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Involvement of Phosphoinositide 3-Kinase Signaling Pathway in Chondrocytic Differentiation of ATDC5 Cells: Application of a Gene-Trap Mutagenesis

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Abstract Gene-trap mutagenesis is based on the notion that the random insertion of a trapping vector may disturb the function of inserted genes. Here, we applied this method to murine mesenchymal ATDC5 cells, which differentiate into mature chondrocytes in the presence of insulin. As the trap vector we used pPT1-geo, which lacks its own promoter and enhancer, but contains a *lacZ–neo* fusion gene as a reporter and selection marker driven by the promoter of the trapped gene. After pPT1-geo was introduced into ATDC5 cells by electroporation, the neomycin-resistant clones were screened for β-galactosidase activity. The selected clones were cultured in differentiation medium to evaluate the chondrogenic phenotype. The clones no. 6–30 and 6–175, which exhibited impaired and accelerated mineralization, respectively, were subjected to further analysis. In clone no. 6–30 in which the gene coding for the p85α subunit of phosphoinositide 3-kinase (PI3K) was trapped, the expression of marker genes of early chondrocytes including collagen type II, aggrecan, and PTH/PTHrP receptor was delayed. The insulin-induced stimulation of growth was reduced in clone no. 6–30 compared with the parental ATDC5 cells. Moreover, treatment of parental ATDC5 cells with a specific inhibitor of PI3K, LY294002, phenocopied clone no. 6–30, suggesting the involvement of PI3K signaling in the chondrogenic differentiation of ATDC5 cells. Clone no. 6–175 with accelerated mineralization was revealed to have a gene homologous to human KIAA0312 trapped, whose function remains unclear. Taken together, the gene-trap in ATDC5 cells might be useful to identify the molecules involved in chondrogenic differentiation. J. Cell. Biochem. 93: 418–426, 2004.

Key words: gene-trap; chondrocyte; ATDC5; phosphoinositide 3-kinase; insulin

Endochondral bone formation is a multistep phenomenon consisting of mesenchymal condensation of undifferentiated cells, proliferation of chondrocytes, and differentiation into hypertrophic chondrocytes, followed by mineralization [de Crombrugghe et al., 2001]. During maturation, chondrocytes change morphologically, and exhibit alterations in the production of extracellular matrix proteins. Hypertrophic chondrocytes are characterized by a high level of alkaline phosphatase (ALP), diminished levels of collagens type II and IX, and the production of type X collagen, a hypertrophic chondrocyte-specific product. Various kinds of signaling molecules, including Indian hedgehog, parathyroid hormone-related protein (PTHrP), and fibroblast growth factors (FGFs)

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have been revealed to regulate the maturation of chondrocytes [Karaplis et al., 1994; Lanske et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999; Liu et al., 2002; Minina et al., 2002; Ohbayashi et al., 2002]. However, the molecular mechanisms underlying the proliferation and differentiation of chondrocytes are still not fully understood.

Gene-trapping is a genome-wide approach applied to clarify the functions of genes by random insertion of a trapping vector which might disturb the function of the inserted genes [Stanford et al., 1998; Stanford et al., 2001]. In essence, a gene-trap vector consists of a promoterless reporter gene, a selectable marker and a splice acceptor site immediately upstream of the reporter gene [Stanford et al., 2001]. When the gene-trap vector is appropriately inserted in the endogenous gene, a fusion transcript including the upstream coding sequence of the gene and the reporter gene is generated by the endogenous *cis*-acting promoter and enhancer element of the trapped gene. This event is associated with the conversion of the trapped gene into a mutated gene and allows one to examine its expression pattern by visualization of the reporter activity. This approach is usually applied in embryonic stem (ES) cells to analyze the effect of gene trapping in individuals such as gene-trapped mice. At present, several large-scale, gene-trap screens are being carried out with various vectors, which aim to generate a public resource of mutagenized ES cells [Stanford et al., 2001].

