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Osteoblast Proliferation or Differentiation Is Regulated by Relative Strengths of Opposing Signaling Pathways

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Skeletal development requires the correct balance of osteoblast proliferation, survival, and differentiation which is modulated by a network of signaling pathways and transcription factors. We have examined the role of the AKT (PKB), and ERK1/2 signaling pathways in the osteoblast response to FGFs, which inhibit differentiation, and to IGF-1 and Wnt signaling, which promote it. Using osteoblastic cell lines as well as primary calvarial osteoblasts, we show that ERK1/2 and AKT have distinct effects in FGF-induced osteoblast proliferation and differentiation. ERK1/2 is a primary mediator of FGF-induced proliferation, but also contributes to osteoblast differentiation, while AKT is important for osteoblast survival. Signaling by IGF-1, that promotes osteoblast differentiation, strongly activates AKT and weakly ERK1/2, while the opposite results are obtained with FGF, which inhibits differentiation. By introducing a constitutively active form of AKT, we found that increased AKT activity drives osteoblasts to differentiation. Increasing the AKT signal in osteoblasts that harbor FGFR2 activating mutations, found in Crouzon (342Y) and Apert (S22W) syndromes, is also able to drive differentiation in these cells, that normally fail to differentiate. Wnt signals, that promotes differentiation, also induce AKT phosphorylation, and cells expressing active AKT have increased levels of stabilized beta-catenin, a central molecule in Wnt signaling. Our results indicate that the relative strengths of ERK and AKT signaling pathways determine whether osteoblasts are driven into proliferation or differentiation, and that the effects of AKT may be due, in part, to synergy with the Wnt pathway as well as with the Runx2 transcription factor.

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The flat bones of the skull are formed by the process of intramembranous ossification during which mesenchymal cells condense directly to osteoprogenitors and undergo an orderly spatial and temporal pattern of differentiation into mature, bone forming osteoblasts. These cells secrete a matrix (osteoid), which is eventually mineralized to form the calvarial flat bones that are separated by flexible sutures (Morriss-Kay and Wilkie, 2005). Activating mutations in FGF receptors are the cause of several human autosomal dominant craniofacial disorders whose unifying feature is craniosynostosis, or premature fusion of the cranial sutures. In craniosynostosis disorders, the regulated progression of osteoblast proliferation, differentiation and apoptosis is perturbed. While it is clear that excessive or unregulated FGF signaling gives rise to defects in bone formation, the specific osteoblast response that causes the defect is not well defined (Marie et al., 2005; Ornitz, 2005).

We and others have previously shown that FGF signaling has a different effect on mature and immature osteoblasts. Immature osteoblasts are induced to proliferate and the proliferative effect is lost as the cells differentiate (Debiais et al., 1998; Mansukhani et al., 2000). Osteoblasts in differentiating conditions continuously exposed to FGF undergo increased apoptosis and their differentiation is blocked. Similar results are produced by the constitutive expression of the craniosynostosis-linked mutations FGFR2/S252W (Apert) or FGFR2/C342Y (Crouzon) (Mansukhani et al., 2000). In this study we have examined the contribution of the signal transduction pathways ERK and AKT in the proliferation, differentiation and apoptosis of osteoblasts, and in the FGF response.

The ERK MAP kinase signal transduction pathway is activated by growth factors and is associated with cell proliferation, differentiation and survival. The PI3-kinases are important signal transducers of responses to hormones and growth factors. AKT (PKB), is a downstream target of phosphphatidylinositol-3-kinase (PI3-K) and is a critical mediator of cell proliferation and survival (Datta et al., 1999; Brazil and Hemmings, 2001; Brazil et al., 2004). AKT can phosphorylate a variety of targets leading to activation or inhibition of their function. The anti-apoptotic effects of AKT activation are linked to its ability to inhibit pro-apoptotic proteases (caspase-9), inactivation of the Forkhead/FOXO transcription factors that activate several pro-apoptotic molecules, and by inhibiting the Bcl-2 family protein, BAD (Brazil and Hemmings, 2001; Tran et al., 2003). Furthermore, the AKT pathway is known to play an important role in bone development (Liu et al., 2007; Peng et al., 2003).

Abbreviations: MAPK; mitogen-activated protein kinase; FBS; fetal bovine serum; BrdU; bromodeoxyuridine; PBS; phosphate-buffered saline; SD; standard deviation.

