p85α Regulates Osteoblast Differentiation by Cross-talking with the MAPK Pathway*

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Class IA phosphoinositide 3-kinase (PI3K) is involved in regulating many cellular functions including cell growth, proliferation, cell survival, and differentiation. The p85 regulatory subunit is a critical component of the PI3K signaling pathway. Mesenchymal stem cells (MSC) are multipotent cells that can be differentiated into osteoblasts (OBs), adipocytes, and chondrocytes under defined culture conditions. To determine whether p85α subunit of PI3K affects biological functions of MSCs, bone marrow-derived wild type (WT) and p85α-deficient (p85α−/−) cells were employed in this study. Increased cell growth, higher proliferation rate and reduced number of senescent cells were observed in MSCs lacking p85α compared with WT MSCs as evaluated by CFU-F assay, thymidine incorporation assay, and β-galactosidase staining, respectively. These functional changes are associated with the increased cell cycle, increased expression of cyclin D, cyclin E, and reduced expression of p16 and p19 in p85α−/− MSCs. In addition, a time-dependent reduction in alkaline phosphatase (ALP) activity and osteocalcin mRNA expression was observed in p85α−/− MSCs compared with WT MSCs, suggesting impaired osteoblast differentiation due to p85α deficiency in MSCs. The impaired p85α−/− osteoblast differentiation was associated with increased activation of Akt and MAPK. Importantly, bone morphogenic protein 2 (BMP2) was able to intensify the differentiation of osteoblasts derived from WT MSCs, whereas this process was significantly impaired as a result of p85α deficiency. Addition of LY294002, a PI3K inhibitor, did not alter the differentiation of osteoblasts in either genotype. However, application of PD98059, a Mek/MAPK inhibitor, significantly enhanced osteoblast differentiation in WT and p85α−/− MSCs. These results suggest that p85α plays an essential role in osteoblast differentiation from MSCs by repressing the activation of MAPK pathway.

Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells that were originally isolated as colony forming adherent fibroblast-like cells or colony-forming unit fibroblastic cells (CFU-Fs) from bone marrow suspension (1–5). MSCs are multipotent cells that are capable of differentiating into multiple cell lineages including osteoblasts, chondrocytes, adipocytes, smooth muscle cells, skeletal, and cardiac myocytes, endothelial cells, and neurons. MSCs persist into adulthood and provide a supply of osteoblasts for normal adult bone remodeling (6–10). The majority of MSCs allocated for the osteoblast lineage likely reside in bone marrow and comprise approximately one of 100,000 bone marrow cells (11). While osteoblast differentiation is one of the main characteristics of MSCs (1–5), the signaling pathway(s) involved in regulating this process are poorly understood.

Class IA phosphatidylinositol-3-kinases (PI3Ks) are critical regulators of a broad range of cellular processes including cell proliferation, differentiation, survival, and migration (12, 13). Based on primary sequence, substrate preference, and regulation, the PI3K family of lipid kinases can be divided into three major classes (12, 14–16). While class IA PI3Ks are heterodimeric enzymes composed of a catalytic subunit (p110α, p110β, or p110δ) complexed with one of five regulatory subunits (p85α, p55α, p50α, p85β, or p55γ), the class IB enzyme is a dimer made of p110γ catalytic subunit and p101 or p84 regulatory subunit (14, 16, 17). The class I catalytic subunit polypeptides p110α, p110β, p110δ, and p110γ are encoded by PIK3CA, PIK3CB, PIK3CD, and PIK3CG, respectively (14, 18). The regulatory subunits are encoded by five genes: PIK3R1 encodes p85α, p55α, and p50α; PIK3R2 encodes p85β; PIK3R3 encodes p55γ; PIK3R5 encodes p101; and PIK3R6 encodes p84 (14, 18). p85 subunits contain Src homology 2 (SH2) domains, which bind phosphorylated tyrosine (pTyr) in a specific amino acid sequence context. p85 subunits provide at least three functions to p110 proteins: stabilization, inactivation of their kinase activity in the basal state and recruitment to pTyr residues in receptors and adaptor molecules. Engagement of the p85 SH2 domains by pTyr relieves the p85-mediated inhibition of p110 isoforms and also brings them in contact with their lipid substrates in the membrane.

