Mesenchymal stem cell mechanotransduction is cAMP dependent and regulated by Adenylyl Cyclase 6 and the Primary Cilium

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Running title
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Abbreviations and symbols

MSC: Mesenchymal Stem Cell
OFS: Oscillatory Fluid Shear
AC: Adenylyl Cyclase
AC6: Adenylyl Cyclase 6
cAMP: Cyclic Adenosine 3′,5′-monophosphate
MDL: MDL-12,330A hydrochloride
FSK: Forskolin
Cox2: Cyclo-oxygenase 2
Opn: Osteopontin
Runx2: Runt-related Transcription Factor 2
qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction
Pa: Pascal
Hz: Hertz
siRNA: Small interfering RNA
Abstract

Mechanical loading is a potent stimulus regulating bone adaptation, that requires the replenishment of the osteoblast from a progenitor population. One such progenitor is the mesenchymal stem cell which undergoes osteogenic differentiation in response to oscillatory fluid shear. Yet, the identification of the mechanism mediating stem cell mechanotransduction, and thus the ability to target this therapeutically, is poorly understood. In this study, we demonstrate that mesenchymal stem cells utilize cAMP as a 2nd messenger in mechanotransduction, that 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. Lastly, 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 which can be targeted therapeutically, demonstrating a potential mechanotherapeutic for bone loss diseases such as osteoporosis.
Introduction

Bone has long been established as a mechanosensitive organ, which 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), Osteopontin (Opn) and Runt-related transcription factor 2 (Runx2) expression at early time points, 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).

Secondary messengers are one of the initiating biochemical components of intracellular signalling cascades triggered by a biophysical stimulus. Intracellular calcium (Ca$^{2+}$) has been the predominate 2nd 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 mesenchymal stem cells have demonstrated intracellular calcium 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 (dos Santos et al.) is another 2nd 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 signaling is biphasic in osteocytes undergoing an initial decrease followed by increase after 30 minutes of OFS (Kwon et al., 2010). A recent study in human MSCs demonstrated that activation of cAMP signaling augmented MSC osteogenesis in an ectopic bone formation model in immune-deficient mice (Siddappa et al., 2008), indicating that MSCs may too utilize cAMP as a potential pre-osteogenic signal, however the role of cAMP as a 2nd messenger in MSC mechanotransduction is poorly understood.

Cyclic AMP is universally generated by adenylyl cyclases (ACs), a family of enzymes that catalyze the cyclization of adenosine triphosphate (ATP) into cAMP (Kamenetsky et al., 2006). Adenylyl cyclase’s comprise of a family of nine distinct transmembrane isoforms (AC1-AC9), where each of the transmembrane ACs 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) 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 extra-cellular signalling (Singla and Reiter, 2006, Lee et al., 2015). Extending into the extracellular milieu, the primary cilium are ideally positioned to relay both biochemical (HH (Singla and Reiter, 2006), TGFβ (Clement et al., 2013, Labour et al., 2016), BMP (Monnich et al., 2018)) and biophysical (fluid shear (Hoey et al., 2012b), compression (Wann et al., 2012), 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, Hoey et al., 2012b), with these findings verified using \textit{in vivo} models (Temiyasathit et al., 2012, Chen et al., 2016b), 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 calcium signalling with initial studies by Praetorius and Spring (Praetorius and Spring, 2001) being verified by advanced cilia localized genetically encoded calcium indicators (Jin et al., 2014, Lee et al., 2015).

Interestingly, work has recently emerged suggesting that primary cilia are not calcium-responsive mechanosensors as previously thought (Delling et al., 2016). Although these discrepancies may be associated to differences in calcium sensors utilized, it has raised the possibility of alternative second messengers in cilia mechanotransduction, namely cAMP. Previous work has demonstrated that 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 compartmentalized cAMP is regulated by ACs which localizes to the ciliary microdomain (Moore et al., 2016). Moreover, AC6 was shown to localize 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 ciliary may also be a cAMP-responsive mechanosensor and that molecular mechanisms may be cell type dependent. However, despite the progress in cilia-mediated mechanotransduction in mature bone cells, the molecular mechanism of cilia-mediated mechanotransduction in stem cells remains unknown.