The clonal cell line, ATDC5, is a commonly used cell model of endochondral ossification [Akiyama et al., 1996; Syukunami et al., 1996; Syukunami et al., 1997]. ATDC5 was originally isolated from the murine feeder-independent teratocarcinoma stem cell line AT805 on the basis of chondrogenic potential [Atsumi et al., 1990]. In the presence of insulin, the confluent monolayer culture of ATDC5 cells differentiate into proliferating chondrocytes through a cellular condensation process and undergo cellular hypertrophy and mineralization in the absence of β-glycerophosphate [Syukunami et al., 1997]. In other words, ATDC5 cells reproduce the multistep chondrocytic differentiation process encompassing the stages from mesenchymal condensation to calcification in vitro, which enables the study of the molecular mechanisms underlying endochondral bone formation.

In the present study, to identify the genes involved in chondrocytic differentiation, we applied a gene-trap approach in ATDC5 cells. One of the trap clones, which exhibited impaired mineralization, was identified to have trapped the gene encoding the p85α subunit of phosphatidylinositide 3-kinase (PI3K). Here, we demonstrate the usefulness of gene-trap mutagenesis in ATDC5 cells for investigating the molecular mechanisms of chondrocytic differentiation.

MATERIALS AND METHODS

Cell Culture

For chondrogenic induction, parental ATDC5 cells or the trap clones were inoculated into six-well culture plates (5×10^5 cells/well) and cultured in a 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 (DMEM/F12) medium containing 5% fetal bovine serum (FBS: ICN Biomedicals, Aurora, OH), insulin–transferrin–selenium-G supplement (ITS: Invitrogen, Carlsbad, CA; insulin 10 μg/ml, sodium selenite 6.7 μg/ml, and transferrin 5.5 mg/ml as a final concentration) at 37°C in a 5% CO2 atmosphere. After 3 days later, the medium was changed to alpha minimal essential medium (αMEM) supplemented with 5% FBS and ITS, and the culture plates were sealed with adhesive tape to facilitate mineralization. The medium was replaced every 3 days.

To evaluate the proliferation, parental ATDC5 cells, or the trap clones were inoculated into six-well culture plates at a density of 1×10^4/well, and cultured in DMEM/F12 containing 5% FBS in the presence or absence of insulin (10 μg/ml; Sigma, St. Louis, MO). The cell number in each well was then sequentially determined after trypsinization. In some experiments, the effects of specific PI3K inhibitor LY294002 (Sigma) were examined.

Gene-Trapping

As the trap vector, we used pPT1-geo, which lacks its own promoter and enhancer, but contains an *Escherichia coli* (*E. coli*) *lacZ* gene encoding β-galactosidase as a reporter fused to an *E. coli* neomycin resistance gene as a selection marker, which was designated *lacZ–neo* [Mainguy et al., 2000; Fig. 1]. The fusion gene *lacZ–neo* is assumed to be driven by the promoter of the trapped gene. Since the trap vector contains a splice acceptor site from the murine engrailed-2 gene upstream of *lacZ–neo*,

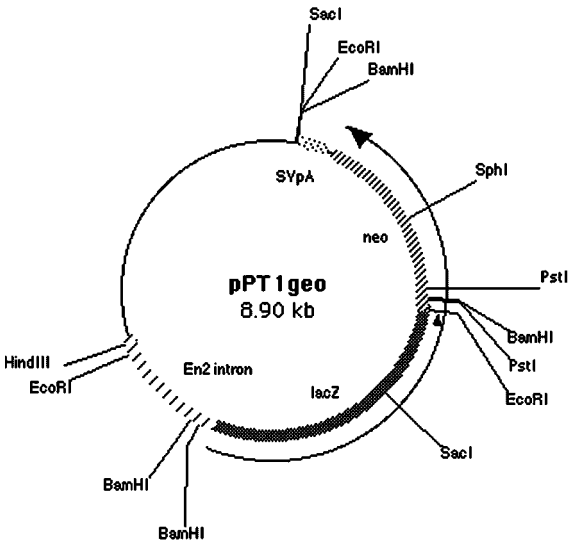

Fig. 1. Structure of the trapping vector pPT1-geo. It lacks its own promoter and enhancer, but contains a *lacZ–neo* fusion gene as a reporter and a selection marker driven by the promoter of the trapped gene.

its integration in the intron leads to a fusion transcript being generated from the upstream exon of the trapped gene, the exon of engrailed-2 and *lacZ–neo* on transcriptional activation of the trapped gene. After pPT1-geo linearized with *Hind*III digestion was introduced into ATDC5 cells using the Gene Pulser II electroporation system (BioRad, Helcules, CA), the neomycin-resistant clones were selected in the presence of G418 (400 μg/ml, Invitrogen). Then the neomycin-resistant clones (815 clones in total) were screened for β-galactosidase activity. Among them, 26 clones with a 100-fold higher level of β-galactosidase activity than the parental ATDC5 cells were then subjected to chondrogenic induction, followed by alcian blue and alizarin red staining to evaluate the extracellular matrix production and mineralization, respectively.

