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# The primary cilium undergoes dynamic size modifications during adipocyte differentiation of human adipose stem cells

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ABSTRACT

The primary cilium is an organelle present in most of the cells of the organism. Ciliopathies are genetic disorders of the primary cilium and can be associated with obesity. We have studied the primary cilium during adipocyte differentiation of human adipose stem cells (hASC). We show here that the size of the primary cilium follows several modifications during adipocyte differentiation. It is absent in growing cells and appears in confluent cells. Interestingly, during the first days of differentiation, the cilium undergoes a dramatic elongation that can be mimicked by dexamethasone alone. Thereafter, its size decreases. It can still be detected in cells that begin to accumulate lipids but is absent in cells that are filled with lipids. The cilium elongation does not seem to affect the localization of proteins associated with the cilium such as Kif3-A or Smoothened. However, Hedgehog signaling, an anti-adipogenic pathway dependent on the primary cilium, is inhibited after three days of differentiation, concomitantly with the cilium size increase. Together, these results shed new light on the primary cilium and could provide us with new information on adipocyte differentiation under normal and pathological conditions.



## 1. Introduction

The primary cilium is an organelle present in one copy in most of the cells of the organism [1–3]. It is composed of a basal body and an axoneme made of 9 doublets of tubulin. The cilium has different functions. It is used as a mechanoreceptor in kidney cells, and bears the photoreceptors in rods and cones. This organelle has also important signaling transduction properties. Signaling pathways, such as Hedgehog, Wnt and TGF, are, to some extent, dependent upon the primary cilium. The cilium itself is regulated. It disappears during cell proliferation. Indeed, during mitosis, centrioles of the basal body are recruited to form the centrosome which nucleates the mitotic spindle. When cells enter the G0 phase of the cell cycle the centrioles migrate to the plasma membrane, in vicinity to the nucleus, to re-form the basal body and the axoneme of the cilium. The primary cilium also disappears during specific biological processes, such as the differentiation of mesenchymal stem cells. Indeed, we have shown that osteoblastic differentiation of human adipose stem cells (hASC) is associated with a loss of the cilium [4]. Marion et al. have shown that the cilium disappears during adipocyte differentiation [5]. More recently, Fu et al. showed that the cilium disappears during myogenic differentiation [6].

The importance of the cilium in adipogenesis is supported by the phenotype of patients suffering from Bardet–Biedl and Alström syndromes [7,8]. Indeed, these ciliopathies are associated with obesity. It is thus important to understand the biological regulations of the primary cilium during adipocyte differentiation.

Although it has previously been shown that adipocytes do not possess a primary cilium [5] the precise details of the behavior of the primary cilium during adipocyte differentiation was still unknown. Here, we reveal that hASC possess a primary cilium, the size of which undergoes several modifications during the adipocyte differentiation process. We show that the cilium undergoes a

*Abbreviations:* hASC, human adipose stem cells; Hh, Hedgehog; IBMX, isobutyl methyl xanthine.

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dramatic increase in size during the first days of differentiation. Interestingly, the cilium can still be detected in cells that begin to accumulate lipids. It disappears when cells are filled with lipids. This modulation of cilium size could have important functions in adipogenesis under normal and pathological conditions.

## 2. Material and methods

### 2.1. Material

Shh-conditioned medium was obtained from an HEK 293 cell line stably transfected with Shh-N expression vector (ATCC #CRL-2782). Antibodies: monoclonal anti-acetylated tubulin was from Sigma–Aldrich (Saint Quentin Fallavier, France), guinea pig anti-perilipin 1 was from Acris antibodies (Herford, Germany), rabbit anti-Kif-3A was from Abcam (Cambridge, UK), rabbit anti-Smoothened was from Santa Cruz Biotechnologies (Ozyme, St Quentin en Yvelines, France). Anti-mouse antibody coupled to Alexa fluor 488, anti-rabbit antibody coupled to Alexa fluor 647 and anti-guinea pig antibody coupled to Alexa fluor 594 were from Life Technologies (Saint Aubin, France).