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In his report we show that signaling by IGF-1, which is known to promote osteoblast differentiation, strongly activates AKT while FGF signaling, that inhibits differentiation, causes a strong and sustained ERK1/2 activation. We found that ERK is required for FGF-induced proliferation, while AKT, as in many other cell types, plays a role in osteoblast survival. Furthermore, we find that ERK signaling negatively regulates AKT activity and that ERK activation is reduced in cells expressing constitutively active AKT. We examined the hypothesis that the potent differentiation-inducing signals of AKT in osteoblasts act via synergize with the Wnt-beta catenin pathway which are known to enhance osteoblast differentiation and bone mass. Our results suggest that the effects of AKT may be due, in part, to synergy with differentiation-inducing signals of the Wnt pathway, and that the relative strengths of opposing AKT and ERK signaling pathways affect the osteoblast response.

Materials and Methods

Reagents and antibodies

Antibodies against phospho-AKT (Ser 473), AKT, phospho-p44/42 MAPK (ERK1/2), phospho-FOXO (Ser256), phospho-GSK3 (Ser 9) were from Cell Signaling Technology (Beverly, MA). The antibody against ERK2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and against active beta catenin from UBI (Upstate Biotechnology, Lake Placid, NY). The anti-Cbfa1 polyclonal antibody was obtained from of Dr. G. Karsenty, Columbia University. The horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Promega (Madison, WI). Anti-BrdU monoclonal antibody was from Amersham/GE Gealthcare (Fairfield, CT) (RPN-202). Anti-mouse secondary antibody Cy3-conjugated was from Jackson Laboratories (West Grove, PA). MEK1/2 inhibitor (U0126) was from Cell Signaling Technology and PI-3 kinase inhibitor (LY294002) was from Calbiochem (La Jolla, CA). Quantitative RT-PCR analysis was performed as previously described (Mansukhani et al., 2005). Specific primers used:

OPN/Bsp1: DIR: 5′ AAGGAGTCCCTCGATG REV 5′-CTGA-TGTTCCAGGCTGG-3′; osterix: DIR 5′-GCTCAGGT-ACAGTGAGC-3′ REV: 5′-ACCATGACGACAAGGG-3′; cbfa1: DIR 5′-AGAAGAGCCAGGCAGGTGCT-3′ REV: 5′-TTCG-TGGGTTGGAGAAGCG-3′; beta–actin: DIR: 5′-GGCCGCC-CTAGGCACCAG-3′; REV: 5′-CTCTTGATGTCACGC-ACGATTTCCC-3′.

Cell culture and preparation of primary osteoblasts

OB1, OB5, primary osteoblasts, 293 gag-pol expressing (293GP) cells were grown in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Primary calvarial osteoblasts were isolated from newborn mice as previously described (Mansukhani et al., 2000). For differentiation, cells were cultured in DMEM containing 10% FCS, ascorbic acid (AA; 100 μg/ml), and β-glycerophosphate (BGP, 4 mM) and the medium was changed every 3 days.

Cloning and retroviral infections

MEK1(AF), was obtained from Dr. J. Han, Scripps Institute, CA. cDNAs were digested with *Ssp*I and *Xba*I, and subcloned into pSF1N retroviral expression vector encoding green fluorescence protein, pSF1n-GFP, (from Riccardo Priore, New York University), digested with Hind III, generating pSF1n-GFP/MEK1(AF). pBabe/Myr-AKT, (which contains the myristilation sequence of c-src fused to the NH2 terminus of AKT), and pBabe/Myr (K179M), the dominant negative AKT, (Myr-AKT-K179M), retroviral expression vectors were a gift from Dr. M. Pagano (New York University School of Medicine). Retrovirus construct FGFR2/Cr contains mouse FGFR2/Crouzon(C342Y). Retrovirus production was done in 293GP cells. Cells were plated in 100 mm plate and at 60% confluence, were cotransfected with 5 μg of the indicated plasmid and 5 μg of pCMV-VSV-G env plasmid by calcium phosphate precipitation (Chen and Okayama, 1988). The following day the medium was changed and 48 h later the viral supernatant was collected. About 2×10^5 OB1, OB1/Cruzon, or OB5 cells were infected with 1 ml of viral supernatant in presence of 8 μg/ml of polybrene for 4–6 h. For pools and clones (e.g., AKTMyr#7), selection was done for 10 days in complete medium + 2 μg/ml puromycin.

