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Adipogenic Differentiation of hMSCs is Mediated by Recruitment of IGF-1R Onto the Primary Cilium Associated with Cilia Elongation

Melis T. Dalbay[1], Stephen D.Thorpe[1]*, John T. Connelly[2], J. Paul Chapple[3] and Martin M. Knight[1]

[1]Institute of Bioengineering and School of Engineering and Materials Science, Queen Mary University of London, London, United Kingdom; [2]Institute of Bioengineering and Institute of Cellular and Molecular Sciences, Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary, University of London, London, UK; [3]Centre for Endocrinology, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary, University of London, London, UK.

*Corresponding Author: Stephen D. Thorpe, PhD, Address: School of Engineering and Materials Science, Queen Mary University of London, Mile End Rd, London, E1 4NS, UK., Tel: +44 (0)20 7882 8868, Fax: +44 (0)20 7882 3390, Email: s.thorpe@qmul.ac.uk; Acknowledgements: Funding provided by QMUL research studentship and Marie Curie Intra European Fellowship

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ABSTRACT

Primary cilia are single non motile organelles that provide a highly regulated compartment into which specific proteins are trafficked as a critical part of various signaling pathways. The absence of primary cilia has been shown to prevent differentiation of human mesenchymal stem cells (hMSCs). Changes in primary cilia length are crucial for regulating signaling events, however it is not known how alterations in cilia structure relate to differentiation. This study tested the hypothesis that changes in primary cilia structure are required for stem cell differentiation. hMSCs expressed primary cilia which were labelled with acetylated alpha tubulin and visualized by confocal microscopy. Chemically induced differentiation resulted in lineage specific changes in cilia length and prevalence independent of cell cycle. In particular adipogenic differentiation resulted in cilia elongation associated with the presence of dexamethasone, whilst insulin had an inhibitory effect on cilia length. Over a seven day time course adipogenic differentiation media resulted in cilia elongation within two days followed by increased nuclear PPARγ levels, an early marker of adipogenesis. Cilia elongation was associated with increased trafficking of IGF-1Rβ into the cilium. This was reversed upon inhibition of elongation by IFT-88 siRNA transfection which also decreased nuclear PPARγ. This is the first study to show that adipogenic differentiation requires primary cilia elongation associated with the recruitment of IGF-1Rβ onto the cilium. This study may lead to the development of cilia-targeted therapies for controlling adipogenic differentiation and associated conditions such as obesity. STEM CELLS *2014; 00:000–000*

INTRODUCTION

Primary cilia are single non-motile organelles that typically protrude from the cell surface and are expressed by most mammalian cell types including stem cells [1]. They play a critical role in the regulation of cell and tissue development and homeostasis [2, 3] via a variety of signaling pathways including Wnt [4], hedgehog [5, 6], notch [7], mechanotransduction [8] and growth factor signaling [9].

Primary cilia consist of a microtubule structure called the axoneme that is covered by a specialized lipid bilayer. The axoneme extends from the basal body, which originates from the most mature of the two centrioles.

Proteins are transported on and off the cilium by molecular motors through the process of intraflagellar transport (IFT) [10]. Cilia assembly and disassembly is therefore regulated by IFT which controls the transport of tubulin to and from the tip of the cilium and hence controls the cilia length [11]. As well as transport of structural proteins, IFT is also involved in trafficking of cilia signaling proteins. This process is critical to the role of primary cilia in acting as a signaling hub that modulates mechanical, osmotic and chemical signaling [12]. Mutations occurring in IFT proteins may therefore influence cilia structure and impair signaling pathways, resulting in a range of developmental and degenerative disorders, collectively termed ciliopathies [13].

Recent studies have shown that differentiation of mesenchymal stem cells requires the presence of primary cilia and associated IFT. In particular primary cilia are involved in differentiation along osteogenic [8, 14-18], adipogenic [16, 17, 19], chondrogenic [17, 20] and neurogenic [21] lineages. Interestingly the length of primary cilia differs between various differentiated cell types. For example epithelial cells typically have longer cilia than articular chondrocytes. However it is not known how primary cilia structure changes during differentiation and whether any such changes are important in regulation of differentiation. Recent evidences suggest that the length of primary cilia influences cilia signaling pathways including mechanotransduction, hedgehog and wnt signaling [22-24]. The mechanisms through which this occurs are unclear. Besschetnova *et al.*, have demonstrated that anterograde IFT velocity is increased in longer cilia [25], however, Ludington *et al.*, show that this is associated with a reduction in the injection of IFT cargoes thereby affecting the trafficking of signaling molecules and receptors into the ciliary compartment [26]. In addition, other studies show that the process of cilia elongation or shortening also regulates cilia function associated with dynamic changes in trafficking [27]. We therefore hypothesize that biochemical differentiation conditions induce alterations in primary cilia length and these structural changes are important in facilitating the differentiation of human mesenchymal stem cells (hMSCs). We show that differentiation of hMSCs results in changes in cilia length and prevalence in a lineage specific manner. We then focus on adipogenic differentiation conditions which increase cilia length, and demonstrate that this elongation and associated alterations in trafficking are required for adipogenesis. Finally we examine the mechanism and reveal that cilia elongation drives increased ciliary expression of insulin like growth factor-1 receptor (IGF-1R) and that subtle inhibition of this elongation by knock down of IFT88 prevents adipogenic differentiation.