### 2.2. Growth and adipocyte differentiation of hASC

Establishment and characterization of the adipocyte differentiation of hASC have been previously described [9]. These cells are multipotent, display a normal karyotype and are not transformed. hASC were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2.5 ng/ml hFGF-2, 60 μg/ml penicillin, and 50 μg/ml streptomycin. At day 2 post-confluence (designated day 0), cells were induced to differentiate into adipocytes in the presence of DMEM/Ham's F12 media supplemented with 0.85 μM insulin, 10 μg/ml transferrin, 1 μM dexamethasone, 100 μM isobutyl-methyl xanthine (IBMX), and 0.1 μM rosiglitazone. Three days later, the medium was changed (dexamethasone and IBMX were omitted). Media were changed twice a week.

### 2.3. Immunocytochemistry

Cells were seeded on cover slips and treated as described in the text. Cells were rinsed twice with PBS and fixed with Roti-Histofix (Roth, Lauterbourg, France) for 20 min at room temperature or with methanol at −20 °C. Fixed-cells were incubated in PBS with 3% bovine serum albumin (BSA), 0.1% tween-20 and 0.1% triton X-100 for 30 min at room temperature. Cells were then incubated with the appropriate antibodies in the same buffer for 90 min at room temperature. After 3 washes in PBS, cover slips were incubated with the appropriate secondary antibody coupled to Alexa Fluor (1:600) for 45 min at room temperature. At least ten representative fields were examined for each condition. The total number of cilia was counted along with total number of nuclei for at least 50 cells. Cilia length was measured using Fiji [10].

### 2.4. RNA extraction and analysis

Total RNAs were extracted with the TRI-Reagent kit (Euromedex, Souffelweyersheim, France) according to manufacturer's instructions. Total RNA was subjected to real-time quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis as described in Ref. [11] except that Sybr Green was from Takara (Ozyme, St Quentin en Yvelines, France). Primers were designed using Primer Express software (Applied Biosystems, Courtaboeuf, France) and validated by testing PCR efficiency using standard curves (85% ≤ efficiency ≤ 115%). Gene expression was quantified using the comparative $C_T$ (threshold cycle) method on a StepOnePlus system (Applied Biosystems); TBP was used as reference.

Primers used (Forward; Reverse):

TBP: CACGAACCACGGCACTGATT, TTTTCTTGCTGCCAGTCTGGAC;
Gli1: TGCAGTAAAGCCTTCAGCAATG, TTTTCGCAGCGAGCTAGGAT;
PPARγ: AGCCTCATGAAGAGCCTTCCA; AGCCTCATGAAGAGCCTTCCA,
PLN: ACCATCTCCACCCGCCTC; GATGGGAACGCTGATGCTGT;
BBS10: TGAAAACATTCAAACCCATGGA: CGTTAGGAGAGCCTGGGAAA;
BBS12: GCTCAGGGTGGCTGCATAAT, TTGGTCTGTATATTGCCAGAGATGA;
Kif3A: TACTGGACAGCGCCTAAAGGA; CCAAGGCAGAAATTACATTACCAA;
IFT88, TGACATCTGCAAAACTCATTGCT; TCCACGCACCAATCATAACCT;
SREBP: GGAGGGGTAGGGCCAACGGCCT; CATGTCTTCGAAAGTGCAATCC.

### 2.5. Electron microscopy

After hours of fixation, the samples were rinsed in distilled water, dissected and dehydrated with a series of ethanol solutions of increasing concentrations. Samples were then dried using hexamethyldisilane (Carl Roth, Karlsruhe, Germany) and sputter-coated with 3-nm gold–palladium coating prior analysis with the scanning electron microscope, JEOL 6700F. The pictures were collected at low voltage 1–3 kV.

### 2.6. Statistics

Data are shown as means ± SD. Statistically differences between groups were analyzed using unpaired Student's t test.

