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Suppression of the PI3K Subunit p85α Delays Embryoid Body Development and Inhibits Cell Adhesion

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ABSTRACT

Phosphatidylinositol-3-kinases (PI3Ks) exert a variety of signaling functions in eukaryotes. We suppressed the PI3K regulatory subunit p85α using a small interfering RNA (*Pik3r1* siRNA) and examined the effects on embryoid body (EB) development in hanging drop culture. We observed a 150% increase in the volume of the treated EBs within 24 h, compared to the negative controls. Fluorescence Activated Cell Sorting (FACS) assays showed that this increase in volume is not due to increased cellular proliferation. Instead, the increase in volume appears to be due to reduced cellular aggregation and adherence. This is further shown by our observation that 40% of treated EBs form twin instead of single EBs, and that they have a significantly reduced ability to adhere to culture dishes when plated. A time course over the first 96 h reveals that the impaired adherence is transient and explained by an initial 12-hour delay in EB development. Quantitative PCR expression analysis suggests that the adhesion molecule integrin-β1 (ITGB1) is transiently downregulated by the p85α suppression. In conclusion we found that suppressing p85α leads to a delay in forming compact EBs, accompanied by a transient inability of the EBs to undergo normal cell–cell and cell–substrate adhesion. J. Cell. Biochem. 112: 3573–3581, 2011.

KEY WORDS: ADHESION; EMBRYOID BODY DEVELOPMENT; EMBRYONIC STEM CELLS; PHOSPHATIDYLINOSITOL-3-KINASE; INTEGRIN

The phosphatidylinositol-3-kinase (PI3K) family is specific to eukaryotes, whereby the role of class IA PI3Ks is to phosphorylate the second messenger lipid PIP_2 to PIP_3, and these in turn exert a variety of signaling functions involved in regulation of cellular survival, proliferation, differentiation, migration, and trafficking [Kong and Yamori, 2009]. The most ancient role of PI3K probably was to mark specific cellular membranes for trafficking events, and isoforms of PI3K have evolved for the dedicated purpose of signal transduction to control physiological cell functions of multicellular organisms [Katso et al., 2001]. Unsurprisingly, it has become evident over the past decade that the PI3K signaling pathway is one of the most highly mutated systems in human cancers [Engelman et al., 2006; Memmott and Dennis, 2009].

There are three distinct classes of PI3Ks: Class I (subdivided into IA and IB), class II and class III [Vanhaesebroeck et al., 1997]. The focus of this article is on class IA PI3Ks, each of which consists of one catalytic subunit (p110α, p110β, or p110δ, encoded by three different genes) and one regulatory subunit (p50α, p55α, p85α, p85β, or p55γ). These α, β, and γ regulatory subunits are also encoded by three different genes: *Pik3r1*, *Pik3r2*, and *Pik3r3*, respectively [Antonetti et al., 1996; Xia and Serrero, 1999].

The gene locus *Pik3r1* encodes p85α, p55α, and p50α regulatory subunits [Vanhaesebroeck et al., 1997]. It used to be thought that the p55α and p50α subunits are generated from alternative splicing of p85α mRNA [Fruman et al., 1996; Inukai et al., 1996]. However, it has since been shown that p55α and p50α have their own promoters within the gene locus *Pik3r1* [Abell et al., 2005]. They share a common carboxy-terminal domain, but the amino-terminal ends are unique. Starting at the carboxy-terminal end, the p85α, p55α, and p50α regulatory subunits are composed of two Src homology 2 (SH2) domains which bind tyrosine-–phosphorylated adaptor proteins, activating the kinase activity of the p110 subunits. The p85α, p55α, and p50α subunits furthermore contain an inter-SH2 domain which binds to and thus forms a dimer with p110. The

Abbreviations: PI3K, Phosphatidylinositol-3-kinase; PIP_2, Phosphatidylinositol (4,5)-diphosphate; PIP_3, Phosphatidylinositol (3,4,5)-triphosphate; *Pik3r1*, Phosphatidylinositol 3-kinase regulatory subunit 1; CFSE, Carboxyfluorescein diacetate succinimidyl ester; ESC, embryonic stem cell; EBs, embryoid bodies.

Additional supporting information may be found in the online version of this article.

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functions of two further domains specific to p85α, namely the BRC homology (BH) and the SH3 domains, are not fully understood. Another difference in p85α is its unique amino-terminal sequence of 340 amino acids, whereas p55α and p50α have terminal sequences of only 34 and 6 amino acids, respectively.

The specific roles of the *Pik3r1* regulatory subunits p85α, p55α, and p50α are not currently known. When co-expressed in cells, each *Pik3r1* regulatory subunit can bind with each of the p110 catalytic subunits. There is evidence that the expression of p85α, p55α, and p50α subunits are differentially regulated in response to a variety of physiological processes [Kok et al., 2009].

Examination of p85α, p55α, p50α mRNA shows that p85α is expressed more abundantly than p55α mRNA in most tissues, with the exceptions of brain and skeletal muscle [Inukai et al., 1996]. p50α is highly expressed in the liver and is also expressed in the kidney and brain [Fruman et al., 1996]. In ESCs, only p85α and neither p55α nor p50α are expressed [Hallmann et al., 2003]. An analysis of p85α protein expression in differentiated cell types has suggested an association between p85α and cell type specific cellular differentiation [El Sheikh et al., 2003]. An as yet poorly explored component in the PI3K system is the role of regulatory subunit p85α, which has recently been implicated in cancer [Jücker et al., 2002; Li et al., 2008].