MATERIALS AND METHODS

hMSC Culture and Differentiation

Human bone marrow-derived mesenchymal stem cells (hMSCs) were obtained from Stem Cell Technologies (Manchester, UK) and maintained in basal media (BM) consisting of low glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Paisley, UK) with 10% fetal bovine serum and 1% Penicillin-Streptomycin (both Sigma-Aldrich, Gillingham, UK), with the addition of 1 ng/ml fibroblast growth factor-2 (FGF-2;PeproTech, London, UK). Media was exchanged every 3-4 days. For all studies, hMSCs at passage 4-7 were plated at 5×10^3 cells/cm^2 in either 24 well plates on FBS coated glass coverslips (thickness 1.5) for imaging studies or in 6 well plates for protein isolation. Five days post seeding, media was changed to differentiation media. Adipogenic differentiation media (AM) consisted of BM with the addition of 1 µM dexamethasone, 500 µM 3-Isobutyl-1-methylxanthine (IBMX), 100 µM indomethacin and 10 µg/mL insulin (all Sigma-Aldrich). Osteogenic media (OM) consisted of BM with the addition of 100 nM dexamethasone, 50 µM L-ascorbic acid and 10 mM β-glycerophosphate (all Sigma-Aldrich). Chondrogenic media (CM+) consisted of high glucose DMEM (Gibco) with 1% Penicillin-Streptomycin, 1mM sodium pyruvate, 1.5 mg/mL bovine serum albumin (BSA), 40 µg/mL L-proline, 4.7 µg/mL linoleic acid, 50 µg/mL L-ascorbic acid, 100nM dexamethasone (all Sigma-Aldrich) and 1X insulin-transferrin-selenium G supplement (Gibco) with the addition of 10 ng/mL transforming growth factor-β3 (TGF-β3; PromoCell, Heidelberg, Germany). Chondrogenic media without TGF-β3 (CM-) was used as a basal media control for chondrogenic differentiation. Media was exchanged every 3-4 days.

siRNA Transfection

Transfections were performed on hMSCs 5 days post seeding in antibiotic free BM using DharmaFECT 1 transfection reagent (GE Dharmacon, Lafayette, CO, USA) according to manufacturer's protocol. hMSCs were transfected with 25 nM SMART pool ON-TARGETplus human siRNA to intraflagellar transport protein-88 (IFT88) or ON-TARGETplus non-targeting pool siRNA (both GE Dharmacon). Transfection media was exchanged for AM 24 hrs post transfection. At day 2 and day 5 of adipogenic differentiation induction, hMSCs were either fixed for immunocytochemistry or lysed for protein analysis. For day 5 samples, transfections were repeated in antibiotic-free AM in the same manner 72 hrs post the first transfection (day 2 of differentiation induction).

Immunocytochemistry and staining

In order to improve cilia prevalence in BM, OM and AM cultures containing FBS [28], serum was withdrawn from culture media 24 hrs prior to fixation as adopted in numerous previous studies. Cells were fixed in 4% PFA

for 10 min and washed in phosphate buffered saline (PBS). To assess adipogenic differentiation, lipid droplets were stained using 0.2% Oil Red O in 60% isopropanol for 5 min. Osteogenic differentiation was assessed by detection of alkaline phosphatase activity. In this case coverslips were fixed using 4% PFA for 2 minutes, washed in PBS and immediately stained using an alkaline phosphatase detection kit according to manufacturer's instructions (Merck Millipore, Watford, UK). Chondrogenic differentiation was assessed via collagen type II immunohistochemistry. For this and other immunohistochemistry staining fixed cells were washed with PBS and permeabilized with 0.5% Triton-X100 for 10 min and washed with PBS. Samples were blocked in 5% goat serum with 1 mg/mL BSA in PBS for 30 min at room temperature followed by incubation with primary antibodies at 4 °C overnight. Primary antibodies used were mouse monoclonal anti acetylated α-tubulin (1:2000; Sigma-Aldrich), rabbit polyclonal anti-pericentrin (1:500; Abcam, Cambridge, UK), rabbit polyclonal anti-collagen type II (1:100; Abcam), mouse monoclonal anti-Ki67 (1:500; Sigma-Aldrich), rabbit polyclonal anti-peroxisome proliferator-activated receptor γ (PPARγ; 1:100; Santa Cruz Biotechnology, Heidelberg, Germany) and rabbit polyclonal anti-IGF-1Rβ (1:100; Santa Cruz Biotechnology). Secondary antibodies were Alexa Fluor 488 F(ab')$_2$ fragment of goat anti-mouse IgG (H+L), Alexa Fluor 488 goat anti-rabbit IgG (H+L), Alexa Fluor 594 F(ab')$_2$ fragment of goat anti-rabbit IgG (H+L), Alexa Fluor 633 goat anti-rabbit IgG (H+L) (all 1:1000; Molecular Probes, Paisley, UK). F-actin was stained using Alexa Fluor 555 phalloidin (1:50) and nuclei were stained with Hoechst 33342 (1:5000; both Molecular Probes). Immunofluorescently stained coverslips were mounted using ProLong Gold Antifade Mountant (Molecular Probes).

Confocal Microscopy

Stained coverslips were imaged using a Leica SP2 confocal microscope (Leica Microsystems, Milton Keynes, UK), with multiple z-sections taken through the thickness of the cell at 500 nm intervals. Maximum projections of z-stacks were imported into image J for measurement of cilia length using acetylated α-tubulin and pericentrin to identify primary cilia. Cilia length is given in micrometers (μm). Image J was also used to quantify ciliary IGF-1Rβ and nuclear PPARγ intensity by manually tracing around each cilium and nucleus and measuring the mean intensity in the corresponding fluorescent channel. Intensity values are presented in arbitrary units (AU) as obtained from the confocal software. Mean values of 100-110 measurements per sample were plotted in each graph.

Western blots

hMSCs were lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (New England Biolabs, Herts, UK) containing protease inhibitors (Roche, Burgess Hill, UK). Total protein was measured using Bradford reagent (Sigma-Aldrich). Protein supernatants were reduced in Lamelli buffer, separated on a Mini-PROTEAN TGX Any kD gel and transferred to nitrocellulose membranes (all Bio-Rad Laboratories, Hemel Hempstead, UK). Membranes were blocked in 5% milk for 60 min before overnight incubation with primary antibodies; rabbit polyclonal anti-PPARγ (1:500; Santa Cruz), rabbit polyclonal anti-IFT88 (1:500; Proteintech, Manchester, UK) and mouse monoclonal anti-β actin (1:10,000 Abcam). Secondary antibodies used were IRDye 680RD goat anti-mouse IgG (H+L) and IRDye 800CW donkey anti rabbit IgG (H+L) (both 1:15,000; LI-COR Biotechnology, Cambridge, UK). Membranes were imaged using an Odyssey infra-red imaging system (LI-COR Biotechnology).

Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). When data sets adhered to a normal distribution, 2-sample t-test or ANOVA was used as indicated in figure legends. For non-parametric data sets, Mann-Whitney U tests were used to compare conditions. Data are presented as mean ± standard error.