Identification of the Trapped Genes by 5′-Rapid Amplification of cDNA End (RACE)

Total RNA was extracted from trap clones using TRIZOL reagent (Invitrogen), and messenger RNA purified with oligo(dT) latex (Oligotex^{TM}-dT30 Super mRNA Purification Kit; Takara Biomedicals, Shiga, Japan). To identify the trapped genes, 5′-RACE was performed utilizing the 5′-RACE System for RACEs (Invitrogen), according to the manufacturer′s instructions with some modifications. Briefly, first-strand cDNA was synthesized from mRNA (1 μg) using SuperScript II reverse transcriptase (Invitrogen) with a primer specific to *lacZ* cDNA in pPT1-geo; LacZ-GSP1, 5′-TGGCGAAAGGGGGATGTG-3′. After the first strand of cDNA was synthesized, the original mRNA template was removed by treatment with RNase, and the unincorporated dNTPs and the primer were separated from the cDNA using a GlassMAX Spin Cartridge (Invitrogen). Then a homopolymeric tail was added to the 3′-end of the cDNA using TdT and dCTP, followed by PCR amplification using Taq polymerase (Promega, Madison, WI) and the following set of primers; 5′RACE Abridged Anchor Primer, 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′ (I; inosine) and LacZ-GSP2, 5′-ATGTGCTGCAAGGCGATTAAGTTG-3′. The PCR product then served as the template for the second round of PCR using the primers LacZ-GSP3, 5′-CCAGGGTTTTCCCAGTC-3′, and 5′RACE-AUAP, 5′-GGCCACGCGTCGACTAGTAC-3′. The product of the second PCR was then cloned into pT7-Blue vector (Novagen, Madison, WI) and sequenced using an automated sequencer (377A model; PE Applied Biosystems, Tokyo, Japan).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA (2.5 μg) prepared as above was reverse transcribed using random hexamer (Promega) and SuperScript II (Invitrogen). PCR was performed using Taq polymerase (Promega) and murine-specific primer sets as follows; murine type II collagen pro α1 chain, sense, 5′-TCTCCACTCTTCTAGTTCCT-3′ and antisense, 5′-TTGGGTCATTTCCACATGC-3′; murine aggrecan, sense, 5′-ACTATGACCACTTTACTCTTGG-3′ and antisense, 5′-TGGCGATGATGGCGCTGTTCTG-3′; PTH/PTHrP receptor sense, 5′-ATGCTCTTCAACTCCTTCCAG-3′ and antisense, 5′-ACTGGCTTCTTGGTCCATCTG-3′; GAPDH sense, 5′-CCCATCACCATCTTCCAGGA-3′ and antisense, 5′-TTGTCATACCAGGAAATGAGC-3′. Amplification of the expected fragments was confirmed by sequencing of the products.

Measurement of ALP Activity

ALP activity was measured by the Lowry method using *p*-nitrophenylphosphate as a substrate in an alkaline glycine buffer containing 10 mM $MgCl_2$ and standardized to the protein amount [Lowry et al., 1954].

