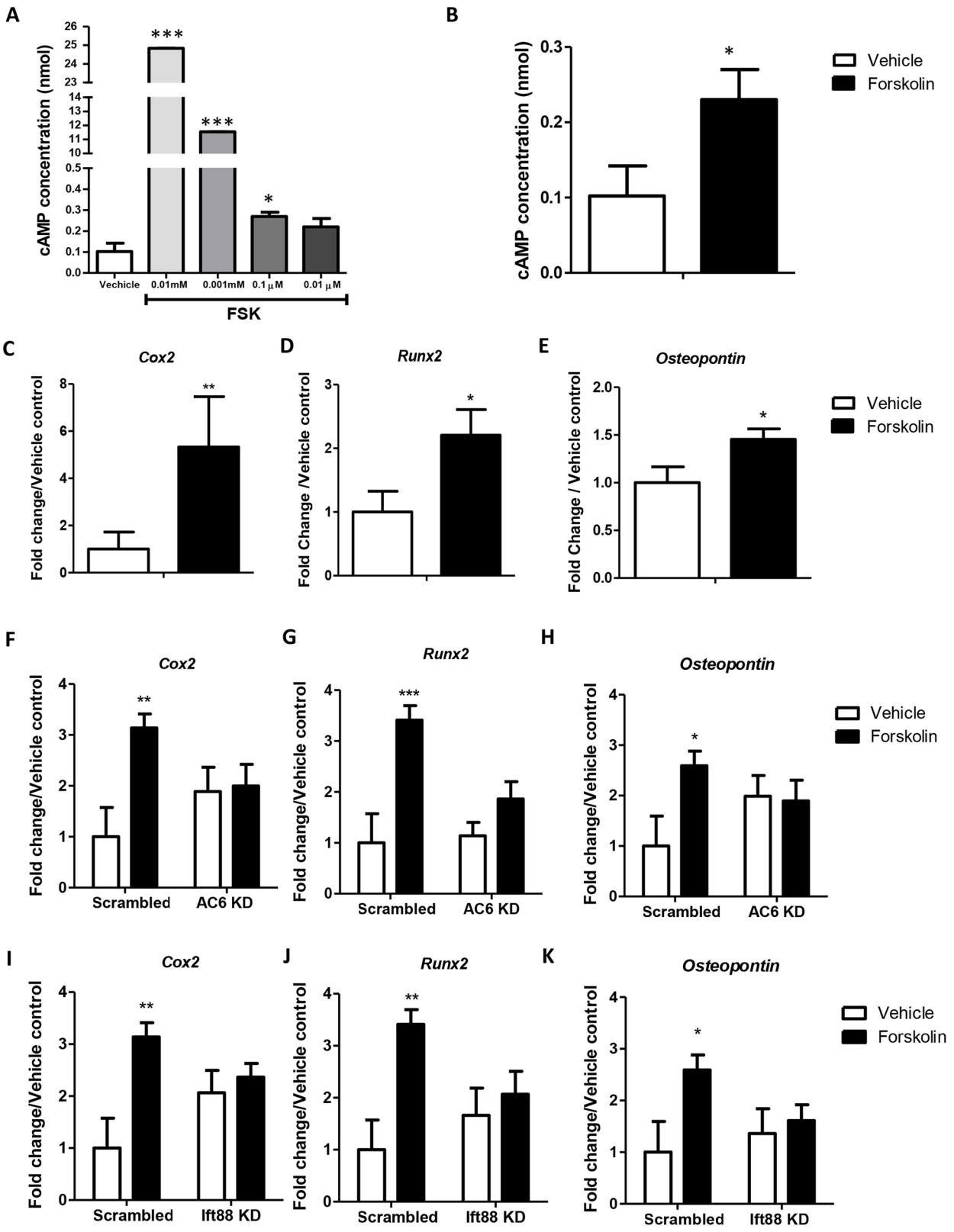

Fig. 7. Cyclic AMP signalling can be biochemically activated with an adenylyl cyclase activator demonstrating artificial mechanotransduction. (A,B) Effect of forskolin (FSK) treatment on cAMP concentration in MSCs (N=3, n=6). (C–E) Effect of forskolin treatment on expression of osteogenic genes *Cox2* (C), *Runx2* (D) and *Opn* (E) (N=3, n=9). (F–H) AC6 is required for forskolin-induced increases in expression of osteogenic genes *Cox2* (F), *Runx2* (G) and *Opn* (H) in MSCs after 2 h (N=2, n=6). (I–K) IFT88 is required for forskolin-induced increases in expression of osteogenic genes *Cox2* (I), *Runx2* (J) and *Opn* (K) in MSCs after 2 h (N=2, n=6). DMSO concentration for shown is 0.01%. Statistical tests: unpaired two-tailed Student's *t*-test with Wilcoxon correction (A–E), two-way ANOVA with Bonferroni post-hoc (F–K). Values are means±s.e.m. for three independent replicates. *P<0.05, **P<0.01, ***P<0.001.

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expressed within progenitors have not been established. Using qRT-PCR, MSCs were found to express only seven of the nine adenylyl cyclase isoforms, which is similar to the number seen in the differentiated osteocyte (Kwon et al., 2010). In our study, MSCs are shown to express *Ac1*, *Ac3*, *Ac4* and *Ac6–Ac9*, while *Ac2* and *Ac5* were not detected. This contrasts with osteocytes, which do not express *Ac1* or *Ac8* (Kwon et al., 2010). These differences indicate a preferential change in adenylyl cyclase expression with lineage commitment. Of the four highly expressed adenylyl cyclases in MSCs (*Ac3*, *Ac6*, *Ac7* and *Ac9*), *Ac6* has been shown to have an important role in bone mechanoadaptation both *in vitro* and *in vivo* (Kwon et al., 2010; Lee et