## 3. Results

### 3.1. The primary cilium undergoes a transient elongation during adipocyte differentiation

We studied the primary cilium throughout adipocyte differentiation. To do this, hASC were grown and adipocyte differentiation was induced as described in Materials and Methods. Cells were fixed at proliferation (Supplementary Fig. 1), at day 0 (two days after cell confluence) and 3, 7, and 14 days after the induction of differentiation. Acetylated alpha tubulin, which is concentrated in the axoneme and widely recognized as a specific marker of the primary cilium, was detected by immunocytochemistry. Adipocyte differentiation was assessed through perilipin expression, a protein that surrounds lipids droplets in adipocytes (Fig. 1A). The size of the cilium (Fig. 1B), and the percentage of cells displaying a primary cilium (Fig. 1D) were quantified. As expected, proliferating cells do not exhibit a primary cilium (Supplementary Fig. 1 and Fig. 1D). At day 0, most of the cells (76%) exhibit a primary cilium. At this point, the size of the cilium is relatively homogenous, with more than 80% of the cilia ranging between 2 and 4 μm. Interestingly, after 3 days, the size of the cilium increases and is more heterogeneous. Most of the cells display a cilium between 4 and 8 μm and more than 20% of the cilia are longer than 8 μm. This increase in size is still apparent at day seven, when some cells begin to accumulate lipids. It can be noted that several perilipin-positive cells maintain a cilium. At day 14, only 20% of the cells display a primary cilium with a size similar to the one observed at day 0. At this point, cells exhibiting a primary cilium do not accumulate lipids; and conversely, all perilipin-positive cells are missing a cilium.