In ESCs derived from a p85α null mouse [Fruman et al., 1999, Hallmann et al., 2003], Hallmann and colleagues found reduced proliferation in undifferentiated stem cells, although differentiation itself did not appear to be affected. These experiments did not extend to identifying potential time delays in development.

Here, we examined the role of the regulatory subunit p85α during the early stages of murine ESC differentiation. We suppressed p85α levels using small interfering RNA (siRNA) at the single cell stage, and then monitored the development into embryoid bodies (EBs) over a time course.

MATERIALS AND METHODS

ES CELL CULTURE

The murine IOUD2 ES cell line [Chambers, 2004] (kindly provided by Professor Austin Smith, Cambridge) was cultured on 0.1% gelatin coated flasks in maintenance medium consisting of knockout DMEM medium (Invitrogen by Life Technology, Darmstadt, Germany), 10% Knockout serum replacement (Invitrogen), 1% pretested fetal calf serum (Cambrex/Lonza Walkersville Inc., Walkersville, MD), 1% Non essential amino acids (Invitrogen), 2 mM Glutamine (Invitrogen), 10 ng/ml LIF, 0.1 mM β-mercaptoethanol (Invitrogen).

ES CELL DIFFERENTIATION AS EBs

ES cells were differentiated as EBs by withdrawing LIF [Doetschman et al., 1985]. The adherent ES cells were trypsinised and plated as "hanging drops". Each droplet of 20 μl contained 800 individual cells in alpha-MEM medium containing 10% fetal bovine serum (pretested for mesodermal differentiation), 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol (EB differentiation medium). After 48 h of culture as hanging drops, the drops were transferred into a cell culture dish and the newly formed EBs were allowed to attach [Wobus et al., 2002].

SUPPRESSION BY SMALL INTERFERING RNA (siRNA)

IOUD2 ES cells were transfected with *Pik3r1* siRNA (s71608, Ambion by Life Technology, Darmstadt, Germany), or Silencer Select negative control #1 siRNA (Ambion/Applied Biosystems) using DharmaFECT1 transfection reagent (Dharmacon/ThermoFisher Scientific Inc., Waltham, MA) following the manufacturer's protocol. The *Pik3r1* siRNA is designed to suppress all three subunits. However, since Hallmann et al. [2003] have shown that p50α and p55α are not expressed in ES cells, downregulation by the *Pik3r1* siRNA should be directed specifically to p85α. Briefly, cells were seeded in maintenance medium or in EB differentiation medium and incubated for 24 h before transfection of siRNA was performed. For transfection, 5 μl of DharmaFECT1 reagent was added to 245 μl of serum free transfection medium (knockout DMEM or alpha-MEM medium supplemented with 1% L-glutamine and 0.1 μM β-mercaptoethanol, respectively) and incubated for 5 min at room temperature. *Pik3r1* siRNA was diluted in transfection medium and incubated for 5 min at room temperature. The DharmaFECT1 and specific siRNA (*Pik3r1* siRNA for test cells, Silencer Select siRNA for negative control cells) were mixed and after 20 min of incubation, the mixture was diluted with transfection medium and added to IOUD2 cells at the final concentration stated in each experiment. The siRNA mediated gene suppression was then measured by quantitative PCR (qPCR).

QUANTITATIVE PCR

RNA was extracted from IOUD2 ES cells using the RNeasy Plus Kit (Qiagen, Hilden, Germany). Using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), 1 μg RNA was reverse transcribed. Expression levels of the genes for p85α (*Pik3r1*), p21 protein (Cdc42/Rac)-activated kinase 4 (*PAK4*), cadherin 1 (*CDH1*), fibronectin 1 (*FN1*), integrin-β2 (*ITGB2*), integrin-α1 (*ITGA1*), integrin-α2 (*ITGA2*), integrin-α5 (fibronectin receptor α5) (*ITGA5*), integrin-α6 (*ITGA6*), laminin-α5, integrin-β1 (fibronectin receptor β1) (*ITGB1*), and of the reference genes *GAPDH* and *Polr2f*, were determined by qPCR analysis of 0.5 μl cDNA on a 7900HT Fast Real-time PCR System using TaqMan Gene Expression Assays-on-Demand kits and TaqMan Gene Expression Master Mix (all Applied Biosystems; assay numbers Mm00803163_g1, Mm99999915_g1, Mm01300222_m1, Mm00712546_m1, Mm01247357_m1, Mm01300222_m1, Mm01256744_m1, Mm00434513_m1, Mm01306375_m1, Mm00434371_m1, Mm00439797_m1, Mm00434375_m1, Mm01222029_m1, Mm01253227_m1, Mm99999915_g1, respectively) in 20 μl of reaction volume. Ct values were normalized to *Polr2f* or *GAPDH* expression. Relative expression values for amplified products were calculated using the delta-delta-Ct method as previously described in Meier-Stiegen et al. [2010].

WESTERN BLOT

30 μg of protein lysates from the cells were separated on a SDS–PAGE and blotted onto a nitrocellulose membrane. Immunoblots were carried out using antibodies against p85α (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-1637; 1:300) and actin (Santa Cruz, sc-1616-R; 1:5,000) as a loading control. The detected proteins were visualized with the ECL Advance chemiluminescence

system (GE Healthcare, Buckinghamshire, UK) using a LAS-3000 documentation system (Fujifilm, Tokyo, Japan). 16 bit digital images were quantified using ImageQuant 5.2 Software (Molecular Dynamics), and expression levels of p85α were calculated by normalization against loading control. Relative expression of control cells was set to 100%.