Western Blotting

Whole cell extracts were harvested in RIPA buffer [1% Triton, 1% Na deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-Cl (pH 7.4), 5 mM EDTA, and protease inhibitor cocktail (Complete™; Roche Diagnostics GmbH, Mannheim, Germany)]. The cell lysates containing 10 μg of each protein were then subjected to SDS–PAGE, and were transferred to PVDF membrane (Biorad). After blocking with Block Ace reagent (Dainippon Pharmaceuticals, Osaka, Japan), the membranes were incubated with the following primary antibodies; anti-PI3K p85α monoclonal antibody (B-9, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-β galactosidase polyclonal antibody (Rockland, Gilbertsville, PA). After incubation with the corresponding secondary antibodies, the proteins were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

Southern Blot Analysis

Genomic DNA was extracted from parental ATDC5 cells and trap clones, and digested with the enzymes *Sph*I or *Pst*I. The digested DNA was then electrophoresed, transferred to a Hybond-N+ membrane (Amersham Biosciences), and probed with a radiolabeled fragment of *lacZ* cDNA prepared by *Eco*RI / *Sac*I digestion of pPT1-geo.

RESULTS

Isolation of A Clone With the Gene Encoding the PI3K p85α Subunit Trapped

As described in "Materials and Methods," we obtained 815 neomycin-resistant clones. Since trap vector pPT1-geo possesses the *lacZ–neo* fusion gene which should be driven under the control of the trapped gene promoter, the trapped clones can be isolated under the selection with neomycin only when the trapped genes are expressed in ATDC5 cells. Among them, 26 clones with strong β-galactosidase activity were subjected to chondrogenic induction in the presence of insulin. We performed alcian blue and alizarin red staining of these cells at 5 and 7 weeks of culture, respectively. In alizarin red staining, 8 clones exhibited accelerated mineralization, while 14 clones showed delayed mineralization compared with the parental ATDC5 cells (data not shown). One of the clones, no. 6–30, accumulated less of a cartilageous matrix than the parental ATDC5 cells as shown by alcian blue staining (Fig. 2). In addition, alizarin red, which stains mineralized matrices, revealed reduced mineralization in this clone (Fig. 2). 5′-RACE analysis showed that the trapping vector was inserted in intron 1 of the gene encoding the p85α subunit of PI3K in the clone no. 6–30. On the other hand, the clone no. 6–175 with accelerated mineralization (Fig. 2) was revealed to have a gene homologous to human KIAA0312 trapped, whose function remains unclear. Since we obtained both clones with delayed mineralization and accelerated mineralization, we assume that trapping of the genes was responsible for the phenotypical change of these clones. In the clone no. 6–30, the trapped allele was assumed to express a fusion protein containing the N-terminal Src homology 3 (SH3) region of the p85α subunit transcribed from exon 1A and the product of the *lacZ–neo* fusion gene, but lacking the SH2 domains and the domain responsible for interaction with p110 (Fig. 3). Western blot analysis using monoclonal antibody against p85α which recognizes amino acids 333–430 mapping within the amino terminal SH2 domain, demonstrated the reduced expression of p85α in the trap clone no. 6–30, since the fusion protein from the trapped allele was not recognized by the antibody (Fig. 4, left panel). Because one of the p85α alleles remained intact, residual expression of the protein was detected in the

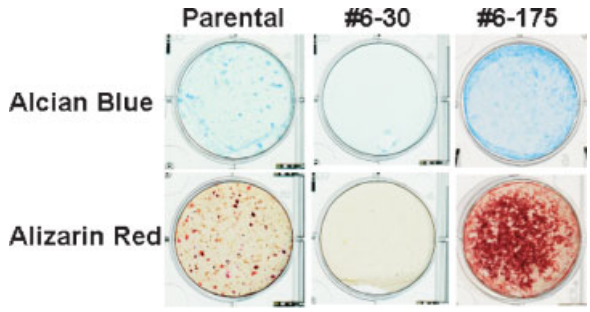

Fig. 2. Alcian blue and alizarin red staining of parental ATDC5 cells and the trap clones no. 6–30 and 6–175 cultured in the presence of insulin. The cells were inoculated into six-well culture plates (5×10^5 cells/well) and cultured in DMEM/F12 medium containing 5% FBS and 10 μg/ml of insulin at 37°C in a 5% CO_2 atmosphere. After 3 days, the medium was changed to αMEM containing 5% FBS and 10 μg/ml of insulin, and the culture plates were sealed with adhesive tape to facilitate the mineralization. The medium was replaced every 3 days. Alcian blue and alizarin red staining were performed on day 35 and day 49, respectively.