al., 2014), while *Ac3*, *Ac7* and *Ac9* have predominately been associated with the brain (Kamenetsky et al., 2006). Therefore, we focused specifically on AC6 within this study. Lee et al. (2014) showed that mice with a global knockout of AC6 had no skeletal phenotype abnormalities; however, they exhibited diminished loading-induced bone formation, demonstrating a critical role for *Ac6* in skeletal mechanoadaptation. Additionally, primary mature bone cells from these mice had an attenuated fluid shear-induced increase in *Cox2* mRNA expression compared to controls. However, it is currently unclear if AC6 plays a role in MSC mechanotransduction. Therefore, we further investigated the expression pattern of AC6 specifically in MSCs through the use of immunocytochemistry, and AC6 was found to be abundantly expressed throughout the plasma membrane of MSCs. Knocking down AC6 expression in MSCs did not affect basal cAMP levels but abolished the increase in cAMP signalling in response to fluid shear, demonstrating a similar mechanotransduction-specific role as in the AC6 knockout animal. This trend was also evident when examining early osteogenic signalling in MSCs, suggesting the defect in loading-induced bone formation *in vivo* may be attributable to a defect in mechanotransduction in many cell types along the osteogenic lineage. Interestingly, the complete loss of the cAMP and early osteogenic responses to OFS following *Ac6* knock down mirrors that seen following generic adenylyl cyclase inhibition, indicating that AC6 is the dominant adenylyl cyclase mediating MSC mechanotransduction. Hence, our data demonstrates a novel role for AC6 in MSC mechanotransduction, mediating fluid shear-induced cAMP second messenger signalling and downstream early osteogenesis.