Proliferation and apoptotic assays

Cells (1×10^4) were seeded onto glass coverslips in 24-wells plates (Costar) in DMEM + 10% FCS. After 24 h the cells were either serum starved in 2% FCS, or induced to differentiate with AA and BGP in the absence or presence of FGF1 (10 ng/ml) and heparin (5 μg/ml). The cells were labeled with 1 μg/ml of bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) for 4–6 h at 37°C, fixed in 3.7% paraformaldehyde for 10 min at room temperature, permeabilized for 10 min with 0.5% Triton in PBS and treated for 15 min with 1.5 N HCl. After several washes, analysis of BrdU incorporation was performed using an anti-BrdU monoclonal antibody (Amersham) followed by anti-mouse secondary antibody conjugated with Cy3 (1:400). Cell nuclei were stained with 0.5 μg/ml of Hoechst (Sigma) in PBS for 20 min prior to mounting on slides. The fluorescence was visualized using an Axioplan 2 Zeiss microscope equipped with a digital camera. The frequency of S phase cells was calculated as a ratio of BrdU positive nuclei to the total Hoechst stained nuclei. Hoechst staining was also used to detect apoptosis along with TUNEL assay staining kit (Roche (Indianapolis, IN)). Apoptotic nuclei were counted as a proportion of total nuclei.

Alkaline phosphatase and mineralization staining

Cells were fixed with citrate-acetone and histochemically stained for alkaline phosphatase (ALP) according to the manufacturer's instructions (85L-2, Sigma). To visualize mineralized nodules, cells were fixed and stained with Alzarin Red (75 μg/ml) overnight and washed with H_2O.

Cell lysate preparation and Western analysis

Cells were washed once with cold phosphate-buffered saline and lysed either in RIPA buffer (10 mM Tris–HCl at pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, 1% Triton X-100) or HNTG buffer (50 mM HEPES at pH 7.5, 750 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 105 glycerol, 15 Triton X-100) in presence of protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, leupeptin, and pepstatin, 1 mM sodium orthovanadate, 10 mM sodium fluoride). Lysates were clarified by centrifugation at 13,000*g* for 20 min at 4°C. Protein concentration was determined by the Bradford methods (Biorad, Hercules, CA). Total protein extracts (10–20 μg) were resuspended in Laemmli sample buffer, boiled for 5 min, separated by SDS–polyacrylamide gels (SDS–PAGE) as previously described (Raucci et al., 2004).

Results

Activation of the ERK1/2 and AKT pathways by FGF and IGF-1

The murine OB1 osteoblast cell line can be induced to differentiate and express osteopontin, ALP, and bone sialoprotein, markers of osteoblast differentiation (Mansukhani et al., 2000). We have previously shown that FGF stimulation induces proliferation of immature OB1 cells but that this proliferative response is lost as the cells differentiate. With long-term exposure to FGF, differentiation is inhibited and the cells undergo increased apoptosis (Mansukhani et al., 2000).

In order to determine which of the known FGF-induced signal transduction pathways may be responsible for the FGF response of OB1 cells, we studied the activation of the ERK1/2, and the PI3-kinase-AKT pathways. For all our experiments we used FGF1, a universal ligand for all FGF receptor isoforms. Osteoblasts were stimulated with FGF1 for different times, and cellular lysates were analyzed by Western blot using phospho-specific antibodies that recognize active AKT or ERK1/2. We compared the kinetics of ERK and AKT activation by FGF with IGF-1 in OB1 as well as in OB5 cells, which are spontaneously immortalized murine calvarial osteoblasts (Mansukhani et al., 2005). Although FGF and IGF both signal through tyrosine kinase receptors, FGF signaling inhibits osteoblast differentiation while IGF is known to stimulate this process (Yeh et al., 1997; Zhang et al., 2002). Figure 1A shows that in OB1 or OB5 cells, FGF induces strong and sustained ERK1/2 activation that is detectable at 15 min and persists up to 2 h, while induction of AKT activation was detectable but very weak. In contrast to FGF, IGF-1 stimulation leads to a robust induction of AKT phosphorylation which is still sustained at 1 h, while ERK phosphorylation is weak and transient and no longer detectable by 30 min. Similar results were obtained in mouse primary calvarial osteoblasts (data not shown). Thus the relative signaling strength of these two pathways differs, with IGF-1 preferentially activating the AKT pathway, and FGF stimulation favoring the ERK pathway. Highlighting the opposing effects of these pathways on osteoblasts, Figure 1B shows that, unlike FGF, IGF-1 does not stimulate proliferation of OB1 cells, and slightly reduces the extent of BrdU incorporation by FGF ($P = 0.05$). While FGF enhances apoptosis, IGF-1 treatment reduces the extent of apoptosis in differentiating OB1 cells and reduces the extent of apoptosis induced by FGF ($P = 0.05$).

