Growth arrest induces primary-cilium formation and sensitizes IGF-1-receptor signaling during differentiation induction of 3T3-L1 preadipocytes

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Summary

The first stage of 3T3-L1 adipocyte differentiation is growth arrest, which is achieved by contact inhibition at confluence. In growth-arrested confluent 3T3-L1 preadipocytes, α-tubulin acetylation and primary-cilium formation were induced. The blockade of primary-cilium formation by suppressing IFT88 or Kif3a inhibited 3T3-L1 adipocyte differentiation. IGF-1 (IGF-I)-receptor signaling, which is essential for differentiation induction, was sensitized by the formation of a primary cilium in confluent 3T3-L1 preadipocytes. The receptor located in primary cilium was more sensitive to insulin stimulation than that not located in cilia. During cilium formation, insulin receptor substrate 1 (IRS-1), one of the important downstream signaling molecules of the IGF-1 receptor, was recruited to the basal body at which it was phosphorylated on tyrosine by the receptor kinase in cilia. Akt-1, an important signal molecule of the IGF-1 receptor in adipocyte differentiation, was also activated at the basal body. These IGF-1-receptor signaling processes were all inhibited in IFT88- or Kif3a-knockdown cells. Thus, the primary cilium and its basal body formed an organized signaling pathway for the IGF-1 receptor to induce adipocyte differentiation in confluent 3T3-L1 preadipocytes.

Supplementary material available online at
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Key words: Primary cilium, IGF-1 receptor, α-tubulin acetylation, Growth arrest, Adipocyte differentiation, 3T3-L1 cell

Introduction

In vitro preadipocytes, e.g. 3T3-L1 and 3T3-F442A, can be induced to differentiate into adipocytes. The in vitro adipocyte differentiation faithfully recapitulates most of the aspects of adipogenesis in vivo (Cowherd et al., 1999; Gregoire et al., 1998; Rosen and Spiegelman, 2000). IGF-1 (IGF-I)-receptor signaling (along with glucocorticoid and cAMP) is essential for 3T3-L1 preadipocyte differentiation induction (Mackall et al., 1976; Rosen et al., 1979; Smith et al., 1988; Jin et al., 2000). Immediately after induction, growth-arrested confluent 3T3-L1 preadipocytes re-enter the cell cycle (called mitotic clonal expansion) and start the adipocyte differentiation program (Cowherd et al., 1999; Gregoire et al., 1998; Rosen and Spiegelman, 2000). IGF-1-receptor signaling induces both the clonal expansion and adipocyte differentiation through the activation of phosphatidylinositol-3-kinase–Akt and MEK-ERK signaling cascades (Qiu et al., 2001; Xu and Liao, 2004). On the plasma membrane, IGF-1-receptor signal transduction is facilitated by its localization in membrane lipid rafts, which enable a close interaction between the receptor tyrosine kinase and intracellular signaling molecules (Brown and London, 1998; Huo et al., 2003; Hong et al., 2004). The membrane microenvironment is important for IGF-1-receptor signaling.

During the adipocyte differentiation process, 3T3-L1 cells are shifted from dividing preadipocytes to growth-arrested adipocytes. Apart from the growth arrest in terminally differentiated adipocytes, which is probably caused by the expression of CCAAT/enhance-binding protein α (C/EBPα) in adipocytes (Johnson, 2005), growth arrest in 3T3-L1 preadipocytes is a prerequisite step for differentiation induction (Ailhaud et al., 1989; Rosen and Spiegelman, 2000). Only in growth-arrested 3T3-L1 preadipocytes, which can be achieved by contact-inhibition at confluence, does hormonal regimen (insulin, dexamethasone and isobutylmethylxanthine) induce adipocyte differentiation.

When cells become confluent or enter G0 phase, the formation of the primary cilium is induced (Satir and Christensen, 2007; Plotnikova et al., 2008). Recently, many studies have indicated that the primary cilium is involved in intracellular signal transduction (Singla and Reiter, 2006). It is required for the signal transduction of G-protein-coupled olfactory receptor (Boekhoff et al., 1990), platelet-derived growth factor (PDGF) receptor α (Schneider et al., 2005), hedgehog and Wnt signals (Rohatgi et al., 2007; Corbit et al., 2008). The results from these studies prompted us to investigate the function of the primary cilium in IGF-1-receptor signaling for adipocyte differentiation induction in growth-arrested 3T3-L1 preadipocytes. Here we show that α-tubulin acetylation and the formation of a primary cilium are induced by confluence. The primary cilium promotes adipocyte differentiation by sensitizing the IGF-1 receptor and its signal transduction.

Results

Primary-cilium formation is induced in confluent 3T3-L1 preadipocytes and is essential for adipocyte differentiation

Growth arrest is the first stage of 3T3-L1 preadipocyte differentiation induction (Ailhaud et al., 1989; Rosen and Spiegelman, 2000). In order to induce adipocyte differentiation, 3T3-L1 preadipocytes were cultured to growth arrest by contact-

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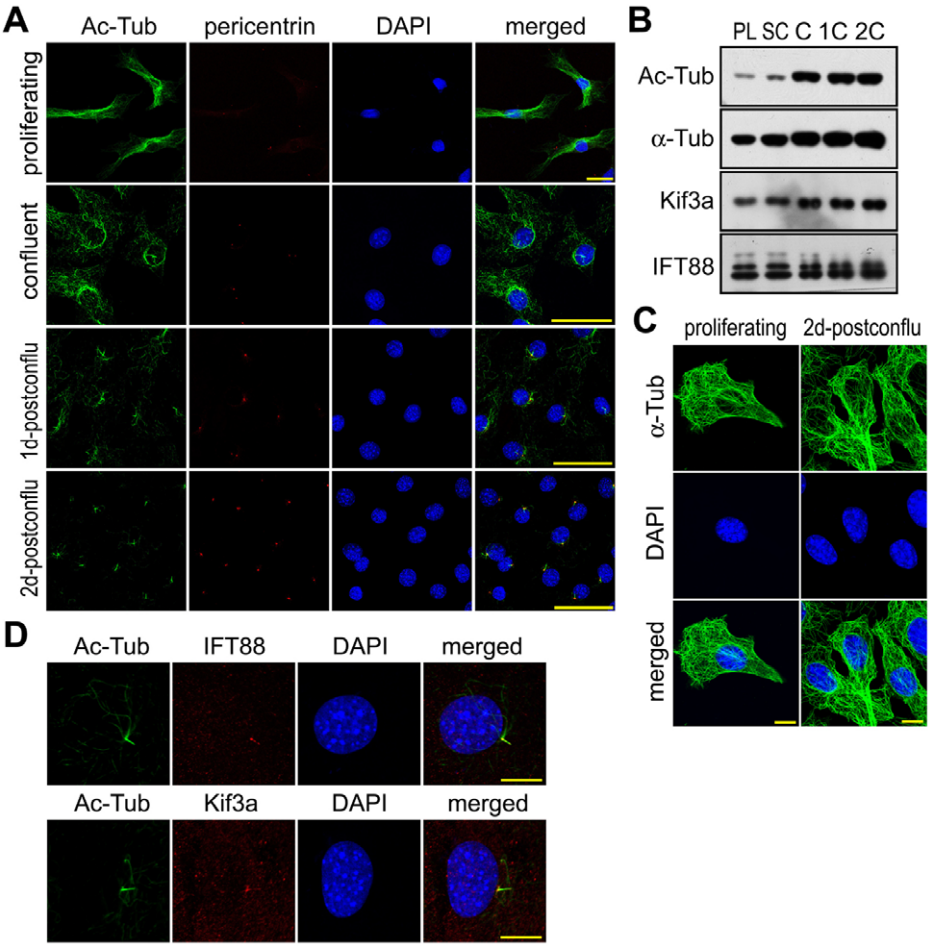