CFSE PROLIFERATION ASSAY

Cells were washed with PBS containing 0.1% FCS and incubated in 10 μM CFSE (Carboxyfluorescein diacetate succinimidyl ester) in 1 ml of PBS supplemented with 0.1% of FCS for 5 min at 37°C. Five milliliters of cold maintenance or differentiation transfection medium, respectively, were added to each flask and the cells were incubated on ice for 5 min. The cells were then washed twice, and kept in complete maintenance or differentiation medium, respectively, at 37°C until analyzed by flow cytometry. The time point for harvesting the cells for FACS analysis was either 24 h or 48 h after siRNA treatment.

FLOW CYTOMETRY

The cells were detached by incubation with dissociation buffer for 15 min at 37°C. Subsequently, the cells were centrifuged and resuspended in ice cold flow cytometry buffer (PBS containing 2% FCS). Cells were analyzed with a FACSCanto machine (BD Biosciences, Heidelberg, Germany). Data were collected from batches of 10,000 cells. Data analysis was performed using DIVA software (BD Biosciences, Heidelberg).

RESULTS

EFFICIENT KNOCKDOWN OF PI3K SUBUNIT p85α IN ESCs

In order to use the *Pik3r1* siRNA to examine the role of p85α in ESCs and during EB differentiation, it was important initially to establish the level of knockdown that can be achieved within the experiments. We found that optimal *Pik3r1* siRNA concentrations to treat the IOUD2 cells were 12.5 nM and 25 nM concentrations, which after 24 h resulted in gene knockdowns of 67 and 72%, respectively (Fig. 1A). After 48 h, knockdown increased to 73% (Fig. 1B). Knockdown of p85α was also confirmed at the protein level (Fig. 1C). To minimize the risk of side-effects, 12.5 nM was chosen as the working concentration.

We proceeded to examine the effect of *Pik3r1* siRNA on the undifferentiated ES cells. The cells were analyzed 24 and 48 h after transfection. Cell proliferation was examined firstly by counting the cells and secondly by staining the cells with the proliferation marker CFSE and subsequent Fluorescence Activated Cell Sorting (FACS) analysis at different time points. The cell counts at the 24-hour and 48-hour time points show that the number of cells was increasing. However, the increase in cell number was at a reduced rate compared to the negative control cultures (Fig. 2A). To determine if this was due to a reduction in proliferation, CFSE staining was performed. The results from the CFSE staining (Fig. 2B) show that after 24 h of *Pik3r1* knockdown proliferation was slightly reduced compared to the negative control. After 48 h of *Pik3r1* knockdown however, a clear reduction in the proliferation compared to the negative control cells was observed. Taken together, the results presented in Figure 2

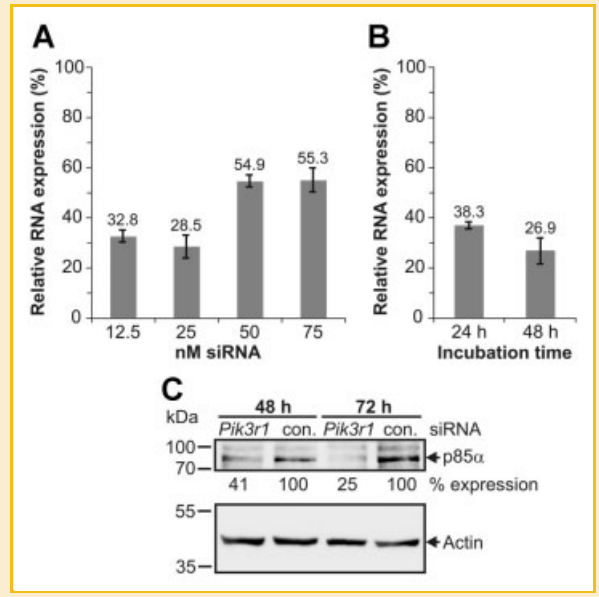

Fig. 1. *Pik3r1* siRNA knockdown measured at the RNA and protein levels. Undifferentiated IOUD2 ES cells were treated with *Pik3r1* siRNA or Silencer Select negative control siRNA and the levels of *Pik3r1* were measured to examine gene expression knockdown. A: Remaining *Pik3r1* RNA measured by qPCR at four concentrations of *Pik3r1* siRNA after 24 h. The bars display the expression as a percentage of the negative control cells' expression. Results are an average of three independent experiments. B: Remaining *Pik3r1* RNA measured up to 48 h of treatment with 12.5 nM *Pik3r1* siRNA. Results are an average of three independent experiments. C: *Pik3r1* knockdown measured at the protein level by Western blot. Bands of p85α and Actin were quantified, and remaining expression of p85α was calculated by normalization to loading control and control cells, which were set to 100%.

indicate that the knockdown of *Pik3r1* in undifferentiated ES cells leads to a decrease in proliferation rate. Our gene knockdown results are in close agreement with the *Pik3r1* gene disruption results of Hallmann et al. [2003], who observed reduced proliferation with a G_0/G_1 cell cycle arrest in undifferentiated ES cells following irreversible heterozygous and homozygous p85α gene knockout, thus validating our approach.