RESULTS

Primary cilia length changes during hMSC differentiation in a lineage specific manner

To investigate changes in primary cilia length and prevalence during differentiation, human bone marrow-derived MSCs were seeded on glass coverslips and cultured in BM for five days. The media was then replaced with either AM, OM, CM- or CM+, with some samples maintained in BM as an undifferentiated control. As biochemical induction of chondrogenesis involves the use of a serum-free media formulation containing TGF-β3 (CM+), a TGF-β3 free equivalent media (CM-) was used as a control basal media for chondrogenic differentiation. Cells were cultured in differentiation conditions for a further seven days prior to fixation (Day7). In addition, separate cells were fixed prior to differentiation (Day 0). Differentiation along each lineage was confirmed at day 7 with adipogenic differentiation inducing lipid droplet accumulation demonstrated by oil red O staining, osteogenic hMSCs stained positive for alkaline phosphatase (ALP) and chondrogenic hMSCs stained positively for collagen type II (Fig. S1).

Primary cilia were labeled with acetylated α-tubulin (green) and the basal body with pericentrin (red) (Fig. 1A). While there was no significant change in cilia length for hMSCs cultured in BM over the seven day period, primary cilium length was observed to increase by day 7 following differentiation in osteogenic media (OM) and adipogenic media (AM). Chondrogenic differentiation media (CM+) produced no change in length compared to basal control. However when compared to the TGF-β3-free chondrogenic control media (CM-), chondrogenic differentiation in the presence of TGF-β3 significantly

shortened the primary cilium (p<0.001; Fig. 1B). At day 0, 87.8±2.4% of hMSCs expressed a primary cilium and this increased to 96.4±1.6% over the 7 day culture period (p<0.05; Fig. 1C). At day 7, adipogenic differentiation resulted in no change in percentage ciliation. By contrast, osteogenic differentiation resulted in decreased ciliation to a mean value of 87.4±3.0%. Cells grown in chondrogenic media with or without TGF-β3 displayed the greatest reduction in primary cilia expression with 75±5.4% and 70.4±5.3% of cells presenting a primary cilium respectively (Fig. 1C). As cilia are resorbed at cell division [29], we investigated whether the observed differences in ciliation were associated with corresponding differences in hMSC proliferation. Based on Ki67 expression, hMSC proliferation was consistently below 10% with no significant differences in proliferation between any of the groups (Fig. 1E), indicating that changes in primary cilium length during differentiation were not the result of differences in hMSC proliferation.

Nuclear localization of PPARγ increases following primary cilia elongation during adipogenic differentiation

In order to investigate the role of primary cilia length changes on hMSC differentiation, we focused on differentiation down the adipogenic lineage which was associated with ~60% cilia elongation compared to undifferentiated hMSCs. We began by investigating cilia length and associated changes in the nuclear expression of the adipogenic marker PPARγ with time over the first 7 days of differentiation (Fig. 2A). In BM cilia length of hMSCs remained approximately constant with a mean length of approximately 3μm. By contrast after 2 days of adipogenic induction, primary cilia were significantly longer than non-differentiated hMSCs in BM and remained longer throughout the 7 day culture period (Fig. 2A & 2C). However there were fluctuations in cilia length in adipogenic conditions with reduced levels of cilia elongation at day 4 and day 7 (Fig. 2C & Fig. S6A). While total PPARγ expression levels were similar in hMSCs grown in BM and AM (Fig. S2), we observed greater levels of nuclear PPARγ localization consistent with adipogenic differentiation at all time-points (p<0.001) with a marked increase from day 4 of differentiation following the initial increase in cilia length at day 2-3 (Fig. 2E). Thus, there is a temporal mismatch between the cilia length and subsequent nuclear PPARγ expression, since elongation precedes subsequent differentiation. Indeed there was no correlation between primary cilia length and nuclear PPARγ intensity on an individual cell basis at any single time point (Fig. S3).

Adipogenic differentiation was also associated with actin cytoskeletal changes. hMSCs grown in BM contained organized, dense F-actin stress fiber bundles. By contrast, hMSCs cultured in AM did not exhibit such organized F-actin bundles (Fig. 2B). These changes in actin organization associated with adipogenic differentiation are in agreement with previous reports [30, 31].

Cilia prevalence was maintained between 75-100% for both culture groups over the 7 day period (Fig. 2D). Cilia prevalence was lowest at day 2 of adipogenic induction (75.8±5.1%) and increased at later time points (Fig. 2D & S6B). Assessment of cell proliferation based on Ki67 expression showed that the rate of cell division remained less than 10% and was not significantly altered with culture duration or differentiation (Fig. S4). Thus temporal changes in cilia length with adipogenic differentiation cannot be attributed to differences in cell proliferation.

The role of adipogenic media components in cilia elongation and differentiation

To gain some insight into the mechanistic role of primary cilia elongation in adipogenic differentiation, we investigated the role of insulin on cilia length and prevalence. Insulin is a known stimulator of both insulin receptor (IR) and IGF-1R signaling necessary for adipogenic differentiation. In particular insulin is required for later events in adipogenesis such as maintenance of adipogenic phenotype by binding to insulin receptors (IR), IGF receptors (IGFR) [32-34]. Here we cultured hMSCs for 48 hrs in either basal media (BM) or adipogenic media (AM), both with (BM+Ins, AM) and without (BM, AM-Ins) insulin. An additional group (AM-/+Ins) was cultured for 24 hrs in insulin-free AM (AM-Ins) followed by 24 hrs in standard AM including insulin. In all cases FBS was removed 24 hrs prior to fixation. For hMSCs grown in AM, primary cilia length increased compared to cells in BM, however, primary cilia were significantly longer when insulin was not included in the adipogenic media (AM-Ins) (Fig. 3A & B). The addition of insulin after 24 hrs (AM-/+Ins) reduced cilia length compared to AM-Ins. Similarly, addition of insulin to BM alone (BM+Ins) did not induce any cilia elongation in contrast to the elongation observed in full adipogenic media (Fig. 3A &3B). This increase in primary cilia length corresponded to increased nuclear PPARγ accumulation in cells that were grown in AM, AM-Ins and AM-/+Ins (Fig. 3A & 3C). In addition, hMSCs cultured in BM+Ins did not exhibit an increase in nuclear PPARγ associated with the absence of cilia elongation (Fig. 3A & 3C). Our data suggests that primary cilia elongation in the biochemical induction of adipogenic differentiation is due to one or a combination of dexamethasone, IBMX and indomethacin, with insulin providing negative feedback on the regulation of primary cilia length.