Fig. 1. Primary-cilium formation in growth-arrested confluent 3T3-L1 preadipocytes. (A) Growth-arrest-induced cilium formation. Proliferating, confluent, and 1-day or 2-day post-confluent 3T3-L1 preadipocytes were stained for acetylated α-tubulin (Ac-Tub) and pericentrin. Scale bars: 50 µm. (B) Western blot of cilium-related proteins. 3T3-L1 preadipocytes were harvested at 24 hours after plating (PL), sub-confluence (SC), confluence (C), 1 day post-confluence (1C) and 2-day post-confluence (2C). Acetylated α-tubulin, α-tubulin (α-Tub), Kif3a and IFT88 were analyzed by western blot. (C) Microtubules in proliferating and 2-day post-confluent 3T3-L1 preadipocytes were stained with anti-α-tubulin antibody. Scale bars: 10 µm. (D) Localization of IFT88 and Kif3a in primary cilium. A 2-day post-confluent 3T3-L1 preadipocyte was stained for acetylated α-tubulin with IFT88 or Kif3a. Scale bars: 10 µm.

inhibition at confluence. In confluent 3T3-L1 preadipocytes, α-tubulin acetylation was induced and primary cilia were formed (Fig. 1A,B). The formation of a primary cilium by acetylated α-tubulin in confluent preadipocytes did not induce visible reorganization in the microtubule network (Fig. 1C). The α-tubulin protein also maintained a relatively constant level (Fig. 1B). During the transition from proliferation to confluence, the intraflagellar transportation protein IFT88 (Pazour et al., 2000) exhibited little change in protein

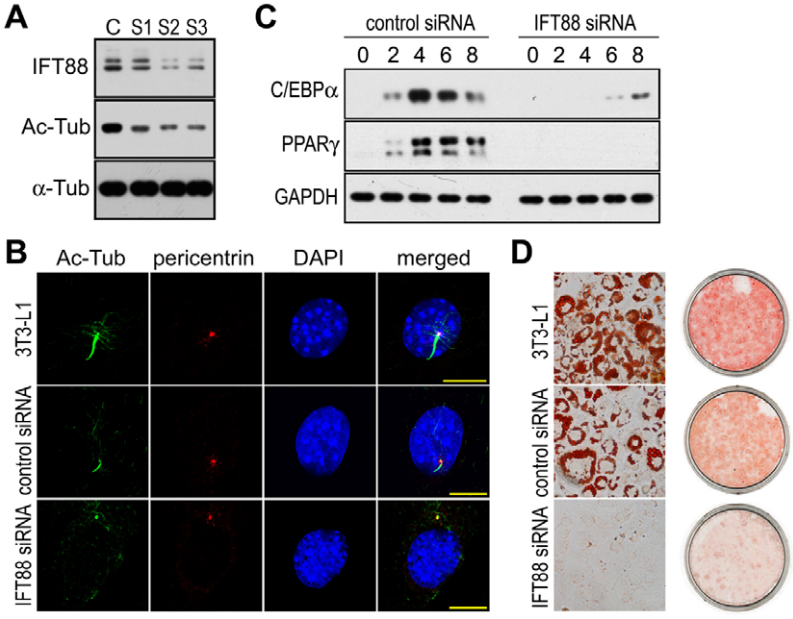

Fig. 2. Inhibition of adipocyte differentiation by blocking primary-cilium formation. (A) IFT88 expression. 2-day post-confluent 3T3-L1 preadipocytes infected with *IFT88* RNA-interference retrovirus (S1, S2 or S3) or control virus (C) were analyzed for IFT88 and α-tubulin acetylation (Ac-Tub). (B) Primary-cilium formation is blocked by IFT88 knockdown. 3T3-L1, wild-type preadipocyte; control siRNA, preadipocyte infected with control virus; IFT88 siRNA, preadipocyte infected with IFT88 RNA-interference virus (S3). Scale bars: 10 µm. (C) Expression of adipocyte transcription factors. Numbers (0, 2, 4, 6, 8) indicate the days after differentiation induction. C/EBPα and PPARγ were detected by western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the house-keeping control. (D) Adipocyte differentiation. Standard differentiation protocol was followed and cells were stained with Oil-red-O on day 8.

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level. The protein level of Kif3a, another microtubule motor protein important for cilium formation (Marszalek et al., 1999), increased slightly in confluent cells (Fig. 1B). These two proteins were important for cilium formation and their presence in primary cilium could be observed by immunofluorescence staining (Fig. 1D). Because growth arrest markedly induced α-tubulin acetylation and the cilium was enriched with acetylated α-tubulin, α-tubulin acetylation should be one of the key events in growth-arrest-induced cilium formation.

The formation of primary cilia can be blocked by suppressing IFT88 expression (Pazour et al., 2000). To ascertain the function of the primary cilium in adipocyte differentiation induction, three sequence segments of IFT88 were selected to construct retroviral plasmids for RNA interference and IFT88 was effectively reduced in confluent 3T3-L1 preadipocytes infected by the retrovirus (Fig. 2A). In these IFT88-knockdown cells, the formation of a primary cilium was blocked (Fig. 2B) and the adipocyte differentiation induced by hormonal regimen was largely inhibited (Fig. 2D). The inhibited adipocyte differentiation in IFT88-knockdown cells was further verified by the inhibited expression of two key adipogenic transcription factors, C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) (Fig. 2C). Thus, without primary cilium, adipocyte differentiation could not be induced even though these IFT88-knockdown preadipocytes reached growth arrest at confluence.