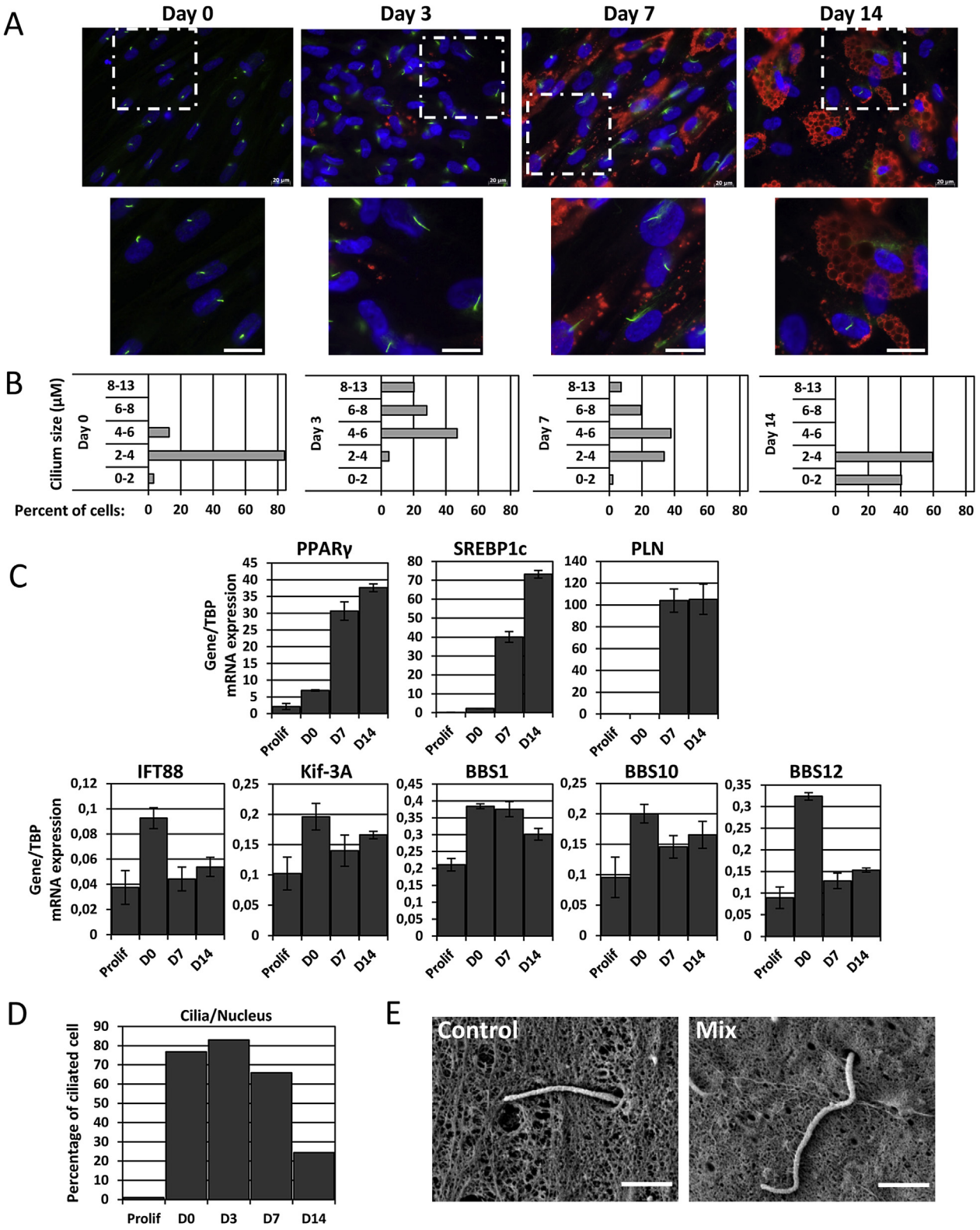


**Fig. 1.** A) hASC were treated for various days with a differentiation mix (MIX). At the indicated time cells were fixed. Acetylated tubulin (in green) and perilipin (in red) were revealed by immunocytochemistry. Nuclei were stained with Hoechst 33258. The white bar represents 20 µm. The insets represent a magnification of the dotted box. B) Cilia were measured. Percentages of cilia belonging in each class size were represented in the histograms. C) RNA were extracted at the indicated time and gene expression was measured using quantitative RT-PCR. D) The percentage of cells displaying a primary cilium from 1A was measured and is represented in this histogram. E) hASC were treated for three days with a control medium or with the differentiation mix (MIX). Cells were fixed and analyzed by scanning electron microscopy. The white bar represents 1 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We then analyzed the cilium size through electron microscopy. hASC were treated for three days with a control or a differentiation medium, fixed and visualized by scanning electron microscopy (Fig. 1E). As observed, hASC exhibit a primary cilium, with a visible ciliary pocket. The size of the cilium was more important in cells treated for three days with the differentiation medium. This confirms the results obtained with immunochemistry and ruled out the possibility that the increase in cilium size was linked to a modification in the composition of acetylated tubulin of the axoneme. It also indicates that the increase in cilium length takes place outside of the cell, and is not linked to an increase of this organelle in the intracellular compartment.

We then compared the time-course between the modulation of cilium size and the expression of specific genes. We tested the expression of adipocyte-specific genes (PPARγ, SREBP1c, Perilipin), and of several cilium-related gene (IFT88, Kif-3A, BBS1, BBS10, BBS12) using quantitative RT-PCR (Fig. 1C). As observed, the kinetic of the increase in cilium size and its disappearance at day 14 does not seem to be paralleled by the expression of the tested genes. Moreover, the loss of the cilium appears later than the expression of the adipocyte specific genes that we tested. The loss of the cilium is a late event in adipocyte differentiation and corroborates the observation that cells that begin to accumulate lipids still maintain a cilium.

These data indicate that the primary cilium is dynamically regulated during the differentiation process. It grows in size during the first days of differentiation. Thereafter, it can be still detected in cells that begin to accumulate triglyceride but is absent in lipid-loaded cells.

### 3.2. Dexamethasone increases cilia length

We then tried to isolate the component of the differentiation medium responsible for the increase in cilia length. hASC were treated with the control medium, or with the control medium supplemented with transferrin, insulin, rosiglitazone, IBMX, dexamethasone, or with the complete differentiation medium. After three days, the cilium was analyzed as described for Fig. 1. As observed in Fig. 2A, only dexamethasone causes an increase of the cilium length. We then performed a dose–response study of dexamethasone. Cells were treated with increasing doses (from 10 nM to 1 μM) of dexamethasone for 3 days. The cilium was then measured (Fig. 2B). The effect of dexamethasone is apparent at 10 nM, with a maximal effect observed at 1 μM (see Supplemental Fig. 2 for photographs).