KNOCKDOWN OF PI3K SUBUNIT p85α DELAYS EB DEVELOPMENT AND INHIBITS CELL ADHESION

EB morphology is altered after treatment of ESC with *Pik3r1* siRNA. We went on to examine the effects of p85α knockdown on EB differentiation. The ESCs were treated with *Pik3r1* siRNA and differentiated in EB cultures using the hanging drop method. Forty-eight hours after siRNA treatment, the *Pik3r1* mRNA knockdown in the EBs was 65%. By this point, the cells were growing in hanging drops, aggregating and forming EBs. The EBs were then plated into a cell culture dish where they attached and continued differentiation.

Comparing the formation of EBs after 24, 36, and 48 h of p85α-suppression, a striking observation was the frequent formation of "twin" aggregations (EBs) within one hanging drop, whereas normally only a single aggregation per hanging drop is formed (Fig. 3A, B). In detail, at 24 h, 40% of the hanging drops of p85α siRNA treated cells contained twin EBs compared to 8% in the

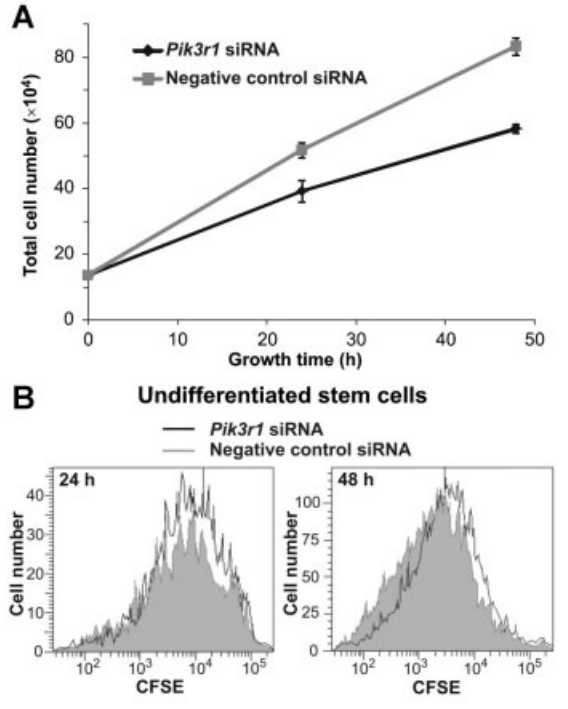

Fig. 2. Reduced ES cell proliferation after treatment with *Pik3r1* siRNA. Undifferentiated IOUD2 ES cells were treated with *Pik3r1* siRNA or Silencer Select negative control siRNA and their proliferation was examined (A). Cell growth was quantified by counting the cells 24 h after treatment and 48 h after treatment with Silencer Select negative control siRNA or *Pik3r1* siRNA. Grey: Silencer Select negative control treated cells. Black: *Pik3r1* siRNA treated cells. B: FACS analysis of undifferentiated ES cells grown as a monolayer show the level of CFSE staining for each of the siRNA treatments. The cells with the lower intensities to the left reflect dilution of the CFSE stain and thus increased proliferation. Grey: Silencer Select negative control treated cells. Black: *Pik3r1* siRNA cells. This experiment was repeated three times with virtually identical results.

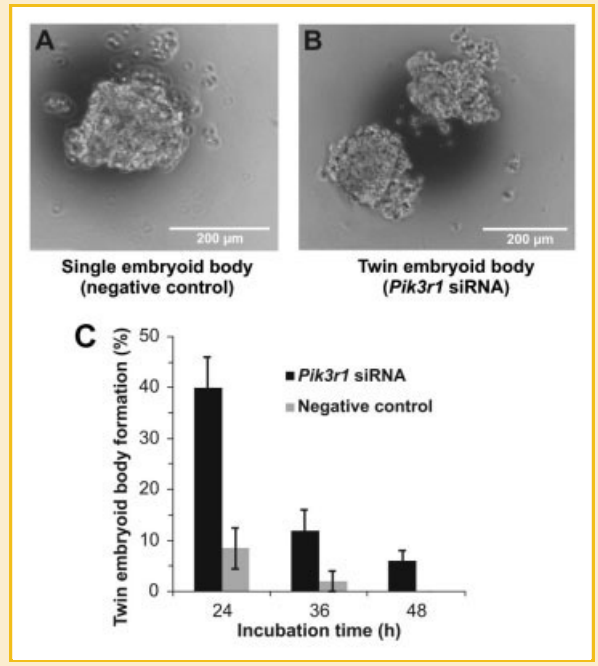

Fig. 3. Increased twin EB formation by suppression of *Pik3r1*. IOUD2 ES cells were treated with Silencer Select negative control siRNA (A) or *Pik3r1* siRNA (B), and subsequently differentiated according to the EB differentiation protocol. The morphology of the EBs was examined and it was observed that a higher proportion of twin EBs formed after *Pik3r1* siRNA treatment. C: Quantification of experiments of A and B. The bars compare the number of twin EBs formed by the *Pik3r1* siRNA treatment (black bars) with the Silencer Select negative control treated EBs (grey bars).

negative control (Fig. 3C). Although the number of hanging drops with twin EBs did reduce over time (at 36 h it was 12% and at 48 h 6%) the incidence of twin EBs remained significantly higher ($P < 0.02$) in hanging drops with p85α siRNA treated cells than in the negative controls throughout the 48 h period.