IGF-1 receptor is sensitized in ciliated confluent 3T3-L1 preadipocytes

As a sensory organelle on cell surface (Singla and Reiter, 2006), primary cilia should be important for receptor signaling during differentiation induction. Of three differentiation inducers (insulin, dexamethasone and isobutylmethylxanthine), only insulin activates the cell-surface IGF-1 receptor during differentiation induction (Smith et al., 1988; Jin et al., 2000). Upon insulin stimulation, the autophosphorylation on tyrosine residues of the IGF-1 receptor distinguishes the activated receptor from the quiescent receptor. Insulin stimulation led to a faster initial IGF-1-receptor activation (Tyr1131 phosphorylation) in ciliated confluent 3T3-L1 preadipocytes than in non-ciliated proliferating preadipocytes (Fig. 3A). Although the IGF-1 receptor was not exclusively localized in the cilium of confluent preadipocytes (Fig. 3B), the receptor in cilia was activated faster than that in other plasma-membrane areas (Fig. 3C,D). During the initial stage of insulin stimulation, the activated IGF-1 receptor appeared in primary cilium first (Fig. 3C,D). After 15 minutes of insulin stimulation, IGF-1 receptor on other plasma-membrane areas was also activated (supplementary material Fig. S1). The receptors not in cilium could still be activated by insulin stimulation, but they were not as sensitive as those in cilium.

In primary cilia, the phosphorylation on other tyrosine residues (Tyr1135/1136 and Tyr1161) in the activated IGF-1 receptor could also be detected (supplementary material Figs S1 and S2). To verify the specificity of antibody staining, the corresponding tyrosine-phosphorylated peptide for the anti-phospho-IGF-1-receptor (Tyr1131/1135/1136 phosphorylation) antibody was used to block the antibody. The staining of Tyr1131/1135/1136-phosphorylated IGF-1 receptor in cilium was completely blocked by this blocking peptide (supplementary material Fig. S2).

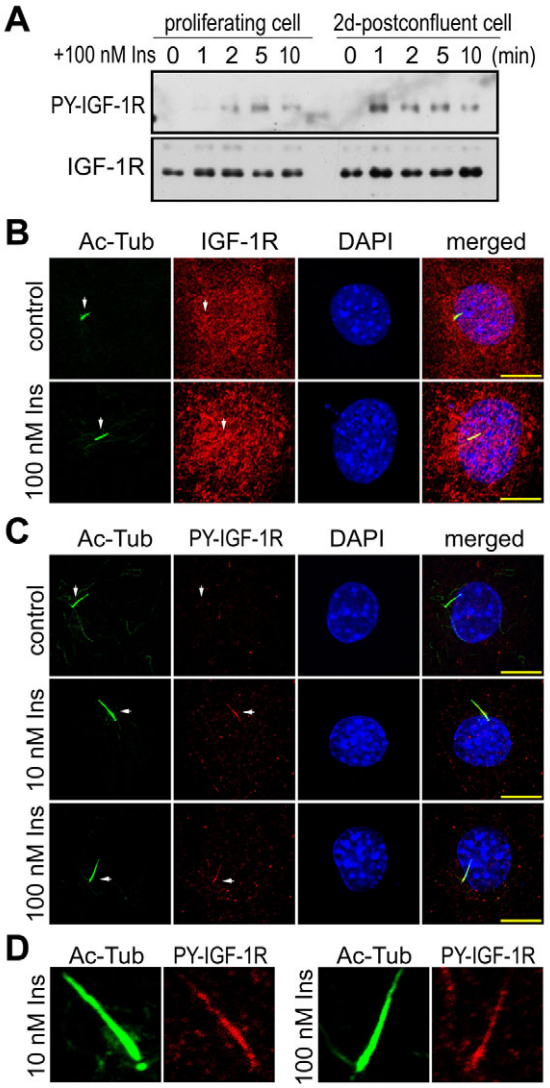

Fig. 3. IGF-1-receptor activation in ciliated 3T3-L1 preadipocytes. (A) IGF-1-receptor activation. Proliferating or 2-day post-confluent 3T3-L1 preadipocytes were treated with 100 nM insulin for the indicated time. The activated receptor was western blotted for Tyr1131 phosphorylation (PY-IGF-1R). (B) Immunofluorescence staining of IGF-1 receptor and primary cilium. 2-day post-confluent 3T3-L1 preadipocytes treated with 100 nM insulin for 1 minute or not (control) were stained for acetylated α-tubulin (Ac-Tub) and IGF-1-receptor β-subunit (IGF-1R). Arrowheads indicate the primary cilium. (C) Immunofluorescence staining of activated IGF-1 receptor. A 2-day post-confluent preadipocyte was treated with 10 nM or 100 mM insulin for 1 minute, or untreated (control), and stained for acetylated α-tubulin and Tyr1131-phosphorylated IGF-1 receptor β-subunit. Arrowheads indicate the primary cilium. (D) Enlarged image of primary cilium and Tyr1131-phosphorylated IGF-1 receptor. Scale bars: 10 μm.

Activated IRS-1 and other signaling molecules are associated with the basal body

IRS-1 is a direct downstream substrate of IGF-1-receptor tyrosine kinase as well as insulin-receptor tyrosine kinase (White and Yenush, 1998). During primary-cilium formation, IRS-1 was recruited to the basal body (Fig. 4A). The recruitment of IRS-1 at the basal body was insulin-independent (Fig. 4A,B) and it was verified by double-staining for the centrosome and basal-body marker protein γ-tubulin, and IRS-1 (supplementary material Fig. S2). The corresponding peptide for anti-IRS-1 antibody completely blocked IRS-1 staining at the basal body (supplementary material Fig. S2).