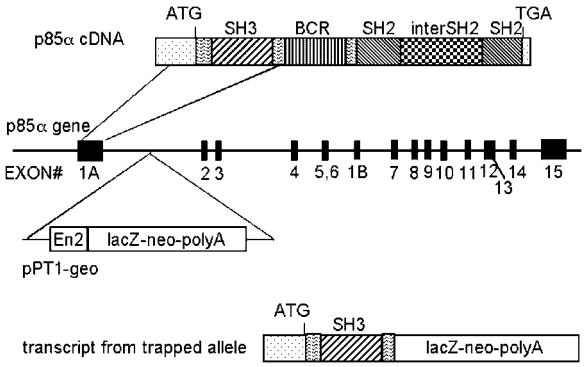

Fig. 3. Schematic representation of the genomic organization of the murine *p85α* gene and the insertional mutation resulting from gene-trapping with the vector pPT1-geo. The locations of exons (solid boxes) and introns (horizontal lines between exons) are indicated. SH3; Src homology 3 domain, BCR; the breakpoint cluster region homology domain, SH2; Src homology 2 domain. En2; murine engrailed-2 exon, lacZ–neo–polyA; fusion gene of *lacZ–neo*. ATG indicates an initiation codon, and TGA is the termination codon.

clone. When the antibody against β-galactosidase was utilized as the primary antibody, only one band was detected corresponding to the fusion of part of p85α and the *lacZ*-neo product, indicating that only one fusion gene was expressed (Fig. 4, right panel, arrowhead). Although Southern blot analysis using *lacZ* cDNA as the probe suggested that the trap vector had been

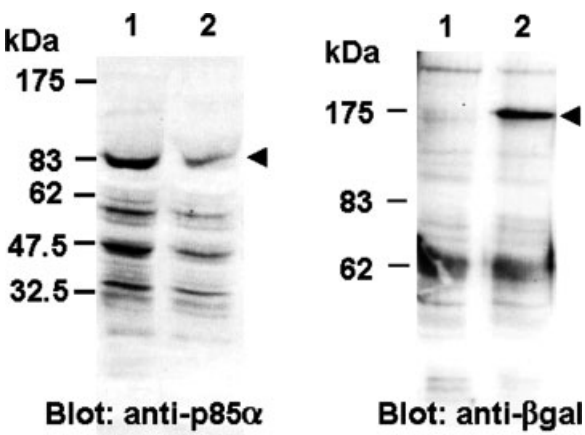

Fig. 4. Detection of the p85α subunit by Western blot analysis. Whole cell lysates obtained from the parental ATDC5 cells (**lane 1**) and the trap clone no. 6–30 (**lane 2**) were subjected to SDS–PAGE, followed by immunoblotting using antibody against amino acids 333–430 mapping within the SH2 domain of p85α (**left panel**) or antibody against β-galactosidase (**right panel**). The arrowhead in the left panel shows the signal corresponding to the wild-type protein of p85α. On the other hand, the arrowhead in the right panel indicates the signal corresponding to the fusion protein of part of p85α and the lacZ–neo product.

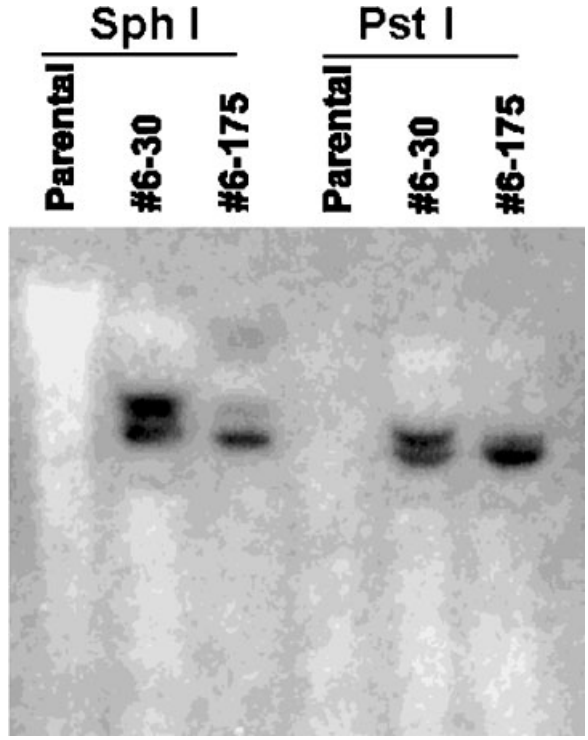

Fig. 5. Southern blot analysis. Genomic DNA extracted from the parental ATDC5 cells or the trap clones no. 6–30 and 6–175 was digested with the restriction enzymes SphI or PstI, and subjected to Southern blot analysis using a radiolabeled fragment of lacZ cDNA prepared by *Eco*RI/*Sac*I digestion of pPT1-geo as the probe.