The primary cilium, an organelle required for fluid shear-induced MSC osteogenesis *in vitro* and *in vivo* (Hoey et al., 2012b; Chen et al., 2016a), localises AC6 and is required for fluid shear-induced cAMP signalling. Recently, it has been shown that several adenylyl cyclases localise to primary cilia in numerous cell types. Most notably, AC6 is localised to the primary cilium in osteocytes (Kwon et al., 2010). Furthermore, mouse embryonic fibroblasts and IMCD3s have elevated basal ciliary cAMP levels as a result of AC5 and/or AC6 localised activity, indicating a link between AC6, cAMP and the primary cilium (Moore et al., 2016), leading us to further investigate the role of AC6 in the previously demonstrated primary cilia-mediated MSC mechanotransduction. In this study, we show that AC6 preferentially localises to the primary cilium. This specific localisation to an area of high strain may potentially increase the mechanosensitivity of the cell. Furthermore, due to the discrete microdomain of the cilium and the specific localisation of a plethora of signalling molecules, localisation of AC6 to this signalling centre may amplify and enhance the rate of AC6-mediated mechanosignalling. Interestingly, in MSCs which do not possess primary cilia, fluid shear-induced increases in cAMP are lost, which is consistent with AC6 knockdown. This therefore suggests that the localisation of AC6 to the cilium is functionally significant and demonstrates a potential Ca^{2+}-independent mechanism of cilia-based mechanotransduction (Malone et al., 2007; Delling et al., 2016). In summary, primary cilia mediate MSC mechanotransduction via cAMP signalling that is likely dependent on AC6 localisation to the ciliary microdomain, demonstrating a novel molecular mechanism of cilia-mediated MSC mechanotransduction.

Biochemical activation of adenylyl cyclases elicits a cAMP second messenger and early osteogenic gene response that mirrors that seen with OFS in MSCs. The adenylyl cyclase agonist forskolin, a diterpene that stimulates cAMP production by directly activating adenylyl cyclases (Lorenzo et al., 1986), induced a cAMP response in MSCs similar to that elicited by OFS. Furthermore, forskolin induced an early osteogenic response in terms of *Cox2*, *Runx2* and *Opn* expression, as seen following OFS, revealing itself as a potential novel therapeutic that mimics the beneficial effect of mechanical loading. This potential therapeutic role for forskolin is consistent with previous findings where cAMP activation enhanced bone formation of transplanted MSCs *in vivo*, where both 8-bromo-cAMP and forskolin resulted in increased bone formation in pre-treated MSCs (Doorn et al., 2012). Additionally, direct activation of PKA with cAMP and forskolin in rat and human osteoblast-like cell lines led to activation of the osteocalcin promoter (Boguslawski et al., 2000). Ablation of AC6 in MSCs was found to inhibit the forskolin-induced increases in osteogenic gene expression, although this was somewhat surprising as forskolin is recognised as a broad-spectrum adenylyl cyclase agonist. Similar results have been found in vascular smooth muscle cells (VSMCs) (Gros et al. (2006) demonstrated that the downstream consequences of cAMP signalling are not common to all isoforms of adenylyl cyclases in VSMCs, with siRNA directed at *Ac6* sufficient to reduce forskolin-activated adenylyl cyclase activity. Moreover, knockdown of *Ac6* alone could inhibit the forskolin-induced increases in cAMP, synthesis and arborisation observed in control cells and cells overexpressing AC6 (Gros et al., 2006). Our findings agree with this study in that a knockdown of *Ac6* was sufficient to inhibit the effect of forskolin on MSC early osteogenesis. Similarly, we found that the inhibition of primary cilia formation via siRNA targeting *Ift88* blocked the forskolin-induced increases in *Cox2*, *Runx2* and *Opn* expression. This data suggests that both the primary cilium and the ciliary localised AC6 are required for forskolin-induced alterations in osteogenic gene expression. Together with our observations, this demonstrates that biochemically targeting mechanotransduction mechanisms, such as the one identified in this study, is a potential strategy to enhance early osteogenic signalling in MSCs and bone formation by mimicking the beneficial effects of physical loading.