The striking inverse relationship between AKT and ERK1/2 phosphorylation observed in IGF or FGF treated cells led us to investigate whether these pathways may interfere with each other, such that inhibition of ERK signaling would potentiate the activation of AKT by FGF, and inhibition of AKT signals would enhance activation of ERK1/2. We treated OB1 cells with UO126, a MEK1/2 inhibitor, which prevents ERK1/2 phosphorylation, and then subjected them to FGF stimulation. The results shown in Figure 1C clearly indicate that inhibition of the ERK pathway results in stronger AKT phosphorylation induce by FGF that is detectable up to 60 min. In the converse experiment,, OB1 cells treated with the LY294002, an inhibitor of the AKT pathway, showed that inhibiting AKT activation by IGF produced no significant enhancement of ERK activation by IGF.

Effect of ERK1/2 and AKT pathways on FGF-induced proliferation of OB1 cells

To assess the contribution of ERK1/2 and AKT activation to the proliferative effect of FGF on OB1 cells we used chemical inhibitors that are known to specifically prevent the activation of these pathways. We used the MEK1/2 inhibitor, U0126, and the PI3-kinase inhibitor, LY294002, to prevent the activation of ERK1/2 and AKT respectively. The inhibitors at concentrations up to 30 μM blocked the activation of the respective pathways while maintaining specificity (data not shown).

We performed BrdU incorporation assays to test the effect of these inhibitors on FGF-induced proliferation of OB1 cells that had been serum starved for 24 h. In accordance with our previous results (Mansukhani et al., 2000), FGF1 induced a modest increase in proliferation of OB1 cells Increasing concentrations of the MEK1/2-ERK1/2 inhibitor, U0126,

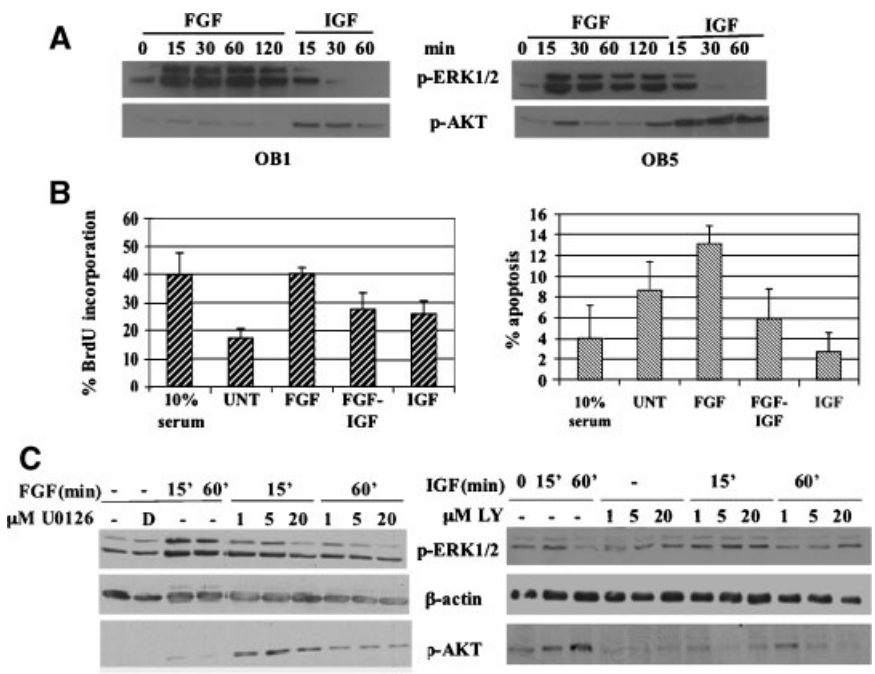


Fig. 1. Signaling pathways in osteoblasts. A: Comparison of AKT and ERK activation in OB1 and OB5 osteoblasts treated with FGF or IGF (100 ng/ml) for the indicated times. B: Analysis of BrdU incorporation and apoptosis (TUNEL assay) in OB1 cells after 24-h treatment with FGF, IGF or 1:1 FGF and IGF at 100 ng/ml each. For the apoptosis assay, cells were differentiated for 24 h prior to treatment. The data shown are the mean of three independent experiment ± SD. Statistical differences between treatments were measured by ANOVA. *P*-values at or below 0.05 were considered significant. C: Effect of specific pathway inhibitors on OB1 cells. Effect of the MEK inhibitor, U0126, on AKT activation by FGF (10 ng/ml) for 15 and 60 min. D, DMSO vehicle (left part). Effect of PI3K-AKT inhibitor LY294002 (LY) on ERK1/2 activation by IGF (10 ng/ml) for 15 and 60 min.