inserted into two locations in the genome of the clone no. 6–30 (Fig. 5) and FISH analyses confirmed it (data not shown), only one product was amplified by 5′-RACE, suggesting that an insertion other than that into the *p85α* gene was likely to occur in non-coding DNA or a gene that was not expressed in ATDC5 cells. As to the clone no. 6–175, integration of the trap vector into a single location was detected in the Southern analysis (Fig. 5).

Impairment of the Insulin-Induced Stimulation of Proliferation in no. 6–30

Parental ATDC5 cells and the trap clone no. 6–30 were cultured in the presence or absence of 10 μg/ml of insulin, and the proliferation of these cells was evaluated. In the absence of insulin, there was no significant difference in growth between parental ATDC5 cells and the trap clone no. 6–30 (Fig. 6). In the presence of insulin, the growth was accelerated both in parental ATDC5 cells and in no. 6–30. However, the insulin-induced stimulation of proliferation

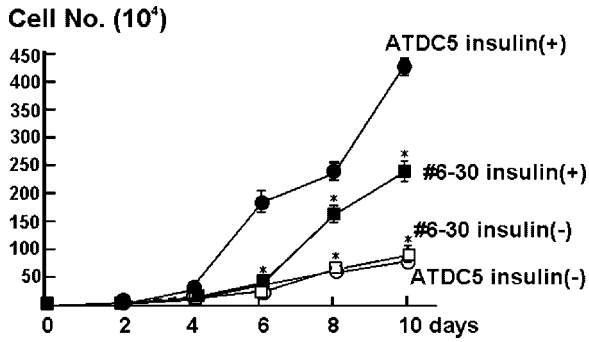

Fig. 6. Growth curves of the parental ATDC5 cells and the clone no. 6–30 cultured in the presence (closed circle; parental ATDC5, closed rectangle; no. 6–30) or absence (open circle; parental ATDC5, open rectangle; no. 6–30) of 10 μg/ml of insulin. The data are expressed as mean ± SE (n = 3). *Significantly different from the values in the insulin-treated parental ATDC5 cells ($P < 0.001$). The experiments were performed three times with similar results.

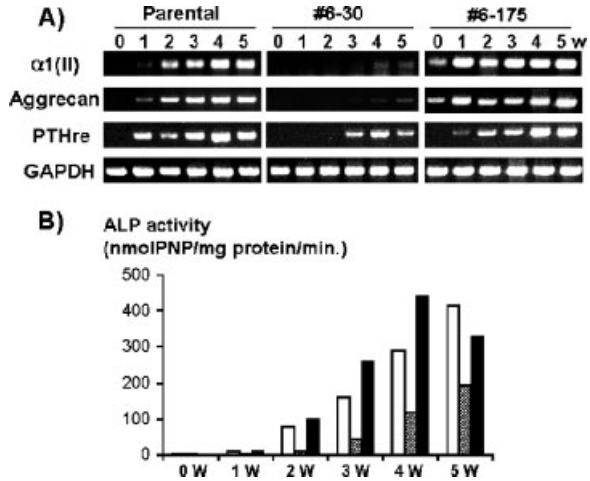

Fig. 7. **A**: RT-PCR analysis for the expression of marker genes of early chondrocytes. Total RNA was extracted from the parental ATDC5 cells or the trap clones no. 6–30 and 6–175 cultured in the presence of insulin (10 μg/ml) for the period indicated, and was subjected to RT-PCR analysis for the expression of type II collagen proα1 chain [α1(II)], aggrecan, PTH/PTHrP receptor (PTHre), and GAPDH. **B**: ALP activity in the parental ATDC5 cells (open columns), the trap clones no. 6–30 (hatched columns), and no. 6–175 (closed columns). Cell lysates were harvested from the cells cultured in the presence of insulin for the period indicated, and subjected to measurement of ALP activity. The experiments were repeated three times, and similar results were obtained.

was markedly reduced in no. 6–30 compared to the parental cells (Fig. 6).