Although there are studies that show PI3K is involved in mesenchymal stem cell signal transduction (19–22), there is no evidence to show the direct impact of PI3K isoform specific regulation of mesenchymal stem cell functions. Utilizing mice lacking the expression of class I PI3K regulatory subunit, p85α, bone morphogenetic protein; CFU-F, colony-forming unit fibroblastic cell; BMMNC, bone marrow mononuclear cell.

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The abbreviations used are: MSC, mesenchymal stem cell; PI3K, phosphoinositide 3-kinase; OB, osteoblast; ALP, alkaline phosphatase; BMP,
confirm the mouse genotypes and Western blot was used to verify loss of p55 allowed selective disruption of p85 described and shown in Fig. 1 (24). The targeting strategy typed by polymerase chain reaction (PCR) as previously wise stated.

Sigma. BMP2 was purchased from R&D (Minneapolis, MN). MSCs were generated from 6–8-week old WT and pho-Erk were purchased from Cell Signaling Technology, Inc. Anti-phospho-Akt, and anti-phospho-Erk were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-β-actin antibody was purchased from Sigma. BMP2 was purchased from R&D (Minneapolis, MN). All other chemicals were purchased from Sigma unless otherwise stated.

Isolation and Expansion of MSCs—Bone marrow-derived MSCs were generated from 6–8-week old WT and p85α−/− mice as described previously (25). Briefly, bone marrow cells were collected by flushing the femurs and tibias with Iscove's modified of MEM (Invitrogen) containing 2% fetal calf serum (FCS) using a 23-gauge needle. Bone marrow mononuclear cells (BMMNCs) were separated by low density gradient centrifugation. Following washing with Iscove's MEM, cells were resuspended and adjusted to a concentration of 2 × 10^6 cells/ml in mouse MesenCult basal medium supplemented with MesenCult Supplemental (Stem Cell Technologies Inc.). 10 ml of the single cell suspension was added into a 10-cm tissue culture plate as previously described (26). Cells were plated into a flask at 2 × 10^6/ml in 10 ml of complete MesenCult medium. Once the culture reached 80–90% confluence, cells were trypsinized and replated at 5 × 10^5 cells/75 cm². MSCs at passage 5 to passage 10 were used in the described experiments.

we demonstrate that p85α subunit plays a critical role in modulating mesenchymal stem cell functions.

EXPERIMENTAL PROCEDURES

Animals and Materials—p85α−/− mice in a C57BL/6J strain have been described previously (23). p85α−/− mice were genotyped by polymerase chain reaction (PCR) as previously described and shown in Fig. 1 (24). The targeting strategy allowed selective disruption of p85α expression while leaving p55α and p50α isoforms intact. These mice were maintained under specific-pathogen-free conditions in the Indiana University Laboratory Animal Research Center, Indianapolis, IN. 6–8-week-old wild type (WT) and p85α−/− mice were used in the study. Anti-p85α antibody, anti-p85β antibody, anti-p110α antibody, and anti-p110β antibody were purchased from Millipore Corp. (Billerica, MA), Anti-phospho-Akt, and anti-phospho-Erk were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-β-actin antibody was purchased from Sigma. BMP2 was purchased from R&D (Minneapolis, MN). All other chemicals were purchased from Sigma unless otherwise stated.

CFU-F Assay—To measure the frequency of MSCs in bone marrow, the CFU-F assay was performed as previously reported with minor modification (26). Briefly, BMMNCs were separated by Ficoll-Hypaque density gradient centrifugation from bone marrow cells. 1 × 10⁶/ml BMMNCs were plated into 6-well tissue culture plates in triplicate for each condition in 2 ml of complete MesenCult medium and incubated at 37 °C, 5% CO₂. At day 14 of culture, medium was removed from each well followed by two washes of PBS and subsequently stained with HEMA-3 quick staining kit (Fisher Scientific) according to the manufacturer’s instructions. Colonies with more than 50 cells were counted microscopically at 20× magnification by a phase contrast microscope (Nikon, Fryer INC. Chicago, IL). Colonies that morphologically differed from MSCs were excluded from the results.