Another observation which was noted during this experiment was that the p85α-suppressed cells showed a delay in EB formation (Fig. 4). After 24 h, the negative control cells were aggregating and forming a sphere within each drop, by 36 h the defined spherical shape of a classic EB had developed, and by 48 h the EBs were proliferating and increasing in size. In contrast, the cells treated with the *Pik3r1* siRNA were not truly aggregating: After 24 h, the cells appeared to have come together only due to gravity, but they did not appear to adhere to each other. By 36 h the cells were beginning to aggregate and finally formed spherical EBs by 48 h.

This delay in forming EBs was quantified by counting the number of drops which contained spherical and compact EBs (with an observed modal diameter of 240 μm) and the drops which contained only loose cell aggregations (with an observed modal diameter of 330 μm) (see Fig. 4 at 24 h). Negative control cells and *Pik3r1* siRNA treated cells differed significantly in their ability to form EBs at all three time points measured (Fig. 5A). After 24 h, the negative control cells were aggregating and forming the spherical and compact structure of the EB. At 24 h, 87% had begun to form spherical EBs, by 36 h 91% were spherical and by 48 h 93% were spherical (Fig. 5A). The *Pik3r1* treated ES cells were aggregating considerably slower: After 24 h only 11% of the drops contained cells aggregating into spheres. At 36 h, about half of the *Pik3r1* siRNA treated cells had begun to form the spherical structures of EBs (58% were spherical), but even by 48 h only 78% had formed a spherical EB. These results suggest that suppressing p85α expression delays the formation of EBs, which may be due to a disability to attach to each other and to form aggregates.

EB size is altered after treatment of ESC with *Pik3r1* siRNA. EB formation using the hanging drop method normally yields EBs which are very similar in size [Dang et al., 2002; Kurosawa et al., 2003]. During the current analyses we observed that p85α knockdown appeared to influence EB volume. To quantify this effect, we measured the EB diameters over a time course of 48 h. As expected, the mean diameters of the negative control EBs increased throughout the culture (Fig. 5B). In contrast, the p85α-suppressed cells at 24 h initially had a larger modal diameter (334 μm vs. 244 μm), which corresponds ($V = 4\pi r^3/3$) to a 156% larger volume than the negative control EBs. At 36 h, the p85α-suppressed EBs had 57% of the volume of the negative controls. The reduced volume of

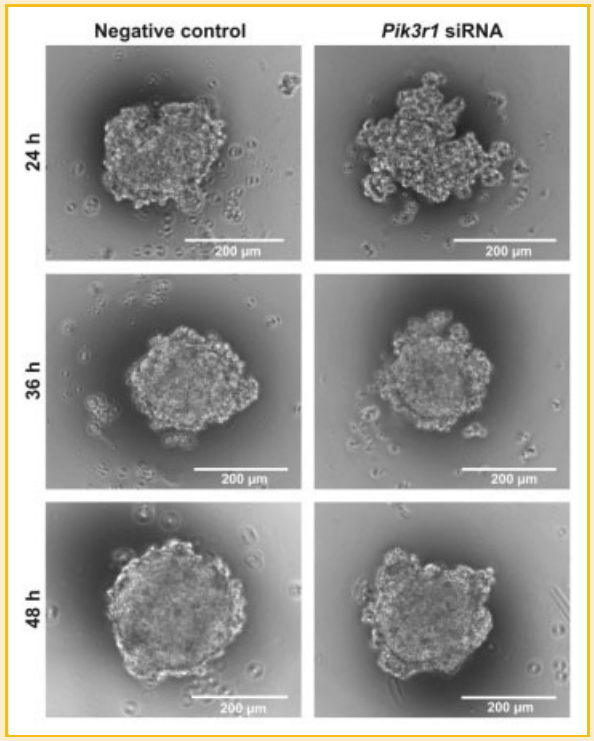

Fig. 4. Delayed EB formation of ES cells suppressed for p85α. The IOUD2 ES cells were treated with *Pik3r1* siRNA for p85α knockdown or with Silencer Select negative control siRNA, and differentiated into EBs. The morphologies of single EBs were compared at 24, 36, and 48 h. The *Pik3r1* gene knockdown in the EBs was 65% (at 48 h), as measured by the average of three independent experiments.

the p85α-suppressed EBs at 36 h was similar to the volume of the negative control EBs at 24 h. At 48 h, two populations were present. About half of the EBs had a smaller volume (68% of the negative control), whereas the other EBs had the same size as the control EBs (Fig. 5B). The size difference between the EBs of the negative control and the *Pik3r1* siRNA treated EBs at 24 h was statistically significant ($P < 1.1 \times 10^{-50}$, n = 104, χ^2-test), while by 36 and 48 h the size differences did not reach the statistical significance level of 5% ($P < 0.095$, n = 125, 36 h; $P < 0.339$, n = 125, 48 h). Looking more closely at Figure 5B at 48 h, it is evident that two populations with distinct EB diameters (c.250 and c.400 μm) have developed, and that their proportions are significantly different ($P < 0.001$) in the p85-suppressed versus the control cells. If we reasonably assume that the smaller EBs are scheduled to develop into larger ones, we can conclude that the developmental time delay due to p85 suppression is still evident at 48 h, in agreement with the cell-to-plate adhesion deficiency we describe below at 72 h. Taken together, these results suggest that there is an initial 12-hour delay in EB formation due to the knockdown of p85α, followed by a return to normal EB development.