Impaired Differentiation in the Trap Clone no. 6–30

The chondrogenic phenotype of no. 6–30 was analyzed by RT-PCR. The expression of collagen type II, aggrecan, and PTH/PTHrP receptor, which are the markers of early chondrogenesis, were suppressed in the trap clone compared with the parental cells (Fig. 7A). In addition, ALP activity was reduced in no. 6–30 compared with the parental cells or trap clone no. 6–175 (Fig. 7B).

Treatment of Parental ATDC5 Cells With PI3K Inhibitor LY294002 Mimicked the Phenotype of the Trap Clone no. 6–30

Treatment of parental ATDC5 cells with the specific inhibitor of PI3K LY294002 (10 μM) markedly inhibited insulin-induced stimulation of proliferation of the parental ATDC5 cells (Fig. 8A). In addition, treatment of parental ATDC5 cells with LY294002 (10 μM) suppressed the accumulation of cartilageous matrix and the mineralization as shown by alcian blue and alizarin red staining, respectively (Fig. 8B).

DISCUSSION

Here, we utilized a gene-trap approach to identify the genes involved in chondrocytic differentiation. Gene-trap mutagenesis is a technique that randomly generates loss-of-function mutations and reports the expression of many mouse genes [Stanford et al., 1998; Stanford et al., 2001]. Although this approach is usually carried out using ES cells to generate and analyze mutant mice derived from mutagenized

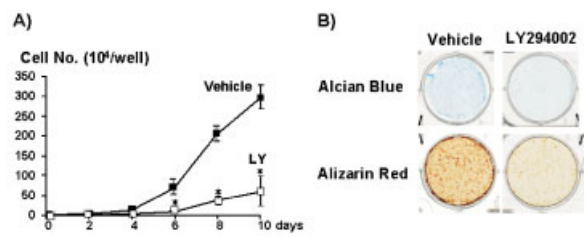

Fig. 8. **A**: Effect of LY294002 on the proliferation of parental ATDC5 cells. ATDC5 cells were cultured with 10 μM of LY294002 (open rectangles) or vehicle (closed rectangles) in the presence of 10 μg/ml of insulin, and the cell number was determined after the indicated culture period. The data are expressed as mean ± SE (n = 3). *Significantly different from the values in the vehicle-treated cells ($P < 0.001$). The experiments were performed three times with similar results. **B**: Effect of LY294002 on matrix accumulation and mineralization of parental ATDC5 cells. ATDC5 cells were cultured with 10 μM of LY294002 or vehicle in αMEM containing 5% FBS and 10 μg/ml of insulin. Alcian blue and alizarin red staining were performed on day 35 and 49, respectively.

ES cell lines, here we applied the strategy to ATDC5 cells that can differentiate into mature chondrocytes in vitro. One of the advantages of gene-trapping in ATDC5 cells is assumed to be that it does not require the large amount of space that is indispensable to screen gene-trapped mice. In addition, ATDC5 cells differentiate into mature hypertrophic chondrocytes with a mineralized matrix in several weeks when cultured in the presence of insulin, which enables one to screen the trap clones by phenotype in a relatively short time. On the other hand, gene-trapping in ATDC5 cells also has limitations: because the wild-type alleles remain, the clones in which some recessive genes are trapped will be missed in the screening by phenotype. However, in cases where the trapped genes have a dose effect or the trapping results in a fusion protein with a dominant-negative effect, one can expect the clones to exhibit a unique phenotype in terms of chondrocytic differentiation.

In the current study, among the clones screened, one with impaired mineralization was revealed to have the gene encoding the p85α subunit of PI3K trapped. In this particular clone, Southern blot analysis revealed that the trap vector was inserted at two locations in the genome (Fig. 5). However, since the only one product was amplified by 5′ RACE, an insertion other than that in the *p85α* gene was likely to occur in non-coding DNA or a gene that was not expressed in ATDC5 cells. Therefore, we assume that the phenotypic change in this clone resulted from the trapping of the *p85α* gene. In addition, treatment of parental ATDC5 cells with the PI3K inhibitor LY294002 mimicked the phenotype of the clone with the *p85α* gene trapped (Fig. 8). These results further support that the insertion of the trapped vector in the *p85α* gene is responsible for the impairment of chondrogenic differentiation of ATDC5 cells.