Therefore, the aim of this study was to determine the molecular mechanism of cilia-mediated mechanotransduction in mesenchymal stem cells, which would facilitate the development of novel mechanotherapies to promote \textit{early osteogenic signalling} in MSCs. Here, with the use of custom built bioreactors, we demonstrate mesenchymal stem cells utilized 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, demonstrating a potential novel anabolic treatment to enhance MSC osteogenesis.
Results

MSCs utilized cAMP signalling as a 2nd messenger in mechanotransduction

To determine whether mesenchymal stem cells utilize 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 cells subjected to 15 min of oscillatory fluid shear significantly increased cellular cAMP levels 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 OFS and returned to basal levels after 30 min OFS (Fig. 1A), demonstrating similar profiles to that seen with calcium signalling. As no change was seen following either 2 or 30 min OFS, our remaining experiments focused on investigating the mechanisms involved in flow-induced increases of cAMP after 15 min OFS.

As cAMP signalling is predominately regulated via ACs, we utilized a general AC inhibitor, MDL-12,330A hydrochloride, to investigate whether this OFS-induced increase in cAMP was AC dependent. Firstly, we performed a dose response study for 15 min to determine the optimum concentration of MDL that would inhibit AC activity, but that did not affect basal cAMP levels. MDL at concentrations of 100 and 200µM, resulted in significant decreases in basal cAMP levels (Fig1B). A concentration of 10µM was chosen as it was the lowest concentration of MDL-hydrochloride and did not elicit any significant changes in cAMP levels (Fig. 1B). We found that treating MSCs with 10µM MDL-hydrochloride blocked the OFS-induced increases of cAMP (p<0.05; Fig. 1C), demonstrating that the OFS-mediated cAMP response was dependent on AC activity. Thereafter, to investigate whether cAMP second messenger signalling is required for fluid shear-induced MSC osteogenesis, AC activity was inhibited as above and osteogenic gene expression was analysed following 2hrs OFS. We found that MSCs display a positive early osteogenic response when subjected to a regime that has previously been shown to elicit osteogenic responses at 2h OFS at 1Pa, 1Hz (Stavenschi et al., 2017), where there was an increase in the expression of the osteogenic genes, Cox2 and Osteopontin (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-hydrochloride this significant increase was lost in all genes.
analyzed (Fig. 1D-F), demonstrating that MSCs utilize 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 (22). Nine membrane-bound isoforms have been identified, with seven of these, AC2-7 and 9 expressed in mature bone cells (20). To determine what AC isoforms are expressed in progenitor cells, utilizing quantitative real-time polymerase chain reaction (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 AC1, AC3-4, AC6-9 are expressed, while 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 (24). To investigate AC6 further, we examined AC6 protein expression and spatial organization 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). Gene expression levels, as quantified by qRT-PCR, 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 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 changes in cAMP activity was found in MSCs following shear (Fig. 3B), demonstrating the specific role of AC6 in regulating mechanically activated cAMP signaling.

**Adenylyl Cyclase 6 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 1Pa, 1Hz as previously described. *Cox2, Runx2* and *Osteopontin* 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. 4A-C). Furthermore, analysis of osteogenic gene expression in MSCs treated with scrambled siRNA and exposed to flow, resulted in a significant 4-, 3.16-, and 3.60-fold increase in Cox2, Runx2 and Osteopontin mRNA levels, respectively, compared to scrambled siRNA no flow controls (p<0.05; Fig. 4A-C). However, similar to that seen with cAMP signalling, depletion of AC6 results in a complete loss of the fluid shear induced increases in osteogenic gene expression (Fig. 4A-C), demonstrating that AC6 is a critical component of MSC mechanotransduction through regulation of cAMP second messenger signalling.

**AC6 is localized to the microdomain of the primary cilium in MSCs**

Given the previously demonstrated role of the primary cilium in MSC mechanotransduction and spatial organization of ACs 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 localized to the primary cilium in MSCs. Utilizing ICC, 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 from the cell surface. As previously demonstrated, AC6 was found throughout the cell membrane but upon co-staining with the primary cilium a distinct spatial organization was revealed with intense staining found along the ciliary microdomain (Fig. 5). No staining was found when AC6 primary antibody was withheld (Fig. S2). This therefore indicates that cilia-mediated MSC mechanotransduction may be dependent on AC6 and its regulation of cAMP signalling.