The presence of dexamethasone in both adipogenic and osteogenic media, both of which induced increased cilia length, prompted us to investigate if dexamethasone alone would be able to induce primary cilia elongation. The presence of dexamethasone in BM (BM + Dex) was sufficient to induce primary cilia elongation to a similar level observed in full adipogenic media (AM) conditions (Fig. 3A & 3B). However it was not sufficient to increase nuclear PPARγ levels (Fig. 3A & 3C). Similarly, culturing hMSCs in adipogenic media without dexamethasone substantially reduced the level of cilia elongation and

did not increase nuclear PPARγ (Fig. 3A-C). Cilia prevalence was similar across all groups (Fig. S5).

Primary cilia elongation increases IGF-1Rβ receptor trafficking into the cilium during adipogenic differentiation of hMSCs

Zhu *et al.* have shown that initial activation of IGF-1Rβ in response to insulin stimulation occurs on the primary cilium [32]. Based on these findings and from our results showing effects of adipogenic media components on cilia length, we hypothesized that primary cilia elongation may be associated with increased IGF-1Rβ receptor localization in the cilium and therefore may be necessary for adipogenic differentiation. To test this hypothesis, IGF-1Rβ intensity in primary cilia of hMSCs was assessed over a seven day period in basal media (BM) and adipogenic media (AM). Representative images of primary cilium (green) with corresponding images of IGF-1Rβ localization (red) are presented in Figure 4A. IGF-1Rβ intensity on the primary cilium (Fig. 4B) corresponds with the changes in primary cilia length during adipogenic differentiation (Fig. S6A), displaying the highest intensity at day 2 where cilia elongation was first observed. Ciliary IGF-1Rβ intensity was greater in hMSCs grown in adipogenic media compared to cells grown in BM at all time-points investigated. Although overall increases in cilia length corresponded to increases in ciliary associated IGF-1Rβ, there was no correlation between primary cilia length and ciliary IGF-R1β on an individual cell basis (data not shown). These results confirm that biochemical adipogenic induction drives cilia elongation and an associated recruitment of IGF-1Rβ into the cilium of hMSCs.

Primary cilia length plays a role in regulating hMSC adipogenic differentiation through increased IGF-1Rβ trafficking into the cilium

Following our observations that ciliary IGF-1Rβ localization and nuclear PPAR γ levels increase, correlating to primary cilia elongation during adipogenic differentiation, we hypothesized that primary cilia elongation and associated IGF-1Rβ recruitment to the cilium may be necessary for adipogenic differentiation. To prevent cilia elongation, siRNA targeting IFT88 protein was transfected into hMSCs before and during adipogenic differentiation induction (Fig. 5). Low concentrations of siRNA (25nM) were used to prevent cilia elongation without inducing a complete ciliary loss. 24 hours post transfection with IFT88 siRNA and non-targeting control siRNA, hMSC media was replaced with either BM or AM for 2 days prior to fixation. As nuclear PPARγ levels rose remarkably from day 4 of differentiation (Fig. 2E), some cells were maintained in culture and were subjected to an additional siRNA transfection 72 hrs after the first transfection. These cells were then fixed after a further 72 hrs correlating to day 5 in BM or AM. Partial silencing of IFT88 was verified by western blot with a reduction, but not complete elimination of IFT88 protein at both day 2 and day 5 (AM+siIFT88) compared to non-targeting controls (AM+siNT) (Fig. 5A). We successfully prevented cilia elongation in hMSCs cultured in AM upon IFT88 siRNA treatment at both day 2 and day 5 (Fig. 5B & C). There was no difference in cilia prevalence between any groups at day 2 while at day 5 cells treated with IFT88 siRNA (AM+siIFT88) exhibited a reduction of approximately 10% in prevalence compared to those grown in BM (90.1±2.8%) or AM alone (92.7±1.6%) (Fig. 5D). The reduction in cilia length following silencing of IFT88 corresponded with decreased IGF-1Rβ localization in the primary cilium (Fig. 5B & E). Furthermore hMSCs treated with IFT88 siRNA and cultured in AM exhibited a significant reduction in nuclear PPARγ levels compared to that of AM and non-targeting siRNA (AM+siNT) control groups (Fig. 6A & B). This demonstrates that cilia elongation stimulates IGF-1Rβ ciliary localization and that this is critical for the biochemical induction of adipogenic differentiation in hMSCs.

DISCUSSION

Primary cilia are involved in mediation of various signaling pathways controlling cell development and metabolism. The requirement for primary cilia in adipogenic and osteogenic differentiation of hMSCs has also been shown by complete knock down of the ciliary trafficking protein, IFT88, resulting in loss of primary cilia and decreased expression of adipogenic and osteogenic markers [17]. Our data shows that primary cilia undergo structural changes during differentiation of hMSCs grown in chemically defined media (Fig. 1). Interestingly, both adipogenic and osteogenic media contain dexamethasone and produced an increase in cilia length. Indeed, we have confirmed that this glucocorticoid steroid drives cilia elongation in hMSCs (Fig. 3). By contrast the presence of TGF-β3 in chondrogenic media causes cilia shortening. Here we focus on the cilia elongation during adipogenic differentiation but changes in cilia structure may also be important for differentiation down various other lineages.

We therefore investigated cilia length and prevalence changes over a seven day time course during adipogenic differentiation and found that elongation occurred within the first 2 days (Fig. 2). This elongation was associated with changes in actin stress fiber organization which have previously been linked to cilia elongation via actin tension [35]. The time course demonstrates that cilia length was increased in adipogenic media throughout the 7 day culture period, although the level of cilia elongation was reduced at day 4 and day 7. Nuclear translocation of PPARγ is required for the transcriptional activation of genes upregulated by adipogenic differentiation [36, 37] . Nuclear levels of PPARγ were increased at day 2 of adipogenic differentiation but were increased further from day 4 onwards, after maximum cilia elongation had occurred. This suggests that cilia elongation precedes maximal differentiation induction,

and is therefore an early event in the differentiation cascade driven directly by the media components.