### 3.3. The increase in cilium length does not modify the localization of cilium proteins but is concomitant with a loss of Hh signaling

We then tested if the increase in the cilium size was associated with a modification in the localization of ciliary proteins. We studied two well described proteins located in the cilium: the kinesin Kif-3A, and smoothened, a transmembrane protein that controls the Hh pathway. hASC were treated for 3 days with or without the differentiation medium and the localization of Kif-3A and Smoothened was analyzed by immunochemistry. As observed in Fig. 3A, Kif-3A is localized at the tip and the base of the cilium while smoothened is detected at the base of the primary cilium. The differentiation medium increases the size of the cilium but did not modify the localization of these proteins. Same results were obtained with dexamethasone alone (data not shown). We then looked at Hh signaling. Indeed, Hh is probably the most well defined signaling pathway the activation of which is dependent upon the primary cilium. Cells were treated or not for three days with the differentiation medium then treated for 24 h with Shh. The expression of Gli-1, a reliable marker of Hh activity [12–14],

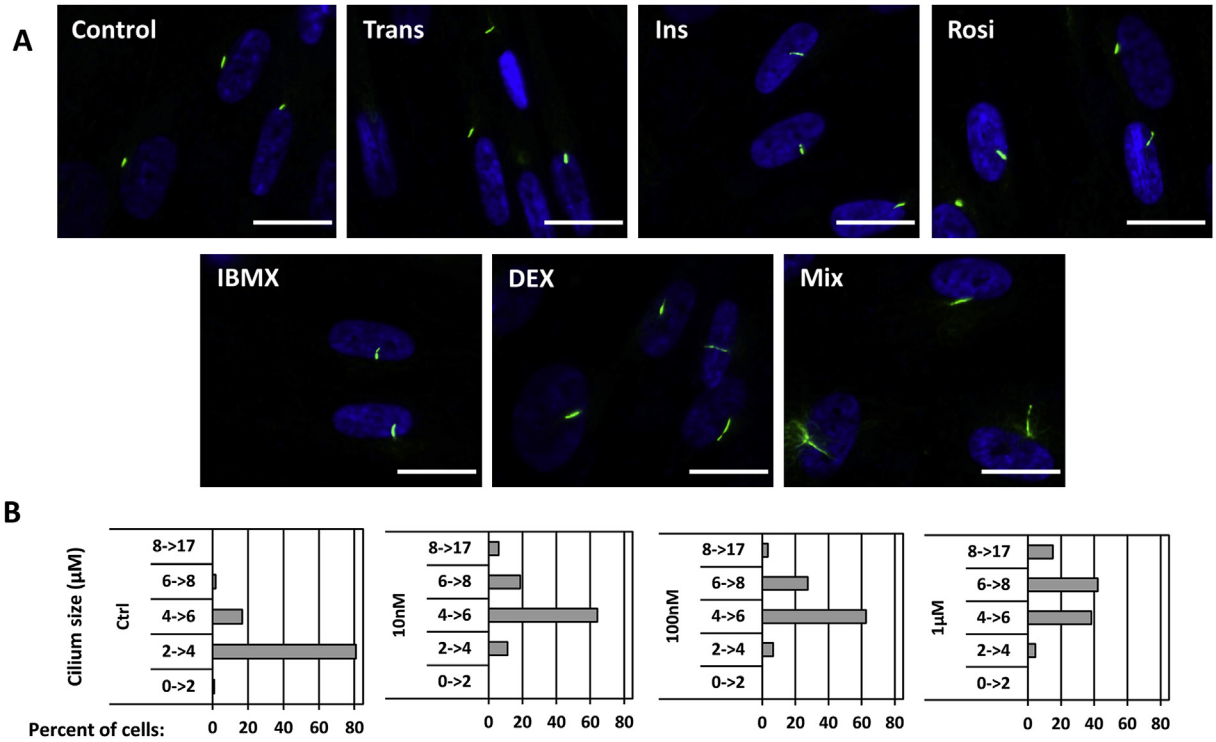


**Fig. 2.** A) hASC were treated with the control medium (Control), or with the control medium supplemented with transferrin (Trans), insulin (Ins), rosiglitazone (Rosi), IBMX, Dexamethasone (Dex), or with the complete differentiation mix (MIX) for three days. Acetylated tubulin (in green) was revealed by immunocytochemistry. Nuclei were stained with Hoechst 33258. The white bar represents 20 μm. B) hASC were treated with a control medium (Ctrl), with 10, 100 nM and 1 μM of dexamethasone (Dex) for three days. Cilia were measured. The percentages of cilia belonging in each class size are represented in the histograms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