**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 (6). Therefore, given the localization 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 signaling was then investigated. The formation of cilia was inhibited through the utilization of siRNA targeting IFT88, which is a principal motor protein required for ciliogenesis. The transfection resulted in significantly diminished Ifi88 mRNA expression which in turn significantly reduced the incidence of primary cilia by 74% as demonstrated by immunocytochemistry (p<0.05; Fig. 6A-D). Mesenchymal stem cells transfected with siRNA
targeting *Ift88* or scrambled siRNA were subjected to OFS, and cAMP signalling was analyzed 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 removal of the primary cilium, 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 utilizes AC6 to mediate this second messenger and downstream osteogenic response, demonstrating a novel molecular mechanism of cilia-mediated mechanotransduction in mesenchymal stem cells.

cAMP signalling can be biochemically activated with an AC activator demonstrating artificial mechanotransduction and mechano therapeutic potential.

Next, we wanted to investigate whether the biochemical activation of this novel MSC mechanotransduction mechanism via ACs could mimic the responses seen to OFS. A dose response study was carried out to determine whether the AC activator, forskolin (FSK), could biochemically mimic the effects of OFS on cAMP signalling. We found that the 0.01mM and 0.001mM of forskolin could induce a 25- and 12-fold increase in cAMP levels following 15min treatment, respectively (p<0.001; Fig. 7A), which while a large increase, does not mirror that seen following OFS. However, cAMP levels increased 2.5-fold compared to the vehicle control in cells treated with 0.1µM FSK (p<0.05; Fig. 7B). As the aim of this study was to biochemically induce the increases in cAMP seen with fluid shear, cells were treated with 0.1µM FSK for further studies. Importantly, 0.1µM FSK treatment was not only able to mimic cAMP signalling following OFS, but also that of the osteogenic response, where treatment with 0.1µM FSK resulted in a significant increase in *Cox2*, *Runx2*, and *Osteopontin* (p<0.05; Fig. 7C-E), to a similar degree to that seen with fluid shear demonstrating artificial mechanotransduction and highlighting FSK as a potential anabolic mechano therapeutic 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 0.1µM FSK as previously described. The expression of *Cox2* and *Osteopontin* in the vehicle control group showed a trend to an increase in expression, however this was not significant (p>0.05; Fig. 7 F-H). Furthermore, analysis of osteogenic gene expression in MSCs treated with scrambled siRNA and
exposed to FSK, resulted in a significant 3.19-, 3.43-, and 2.59-fold increase in Cox2, Runx2 and Osteopontin mRNA levels compared to no treatment controls, respectively (p<0.05; Fig. 7F-H). However, similar to that seen with cAMP signalling and OFS, depletion of AC6 results 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 and this study has demonstrated its role 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 Ift88 siRNA. MSCs transfected with siRNA targeting Ift88 or scrambled siRNA were subjected to treatment of 0.1µM FSK, and the osteogenic gene expression was analyzed as above. The scrambled siRNA treatment did not affect basal or FSK-induced increases in Cox2, Runx2 or Osteopontin in MSCs (3.14-, 3.41- and 2.59-fold, p<0.05; Fig. 7I-K). However, upon removal of the primary cilium, 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.

Discussion

Mechanical loading is a potent stimulus regulating bone adaptation, that requires the replenishment of the osteoblast from a progenitor population. One such progenitor is the mesenchymal stem cell, which undergoes osteogenic differentiation in response to oscillatory fluid shear (Stavenschi et al., 2017). Yet, the identification of the mechanism mediating stem cell mechanotransduction, and thus the ability to target this therapeutically, is poorly understood. In this study, we demonstrate that MSCs utilize cAMP as a 2nd messenger in mechanotransduction, that is 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 which can be targeted therapeutically, demonstrating a potential mechanotherapeutic for bone loss diseases such as osteoporosis.