reduced the FGF proliferative response. The PI3K inhibitor, LY294002, did not appear to significantly affect the percentage of cells incorporating BrdU in response to FGF. All the inhibitors by themselves did not have any effect on the basal proliferation of OB1 cells at 24 h (Figure 2A). Thus, these results indicate that ERK1/2 signaling is required for the FGF-induced proliferation of osteoblast cells.

We also investigated the contribution of steady state ERK1/2 pathway on the proliferation in OB1 cells, grown in complete medium in the absence of additional growth factors, by using dominant negative MEK1 MEK1(AF) to specifically block ERK signaling, (Wang et al., 2002; Li et al., 2006). OB1 cells were infected with retroviruses carrying the vector expressing GFP alone, or dominant negative plasmids GFP-MEK1(AF) for 24 h and a BrdU incorporation assay was performed using GFP to track cells expressing the transduced plasmids. Figure 2B shows the percentage of GFP positive and GFP negative control cells incorporating BrdU. The GFP expressing vector by itself does not interfere with the incorporation of BrdU, while the expression of a constitutively activated FGFR2 (FGFR2/Cr) enhances the proliferative rate of the GFP-positive cells. Expression of the dominant negative MEK1(AF) clearly decreases the proliferation of OB1 cells. Thus, these results suggest that, for OB1 growing in 10% serum, as well as for FGF-induced proliferation of OB1, ERK1/2 is a major mediator of FGF-induced osteoblast proliferation.