We next analyzed the effects of adipogenic media components on cilia length. Adipogenic media is composed of dexamethasone, IBMX, indomethacin with the inclusion of insulin, either from day 0 or after 24 hrs [38]. We observed that hMSCs displayed the longest cilia in adipogenic media without insulin (Fig. 3). In fact insulin had a negative effect on cilia length when added to adipogenic media. Nuclear PPARγ localization increased corresponding to increased cilia length in the absence of insulin but was more enhanced upon supplementation with insulin and in full adipogenic media. Insulin alone in basal media did not induce cilia elongation and resulted in low nuclear PPARγ levels. Our findings correlate with a previous study that showed accumulation of lipid droplets could occur in hMSCs grown in presence of dexamethasone, IBMX and indomethacin without insulin, but not in cells grown in media that contained either insulin or IGF-1 alone [39]. Therefore we propose dexamethasone, IBMX and indomethacin induce adipogenic differentiation through induction of cilia elongation. This agrees with previous studies that suggest that although insulin is also known to be key mediator of adipogenesis, its effects occur primarily after initiation of differentiation and are instead associated with the maintenance of adipogenic differentiation [33, 34].

IGF-1 has been shown to be key in adipogenic induction of 3T3-L1 mouse preadipocytes through binding to its cognate receptor IGF-1R [34]. Furthermore, primary cilia are known to play a role in adipogenic differentiation of mouse preadipocytes through initial activation of cilium localized IGF–1R upon insulin induction followed by phosphorylation of its downstream effector insulin receptor substrate 1 (IRS-1) and Akt at the basal body [32]. We therefore hypothesized that cilia elongation is associated with trafficking of IGF-1R into the cilium. We have shown that there is indeed a significant increase in localization of IGF-1Rβ in the cilium upon induction of cilia elongation with adipogenic media (Fig. 4). We therefore suggest that adipogenesis is initiated by IGF-1 activation of IGF-1Rβ following its recruitment to the cilium associated with cilia elongation. Insulin may then exert a negative feedback on primary cilia length in line with absence of primary cilia in mature adipocytes [40, 41] and possibly in order to activate pathways for terminal differentiation.

Finally we demonstrate that preventing primary cilia elongation in hMSCs by subtly inhibiting IFT88 proteins resulted in decreased ciliary IGF-1Rβ and a subsequent decrease in nuclear PPARγ levels in the presence of adipogenic media. Thus we show for the first time that primary cilia elongation is crucial for adipogenic differentiation of hMSCs through increased IGF-1R trafficking into the cilium and that blocking this elongation impairs adipogenic induction. The presence of primary cilia is important for regulation of fat metabolism and satiety. Loss of primary cilia or mutations in basal body proteins leading to truncated cilia have previously been shown to lead to obesity [42] . Therefore this study also suggests that pharmaceutical manipulation of primary cilia elongation and trafficking could provide a mechanism for preventing adipogenesis and associated obesity.

Conclusion

In this study we show that adipogenic differentiation is associated with an increase in cilia length. However within differentiated cells, there is no correlation between cilia length and ciliary IGF-1Rβ expression or nuclear PPARγ levels (Fig. S3). This indicates that it is the elongation and associated alteration in cilia trafficking that drives adipogenic differentiation rather than the length per se. This is confirmed by disruption of intraflagellar transport with siRNA to IFT88 which prevented induction of cilia elongation blocking IGF-1Rβ recruitment to the primary cilia and subsequent adipogenesis. These studies provide a new insight into the role of primary cilia in stem cell differentiation and highlight, for the first time, how changes in cilia structure are required for differentiation. This has further implications given the expanding range of physical and biochemical factors which modulate primary cilia length and may therefore influence adipogenic differentiation of stem cells.

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Author Contributions

M.T.D.: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; S.D.T.: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; J.T.C.: Interpretation of data, final approval of manuscript; J.P.C.: Interpretation of data, manuscript writing, final approval of manuscript; M.M.K.: Conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Figure 1. Primary cilia length changes during hMSC differentiation in a lineage specific manner. (A) Representative maximum projection confocal images of hMSC primary cilium at day 0 and day 7 of culture in basal media (BM), adipogenic media (AM), osteogenic media (OM), chondrogenic media without TGF-β3 (CM-), chondrogenic media with TGF-β3 (CM+). Primary cilia were labeled for acetylated α-tubulin (green), the basal body stained for pericentrin (red), while nuclei were stained with Hoechst 33342 (blue). Scale bars represent 10 µm. (B) Primary cilia length at day 0 and day 7 of differentiation induction. *n* =100 cilia measured per group. ***: *p*<0.001 vs. Day 7 BM, ###: *p*<0.001 vs. Day 7 CM-; Mann-Whitney U test. (C) Corresponding cilia prevalence at day 0 and day 7 of differentiation induction. *n* = 5 fields per condition; *: *p*<0.05 vs. Day 7 BM; Mann-Whitney U test. (D) Representative immunofluorescent staining of Ki67 (green, top) and DNA (blue, bottom). Scale bar represents 100 µm. (E) Quantification of cell proliferation by Ki67 nuclear staining in hMSCs at day 7 of differentiation. *n* = 5 fields per condition with > 100 cells / field. A one-way ANOVA revealed no significant differences in proliferation rate between the conditions.