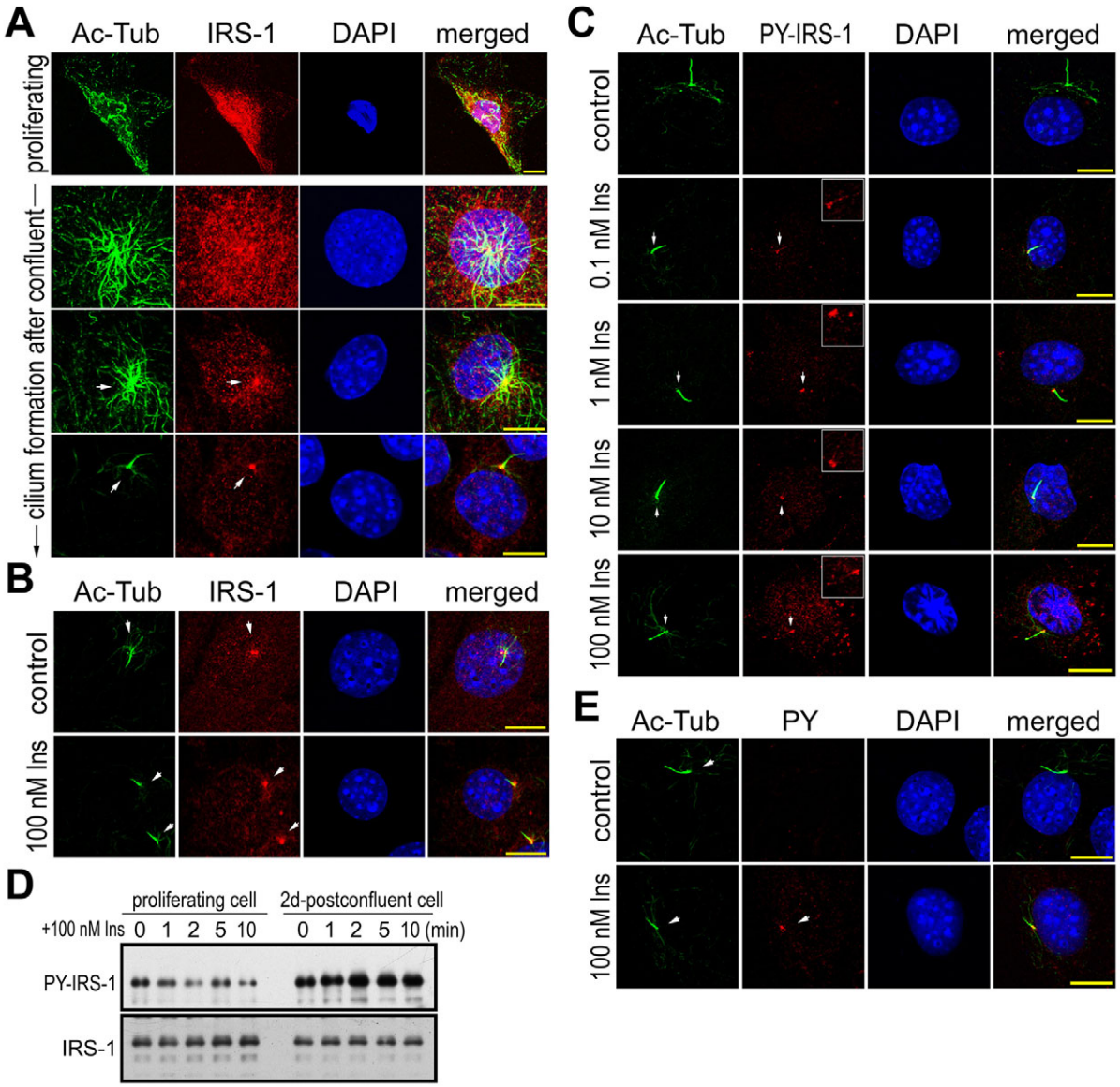

Fig. 4. Recruitment and phosphorylation of IRS-1 at basal bodies. Arrowheads indicate the position of the basal body. (A) Recruitment of IRS-1 to the basal body during cilium formation. Proliferating, confluent and post-confluent 3T3-L1 preadipocytes were fixed and stained for acetylated α-tubulin (Ac-Tub) and IRS-1. (B) IRS-1 at the basal body. 2-day post-confluent 3T3-L1 preadipocytes were treated with insulin or not (control) and were stained for primary cilium (Ac-Tub) and IRS-1. (C) IRS-1 tyrosine phosphorylation at the basal body. 2-day post-confluent 3T3-L1 preadipocytes treated with insulin for 1 minute or not were stained for Tyr989-phosphorylated IRS-1 (PY-IRS-1). (D) Insulin induced IRS-1 tyrosine phosphorylation (western blot). Proliferating or 2-day post-confluent 3T3-L1 preadipocytes were treated with 100 nM insulin for the indicated time and harvested for western blot of IRS-1 and Tyr989-phosphorylated IRS-1. (E) Tyrosine-phosphorylated protein(s) at the basal body after insulin stimulation. Post-confluent preadipocytes treated with insulin or not were stained with anti-phosphotyrosine antibody (PY). Scale bars: 10 μm.

When stimulated with insulin, tyrosine-phosphorylated IRS-1 appeared first at the basal body (Fig. 4C). At very low insulin concentration, the basal body seemed to be the primary place with which the tyrosine-phosphorylated IRS-1 was associated (Fig. 4C). In ciliated confluent 3T3-L1 preadipocytes, insulin stimulation induced more IRS-1 phosphorylation than in non-ciliated proliferating preadipocytes (Fig. 4D). In fact, after insulin stimulation, the basal body was accumulated with tyrosine-phosphorylated protein(s) (Fig. 4E). These observations suggested that the basal body of the cilium mediates the IGF-1-receptor signal to its intracellular signaling molecules.

Akt-1 is the most important intracellular signal activated by the IGF-1 receptor in 3T3-L1 preadipocyte differentiation induction (Xu and Liao, 2004). Non-activated Akt-1 is a cytoplasmic protein (Fig. 5A). However, its activated form (phosphorylation on Ser473) was concentrated at the basal body (Fig. 5B) and the staining of Ser473-phosphorylated Akt-1 at the basal body could be blocked by the peptide corresponding to Ser473-phosphorylated Akt-1 (Fig. 5B). In addition, of two γ-tubulin-stained organelles (one is the basal body and the other should be the centrosome), Ser473-phosphorylated Akt-1 was found at only one (Fig. 5C). After cilium formation in confluent 3T3-L1 preadipocytes, the activation of Akt-1 by insulin was clearly enhanced (Fig. 5D). These results indicated that the activation of Akt-1 by IGF-1 receptor was closely related to the primary cilium and basal body.