Proliferation Assay—To examine the impact of class I regulatory subunit p85α on the proliferation of MSCs, [3H]thymidine (New Life Science Products, Inc.) incorporation assay was performed. Briefly, MSCs from WT and p85α−/− mice were deprived of supplement for 24 h and 1 × 10⁵ cells were plated in 96-flat bottom well plates in 200 μl of α-MEM containing 1% glucose at 37 °C, 5% CO₂, humidified incubator. Cells were cultured for 48 h, and [3H]thymidine was added to cultures 6 h prior to harvest on glass fiber filters with an automated cell harvester (96-well harvester, Brandel, Gaithersburg, MD) and γ emission was measured with a microplate scintillation counter (Packard Bioscience Company, Shelton, CT). Assays were performed in triplicate for each condition. Cell growth was also evaluated by manually counting cell number with a hemocytometer (27). Briefly, MSCs were plated into a flask at a concentration of 2 × 10⁶/ml in 10mls of complete MesenCult medium. Once the culture reached 80–90% confluence, cells were trypsinized and replated at 5 × 10⁵/75 cm². Cell numbers were then recorded and plated at the same density each time. The fold increase in MSCs was calculated for each time point compared with the first point to generate a growth curve.

Cell Cycle Analysis—To investigate if p85α had impact on MSC cell cycle, we conducted flow cytometric analysis and Western blot experiments. MSCs from WT and p85α−/− were plated in 10-cm dishes at 1 × 10⁶ in MSC complete medium and incubated at 37 °C, 5% CO₂ for 24 h. Cells were then changed to serum-free medium and cultured for additional 24 h. For flow cytometry analysis, cells were washed in PBS and fixed in 70% ethanol and washed again in cold PBS. Cells were resuspended in 500 μl PI/Triton X-100 staining solution (to 10 ml of 0. 1% (vi/v) Triton X-100 in PBS add 2 mg of DNase-free RNase A and 0.40 ml of 500 μg/ml PI) and incubated at 37 °C for 15min. Data were acquired on FACS Calibur (BD Biosciences). For Western blot, cells were lysed in lysis buffer (1% Nonidet P-40). Lysates were subjected to immunoblotting to examine expression of CDK2, CDK4, cyclin B, cyclin D, cyclin E, p16, P-40. Lysates were subjected to immunoblotting to examine expression of CDK2, CDK4, cyclin B, cyclin D, cyclin E, p16, and p19. All the antibodies are from Cell Signaling Technologies Inc.

Senescent Assay—Histochemical staining for β-galactosidase activity was utilized to measure the senescence of MSCs as described previously (27). MSCs from WT and p85α−/− mice were plated in chamber slides (2 × 10⁵ cells/chamber) and incubated at 37 °C, 5% CO₂ for 72 h. Cells were stained with a
\textbf{p85α and Mesenchymal Stem Cells}

Senescent Staining kit (Sigma) according to the manufacturer’s instructions. Briefly, cells were washed twice with phosphate-buffered saline (PBS) and fixed for 6 min with fixation buffer (20% formaldehyde, 2% glutaraldehyde, 70.4 mM Na₂HPO₄, 14.7 mM KH₂PO₄, 1.37 M NaCl, and 26.8 M KCl). After 3 washes with PBS, labeling reagents were added into the chamber, and stained at 37°C without CO₂ overnight. Senescent cells displayed a blue color in the cytoplasm. Four thousand cells were counted for each chamber and the percentage of positive cells/4000 cells was determined.

\textit{Differentiation Assays and Histochemical Staining—}To induce osteogenic differentiation, WT, and p85α-/-MSCs were plated at 5 × 10⁶/ml in osteogenic differentiation medium (MesenCult proliferation kit supplemented with 10⁻⁸ mol/liter dexamethasone, 5 µg/ml ascorbic acid 2-phosphate, and 10mmol/liter β-glycerophosphate) in 12-well plate. For Von Kossa staining, cells were maintained in osteogenic differentiation medium for 1, 3, 5, and 7 days. The medium was changed every other day. Von Kossa staining was conducted by fixing cells in 10% buffered formalin for 15 min, washing cells in water, staining cells in 5% silver nitrate under UV for 1 h. For ALP staining, cells were fixed in 10% buffered formalin for 30 min at room temperature, washed with PBS, and incubated with 1% Alcian Blue in 0.1 M HCl (pH 1.0). Chondrogenic cells were visualized as blue-stained cells under the microscope.