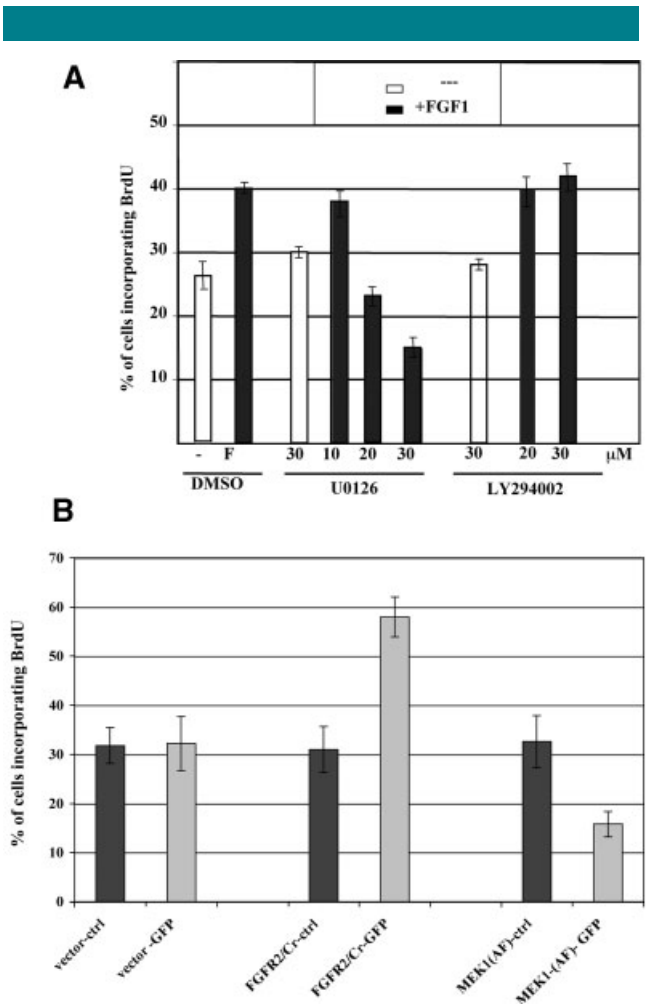


Fig. 2. Effect of ERK1/2 and AKT on proliferation of OB1 cells. A: OB1 cells on coverslips were starved overnight in 2% serum and treated with the indicated concentrations of MEK1/2 inhibitor (U0126) or PI3K inhibitor (LY294002). After 1 h FGF1 (10 ng/ml) and heparin (5 μg/ml) were added for 24 h and the cells were labeled with BrdU for the last 4 h. Percentage of cells incorporating BrdU was determined by counting at least 300 cells. The results shown are the mean of three experiment ± SD. B: Unstarved OB1 cells were infected with the indicated retrovirus constructs tagged with GFP and BrdU incorporation assay was performed 48 h later. The percentage of BrdU-positive cells was determined as a proportion of infected cells (GFP) and of non-green uninfected cells (control) on the same coverslip. The results shown are the mean of three experiment ± SD. Dominant negative constructs, MEK1(AF), FGFR2/Cr is the C342Y activated FGFR2.

ERK1/2 and AKT contribute to osteoblast differentiation

Mouse calvarial osteoblasts are able to differentiate in culture. Mature cells are characterized by increased expression of ALP, an early marker of differentiation, increased matrix production, and eventual mineralization of the extracellular matrix. To determine whether the activity of ERK1/2 and AKT is required for osteoblasts differentiation, we used primary calvarial osteoblasts that differentiate more rapidly than osteoblast cell lines. Figure 3A shows that both ERK1/2 and AKT are phosphorylated during primary osteoblasts differentiation. Cells were then induced to differentiate in the presence and absence of the MEK1/2-ERK/2 inhibitor, U0126 or the PI3K inhibitor, LY294002. The extent of differentiation was assessed by staining for ALP expression at days 3, 7, and 18. Figure 3B shows that by 7 days primary osteoblasts stained strongly positive for ALP and mineralization is detectable by 18 days of differentiation. When U0126 was included in the medium, ALP staining was inhibited at 7 days and the inhibitory effect on mineralization was evident at 18 days. Although it appears that ALP staining is also inhibited in the presence of LY294002, this is primarily due to an increase in apoptosis as a result of blocking the AKT survival pathway and consequent loss of the cells during the experiment. As seen in Figure 3C, the inhibitor U0126 has a minor effect on apoptosis compared to vehicle treated cells, while with the inhibitor LY294002 the apoptosis rate is dramatically increased to 56% at 3 days and by day 7, 90% of cells undergo apoptosis.

Together these results suggest that ERK1/2 activation contributes positively to osteoblast differentiation while AKT is required for osteoblasts survival during the differentiation process. The block in differentiation was also seen in the reduction of osteopontin/BSP1 (spp1) determined by RT-PCR analysis of RNA from cells treated with the inhibitors (data not shown).

To confirm the results obtained with chemical inhibitors on differentiation, we also assessed differentiation of pools of OB1 infected with retroviruses expressing dominant negative constructs that specifically blocked each pathway. Dominant negative MEK1 MEK1(AF), was introduced into OB1 cells as well as into OB5 cells. OB5 cells differentiate faster, and more synchronously than OB1 cells and differences in changes in differentiation are more apparent. We confirmed that pools of cells expressing MEK1(AF) exhibited impaired differentiation (data not shown).

Increased AKT signaling enhances osteoblast differentiation

While it is clear that the AKT pathway is required for osteoblast survival during differentiation, we could not assess whether it plays an additional role in promoting or blocking differentiation. Since FGF blocks differentiation and weakly stimulates the AKT pathway, while IGF, a potent AKT activator, induces differentiation, we determined whether increasing AKT activity affected osteoblast differentiation. A constitutively active form of AKT (AKT-Myr), a wild-type AKT (AKT-wt) or a dominant negative form of AKT (AKT-DN) have been previously