Cell proliferation in developing EBs after treatment with *Pik3r1* siRNA is not related to EB size. In our experiments treating undifferentiated ESCs with *Pik3r1* siRNA, p85α knockdown resulted in reduced proliferation (see Fig. 2B). Thus it seemed unlikely that the initially larger cell aggregates are due to an increase in proliferation and not to a deficiency in aggregation. To exclude this possibility, the proliferation of the EBs was measured by CFSE staining and analyzed by flow cytometry. The results depicted in Figure 5C show that the rate of proliferation at 24 h was similar for both the *Pik3r1* siRNA treated EBs and the negative control EBs. Thus, at 24 h, the increase in EB size after p85α knockdown (Fig. 5C) is not related to altered proliferation (Fig. 5C) but indicate most likely a delayed aggregation of the cells within the EB.

Cell attachment is delayed in developing EBs after treatment with *Pik3r1* siRNA. To further test the capacity of cell adherence, attachment of the EBs to the tissue culture wells was measured at days 3 and 4 of EB development. We observed a pronounced delay for the *Pik3r1* siRNA treated EBs to attach to the plate compared to the negative control (Fig. 6A). In detail, the day 2 EBs were left for 24 h to attach to the culture wells, after which time 82% of the negative control EBs had attached to the plate, compared with only 62% of the EBs treated with *Pik3r1* siRNA. After 48 h, all EBs, both the negative control as well as the *Pik3r1* siRNA treated EBs, had attached. The delay of *Pik3r1* siRNA treated EBs attaching to the plastic plate shows that the p85α knockdown not only reduces aggregation and attachment in cell-to-cell contact but also in cell-to-substrate contact.

Integrin β1expression is reduced after treatment with *Pik3r1* siRNA. As a first step towards unraveling the molecular basis of the p85α-controlled adhesion effects, we performed an exploratory qPCR after 24 and 48 h of EB differentiation in EBs treated with *Pik3r1* siRNA and control EBs (Fig. 6B and Supplementary Table I) on nine gene transcripts for proteins involved directly in cellular adhesion: Laminin-α5, integrin-α2, integrin-β2, integrin-α6, integrin-α5, integrin-β1, fibronectin1, PAK4, and cadherin1. Within this qPCR series, we conservatively considered changes in gene expression by more than a factor of 10 as biologically significant. According to this criterion, *integrin-β1* was significantly reduced during the first 24 h, whose expression diminishes by a factor of 12 (Fig. 6B). At 48 h, *integrin-β1* regained its wildtype expression level. Two further adhesion genes (*CDH1* and *PAK4*) increased their expression significantly, by factors of 10 to 23 (Supplementary Table I), further pointing to a complex mechanism by which p85α controls cellular adhesion during early embryonic development.

DISCUSSION

Signaling by PI3K controls a variety of cellular responses including survival, proliferation, and migration [Kumar and Carrera, 2007; Jiménez et al., 2000]. Interactions of cells with extracellular matrix and their neighbors are crucial for normal development. PI3K has been shown to be critically involved in cellular adhesion to extracellular matrix as well as between adjacent cells [Cantley, 2002]. Importantly, during early embryonic development, in which cell–cell and cell–matrix interactions play a critical role, inhibition of PI3Ks by a pharmacological inhibitor of all classes of PI3K, i.e., class IA, IB, II, and III kinases, or disruption of a common direct downstream effector, PDK1, impairs EB formation (Bone and