MSCs utilise cAMP as a 2nd 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 utilization of cAMP signalling as an important 2nd messenger in the skeleton. Interestingly, when this cAMP response was inhibited MSCs exhibited a loss in flow-induced increases in osteogenic genes, Cox2, Runx2 and Osteopontin, 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 Ca2+ signalling (Thompson et al., 2012, Liu et al., 2015, Reich et al., 1990). However, we recently demonstrated that a complete loss of calcium 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 2nd messenger in MSC mechanotransduction. This alternative mechanism of cAMP signalling agrees with previous work in bone mesenchymal stem cells (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 mesenchymal stem cell 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 ACs within lineage committed cells, while the isoforms that are expressed within progenitors have not been established. Using qRT-PCR MSCs were found to express only 7 of the 9 AC isoforms, which is similar to the number seen in the differentiated osteocyte (Kwon et al., 2010). In our studies, MSCs express AC1, 3-4 and 6-9, while AC2 and 5 were not detected. This contrasts with that seen in
osteocytes that do not express AC1 or 8 (Kwon et al., 2010). These differences in AC expression indicate a preferential change in AC expression with lineage commitment. Of the four highly expressed ACs in MSCs (AC3, 6, 7, and 9), 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, 7 and 9 have predominately been associated with the brain (Kamenetsky et al., 2006). Therefore, we focused specifically on AC6 within this study. Lee et al., (2013) showed that mice with a global knockout of AC6 had no skeletal phenotype, 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 \textit{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, where immunocytochemistry was employed, 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 \textit{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 AC inhibition, indicating that AC6 is the dominant AC mediating MSC mechanotransduction. Hence, our data demonstrates a novel role for AC6 in MSC mechanotransduction, mediating fluid shear induced cAMP 2nd messenger signalling and downstream early osteogenesis.

The primary cilium, an organelle required for fluid shear-induced MSC osteogenesis \textit{in vitro} and \textit{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 ACs localise to primary cilia in numerous cell types. Most notably AC6 is colocalised 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 AC5/6 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 localization 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 localization of a plethora of signalling molecules, localization of AC6 to this signalling center 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 that seen with AC6 knockdown. This therefore suggests that the localisation of AC6 to the cilium is functionally significant and demonstrates a potential calcium independent mechanism of cilia-based mechanotransduction (Malone et al., 2007, Delling et al., 2016). In summary, primary cilium 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 cyclase’s elicits a cAMP 2nd messenger and early osteogenic gene response that mirrors that seen with oscillatory fluid shear in MSCs. The AC agonist forskolin, a diterpene which stimulates cAMP production by directly activating ACs (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 Osteopontin, 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 pretreated 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 somewhat surprising as forskolin is recognized as a broad-spectrum adenylyl cyclase agonist. Similar results have been found in vascular smooth muscle cells (VSMCs). Gros et al. demonstrated that the downstream consequences of cAMP signalling is not common to all isoforms of ACs in VSMCs, with siRNA directed at AC6 was sufficient to reduce forskolin activated AC activity (Gros et al., 2006). Moreover, knockdown of AC6 alone could inhibit the Forskolin-induced increases in cAMP and synthesis and arborization in control and cells with an overexpression of 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 Ift88 siRNA blocked the forskolin-induced increases in Cox2, Runx2 and Osteopontin. This data suggests that the primary cilium, and the ciliary localized AC6 are both required for forskolin-induced alterations in osteogenic gene expression. Together with our observations, this demonstrates that biochemically targeting mechanotransduction mechanisms, such as that 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 2nd messenger to initiate an early osteogenic response post fluid shear stimulation. This was further explored and found that fluid shear-induced cAMP signalling is mediated by adenylyl cyclase 6 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 calcium independent mechanism of cilia-based mechanotransduction in stem cells. Therefore, 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 oscillatory fluid shear, demonstrating mechanotherapeutic potential and a new anabolic treatment for bone loss diseases such as osteoporosis.

Materials and Methods

Mesenchymal Stem Cell Culture

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

**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 (10µg/ml) coated glass slides, assembled between two plates and attached to a programmable syringe pump (New Era Pump Systems Inc. Farmingdale, NY, USA). Oscillatory fluid shear was applied through a 10ml syringe (BD Plastipak, VWR, Dublin, Ireland) at 52.5ml/min and 1Hz frequency subjecting cells to a shear stress of 1Pa. This stimulus was applied for 2 hours. The no flow controls take account of the confined environment experienced by the cells in the chambers, they consist of glass slides prepared in the same way and similarly remain in the chambers for 2 hours but are not attached to the syringe pump.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