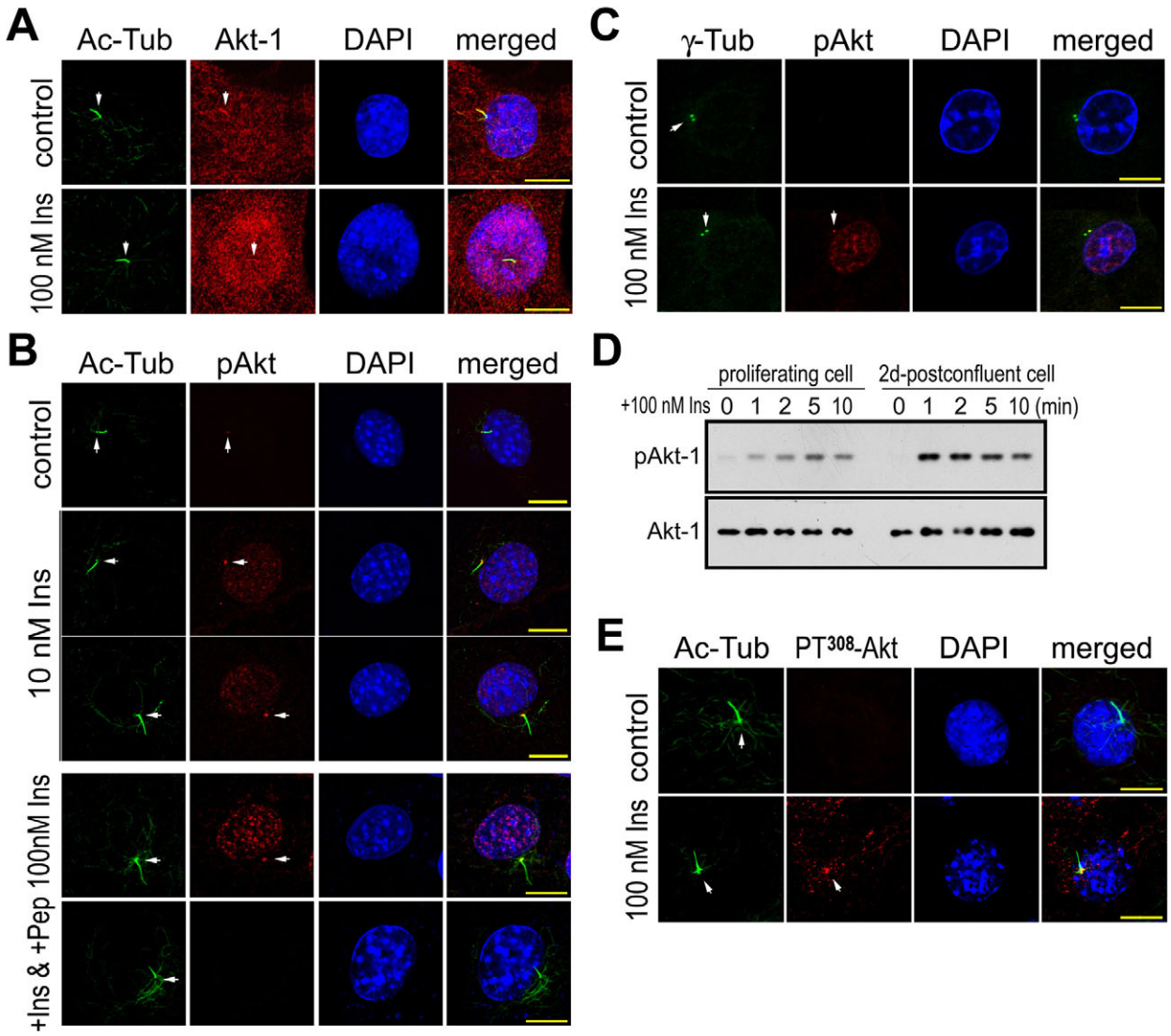

Fig. 5. Akt-1 activation in ciliated 3T3-L1 preadipocytes. Arrowheads indicate the basal body. (A) Primary cilium and Akt-1. Post-confluent preadipocytes treated with insulin or not were stained for primary cilium (Ac-Tub) and Akt-1. (B) Akt-1 activation. Post-confluent preadipocytes treated with insulin for 1 minute or not (control) were stained for primary cilium and Ser473-phosphorylated Akt-1 (pAkt). +Ins & +Pep, cells treated with 100 nM insulin were stained with anti-Ser473-phosphorylated-Akt-1 antibody in the presence of corresponding Ser473-phosphorylated-Akt-1 blocking peptide. (C) Basal body and phosphorylated Akt-1. The basal body was identified by staining for γ-tubulin (γ-Tub). (D) Proliferating or 2-day post-confluent 3T3-L1 preadipocytes were treated with 100 nM insulin for the indicated time and harvested for western blot of Akt-1 and Ser473-phosphorylated Akt-1. (E) Thr308-phosphorylated Akt-1. Cells with insulin treatment or not were stained for primary cilium (Ac-Tub) and Thr308-phosphorylated Akt-1 (PT308-Akt). Scale bars: 10 μm.

The low level of Ser473-phosphorylated Akt-1 detected at the basal body in quiescent preadipocytes might be caused by the residual activation of Akt-1 in serum-containing culture medium (Fig. 5B), because serum starvation could basically eliminate the detection of activated Akt-1 at the basal body (supplementary material Fig. S3). Most importantly, treatment with LY294002, an inhibitor of phosphoinositide 3-kinase (Vlahos et al., 1994), markedly decreased the activation of Akt-1 at the basal body without affecting the activation of IGF-1 receptor in the cilium (supplementary material Fig. S3). As well as Ser473, Thr308 was phosphorylated in activated Akt-1 (Alessi et al., 1996). This Thr308-phosphorylated Akt-1 was also detected at the basal body (Fig. 5E). Thus, the formation of primary cilium not only increased the responsiveness of IGF-1 receptor to ligand stimulation, but also recruited the downstream signaling molecules to the basal body.

IGF-1-receptor signaling is suppressed by the ablation of primary cilia

The inhibition of adipocyte differentiation in non-ciliated IFT88-knockdown confluent preadipocytes suggested that, during differentiation induction, IGF-1-receptor signaling could be inhibited by the ablation of cilium (Fig. 2). Indeed, insulin-induced IGF-1-receptor autophosphorylation was decreased in confluent IFT88-knockdown preadipocytes when compared with the control preadipocyte (Fig. 6A). Without primary cilium, the overall activation of Akt-1 by IGF-1-receptor signaling was markedly decreased and little activated Akt-1 was detected at the basal body (Fig. 6A,B). The inhibited adipocyte differentiation in non-ciliated IFT88-knockdown cells could be rescued by expressing FLAG-tagged human IFT88 that has a different cDNA sequence corresponding to the siRNA fragment (Fig. 6C,D). The expression of C/EBPα and PPARγ was