To induce adipogenic differentiation, WT and p85α-/-MSCs were maintained in adipogenic differentiation medium (MesenCult proliferation kit supplemented with 10⁻⁸ mol/liter dexamethasone, 5 µg/ml ascorbic acid 2-phosphate, 10 mmol/liter β-glycerophosphate, and 10 ng/ml TGFβ3) for 2 weeks. The medium was changed every other day. Cells were then fixed in 10% buffered formalin for 30 min at room temperature, washed with PBS, and incubated with 1% Alcian Blue in 0.1 M HCl (pH 1.0). Adipogenic cells were visualized as red-stained lipid droplets under the microscope.

\textit{RT-PCR—}Reverse transcription-polymerase chain reaction (RT-PCR) was used to examine the expression of osteoblast differentiation markers. Following incubation in differentiation medium for 1, 3, or 7 days, total RNA was extracted by using the RNeasy mini kit (Qiagen, Valencia, CA), and 100 ng of RNA was used for each RT-PCR reaction. RT-PCR was performed using Qiagen one step RT-PCR kit according to the manufacturer’s instruction at week 1 and week 4. The primers used were GGAGAC, CTTGATGCAAAAGCAGCGG, 269 bp for osteonectin; and TCTGCTACTCTGCTGAC, GGGAGCTGCTTGACATCC, 388 bp for osteocalcin (29). All the primers were synthesized by Sigma.

\textit{Western Blot Analysis—}WT and p85α-/-MSCs were plated in 10-cm dishes at 1 × 10⁶ in MSC complete medium. After incubating for 24 h at 37°C, 5% CO₂, cells were depleted with serum for 24 h followed by BMP2 (10 ng/ml) or DMSO stimulation for 10 min. Cells were then lysed in lysis buffer (1% Nonidet P-40). Lysates were subjected to immunoblotting to examine phosphorylation of Akt and Erk.

\textit{Statistical Analysis—}One way ANOVA and t test were used to evaluate statistical differences between WT and p85α-/-MSCs. Statistical significance was defined as \( p < 0.05. \)

\textbf{RESULTS}

\textit{Class IA Regulatory Subunit, p85α Negatively Regulates MSC Proliferation—}p85α has been shown to regulate cell proliferation in multiple cell lineages (17), including in hematopoietic cells (12). However, it remains unclear whether or not p85α impacts MSC proliferation. In the present study, we performed a series of functional assays to evaluate the role of p85α in regulating MSC proliferation. The number of MSCs were scored by counting viable WT and p85α-/-MSCs over a span of 10 continuous passages and the number of MSCs from each passage was recorded, and a growth curve was generated (Fig. 2A). While WT and p85α-/-MSCs grew slowly during early passages; a steady increase in the number of MSCs was observed over 10 passages in both the genotypes. This is consistent with studies by our group and others (27, 30, 31) and after passage 3, p85α-/-MSCs demonstrated faster cell growth compared with WT MSCs at each time point examined (Fig. 2A). This high rate of growth due to p85α deficiency was maintained at later passages as well. Similar findings were observed using a [³H]thymidine incorporation assay (Fig. 2B). These data suggest that p85α is a negative regulator of MSC proliferation.

\textit{p85α-/- Mice Have Increased Colony-forming Unit Fibroblast—}To measure the frequency of the MSC progenitors in the bone marrow of p85α-/- mice in vivo, we performed a CFU-F assay. A significant increase in the frequency and total number of CFU-F/femur was observed in p85α-/- mice compared with WT controls (Fig. 2, C and D). In addition, the average size of CFU-F in p85α-/- cultures was significantly larger than that in WT cultures (Fig. 2E). These results indicate that deletion of p85α results in increased proliferation of MSCs in vitro and frequency of CFU-F in vivo.