Immediately after treatment, TRI reagent (93289, Sigma) 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. 400ng 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, Osteopontin, Ac1-9 and Ift88 were acquired for analysis of flow and forskolin mediated osteogenic changes and validation of knockdowns (4472920; 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 Ltd, Paisley, UK), 0.8 µl of each primer, and 8.4 µl dH2O and sample mix. In the case of Runx2 and Ac1-9, 0.6 µl of each primer was used. Plates were run in an ABI 7500 Fast Thermocycler (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 15s at 95°C and the annealing–extending cycle for 1 min at 60°C for Cox2, Runx2, Osteopontin and Ift88, apart from 18s and Ac1-9, 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 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 (Applied Biosystems, 4331182) 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 outlined 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 adenyl 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 hrs at room temperature. Wells were washed with 200 µl 1X wash buffer four times, before incubating with 100 µl 3,3’,5,5’-Tetramethylbenzidine (TMB) substrate for 30 min at room temperature. Following 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 hours before serum starvation in DMEM low glucose, 0.5% FBS, 1% P/S for 48 hours. After fixation in neutral buffered formalin for 10 min (Sigma), coverslips were permeabilized in 0.1% Triton X-100 and non-specific binding sites were blocked using 1% w/v BSA (Sigma) 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, Cambridge, United Kingdom) was applied for 1 h at room temperature at a dilution of 1:500. Next, Alexa 594 and Alex 488 secondary antibodies (A21203, A11008; Life Technologies, California, USA) were applied in tandem for 1 h at room temperature at 1:500. Finally, DAPI (32670; Sigma) 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, Cambridge, United
Kingdom) 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 100W halogen lamp at 100x (N.A. 1.40 Oil) or the Leica SP7 (Leica
Microsystems, Wetzlar, Germany) scanning confocal microscope at 63x (N.A. 1.40 Oil). Controls
in the 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 Adenylyl Cyclase 6 was 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 (Gibco, Foster City, CA, USA) reduced serum transfection medium. For IFT88
knockdown this was mixed 1:1 with predesigned Stealth RNAi targeting IFT88 (MSS211714,
Invitrogen) at a dilution of 16.7µM in OptiMEM and incubated at room temperature for 15 min
before application, while for AC6 knockdown lipofectamine was mixed 1:1 with Silencer Select
Ambion siRNA (4390825; Bio-Sciences Limited, Dublin, Ireland) at a dilution of 60pmol for 5
min before adding 550µl per slide and incubated for 8 hours at 37°C, following which 10 ml
DMEM (0.5% FBS, 1% P/S) was added to the cells for 24h. The off-target control was 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.
Percentage of ciliated cells were determined and cilia length analysed by epifluorescence
microscopy using an Olympus IX83 fitted with 100x objective. Cilia length were measured using
Image J freeware.

**Biochemical Inhibition and Activation of cAMP Signalling**

Cyclic AMP signalling was diminished through the inhibition of adenylyl cyclase activity by
MDL-12,330A hydrochloride (MDL; M182; Sigma (Zhang et al., 2010)). A dose response study
was performed for 15 min to determine the optimum concentration of MDL that would inhibit AC activity but does not affect basal cAMP levels. Cells were treated for 15 min to replicate OFS conditions for cAMP. Following this, 10µM was chosen to be optimal and therefore was used for further studies investigating the role of AC activity and cAMP signalling in MSC mechanotransduction. MSCs were treated with MDL supplemented medium throughout application of the 1Pa mechanical stimulation, and controls were incubated for the same time frame with vehicle (H₂O) treated medium, for both cAMP and osteogenic gene expression studies.

To modulate cyclic AMP signalling via activation of adenylyl cyclase, the general AC agonist forskolin (FSK; F3917; Sigma (Doorn et al., 2012)) was utilized (Seamon et al., 1981). 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 frame with vehicle (0.01% DMSO) treated medium, for both cAMP and osteogenic gene expression studies. Media was supplemented with 1µM 3-Isobutyl-1-methylxanthine (IBMX) for all cAMP studies.

**Data Analysis**

The relative expression of each gene with reference to 18s was calculated and the results expressed as fold change gene expression relative to the no flow scrambled or vehicle control group along with the standard error of the mean. For the OFS and forskolin with AC6 or Ifit88 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 were 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 conflict of interest with the contents of this article.
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**Author Contributions**

Gillian P. Johnson: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing,

Elena Stavenschi: Collection and/or assembly of data, Data analysis and interpretation.

Kian F. Eichholz: Collection and/or assembly of data.

Michele A. Corrigan: Collection and/or assembly of data.

Sean Fair: Financial support, Approval of manuscript.