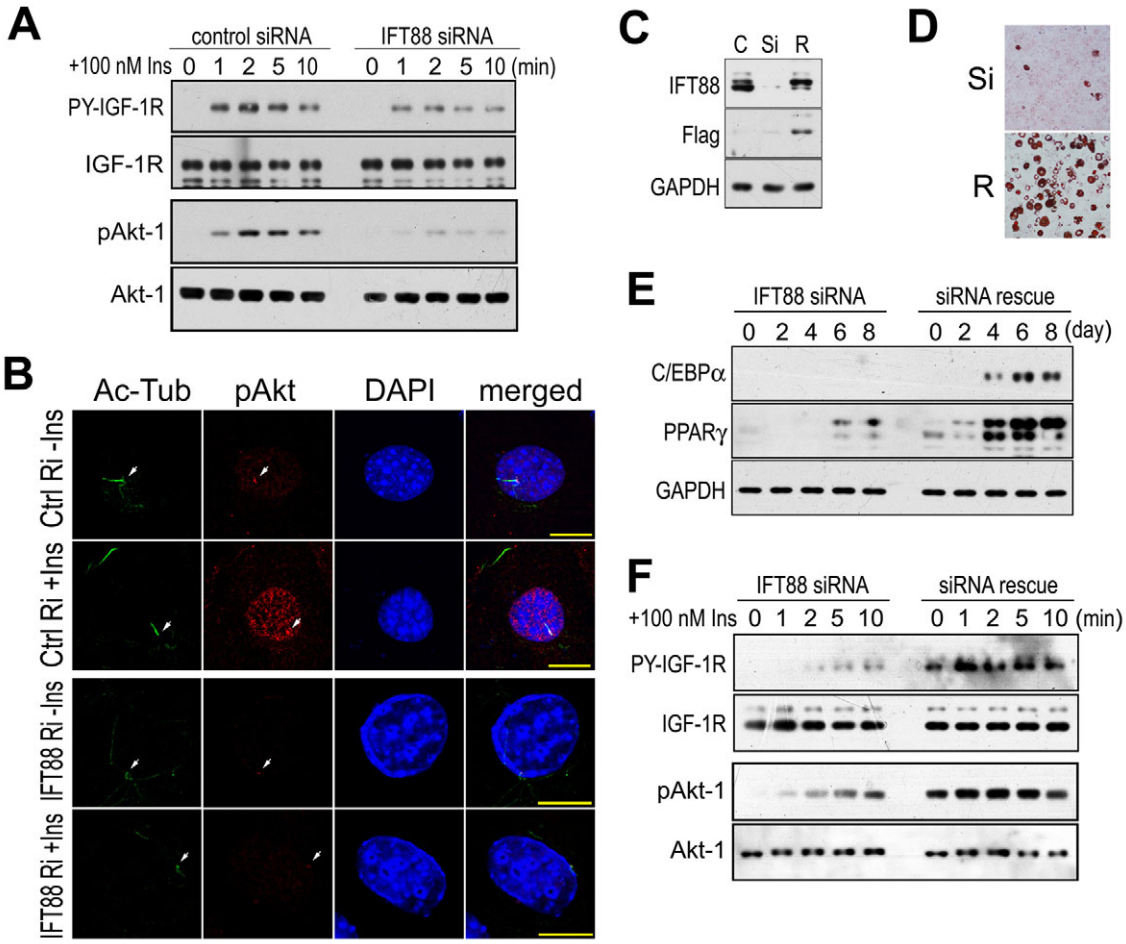

Fig. 6. Inhibition of IGF-1-receptor signaling in IFT88-knockdown preadipocytes. (A) Inhibition of the IGF-1 receptor and Akt-1 activation in IFT88-knockdown cells. 2-day post-confluent IFT88-knockdown preadipocytes (IFT88 siRNA) or control virus-infected preadipocytes (control siRNA) were treated with 100 nM insulin for the indicated time and harvested for western blot of Tyr1131-phosphorylated IGF-1-receptor β-subunit (PY-IGF-1R) and Ser473-phosphorylated Akt-1 (pAkt-1). (B) Inhibition of Akt-1 activation at the basal body by IFT88 knockdown. The arrowheads indicate the basal body. 2-day post-confluent cells were treated with 100 nM insulin for 1 minute (+Ins) or not (–Ins) and stained for primary cilium (Ac-Tub) and Ser473-phosphorylated Akt-1 (pAkt). IFT88 Ri, IFT88-knockdown preadipocyte; Ctrl Ri, control virus-infected preadipocyte. Scale bar: 10 μm. (C) Rescue of IFT88 knockdown by FLAG-tagged human IFT88. C, control-RNAi virus-infected preadipocyte; Si, preadipocyte infected with *IFT88* RNAi virus (S3) and blank expressing virus; R, preadipocyte infected with IFT88 RNAi virus and virus expressing FLAG-tagged human IFT88. (D) Adipocyte differentiation. Standard differentiation protocol was followed and cells were stained with Oil-red-O on day 8. (E) Expression of C/EBPα and PPARγ in rescued cells. Numbers (0, 2, 4, 6, 8) indicate the days after differentiation induction. IFT88 siRNA, cell infected with *IFT88* RNAi virus (S3) and blank expressing virus; siRNA rescue, cell infected with *IFT88* RNAi virus and virus expressing FLAG-tagged human *IFT88*. (F) The activation of IGF-1 receptor and Akt-1 in rescued cells. The labels are the same as in panel A.

restored in FLAG-tagged human-IFT88-rescued cells (Fig. 6E). Most importantly, the inhibited IGF-1-receptor signaling in non-ciliated IFT88-knockdown cells could also be reversed by the expression of FLAG-tagged human IFT88 (Fig. 6F).

In confluent 3T3-L1 preadipocytes, two pericentrin-stained organelles were observed: one is the basal body and the other the centrosome (Fig. 7A). The recruitment of IRS-1 to the basal body during the process of cilium formation was observed (Fig. 4; Fig. 7B). The difference between the basal body and centrosome was most obvious in the association of Ser473-phosphorylated Akt-1 (Fig. 5B,C). In IFT88-knockdown preadipocytes, the remaining basal body and centrosome could still be observed (Fig. 7C). In the overexposed immunofluorescence image, a residual amount of phosphorylated Akt-1 was only detected at one acetylated α-tubulin-stained organelle, which was most probably the remaining basal body, not the centrosome (Fig. 7C).

The disruption of IGF-1-receptor signaling by the ablation of primary cilia could be confirmed in non-ciliated Kif3a-knockdown preadipocytes in which the adipocyte differentiation, IGF-1-receptor signaling and Akt-1 activation were all inhibited (Fig. 8). Kif3a-knockdown preadipocytes exhibited the same phenotype as IFT88-knockdown cell (Figs 6 and 8). Taken together, the formation of the primary cilium and basal body in growth-arrested confluent 3T3-L1 preadipocytes sensitized the IGF-1 receptor and facilitated its downstream signal transduction for adipocyte differentiation.

Discussion

The primary-cilium-dependent signal transduction for PDGF-receptor-α and hedgehog signaling is determined by their cilium localization (Schneider et al., 2005; Rohatgi et al., 2007). However, the IGF-1 receptor was not exclusively clustered in the primary cilium in confluent 3T3-L1 preadipocytes (Fig. 3B). The IGF-1 receptor