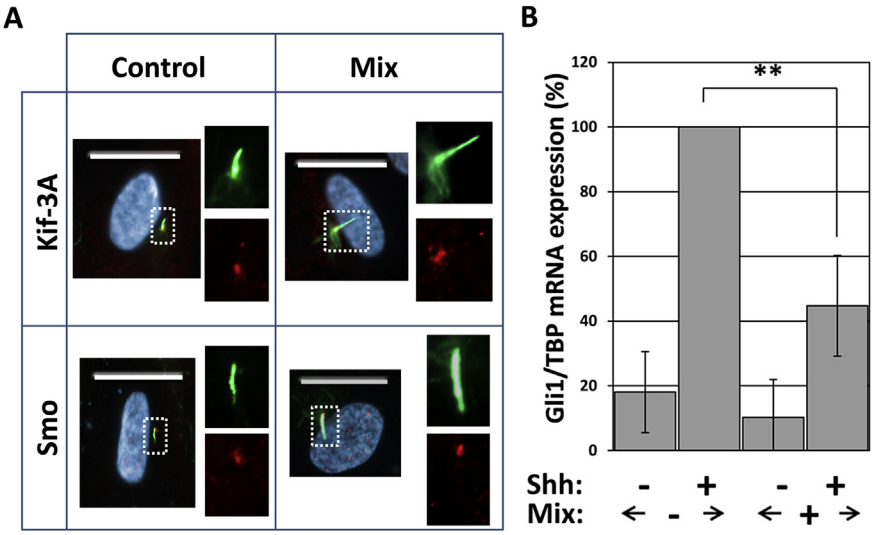


**Fig. 3.** A) hASC were treated with the control medium (Control) or a differentiation mix (MIX) for three days. Acetylated tubulin (in green) and Kif-3A or Smoothened (smo) (in red) were revealed by immunocytochemistry. Nuclei were stained with Hoechst 33258. The white bar represents 20 μm. The insets represent a magnification of the red and green channels in the dotted box. Please note that these insets are not at the same scale between each condition. B) hASC were treated with "−" or without "+" differentiation mix (MIX) for three days. Gli-1 expression was evaluated by real-time quantitative (RT-PCR). Control medium + aShh was taken as 100%. Results are the mean +/− SD of three experiments performed in triplicate. $^{**}p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was then measured by quantitative RT-PCR. As observed in Fig. 3B, Shh treatment induces a 5-fold increase in Gli-1 expression in control cells. A 50% decrease in Gli-1 expression is observed in cells treated for 3 days with the differentiation medium. Thus, cells treated with the differentiation medium are resistant to Hedgehog.

## 4. Discussion

The primary cilium was first described in 1898 [15] but it began to draw particular attention when its involvement in Hedgehog signaling became apparent in 2005 [16]. Since then, its implication in various biological functions has been revealed [1–3]. The involvement of the primary cilium in adipogenesis is supported by the observation that patients suffering from ciliopathies such as Bardet–Biedl and Alström syndroms are obese [7,8]. Although these patients suffer hyperphagia, pair-feeding experiments of *bbs2*$^{-/-}$ and *bbs4*$^{-/-}$ mice have revealed that hyperphagia is not sufficient to explain obesity [17] and, consequently, that adipogenesis is affected in these ciliopathies.

We show here that the cilium undergoes several modifications during adipocyte differentiation: it is present in confluent cells; it grows in size during the first days of differentiation and then disappears when cells are loaded with lipids.