\textit{Deregulation of Cell Cycle in p85α-/- MSCs—}Flow cytometry is a simple and reliable method to evaluate cell cycle changes. In this study, we found that p85α-/-MSCs have less frequency in G1 phase (Fig. 3A). In contrast, increased percentages of G2 and S phases were observed in p85α-/-MSCs (Fig. 3A). This result is in line with the finding above that p85α-/-MSCs are more proliferative than WT MSCs. Further study by Western blot showed that p85α-/-MSCs have much lower expression of p16 and p19 but higher expression of cyclin D and cyclin E. These results suggest that deletion of p85α promotes MSCs exiting from G1 phase and entering into G2-S phase, that is, entrance of cell cycle (Fig. 3D).
Regulates Senescence in MSCs—Senescence is associated with reduction in proliferative capacity that is refractory to mitogenic stimulation and the appearance of senescence-related β-galactosidase activity (27). Long-term in vitro culture of mesenchymal stem cells exhibit reduced proliferation rate, which is associated with replicative senescence (32–35). The molecular mechanism that regulates MSC senescence is not well understood. To test whether the increased proliferation in p85α−/− MSCs is associated with a change in cellular senescence, a senescent assay was performed based upon histochemical staining for β-galactosidase activity. A significant reduction in the number of β-galactosidase-positive cells was observed in p85α−/− MSCs compared with WT MSCs (Fig. 3B). The quantitative results indicate that nearly 50% of WT MSCs were senescent and β-galactosidase positive. In contrast, only 10% β-galactosidase-positive senescent cells were observed in p85α−/− cultures (Fig. 3C). More importantly, as stated above, the two closely related to senescent regulation proteins, p16 and p19 are down-regulated by deletion of p85α (Fig. 3D). These data are consistent with the reduced senescent cells in p85α−/− culture. Collectively, these results suggest that the increased proliferation in MSCs lacking p85α is associated with a decrease in relative senescent activity compared with WT controls. These data suggest that deletion of p85α down-regulates MSC senescent, possibly through down-regulation of p16 and p19 expression, leading to increased MSC proliferation. In addition, as shown in Fig. 3E, deletion of p85α is accompanied by decreased expression of PARP, a substrate of caspase 3 that has been reported to be associated with apoptosis (36, 37).

Deletion of p85α Regulatory Subunit Alters Osteoblast Differentiation from MSCs—MSCs have multipotent differentiation capacity. In this study, we utilized a genetic murine model that has deletion in the class I A PI3K regulatory subunit, p85α, to evaluate the role of the PI3K pathway in modulating multilineage differentiation from MSCs. Deletion of p85α resulted in reduced mineralization of osteoblast as examined by Von Kossa
staining (Fig. 4A, left panels). Deletion of p85α had no effect on chondrocyte differentiation as determined by Alcian Blue staining (Fig. 4A, middle panels), while an increased adipocyte differentiation was observed in p85α−/− cultures as determined by Oil Red O staining (Fig. 4A, right panels).

MSCs are a source of osteoblasts which take part in bone formation. Although many factors have been implicated in regulating osteoblast differentiation and later bone formation (38–40), the molecular mechanisms underlying MSC differentiation into osteoblasts largely remains unclear. To further investigate the role of p85α in regulating osteoblast differentiation, osteoblasts were induced from the MSC cultures under defined conditions in vitro as described under “Experimental Procedures.” Following sequential culture, WT MSCs displayed moderate ALP activity at 24 h (Fig. 4B). On day 3, WT MSCs showed strong ALP activity which peaked on day 7. In contrast, minimum ALP positive cells were observed in p85α−/− cultures at 24 h (Fig. 4A). Although ALP activity increased over time in p85α−/− MSCs, the intensity of ALP staining was significantly less than that of WT cultures at every time point examined (Fig. 4B). Quantification of ALP activity demonstrated a significant difference between WT and p85α−/− MSCs (Fig. 4C).

To confirm the role of p85α in impaired osteoblast differentiation from MSCs, expression of osteogenic specific genes, including osteocalcin, osteopontin, osteonectin, and RUNX2 was examined in WT and p85α−/− MSCs (Fig. 5A). Osteocalcin mRNA expression was increased over time from
day1 to day 7 in WT MSC cultures (Fig. 5A). In contrast, minimum osteocalcin mRNA expression was detected in p85α−/− cells at days 1 and 3 of culture and significantly reduced osteocalcin levels were observed in p85α−/− cultures compared with controls on day 7. mRNA expression of osteonectin and osteopontin was increased to the same extent with time in both WT and p85α−/− cell cultures.