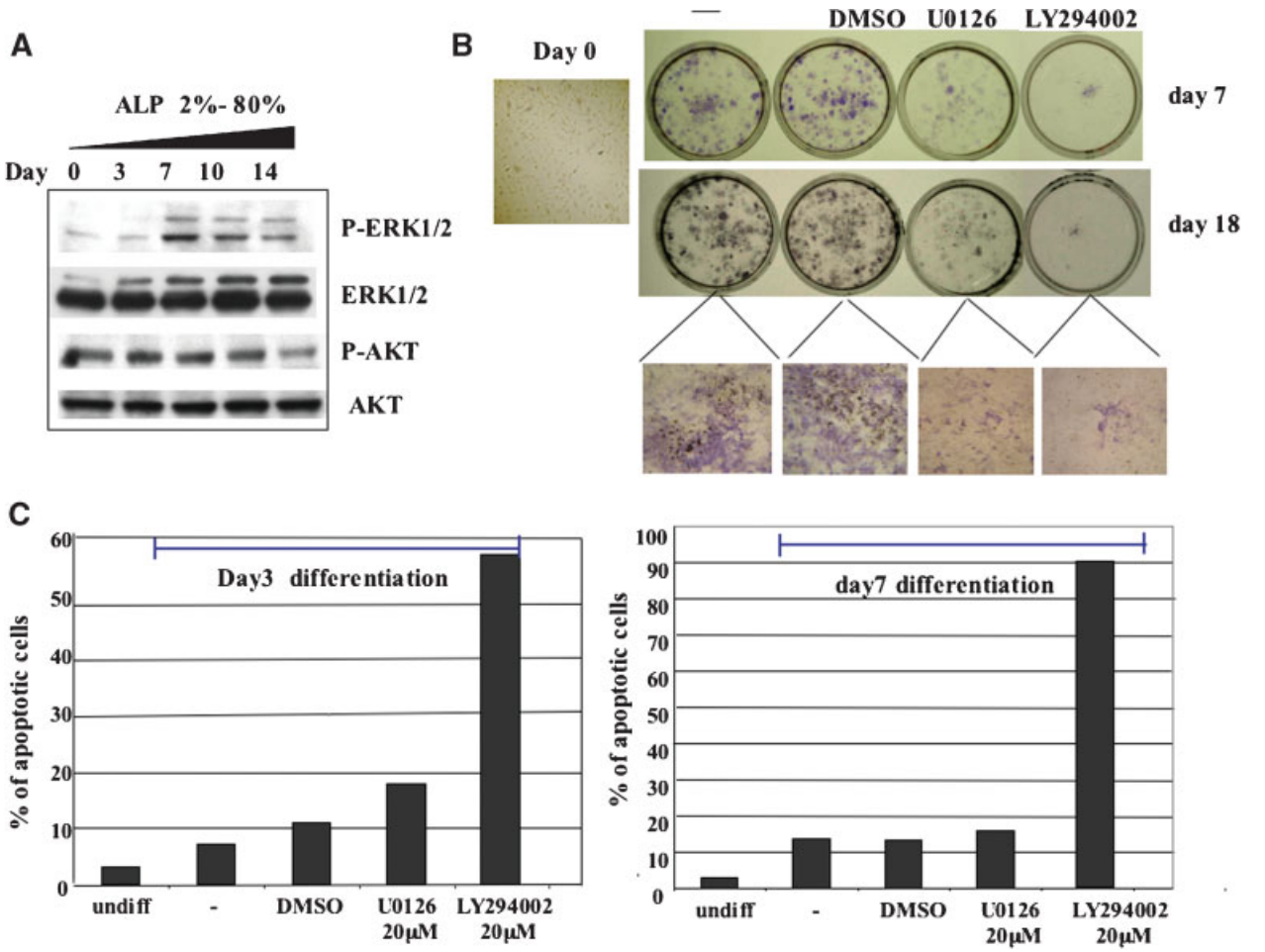


Fig. 3. ERK1/2 and AKT contribute to osteoblast differentiation. A: Primary calvarial osteoblasts were maintained in differentiation medium up to 14 days. Cellular lysates were collected on the indicated days and analyzed by Western blot using the indicated antibodies. Cells were stained for ALP expression at days 0 and 14 and percent of ALP-positive cells was determined. B: Effect of ERK1/2 and AKT inhibitors on ALP expression during osteoblast differentiation. Equal numbers of cells were seeded on 6-well plate and maintained in differentiation medium containing 20 μM U0126, LY294002, or DMSO 0.1%. Plates were fixed and stained for the expression of ALP (purple) at 7 and 18 days. Mineralization (red-brown) nodules were detected by Alizarin Red stain. C: Effect of ERK1/2 and AKT inhibitors on apoptosis during osteoblast differentiation. Cells were treated as in B, and coverslips were fixed and stained with Hoechst. Two hundred nuclei from at least five different microscope fields were counted and the apoptotic nuclei were calculated as percentage of total cells. Experiment was repeated twice with similar results. Results of one experiment are shown.