In summary, this study demonstrates a novel mechanism of MSC mechanotransduction, whereby it was shown that MSCs utilise cAMP as an important second messenger to initiate an early osteogenic response following fluid shear stimulation. This was further explored, and we found that fluid shear-induced cAMP signalling is mediated by AC6 and the primary cilium. These findings add to the growing body of evidence detailing the importance of ciliary-based signalling in MSCs and highlight a Ca^{2+}-independent mechanism of cilia-based mechanotransduction in stem cells. This study highlights this pathway, and the various components within, as potential targets to enhance early osteogenic signalling in MSCs and potential bone formation. Importantly, we have demonstrated proof-of-concept whereby forskolin activated this novel mechanism, eliciting a cAMP and early osteogenic response mirroring that seen with OFS, demonstrating mechanotherapeutic potential and a new anabolic treatment for bone loss diseases such as osteoporosis.

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MATERIALS AND METHODS

Mesenchymal stem cell culture

The murine MSC line C3H10T1/2 was obtained from ATCC (LGC Standards, Teddington, Middlesex, UK). MSCs were tested to confirm lack of mycoplasma contamination with a MycoAlert PLUS detection kit (LT07, Brennan & Co., Dublin, Ireland). MSCs were maintained in Dulbecco's modified Eagle's medium (DMEM; D6046, Sigma-Aldrich) with low glucose (Sigma-Aldrich, Arklow, Ireland) supplemented with 10% fetal bovine serum (FBS; South American origin, Labtech International, Heathfield, East Sussex, UK) and 1% penicillin streptomycin (P/S; P4333, Sigma-Aldrich).

Mechanical stimulation

Oscillatory fluid flow-induced shear stress was applied to MSCs with the use of in-house-designed parallel plate flow chambers as described previously (Stavenschi et al., 2017). MSCs were seeded on fibronectin-coated (10 µg/ml) glass slides, assembled between two plates and attached to a programmable syringe pump (New Era Pump Systems, Farmingdale, NY, USA). Oscillatory fluid shear was applied through a 10 ml syringe (BD Plastipak, VWR, Dublin, Ireland) at 52.5 ml/min and 1 Hz frequency, subjecting cells to a shear stress of 1 Pa. This stimulus was applied for 2 h. The no-flow control setup takes into account the confined environment experienced by the cells in the chambers, consisting of glass slides prepared in the same way and similarly remaining in the chambers for 2 h, but not attached to the syringe pump.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Immediately after treatment, TRI reagent (93289, Sigma-Aldrich) was used to extract RNA per the manufacturer's protocol. The concentration of RNA in each sample was measured using a Nanodrop spectrophotometer and sample purity was checked via 260/280 and 260/230 absorbance ratios. 400 ng of RNA was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Commercially available primers for *18s*, *Cox2*, *Runx2*, *Opn*, *Ac1–Ac9* and *Ift88* were acquired for analysis of flow and forskolin-mediated osteogenic changes and validation of knockdowns (KSPQ12012G, Sigma-Aldrich, St Louis, MO, USA). Quantitative RT-PCR was performed using a 20 µl reaction mix containing 10 µl SYBR Green PCR MasterMix (Invitrogen, Paisley, UK), 0.8 µl of each primer, and 8.4 µl dH2O and sample mix. In the case of *Runx2* and *Ac1–Ac9*, 0.6 µl of each primer was used. Plates were run in an ABI 7500 Fast PCR system (Life Technologies, Carlsbad, CA, USA). The cycle parameters were as follows: Uracil N-glycosylase (UNG) activation was run for 2 min at 50°C, DNA polymerase activation for 10 min at 95°C, the melt cycle was run for 15 s at 95°C and the annealing–extending cycle for 1 min at 60°C for *Cox2*, *Runx2*, *Opn* and *Ift88*, apart from *18s* and *Ac1–Ac9*, which were run at 65°C. A no-template control (NTC) was run in each 96-well plate to confirm the absence of contamination.