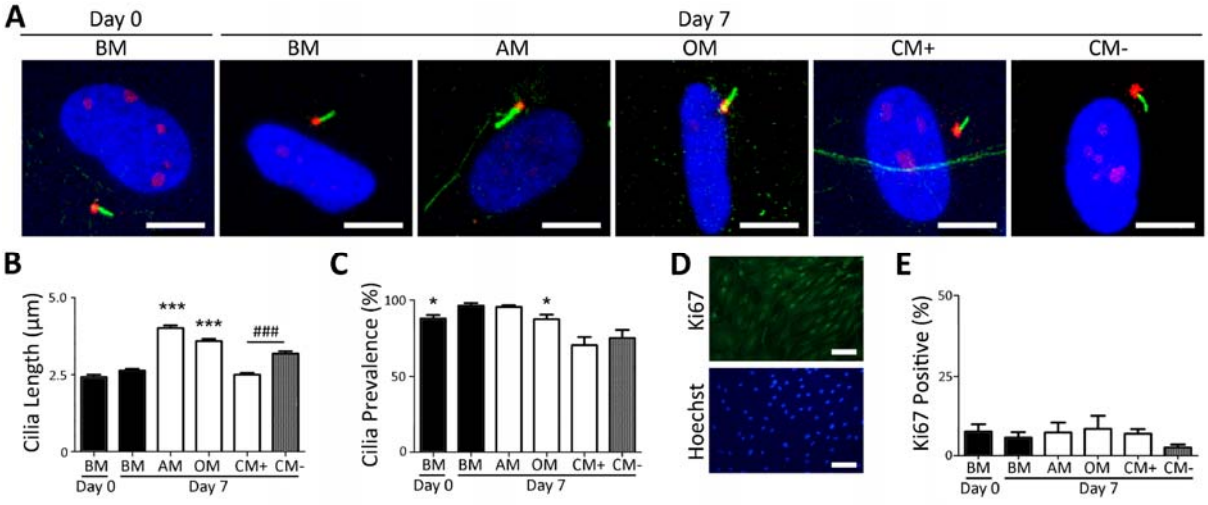

Figure 2. Time course showing nuclear PPARγ localization increases following primary cilia elongation over a 7 day culture period in adipogenic media. (**A**) Representative maximum projection confocal images of hMSCs grown in basal media (BM; top row) and adipogenic media (AM; bottom row) with a single cell inset from day 2 to 7. PPARγ is shown in red, acetylated α-tubulin in green, and the Hoechst stained nucleus in blue. Scale bars represent 20 μm for fields and 10 μm for inset images. (**B**) Representative confocal images showing F-actin (yellow) in hMSCs cultured in BM (top row) and in AM (bottom row) from day 2 to day 7. Hoechst stained nuclei in blue. Scale bars represent 20 μm. Primary cilia length (**C**), prevalence (**D**) and nuclear PPARγ intensity (**E**) for hMSCs cultured in either BM or AM. n=100-110 cilia in (C); *n* = 5 fields in (D); *n* = 100-110 nuclei in (E). *: $p<0.05$ vs. BM, **: $p<0.01$ vs. BM, ***: $p<0.001$ vs. BM; Mann-Whitney U test).