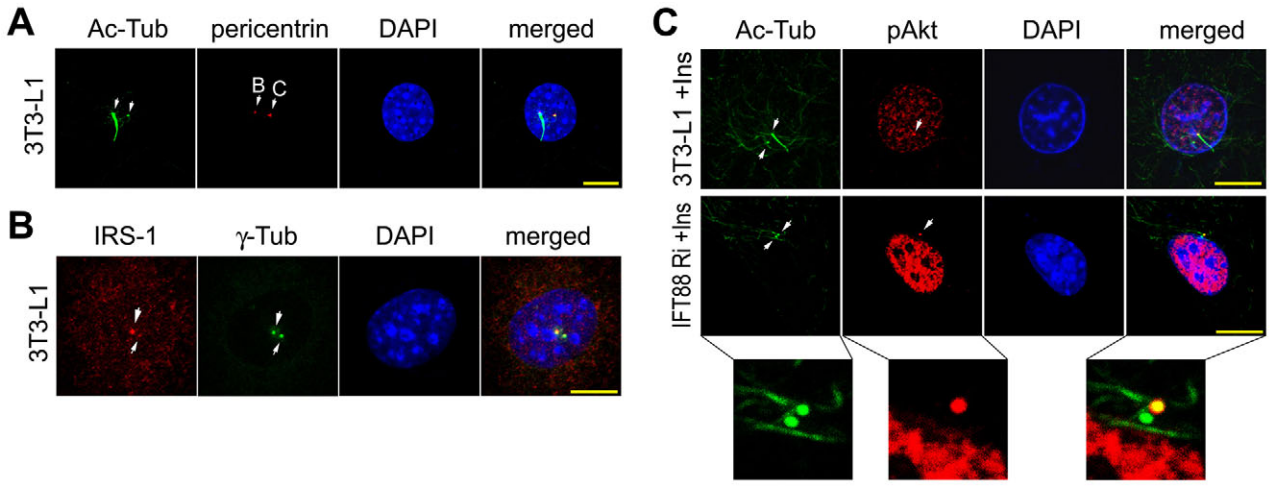

Fig. 7. Signal molecules at the basal body. (A) The basal body and centrosome in 2-day post-confluent 3T3-L1 preadipocytes. In the pericentrin-stained panel, B indicates the basal body and C the centrosome. (B) The association of IRS-1 with γ-tubulin-stained organelles in 2-day post-confluent 3T3-L1 preadipocytes. The basal body (large arrow) and centrosome (small arrow) were detected by staining for γ-tubulin. (C) Staining of activated Akt-1 at the basal body. 2-day post-confluent 3T3-L1 preadipocytes treated with insulin was stained for acetylated α-tubulin and phosphorylated Akt-1. The image of the staining for activated Akt-1 in IFT88-knockdown preadipocyte was overexposed. The insertions are the 5× enlarged images to reveal the details. Scale bars: 10 μm.

that is localized in primary cilium was sensitized (Fig. 3). In ciliated (which is induced by serum starvation) NIH 3T3 cells, the IGF-1 receptor in primary cilia was also more sensitive to ligand stimulation than the receptor in other membrane areas (supplementary material Fig. S4). The specificity of antibody for the detection of activated IGF-1 receptor in cilia was verified by both immunofluorescence staining and western blot analysis (supplementary material Figs S2 and S5). The mechanism by which the IGF-1 receptor is sensitized in cilium is not clear. One possibility is that the membrane of the primary cilium belongs to specific membrane microdomains, such as lipid rafts. We have observed immunofluorescence staining of primary cilium by cholera toxin B subunit, which binds to gangliosides enriched in membrane microdomains (D.Z. and K.L., unpublished data) (Brown and London, 1998; Fujinaga et al., 2003). Currently, we are investigating the possible functions of lipid microdomains in sensitizing the IGF-1 receptor in primary cilium.

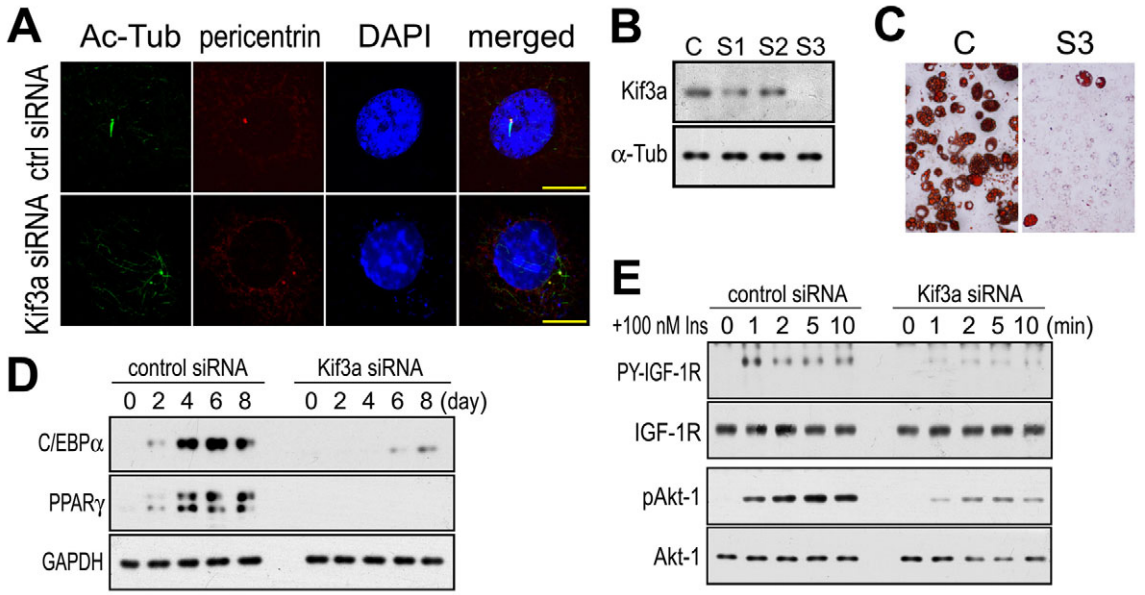

Fig. 8. Inhibition of IGF-1-receptor signaling in Kif3a-knockdown preadipocytes. (A) Primary-cilium formation is blocked by Kif3a knockdown. ctrl siRNA, preadipocyte infected with control virus; Kif3a siRNA, preadipocyte infected with Kif3a RNAi virus (S3). Scale bars: 10 μm. (B) Kif3a expression. 2-day post-confluent 3T3-L1 preadipocytes infected with Kif3a RNAi retrovirus (S1, S2 or S3) or control virus (C) were analyzed for Kif3a expression. S3 retrovirus was used in the subsequent experiment. (C) Adipocyte differentiation. Standard differentiation protocol was followed and cells were stained with Oil-red-O on day 8. (D) Expression of adipocyte transcription factors. The numbers (0, 2, 4, 6, 8) are the days after differentiation induction. C/EBPα and PPARγ were analyzed by western blot. (E) Inhibition of IGF-1 receptor and Akt-1 activation in Kif3a-knockdown cells. 2-day post-confluent Kif3a-knockdown preadipocytes (Kif3a siRNA) or control virus-infected preadipocytes (control siRNA) were treated with 100 nM insulin for indicated time and harvested for western blot of Tyr1131-phosphorylated IGF-1-receptor β-subunit (PY-IGF-1R) and Ser473-phosphorylated Akt-1 (pAkt-1).