### *4.1. What induces the increase in cilium size after three days of differentiation?*

Cell shape and the actin cytoskeleton, in particular actin stress fibers, have been shown to affect the frequency and the size of the primary cilium. Cell displaying stress fibers are less likely to grow a cilium than confined cells [18]. A functional screen for modulators of cilium length has allowed for the identification of actin-regulating proteins as well as proteins involved in vesicle trafficking. In this study, the authors proposed that actin dynamics, rather than global actin cytoskeleton rearrangement, control ciliogenesis [19]. Adipocyte differentiation is associated with a reorganization of the actin cytoskeleton from stress fibers to cortical fibers [20]. This phenomenon could be responsible, by part, for the increase in size of the primary cilium during the first days of differentiation. However, since the cytoskeletal reorganization is maximal after 12 days of differentiation it is not sufficient to explain the modifications sustained by the cilium during adipogenesis. The size-increase of the primary cilium during adipocyte differentiation could be mimicked by dexamethasone alone. Interestingly, although hASC could differentiate to some extent in absence of insulin or IBMX, they failed to differentiate in absence of dexamethasone and rosiglitazone [21]. In hASC, increasing cAMP by IBMX, was not able to modify significantly cilium length after three days, as has been described in other cell types [22–24].

### *4.2. What is responsible for the loss of the cilium during adipogenesis?*

The loss of the cilium has been studied during cell proliferation. During mitosis, an activation of Aurora A is responsible for the activation of HDAC-6 [25]. HDAC-6 deacetylates tubulin leading to a loss of the primary cilium. Since Aurora A is not activated during adipogenesis (data not shown) the loss of the cilium during adipogenesis must follow another pathway. Comparison between kinetics of gene expression and the ratio of cilium-positive cells indicate that the loss of the cilium is a very late event of adipocyte differentiation. The cilium is composed of at least a thousand of proteins [26,27]. We have tested for the expression of some of them (Fig. 1). We observed that the modulation of their expression is unlikely to explain for the loss of the primary cilium. It is interesting to observe that the loss of the primary cilium is observed only in cells filled with large triglycerides droplets. Cells beginning to accumulate lipids still exhibit a primary cilium. Along these lines, it has been observed that several cancer cells lose their primary cilium in a mechanism that is lipid- and SREBP-1c- dependent [28]. SREBP1-c is a transcription factor induced during adipogenesis that controls, among other things, the expression of enzymes related to lipogenesis. Our experiments of lipids treatment of hASC have not allowed us to observe a modification of the primary cilium (data not shown). It can still be envisioned that, in hASC, only an important amount of lipids; or specific lipids, are responsible for the loss of the cilium.

### 4.3. What could be the biological function of the modulation of cilium size?

The function of the cilium during adipocyte differentiation is unclear. Marion et al. showed that depletion of BBS12 decreases the ratio of cilium-positive cells and increases adipocyte differentiation [5]. On the other hand, affecting the primary cilium through a decrease in Alms [29], IFT88 or Kif-3A [30,31] expression inhibit adipocyte differentiation. These observations suggest that the primary cilium controls several steps of adipogenesis and cannot be considered only as a "switch" that triggers or inhibits adipocyte differentiation. This appears consistent with the various modifications in cilium size that we observe during adipogenesis. After three days of treatment, when the cilium reaches its longer size, cells become resistant to Hh activation. The observation that the differentiation medium does not modifies the cellular localization of smoothened indicates that the mechanism of Hh resistance resides elsewhere. So far, we cannot ascertain that cilium elongation and Hh resistance in 3 days-differentiated cells are causally linked. However, we observed previously that Hh signaling inhibits the maturation of hASC [11]. By affecting Hh signaling, cells would protect themselves from an anti-adipogenic effect of Hh. It can also be envisioned that the increase in cilium size could allow for the amplification of specific signaling pathways that remains to be identified. Alternatively, the increase in the cilium size could help to "fish" for pro-adipogenic-circulating molecules. Indeed, because the cilium "floats" freely in the extracellular medium, receptors located at its surface would be more likely in contact with putative ligands in a longer cilium than in a shorter one.

Our results show that the primary cilium undergoes several size modifications during adipocyte differentiation of hASC. This suggests that the cilium could have various functions during adipogenesis. If indeed the increase in size of the cilium, and its loss at the latter stage of differentiation, play a role in adipocyte differentiation it could be interesting to determine if the cilium from adipose stem cells of obese patients or patients suffering from ciliopathies undergoes the same variation than the cilium from adipose stem cells of healthy patients.

## Conflicts of interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.078.

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