David A. Hoey: Conception and design, Data analysis and interpretation, Manuscript writing, Financial support, Final approval of manuscript.
References


**Figure legends**

![Graph A](image1)

**A** MSCs utilize cAMP signaling as a 2nd messenger during mechanotransduction. (A) Effect of oscillatory fluid flow over MSCs at 1Pa, 1Hz for 2, 15 and 30 min on cAMP concentration (N=3, n=6). (B) Dose response test of the AC inhibitor MDL-12,330A hydrochloride on cAMP concentration in MSCs (N=3, n=6). (C) Effect of 10µM MDL-12,330A hydrochloride on cAMP concentration following oscillatory fluid flow at 1Pa, 1Hz for 15 min (N=3, n=9). (D-F) Effect of MDL-12,330A hydrochloride on gene expression following oscillatory fluid flow at 1Pa, 1Hz for two hours (N=2, n=6). All groups are compared to no flow vehicle control. Statistical test employed an unpaired two tailed student t-test with Wilcoxon correction (A), a one-way ANOVA with Bonferroni post-test (B), and a two-way ANOVA with Bonferroni post-tests (C, D, E, F). Values are means ± SEM for three independent replicates. *p<0.05, **p<0.01.
Fig. 2. MSCs express seven of the nine transmembrane Adenylyl Cyclase isoforms, including AC6. (A) qRT-PCR targeting adenylyl cyclases (AC) 1-9 in MSCs. ACs 3, 6, 7 and 9 show the highest expression of the 9 ACs analyzed. AC2 and 5 were not detected (ND; n=3). (B) Adenylyl Cyclase 6 (AC6) expression in MSC verification on a protein level with the use of immunocytochemistry. Cells were stained for AC6 (green) and nuclei counterstained with DAPI (blue). Scale bar 20 µm.

Fig. 3. Adenylyl Cyclase 6 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 (N=3, n=13). (B) Effect of oscillatory fluid flow over MSCs treated with scrambled and AC6 siRNA at 1Pa, 1Hz for 15 min on cAMP concentration (N=3, n=9). All groups are compared to no
flow scrambled control. Statistical tests employed an unpaired two tailed student t-test (A) and a two-way ANOVA with Bonferroni post-test (B). Values are means ± SEM for a minimum of three independent replicates. *p<0.05, ***p<0.001.

Fig. 4. Adenylyl Cyclase 6 is required for fluid shear induced osteogenic gene expression in MSCs. (A-C) Effect of oscillatory fluid flow at 1Pa, 1Hz on osteogenic gene expression after 2 h (N=4, n=9-12). All groups are compared to no flow scrambled control. Statistical tests employed a two-way ANOVA with Bonferroni post-tests. Values are means ± SEM for a minimum of three independent replicates. *p<0.05, ***p<0.001.
Fig. 5. Adenylyl Cyclase 6 is localized to the primary cilium in MSCs. AC6 co-localisation to the primary cilia identified by immunostaining for primary cilia, identified as linear structures enriched in acetylated α-tubulin, (red) and AC6 (green). Nuclei are counterstained with DAPI (blue). N=3, n=77. Scale bar 5µm.
Fig. 6. Primary cilium-mediated mechanotransduction in MSCs is cAMP dependent. (A-C) Intraflagellar transport protein 88 (IFT88) expression was successfully knocked down in MSCs using IFT88 siRNA as verified by immunostaining and qRT-PCR (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; arrow head). Nuclei are counterstained with DAPI (blue). Scale bars represent 5μm and 1μm (insert). (D) Effect of 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 1Pa, 1Hz for 15 min on cAMP concentration (N=3, n=9). Statistical test employed an unpaired two tailed student t-test with Wilcoxon correction (C, D) and a two-way ANOVA with Bonferroni post-tests (E). Values are means ± SEM for three independent replicates. *p<0.05, ***p<0.001.
Fig. 7. Cyclic AMP signaling can be biochemically activated with an AC 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 gene expression (N=3, n=9). (F-H) Adenylyl Cyclase 6 is required for forskolin induced osteogenic gene expression in MSCs.
expression after 2 hours (N=2, n=6). (I-K) Ift88 is required for forskolin induced osteogenic gene expression in MSCs expression after 2 hours (N=2, n=6). DMSO concentration for shown is 0.01%. Statistical test employed an unpaired two tailed student t-test with Wilcoxon correction (A-E). Statistical tests employed a two-way ANOVA with Bonferroni post-tests (F-K). Values are means ± SEM for three independent replicates. *p<0.05, **p<0.01, ***p<0.001
Table 1: Primer sequences and concentrations employed in quantitative PCR analysis.

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