PI3K, a heterodimeric enzyme consisting of a regulatory subunit such as p85α and a 110-kDa catalytic subunit (p110), has been implicated in the regulation of various cellular events, including proliferation, differentiation, and survival in response to many kinds of cytokines and growth factors. Phosphorylated tyrosine residues in receptors and non-receptor proteins such as insulin receptor substrate-1 (IRS-1) interact with the SH2 domain in the regulatory subunit of PI3K, resulting in the activation or recruitment of the enzyme [Toker and Cantley, 1997]. Among the five regulatory subunits of PI3K that have been identified to date, p85α was the first to be cloned [Fruman et al., 1996]. The p85α molecule contains two Src homology 2 (SH2) domains, an N-terminal SH3 domain, and a domain responsible for binding to p110, which is located between the two SH2 domains. The other regulatory subunits p55α and p50α are generated by alternative splicing of the *p85α* gene, and exhibit different PI3K activity-elevating responses to insulin [Pons et al., 1995; Inukai et al., 1996; Inukai et al., 1997].

In our clone with the *p85α* gene trapped, the protein amount of p85α was obviously reduced, which was confirmed by Western blotting using antibody against amino acids 333–430 mapping within the SH2 domain (Fig. 4). Consistent with this result, the insulin-induced stimulation of proliferation was reduced in the trap clone compared with the parental ATDC5 cells (Fig. 6). The decreased response to insulin leads to impaired mesenchymal condensation and impaired expression of the marker genes for early chondrocytes in the trap clone no. 6–30. Moreover, treatment of parental ATDC5 cells with specific inhibitor of PI3K, LY294002, mimicked the phenotype of the trap clone 6–30; suppressed the insulin-induced stimulation of proliferation and impaired the mineralization (Fig. 8). These results indicated the involvement of PI3K in the early stages of the chondrocytic differentiation of ATDC5 cells. In vivo, IGF-I might be the major signal instead of insulin which acts through PI3K in the process of chondrogenesis.

To date, several mouse lines have been generated that lack the product of the *p85α* gene by gene targeting [Fruman et al., 1999; Suzuki et al., 1999; Terauchi et al., 1999]. No phenotypic changes in the skeleton have been reported in these animals. However, the involvement of the PI3K/Akt pathway in bone metabolism has been suggested by a line of in vitro studies [Borgatti et al., 2000; Ghosh-Choudhury et al., 2002; Peng et al., 2003]. Borgatti et al. [2000] reported that exposure to proliferative growth factors induced the nuclear translocation of Akt in osteoblastic MC3T3-E1 cells. It has also been reported that the PI3K/Akt signaling pathway is involved in bone morphogenetic protein-2 (BMP-2)-induced osteoblastic differentiation [Ghosh-Choudhury et al., 2002]. In addition, Peng et al. [2003] have recently reported that mice lacking both Akt1 and Akt2 exhibit

delayed bone development as well as impaired skin development, skeletal muscle atrophy, and impeded adipogenesis. Our findings using the clone where the *p85α* gene was trapped may enhance the importance of the PI3K pathway in mesenchymal cell proliferation and differentiation in response to extracellular stimuli.

In this study, we have also isolated the clone no. 6–175 which exhibited accelerated mineralization (Fig. 2). 5′RACE revealed that a gene homologous to human KIAA0312 has been trapped in this clone. Although the functions of this molecule are still unclear, it contains a HECT domain-like motif characteristic to a subtype of ubiquitin E3 ligase. Whether this molecule really acts as an E3 ligase remains to be elucidated.

In conclusion, a gene-trap approach revealed that PI3K plays some roles in the early steps of chondrocytic differentiation in ATDC5 cells. Although there are some limitations to gene trapping in ATDC5 cells, it is a useful strategy for identifying molecules that might be involved in chondrocytic differentiation.

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