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The association of IRS-1 and activated Akt-1 with the basal body configures the IGF-1 receptor and its downstream signaling molecule into a coordinated cilium and basal-body pathway in confluent 3T3-L1 preadipocytes (Figs 4, 5 and 7). The clustering of tyrosine-phosphorylated protein(s) at the basal body in confluent 3T3-L1 preadipocytes after insulin stimulation suggested the phosphorylation of downstream signaling molecules at the basal body by IGF-1-receptor tyrosine kinase in cilia (Fig. 4E). The coordination of IGF-1-receptor signaling by cilium and basal-body pathway was most evident in the activation of Akt-1, which plays a pivotal role in mediating the receptors signal for adipocyte differentiation (Xu and Liao, 2004) (Figs 5 and 6). That the basal body acts as a station to receive the signal from the receptor in cilia was substantiated by the activation of Akt-1 (Fig. 5B). In this cilium and basal-body configuration, the signal sensed by the receptor in cilia can be efficiently passed to the receiving molecules at the basal body.

Cell division and differentiation often act like two mutually exclusive partners: they are related, but cannot co-exist. 3T3-L1 adipocyte differentiation proceeds through a series steps (proliferation, growth arrest, induction and terminal differentiation) in which the proliferative potential of the cell is gradually diminished (Cowherd et al., 1999; Johnson, 2005). It has been known for a long time that growth arrest in 3T3-L1 preadipocytes is required for differentiation induction (Ailhaud et al., 1989). Thus, cilium formation and sensitization to IGF-1-receptor signaling are two of the pro-differentiation effects induced by growth arrest in confluent 3T3-L1 preadipocytes. The formation of primary cilium and basal body does not create new signal-transduction pathways for IGF-1-receptor signaling in confluent 3T3-L1 preadipocytes, but enhances the existing signal transduction (Figs 3-6). The enhanced signal transduction in confluent preadipocytes could facilitate the adipocyte differentiation induction. However, other factors or cellular modifications induced by growth arrest in confluent 3T3-L1 preadipocytes might also be required for adipocyte differentiation induction. Adipocyte differentiation induction is a synergistic effect of multiple factors.

Regulation of protein acetylation is important in adipocyte differentiation. SIRT1, a nuclear-localized deacetylase, is an inhibitor of adipogenesis by repressing PPARγ activity (Picard et al., 2004). SIRT2, a cytoplasmic deacetylase, regulates 3T3-L1 adipocyte differentiation through FOXO1 acetylation and deacetylation (Jing et al., 2007). Growth-arrest-induced α-tubulin acetylation seemed to be correlated with cilium formation (Fig. 1). In proliferating 3T3-L1 preadipocytes, the low level of α-tubulin acetylation was also induced by serum starvation along with cilium formation (supplementary material Fig. S5). Our current results suggest the involvement of α-tubulin acetylation in the regulation of signal transduction. Thus, from cell surface to nuclei, protein acetylation seems to be a general protein modification for functional regulation.

Materials and Methods

Materials

Anti-phospho-Akt (Ser473) antibody with corresponding Ser473-phosphorylated peptide, anti-IGF-1-receptor β-subunit antibody, anti-IRS-1 antibody with corresponding peptide, and anti-phospho-IRS-1 (Tyr989) antibody with corresponding Tyr989-phosphorylated peptide, anti-C/EBPα antibody and anti-PPARγ antibody were from Santa Cruz. Anti-pericentrin antibody, anti-Kif3a antibody, anti-IFT88 antibody, anti-phospho-Akt-1 (Thr308) antibody, anti-polyglutamylated α-tubulin antibody, anti-Akt-1 with corresponding peptide, anti-phospho-IGF-1 receptor (Tyr1161) antibody and anti-phospho-IGF-1 receptor (Tyr1131/1135/1136) with corresponding phospho-peptide were from Abcam. Anti-phospho-IGF-1 receptor (Tyr1131) antibody was from Cell Signaling. Anti-acetylated α-tubulin antibody, anti-α-tubulin antibody, anti-FLAG antibody, anti-γ-tubulin antibody, horseradish-peroxidase-conjugated secondary antibody, insulin and DAPI were from Sigma. Alexa-Fluor-488- or -546-conjugated secondary antibodies were from Molecular Probes/Invitrogen. The Leica laser scanning confocal microsystem, including the Leica TCS SP2 confocal microscope, Leica confocal scanner and Leica confocal acquisition software, was used with the HCX PL APO 1bd. BL 63.0X/1.4 oil objective at 1.4 numerical aperture at a working temperature of 22°C. The fluorescence medium used was Sigma's DABCO.

Cell culture, differentiation, immunofluorescence staining and western blot

3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. The differentiation induction was carried out as described previously and stained with Oil-red-O (Jin et al., 2000). For insulin treatment, proliferating or 2-day post-confluent cells were treated with insulin (at concentration indicated in each figure) for 1 minute or the indicated time, washed with ice-cold phosphate-buffered saline and then harvested for western blot or fixed for immunofluorescence. Western blot was carried out as described previously (Qiu et al., 2001). For immunofluorescence staining, cells on glass coverslips were fixed with 4% paraformaldehyde at room temperature (~22°C) for 10 minutes or 100% methanol at –20°C for 5 minutes. The staining was carried out following the previously described protocol (Huo et al., 2003). The fluorescence groups conjugated to the secondary antibodies were Alexa fluorophores (Molecular Probes).

The peptide-blocking experiments were carried out by adding the blocking peptide into the blotting buffer during primary-antibody incubation.

IFT88 and *Kif3a* RNA interference, and IFT88 rescue

Three *IFT88* sequence fragments (S1: 174-193 bp, 5′-GGTAAAGATC-AGACCCAGAT-3′; S2: 840-859 bp, 5′-AGCTAAAATGTGTGGCCTAT-3′; S3: 1002-1021 bp, 5′-GCGAAAATTGACGAGGAGAT-3′) were selected, cloned into pSIREN-Retro-Q vector and shuttled into Knockout RNAi Systems (Clontech Laboratories). A general sequence 5′-GTGCGCTGCTGGTGCCAAC-3′ was used as control. Retrovirus was produced in HEK 293T cells following the manufacturers protocol (Clontech Laboratories). Proliferating 3T3-L1 preadipocytes were infected with retrovirus and cultured in medium supplemented with 10 μg/ml puromycin. The infected cells were cultured to 2-day post-confluent for subsequent experiments.

The MSCV retroviral expression system (Clontech Laboratories) was used to construct retrovirus expressing FLAG-tagged human *IFT88*, which has a different cDNA sequence corresponding to the S3 RNAi fragment. 3T3-L1 cells were transfected with IFT88 RNA interference retrovirus (S3) and then with retrovirus expressing FLAG-tagged human IFT88. Cells were selected by puromycin and hygromycin.

Three *Kif3a* sequence fragments (S1: 483-502 bp, 5′-GGTAAAGATCAGA-CCCAGA-3′; S2: 1275-1294bp, 5′-GCGAAAATTGACGAGGAGA-3′; S3: 1858-1877bp, 5′-AGCTAAAATGTGTGGCCTA-3′) were selected and cloned into RNA-interference retrovirus.

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