For AC6 knockdown and related oscillatory fluid shear (OFS) experiments, qRT-PCR reactions were prepared using TaqMan Universal PCR Mix (4304437, Applied Biosystems) with ROX passive dye and a pre-designed TaqMan Gene Expression Assay (4331182, Applied Biosystems) for amplification using the ABI 7500 real-time PCR machine (Applied Biosystems). The relative quantity of each sample was calculated with reference to *18s* and expressed as fold change normalised to the no-flow control group. For validation of AC6 knockdown, the relative quantity of *AC6* was calculated with reference to *Actβ*. All primer sequences and concentrations are listed in Table S1.

Quantification of cAMP signalling

Cyclic AMP activity was analysed using the commercially available Cyclic AMP XP Assay Kit (Cell Signaling Technology, Danvers, MA, USA). Following OFS or treatment with the adenylyl cyclase inhibitor or activator, cells were rinsed with ice-cold phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO, USA) and lysed on ice with 200 µl RLT lysis buffer containing phenylmethylsulfonyl fluoride (PMSF; 1:200; Sigma-Aldrich). Experimental samples (50 µl) were loaded onto the anti-cAMP XP rabbit mAb-coated plate, with 50 µl HRP-linked cAMP solution and incubated on an orbital plate shaker for 3 h at room temperature. Wells were washed with 200 µl 1× wash buffer four times, before incubating with 100 µl 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for 30 min at room temperature. After 30 min, 100 µl stop solution was added. Plate absorbance was read at 450 nm. All samples including the standard curve were run in triplicate.

Immunocytochemistry

MSCs were seeded on fibronectin-coated glass coverslips for 24 h before serum starvation in DMEM low glucose, 0.5% FBS, 1% P/S for 48 h. After fixation in neutral buffered formalin for 10 min (Sigma-Aldrich), coverslips were permeabilised in 0.1% Triton X-100 and non-specific binding sites were blocked using 1% w/v BSA (Sigma-Aldrich) in PBS for 2 h at room temperature. The primary antibody targeting the primary cilium (anti-acetylated α-tubulin, ab24610, Abcam, Cambridge, UK) was applied overnight at 4°C, diluted 1:1500. Next, primary antibody targeting AC6 (anti-AC6, ab14781, Abcam) was applied for 1 h at room temperature at a dilution of 1:500. AlexaFluor 594 and AlexaFluor 488 secondary antibodies (A21203, A11008, Life Technologies) were applied in tandem for 1 h at room temperature at 1:500. Finally, DAPI (32670, Sigma-Aldrich) was applied for 5 min in PBS prior to sample mounting on glass slides using Prolong gold mounting medium (P36934, Invitrogen). For cilium incidence and length studies, the primary antibody for centrioles (anti-pericentrin, ab4448, Abcam) was used in conjunction with acetylated α-tubulin to clearly identify the base of the cilium, at a dilution of 1:1000. Imaging was performed on an Olympus IX83 epifluorescent microscope with a 100 W halogen lamp at 100× (NA 1.40 Oil) or the Leica SP7 (Leica Microsystems, Wetzlar, Germany) scanning confocal microscope at 63× (NA 1.40 Oil). Controls with an absence of primary antibody were used to test for non-specific binding and background staining of the secondary antibodies.

IFT88, primary cilium and AC6 knockdown

The formation of functional primary cilia and expression of adenylyl cyclase 6 were inhibited by siRNA-mediated depletion of IFT88, an intraflagellar transport protein (IFT) required for functional ciliogenesis, and AC6, respectively. Lipofectamine RNAiMAX (Invitrogen) was diluted 1:135 in OptiMEM reduced serum transfection medium (Gibco, Foster City, CA, USA). For IFT88 knockdown this was mixed 1:1 with predesigned Stealth RNAi targeting *Ift88* (MSS211714, Invitrogen) at a dilution of 16.7 µM in OptiMEM and incubated at room temperature for 15 min before application. For AC6 knockdown, lipofectamine was mixed 1:1 with Silencer Select Ambion siRNA (4390825, Bio-Sciences, Dublin, Ireland) at a dilution of 60 pmol for 5 min. 550 µl of the reaction mix was added to cells and incubated for 8 h at 37°C, following which 10 ml DMEM (0.5% FBS, 1% P/S) was added to the cells for 24 h. The off-target controls were Stealth RNAi Negative Control, Medium GC (12935300, Invitrogen) and Silencer Negative Control (AM4641, Thermo-Fisher), respectively. For IFT88 knockdown studies, additional medium was added to each transfection after 24 h. Forty-eight hours following transfection the transfected cells were seeded for experimentation in DMEM (0.5% FBS, 1% P/S). Transfection efficiency was verified 72 h after transfection by qRT-PCR and immunocytochemistry as described above. The percentage of ciliated cells was determined and cilia length analysed by epifluorescence microscopy using an Olympus IX83 fitted with 100× objective. Cilia lengths were measured using ImageJ freeware.