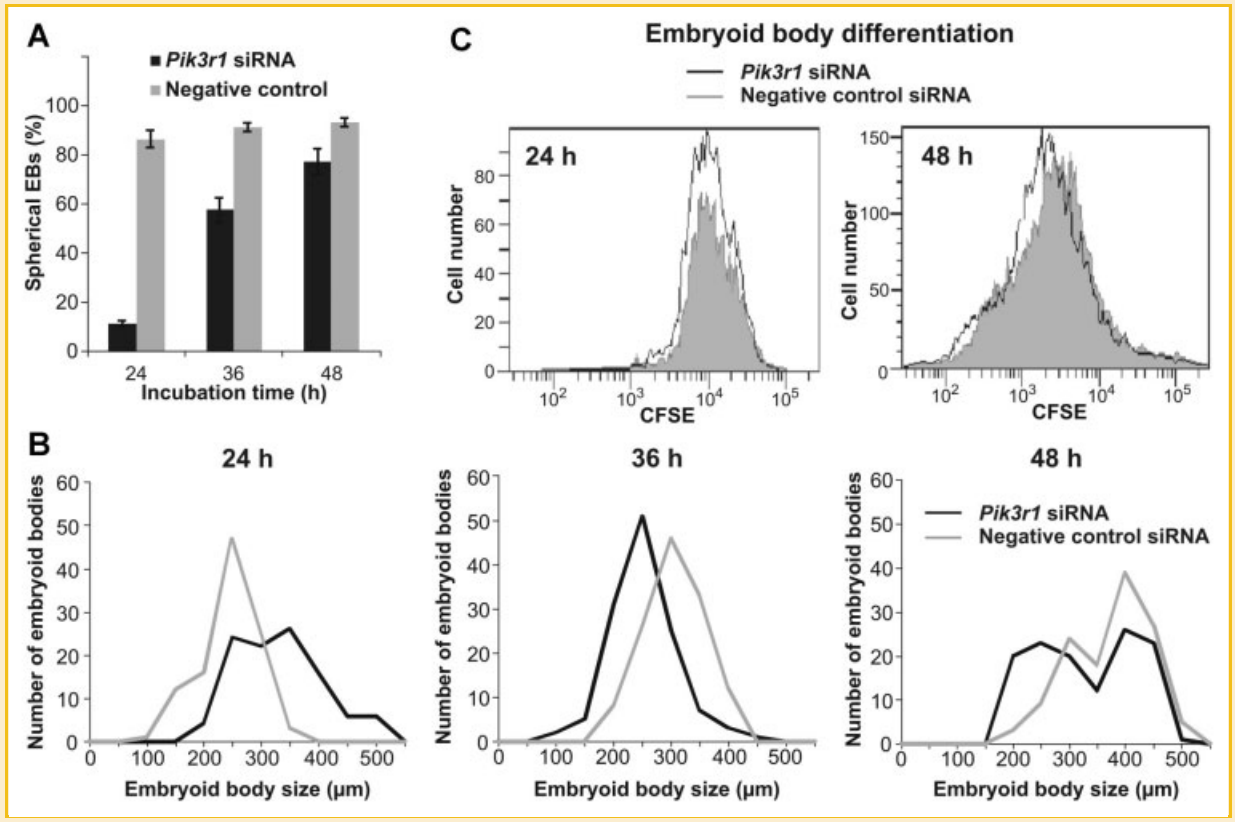

Fig. 5. Altered EB morphology and delayed EB formation of ES cells treated with *Pik3r1* siRNA. IOUD2 ES cells were treated with *Pik3r1* siRNA or Silencer Select negative control siRNA, and subsequently differentiated into EBs. The morphology during EB development was examined at 24, 36, and 48 h. A: EB formation of p85α-suppressed ES cells versus Silencer Select negative control treated cells. The p85α-suppressed ES cells (black bars) show a reduced ability to form spherical EBs compared to Silencer Select negative control treated cells (grey bars) measured over the first 48 h of formation. B: Distribution of EB size at 24, 36, and 48 h. The line graphs compare the distribution of EB diameters in p85α-suppressed EBs and in the negative control treated EBs. C: FACS analysis showing the intensities of CFSE staining the ESCs differentiating as EBs after siRNA treatment. Grey: Silencer Select negative control treated cells. Black: p85α-suppressed cells.

Welham, 2007). Similarly, deficiency of the p110β catalytic subunit of PI3Ks results in very early embryonic lethality, with poor development of blastocysts [Bi et al., 2002]. In the present work, we examined the role of the regulatory subunit p85α in undifferentiated ES cells and in early embryoid development. We provide evidence that *Pik3r1* is involved in early embryoid development: siRNA-mediated knockdown of the p85α subunit encoded by *Pik3r1* results in a delay of EB development with a reduced ability of the developing cells to aggregate and to undergo normal cell–cell and cell–substrate adhesion. Our stem cell results are thus in line with the work of Bone and Welham [2007] who had noted a size reduction of EBs by day 3 of development of cultured mouse ESCs treated with a general inhibitor for PI3K although the effect was not explained further at the time.

To specifically address the role of the PI3K regulatory subunit p85α, we suppressed its levels by siRNA and followed the development of EBs over a time course of 72 h. Our experiments show that the size reduction is due to a 12-hour delay within the first 24 h of EB development. The abnormal EB diameters in the p85α-suppressed cells can be explained by a delay in aggregation: Initially the p85α-suppressed cells make contact with each other but do not aggregate, whereas the control cells do aggregate, forming a tight sphere of cells. By 36 h the p85α-suppressed cells are finally aggregating and form a tightly packed sphere of cells, while the control cells are beyond this stage and growing. During the initial aggregation delay, compensatory pathways are possibly being activated, allowing the EB development to proceed normally, but retaining the 12-hour delay. This compensatory pathway would not be reliant on the splice variants p50α or p55α, as these two variants are not present in ES cells [Hallmann et al., 2003], and in any case p50α and p55α are also suppressed by the siRNA we employed.

Several studies have implicated PI3Ks and specifically also the p85α regulatory subunit in the regulation of ESC self renewal and proliferation [Jirmanova et al., 2002; Takahashi et al., 2003; Hallmann et al., 2003; Paling et al., 2004; Kingham and Welham, 2009]. In line with these studies, proliferation of undifferentiated ESC was slightly reduced in *Pik3r1* siRNA treated ESC compared with control siRNA treated ESC. In contrast, at 48 h after EB differentiation, we observed a slight increase in proliferation after p85α knockdown, although half of the *Pik3r1* siRNA treated EBs were smaller than control EBs. One possible explanation for this discrepancy could reflect the delayed development of EBs, being still in a higher proliferative phase. Alternatively, since it was shown that the outcome of PI3Ks effects on proliferation, i.e., increase or decrease, depends on the levels of PI3K activity [Hallmann et al., 2003], different PI3K activity during EB differentiation, could