Furthermore, similar level of RUNX2 expression was observed in WT and p85α−/− cultures (Fig. 5B). The impaired expression of osteocalcin in p85α−/− cells further demonstrates that p85α is an important regulator for osteoblast differentiation from MSCs.

**p85α Negatively Regulates Akt Activation in MSCs**—Class IA PI3K contains regulatory and catalytic subunits. Deletion of
p85α subunit alters the expression of remaining subunits, including p85β, p110α, p110β, and p110δ. Importantly, the alterations in the expression of remaining subunits vary in different cell types (41). In pan-p85α−/− B cells, p85β expression is increased but p110α, p110β, and p110δ expression is decreased (42). In p85α−/− B cells, p50α and p55α are unaffected; p110δ is decreased (24). However, the expression of p50α and p55α are increased in T cells (24). In the present study, we found that p110α and p110β are down-regulated in p85α−/− MSCs (Fig. 1B). These subunit alterations may deregulate PI3K pathway that in turn may contribute to changes in osteoblast differentiation from MSCs. To evaluate the signaling changes due to p85α deficiency in MSCs, we conducted Western blot analysis to examine the phosphorylation of Akt, a downstream effector of PI3K. Interestingly, the basal level of Akt phosphorylation was significantly increased in p85α−/− MSCs relative to WT controls (Fig. 6). While the phosphorylation of p44/42 MAPK was also increased, BMP2 stimulation had limited impact on the phosphorylation of both Akt and p44/42 in either genotype. These data suggest that p85α regulatory subunit of PI3K negatively regulates the activation of Akt and MAPK, which is associated with reduced osteoblast differentiation from MSCs.

FIGURE 5. Decreased osteocalcin mRNA expression in p85α−/− MSCs. A, mRNA expression of osteoblast differentiation markers in differentiated WT and p85α−/− MSCs. WT and p85α−/− MSCs were differentiated in osteoblast differentiation medium for 1, 3, and 7 days and mRNA expression of osteocalcin, osteopontin, and osteonectin was examined. Shown are representative of two independent experiments with similar results. B, RUNX2 mRNA expression in differentiated WT and p85α−/− MSCs that were differentiated in osteoblast medium for 1, 3, and 7 days. Total RNA was extracted and RT-PCR was performed to measure mRNA expression.

p85α Plays an Important Role in Modulating MSC Differentiation into Osteoblasts—To further determine the role of p85α in mediating MSC differentiation into osteoblasts, we applied BMP2, a growth factor that regulates osteoblast differentiation in the presence or absence of PI3K inhibitor LY294002 or MAPK inhibitor PD98059, and investigated osteoblast differentiation. BMP2 was able to intensify osteoblast differentiation in WT MSCs (Fig. 7A). Addition of LY294002 did not alter osteoblast differentiation in WT MSCs. In contrast, addition of PD98059 to WT MSC cultures enhanced osteoblast differentiation dramatically (Fig. 7A). While addition of BMP2 did not modulate osteoblast differentiation in p85α−/− MSCs; addition of PD98059 partially restored osteoblast differentiation in p85α−/− MSCs (Fig. 7, B and C). These results suggest that hyperactivation of MAPK might negatively impact osteoblast differentiation from MSCs. Furthermore, reduced differentiation of p85α−/− MSCs into osteoblasts is in part due to increased activation of MAPK.

DISCUSSION

PI3K pathway plays a central role in growth factor signaling. The PI3K enzyme is an obligate heterodimer with an SH2-containing regulatory subunit p85 and a catalytic subunit p110 (12). The primary function of the p85 subunit is to bind, stabilize, and inhibit the p110 catalytic subunit until receptor tyrosine kinase activation (43). Despite significant data on the negative regulation of growth factor signaling, no studies have directly addressed the extent to which p85α alone can function to modify mesenchymal stem cell biological functions.