Biochemical inhibition and activation of cAMP signalling

Cyclic AMP signalling was diminished through the inhibition of adenylyl cyclase activity using MDL-12,330A hydrochloride (MDL; M182, Sigma-Aldrich), as previously described (Zhang et al., 2010). A dose-response study was performed for 15 min to determine the optimum concentration of MDL that would inhibit adenylyl cyclase activity but does not affect basal cAMP levels. Cells were treated for 15 min to replicate OFS conditions for cAMP. Following this, 10 µM was chosen to be the optimal dose and therefore was used for further studies investigating the role of adenylyl cyclase activity and cAMP signalling in MSC mechanotransduction. MSCs were treated with MDL-supplemented medium throughout application of the 1 Pa mechanical stimulation, and controls were incubated for the same time with vehicle (H_2O)-treated medium for both cAMP and osteogenic gene expression studies.

To modulate cyclic AMP signalling via activation of adenylyl cyclase, the general adenylyl cyclase agonist forskolin (FSK; F3917, Sigma-Aldrich) was utilised (Seamon et al., 1981; Doorn et al., 2012). Dose-response studies were also carried out, where cells were treated with increasing doses of FSK for 15 min. As with MDL treatment, cells were treated with FSK-supplemented medium for 15 min, and controls were incubated for the same time with vehicle (0.01% DMSO)-treated medium, for both cAMP and osteogenic gene expression studies. Media was supplemented with 1 µM 3-isobutyl-1-methylxanthine (IBMX) for all cAMP studies.

Data analysis

The relative expression of each gene was normalised to expression of *18s* and the results expressed as mean±s.e.m. fold-change gene expression relative to the no-flow scrambled or vehicle control group. For the OFS and forskolin with *AC6* or *Ift88* knockdown studies, a two-way ANOVA analysis was performed with Bonferroni correction post-hoc tests. Dose-response studies for MDL and FSK were analysed using a one-way ANOVA, with Bonferroni post-hoc tests. All other analysis was performed using two-tailed unpaired Student's *t*-test with Wilcoxon correction. All data were analysed using GraphPad Prism 5. Only primers with PCR efficiencies between 90% and 110% were used. In all experiments, $P<0.05$ was considered statistically significant. A power analysis was conducted to determine adequate sample sizes for all experiments (G.Power 3.1.9.2, Dusseldorf). Technical replicates are represented as *N*, while biological replicates are represented as *n*.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualisation: G.P.J., D.A.H.; Methodology: G.P.J., D.A.H.; Software: G.P.J.; Validation: G.P.J.; Formal analysis: G.P.J., E.S.; Investigation: G.P.J., E.S., K.F.E., M.A.C.; Resources: D.A.H., S.F.; Data curation: D.A.H., S.F., E.S., K.F.E., M.A.C.; Writing - original draft: G.P.J., D.A.H.; Writing - review & editing: G.P.J., S.F., D.A.H.; Visualisation: G.P.J., D.A.H.; Supervision: S.F., D.A.H.; Project administration: D.A.H.; Funding acquisition: S.F., D.A.H.

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Supplementary information

Supplementary information available online at
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