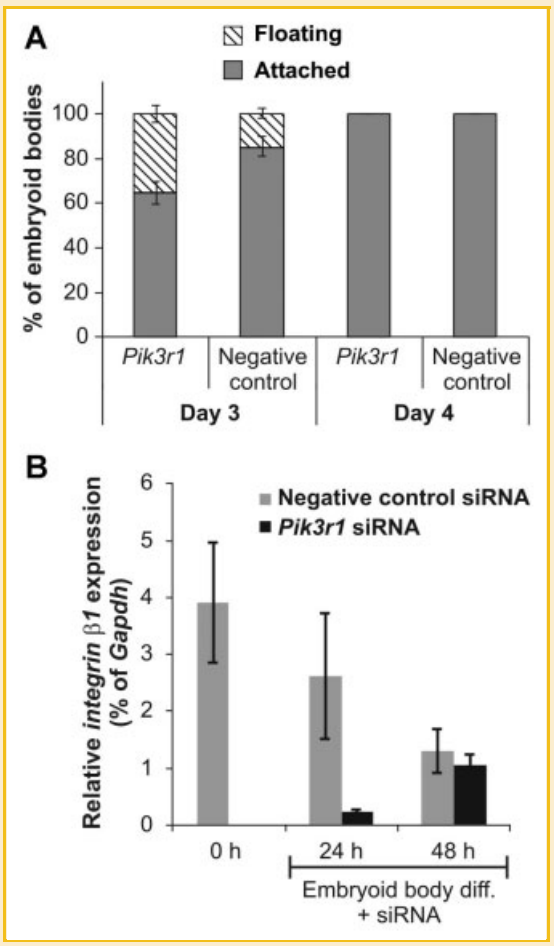

Fig. 6. Reduced attachment to culture dishes and reduced *integrin-β1* expression of EBs suppressed for p85α. IOUD2 ES cells were treated with *Pik3r1* siRNA or Silencer Select negative control siRNA, and subsequently differentiated into EBs. A: Reduced attachment to culture dishes of EBs suppressed for p85α. At day 3, the EBs were plated from hanging drops to culture flasks, where they attached to the plate and continued differentiation. In this experiment the proportion of EBs attached to the plate (grey bar) compared to floating colonies (hatched bars) was measured at days 3 and 4. Error bars indicate standard deviations. Note: On day 4 all colonies were attached in both p85α-suppressed and Silencer Select negative control EBs, i.e., SD = 0. B: Reduced *integrin-β1* expression in ES cells treated with *Pik3r1* siRNA. *Integrin-β1* was measured using qPCR at 24 and 48 h after treatment with *Pik3r1* siRNA and EB differentiation.

potentially influence cell proliferation. In this respect it is interesting to note that PI3Ks have complex functions in the control of cell cycle and cytokinesis [Kumar and Carrera, 2007]. Further work is needed to unravel the mechanism(s) of PI3K activity on proliferation of developing EBs.

Although knockout mice deficient for the p85α regulatory subunits proceed through embryonic development to birth [Terauchi et al., 1999; Fruman et al., 2000] they display several cellular defects of potential significance to our results. In particular, terminally differentiated macrophages from p85α-null mice [Munugalavadla et al., 2005] demonstrated an essential role for the p85α regulatory subunit in regulating adhesion, migration, wound healing, and phagocytosis. Thus, our stem cell results reproduce the macrophage findings as far as the adhesion deficiency phenotype is concerned. Cell motility and cell adhesion depend on the formation of lamellipodia, they provide a mechanical link to connect myosin motors with the initiation of cell adhesion sites [Giannone et al., 2007]. While not needed for cadherin-dependent adhesions [Gavard et al., 2004], analyses of mice deficient in p85α and p85β revealed that the regulatory PI3K subunit p85 is required for PDGF-induced membrane ruffling and lamellipodia formation and thus cellular adhesion [Brachmann et al., 2005].

Future research should focus on the molecular basis of the p85α-controlled adhesion effects we observe. As a first step, we performed an exploratory qPCR (Supplementary Table I) on nine gene transcripts for proteins involved directly in cellular adhesion. One of these gene targets, *integrin-β1*, is significantly reduced in the first 24 h after knockdown of p85α, its expression diminished by a factor of 12. During embryonic development, *integrin-β1* is required for inner cell mass formation and organization [Fässler and Meyer, 1995; Stephens et al., 1995]. In addition, *integrin-β1*-deficient EBs display similar alterations in morphology to the p85α-suppressed cells in that they are irregular in shape, frequently aggregated, and smaller as wild type EBs [Aumailley et al., 2000]. Interestingly, at 48 h, *integrin-β1* regains its wildtype expression level, coinciding with the resumption of normal cell–cell adhesion seen in the morphological experiment at this stage.

Two further adhesion genes (*CDH1* and *PAK4*) change their expression significantly, by factors of 20–23 (Supplementary Table I). Consistent with its role in mediating loss of cell-substratum adhesions and increasing cell motility and migration [reviewed by Wells and Jones, 2010], PAK4 is increased up to about 20-fold after 48 h of p85α knockdown. CDH1 is initially deceased for about fivefold after 24 h with a subsequent 20-fold increase after 48 h, following knockdown of p85α. Since a role for CDH1 in cell aggregation, and for compaction and blastocyst formation was described [Vestweber and Kemler, 1985; Larue et al., 1996; Mohamet et al., 2010], CDH1 is a further likely candidate involved in mediating p85α function(s) for cellular adhesion during EB development. Exploring interactions between the PI3K pathway, integrin-β1, CDH1, PAK4, and cellular adhesion may therefore be a fruitful direction of future enquiry.

In conclusion, within the mammalian PI3K pathway, we find that suppressing the regulatory p85α subunit leads to developmental delay accompanied by a transient inability of the EBs to undergo normal cell–cell and cell–substrate adhesion.

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