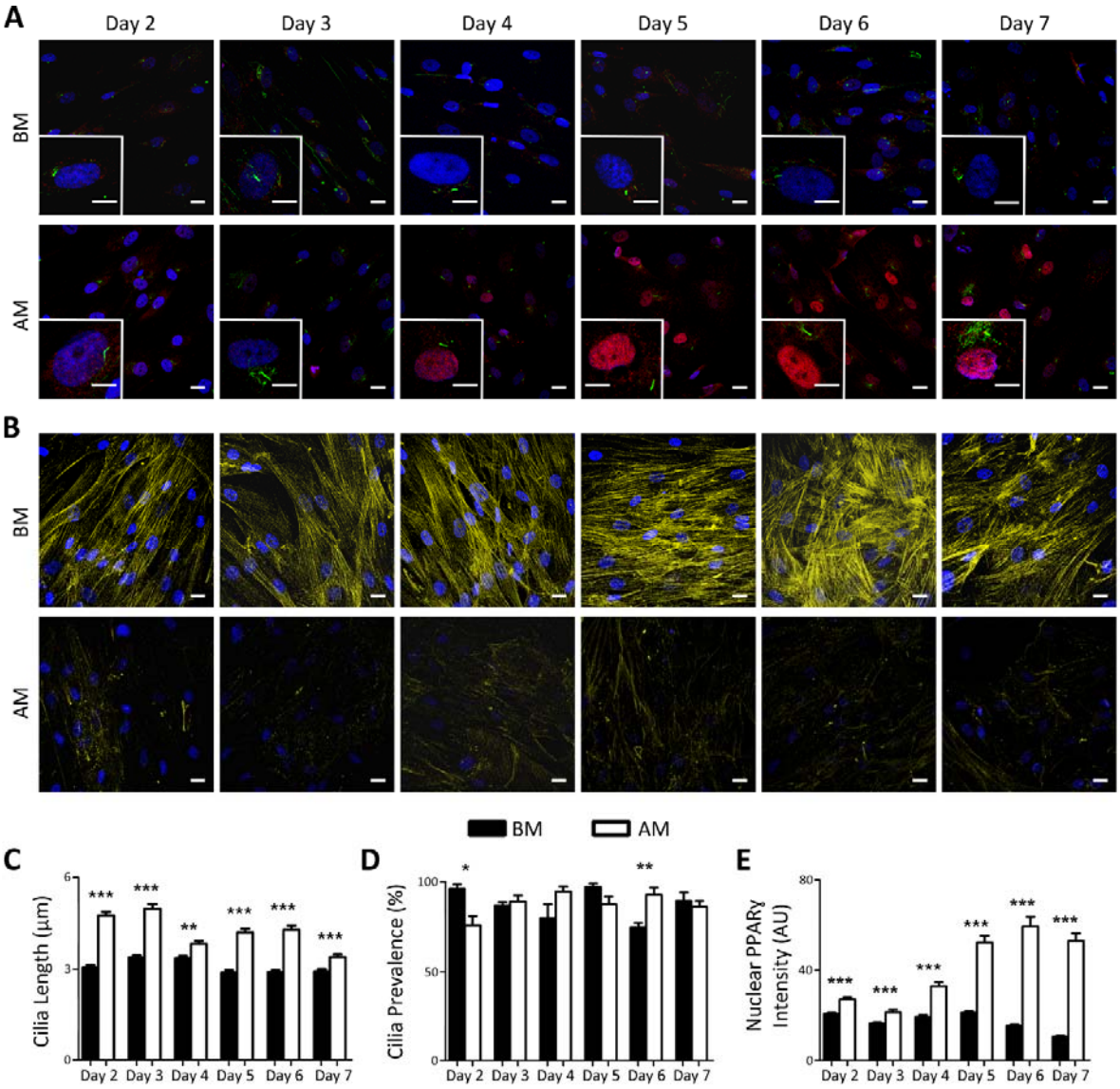

Figure 3. The role of media components in cilia elongation and adipogenic differentiation. (**A**) Representative confocal images showing primary cilia and nuclear PPARγ expression in hMSCs cultured for 2 days in the presence of different media components: BM, complete adipogenic media including insulin (AM), BM with insulin (BM+Ins), adipogenic media without insulin (AM-Ins) ,adipogenic media with no insulin for the initial 24 hrs followed by addition of insulin for 24 hrs (AM-/+Ins), BM with dexamethasone alone (BM+Dex) and adipogenic media without dexamethasone (AM-Dex). PPARγ is shown in red, acetylated α-tubulin in green, and Hoechst stained nuclei in blue. Scale bars represent 10 µm. Cilia length (**B**) and nuclear PPARγ intensity (**C**). *n* = 100-110 cilia per group in (B) and n= 100-110 nuclei per group in (C). *: $p< 0.05$ vs. BM, *** $p<0.001$ vs. BM, #: $p<0.05$ vs. AM, ##: $p<0.01$ vs. AM, ### p<0.001 vs. AM; Mann-Whitney U test.

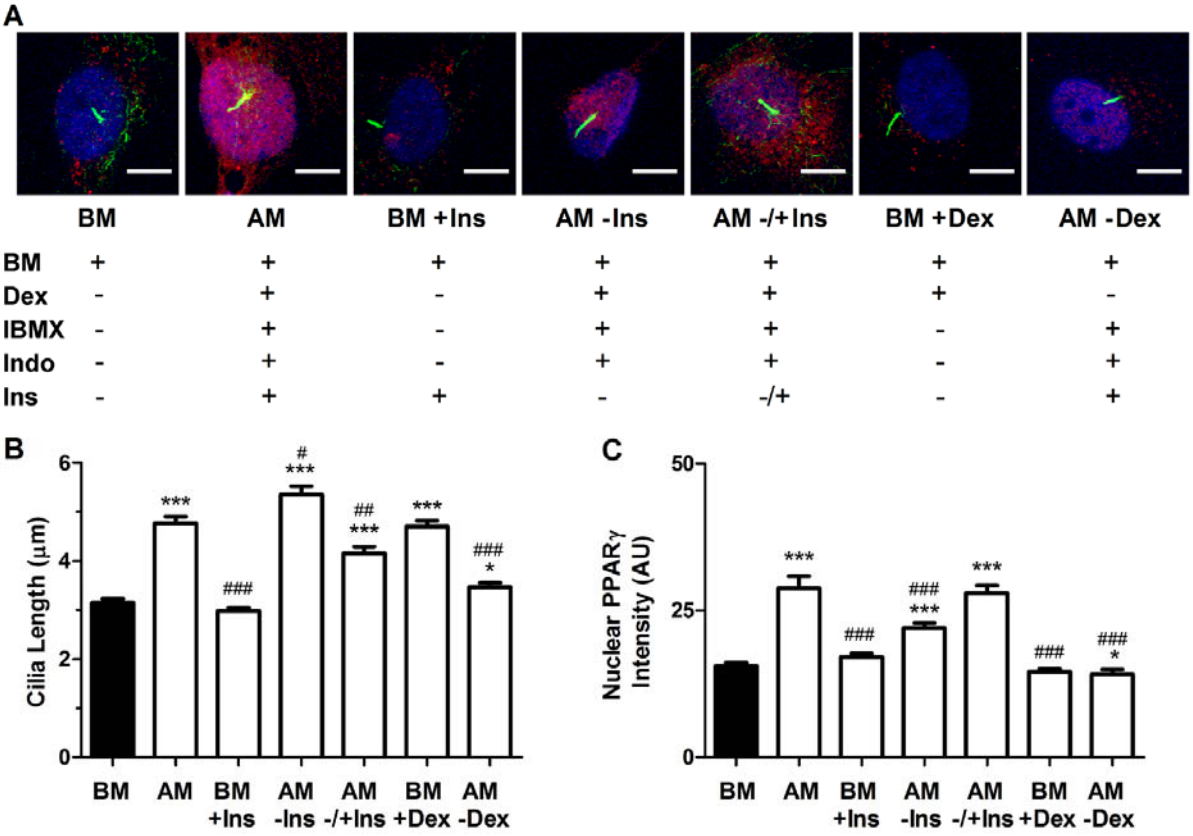

Figure 4. Cilia elongation in adipogenic media is associated with increased IGF-1Rβ localization at the primary cilia. (**A**) Representative images of cilia labeled for acetylated α-tubulin (top, green) of hMSCs cultured in BM and AM and corresponding images labeled for IGF-1Rβ (bottom, red). Scale bars 10 μm. (**B**) Ciliary IGF-1Rβ intensity for BM and AM cultured hMSCs from day 2 to day 7. *n* = 100-110 cilia measured per condition. ***: *p*<0.001 vs. BM; Mann-Whitney U test).