In this study, we demonstrate that deletion of p85α in mesenchymal stem cell results in increased cell proliferation as evidenced by higher CFU-F numbers, rapid cell growth over multiple cell passages, and elevated thymidine incorporation compared with WT MSCs. These results are in line with previous reports that p85α is a negative regulator of the PI3K signaling pathway (44–46). Strong evidence to support this assertion is that there is elevated Akt activity, which promotes cell survival and cell proliferation, in p85α−/− MSCs. Senescent assays show that deletion of p85α reduces the senescent cell number of p85α−/− MSCs. The mechanism(s) of negative regulation by p85α are likely to be complex. Studies have shown that in most cells there is a stoichiometric imbalance between p85 and p110, with the former being in excess of the latter (47, 48). This imbalance leads to a net inhibition of binding of the p85-p110 heterodimer to receptor phosphotyrosines (47). The p85 mono-
p85α also has significant effects independent of its regulation of the p110 catalytic subunit, including the sequestration of insulin receptor substrate proteins and positive regulation of PTEN function (49). While it is unclear which of these mechanisms are operational in p85α−/− MSCs, our results strongly support the notion that loss of p85α in MSCs leads to enhanced Akt and MAPK activation.

It has been reported that PI3-kinase promotes the S-phase entry by increasing Cyclin D1 transcription and translation, preventing its degradation and reducing p27kip1 levels (50). Cyclin D1 and cyclin E are key cell cycle regulators that governs the progression through the G1-S phase of the cell cycle (51). They are frequently overexpressed in breast cancers (51). In this report we showed that both cyclin D and cyclin E are overexpressed in p85α−/− MSCs. Together with the finding of down-regulation of p16 and p19 in p85α−/− MSCs, deletion of p85α may associated with higher proliferation, increased cell numbers in G2-S phase, and decreased senescent in these MSCs.

In all the characteristics of mesenchymal stem cells, the ability to differentiate into different cell lineages is considered the most important. Studies from different groups suggest that two major signaling pathways, Ras/Mek/MAPK and PI3K/Akt, are involved in osteoblast differentiation of mesenchymal stem cells (19–22, 52–57). Although these studies provide valuable information about signal transduction of MSC osteoblast differentiation, the results are somewhat inconsistent. More importantly, many of them are derived by performing pharmacological inhibition of the signaling pathway. There is no genetic evidence to suggest how distinct subunits of PI3K contribute to MSC differentiation. In our study, utilizing p85α−/− MSCs, we found that deletion of p85 leads to a dramatic decrease of ALP activity and mineralization during the process.

**FIGURE 7. Effect of BMP2 and pharmacologic inhibitors on osteoblast differentiation.** A, WT MSCs were cultured in osteoblast differentiating medium in the presence of LY294002 or PD98059 supplemented with or without BMP2. ALP staining was used to analyze ALP activity. A representative photograph of osteoblast differentiation is shown. B, WT and p85α−/− MSCs were cultured in osteoblast differentiating medium in the presence of BMP2 with or without LY294002 or PD98059. ALP staining was used to analyze ALP activity. A representative photograph of osteoblast differentiation is shown. C, quantitative evaluation of osteoblast differentiation in WT and p85α−/− MSCs. Results are the summary of triplicate cultures. Three independent experiments were conducted with similar results.
of osteoblast differentiation. mRNA expression of osteocalcin, an important marker of osteoblast differentiation and calcium deposition, is significantly reduced due to p85α deficiency. Further, our data suggests that while BMP2 enhances osteoblast differentiation of WT MSCs, it does not alter osteoblast differentiation of p85α−/− MSCs. However, inhibition of MAPK activity by PD98059 enhances osteoblast differentiation in both genotypes. This might be associated with the up-regulation of MAPK pathway as evidenced by increased p44/42 phosphorylation and addition of PD98059 restored p85α−/− osteoblast differentiation of MSC to the WT control level. Our results and those observed by others suggest that MSC osteoblast differentiation is negatively regulated by MAPK (22, 58). Inhibition of the ERK1/2 pathway increases ALP activity and mineralization in the BMP4-induced osteogenesis of myeloid-derived suppressor cells (52). LY294002, the PI3K inhibitor, did not dramatically change WT osteoblast differentiation. This may due to the apoptosis induced by LY294002, which has been found from other study (59) as well as in MSC cultures (data not shown). The apoptosis will minimize the cell numbers and make it appear to have less differentiation in the culture. In conclusion, our results demonstrate for the first time that p85α is a negative regulator of MSC growth and plays an important role in regulating osteoblast differentiation by cross-talking with MAPK pathway.

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