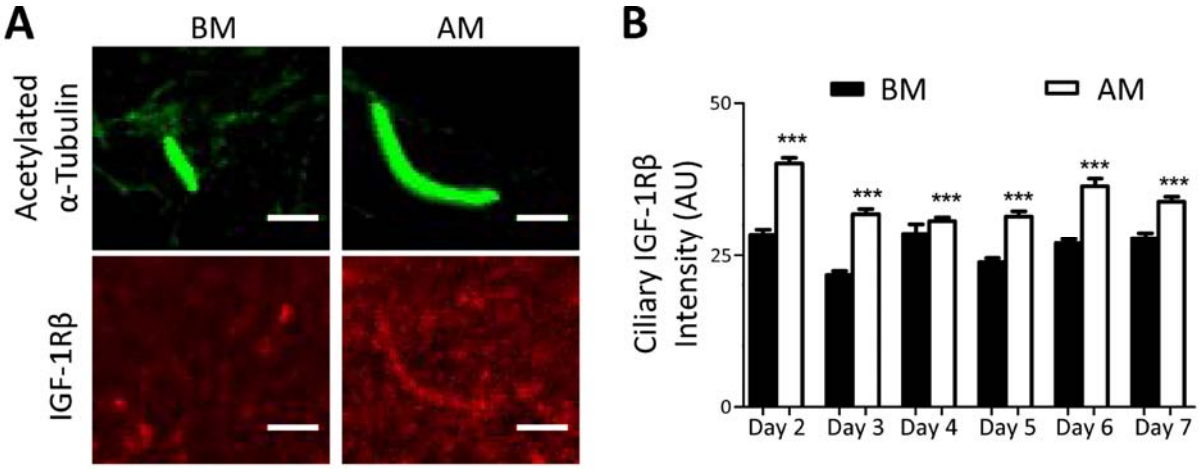

Figure 5. IFT88 siRNA prevents cilia elongation and reduces IGF-1Rβ trafficking into the cilium. (**A**) Western blot for IFT88 and β-actin protein for hMSCs transfected with either IFT88 siRNA (AM+siIFT88) or non-targeting siRNA (AM+siNT) at day 2 and day 5 of differentiation induction (both 72 hrs post transfection with day 5 receiving a second transfection at day 2). (**B**) Representative images of cilia labeled for acetylated α-tubulin (green) and IGF-1Rβ (red) for BM, AM, and AM groups with IFT88 siRNA (AM+siIFT88) or non-targeting siRNA (AM+siNT) (**C**) Cilia length, (**D**) prevalence and (**E**) ciliary associated IGF-1Rβ for each group as described above for day 2 (left) and day 5 (right). *n* = 100-110 cilia measured per group for (C) and (E), *n* ≥ 5 fields per group with ≥ 10 cells/field for (D). *: p<0.05 vs. BM, **: *p*<0.01 vs. BM, ***: *p*<0.001 vs. BM, ##: *p*<0.01 vs. AM, ###: *p*<0.001 vs. AM, +++: *p*< 0.001; Mann-Whitney U test.

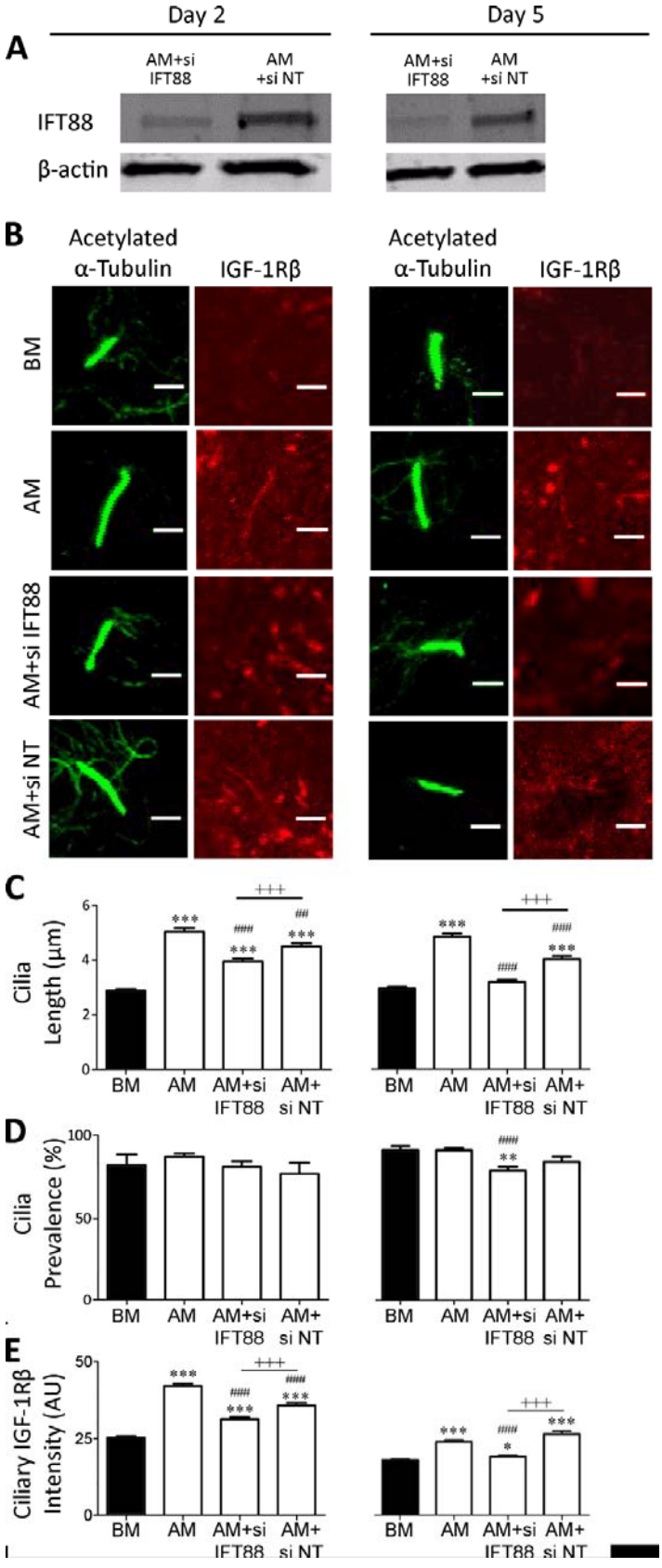

Figure 6. IFT88 mediated primary cilia elongation is critical for adipogenic differentiation of hMSCs. (**A**) Representative maximum projection confocal images showing hMSCs cultured in basal media (BM), adipogenic media (AM), AM with IFT88 siRNA (AM+siIFT88) and AM with non-targeting siRNA (AM+siNT). Cells were analyzed at day five of differentiation induction corresponding to 72 hrs post second transfection at day 2. PPARγ is shown in red, primary cilia in green and Hoechst stained nucleus in blue. Scale bars represent 20 µm for fields (top) and 10 µm for single cell images (bottom). (**B**) Nuclear PPARγ expression for conditions described above. *n* =100-110 nuclei per condition. **: $p<0.01$ vs. BM, ***: $p<0.001$ vs. BM, #: $p<0.05$ vs. AM, ###: $p<0.001$ vs. AM, +: $p<0.05$, +++: $p<0.001$; Mann-Whitney U test.