described (Kohn et al., 1996; Aoki et al., 1998). These constructs were introduced into OB1 or into OB5 cells using retroviral vectors. Cells were transferred to differentiation medium, and 7 days later they were fixed and stained for the expression of ALP. As shown in Figure 4A, only ~30% of a pool of OB5 expressing vector alone or wild-type AKT (wt AKT) are positive for ALP, while cells expressing the constitutively activated form of AKT (AKT-Myr) clearly show increased ALP. The potent differentiation inducing ability of Myr-AKT was also evident in OB1 cells that differentiate less well than OB5, as well as in OB1/Crouzon cells that express activated FGFR2, and that usually do not differentiate. This result suggests that increasing AKT signaling can overcome the inhibitory effect of FGF on differentiation. The increased differentiation of OB1 or OB5 cells expressing AKT-Myr was confirmed by assessing the mRNA expression of differentiation markers ALP and bonesialoprotein (Bsp1) by Northern analysis at 0 and 5 days of differentiation. Both BSP1 and ALP were increased in OB5-AKT-Myr cells relative to the control cells expressing vector alone, AKT-wt or AKT-DN (Fig. 4B).

We also created stable clones of OB5 cells expressing AKT-Myr and confirmed that high-expressing clones differentiated better than the parental or low-expressing clones (data not shown). Figure 4C shows a comparative time course of differentiation of OB5 cells, and of OB5 cells expressing AKT-Myr or the dominant negative form of AKT. The accelerated differentiation exhibited by the AKT-Myr cells is in stark contrast to the poor differentiation of the AKT-DN cells. We then examined the expression of two transcription factors, Cbfa1/Runx2 and osterix that are required for osteoblast differentiation (Nakashima and de Crombrugghe, 2003). It has been reported that signaling through AKT can potentiate osteoblast differentiation by cooperation with the transcription factor Runx2/Cbfa1 (Fujita et al., 2004). Figure 4D shows that the mRNA level of cbfa1 is elevated threefold in AKT-Myr cells by 5 days of differentiation ($P = 0.01$), while the levels of osterix, which lies downstream of cbfa1 in the osteoblast differentiation program, is strongly induced in AKT-Myr expressing cells prior to differentiation and remains elevated at day 5. Thus AKT activation could

accelerate osteoblast differentiation by inducing factors required for the process.

We also examined the effect of AKT-Myr on a known downstream effector of the AKT pathway (Tran et al., 2003). Figure 5A shows that the AKT target FOXO3, a pro-apoptotic transcription factor known to be inactivated by AKT, is phosphorylated in OB1, OB5, or OB1/Crouzon cells expressing AKT-Myr. Interestingly, the basal level of FOXO3 phosphorylation is higher in OB5 cells, which differentiate better than OB1 cells. The effect of AKT-Myr and AKT-DN on apoptosis was monitored during differentiation. At 3 and 7 days of differentiation, AKT-Myr expressing cells have a lower rate of apoptosis than control cells, while AKT-DN cells have increased apoptosis (Fig. 5B). This trend is also evident in OB1/Crouzon cells as well which we have previously shown to have higher apoptosis. Thus AKT signaling increases osteoblast differentiation and survival during differentiation.

AKT signaling affects molecules in the Wnt pathway

Another downstream target of AKT is the enzyme GSK3β which is inactivated by phosphorylation on serine 9 (Brazil et al., 2004; Jope and Johnson, 2004). GSK3β is a negative regulator of the canonical Wnt β-catenin pathway, and Wnt signaling has been shown to promote differentiation in osteoblasts (Westendorf et al., 2004). Active GSK3β phosphorylates β-catenin on conserved serine/threonine residues in the N-terminus, that target it for degradation, and Wnt pathway activation leads to the inactivation of GSK3β. The stabilized β-catenin is translocated to the nucleus and complexes with the LEF/TCF family of transcription factors to regulate Wnt target genes (Clevers, 2006; Nelson and Nusse, 2004). Although GSK3 inactivation by Wnt and AKT are thought to occur via distinct mechanisms, we found that Wnt3A treatment of OB1 osteoblasts also induced GSK3β phosphorylation on serine 9, and also caused a very strong phosphorylation of AKT (Fig. 6A). This finding is in line with the notion that, like Wnt3A, AKT activity promotes differentiation. We therefore examined whether these pathways cooperate and whether increased AKT activity in osteoblasts and consequent inactivation of GSK3 would result in enhancement of β-catenin/Wnt activity.

We examined whether GSK3β was phosphorylated and whether β-catenin was stabilized and increased in AKT-Myr cells. Figure 6B shows that p-GSK3 is increased in AKT-Myr

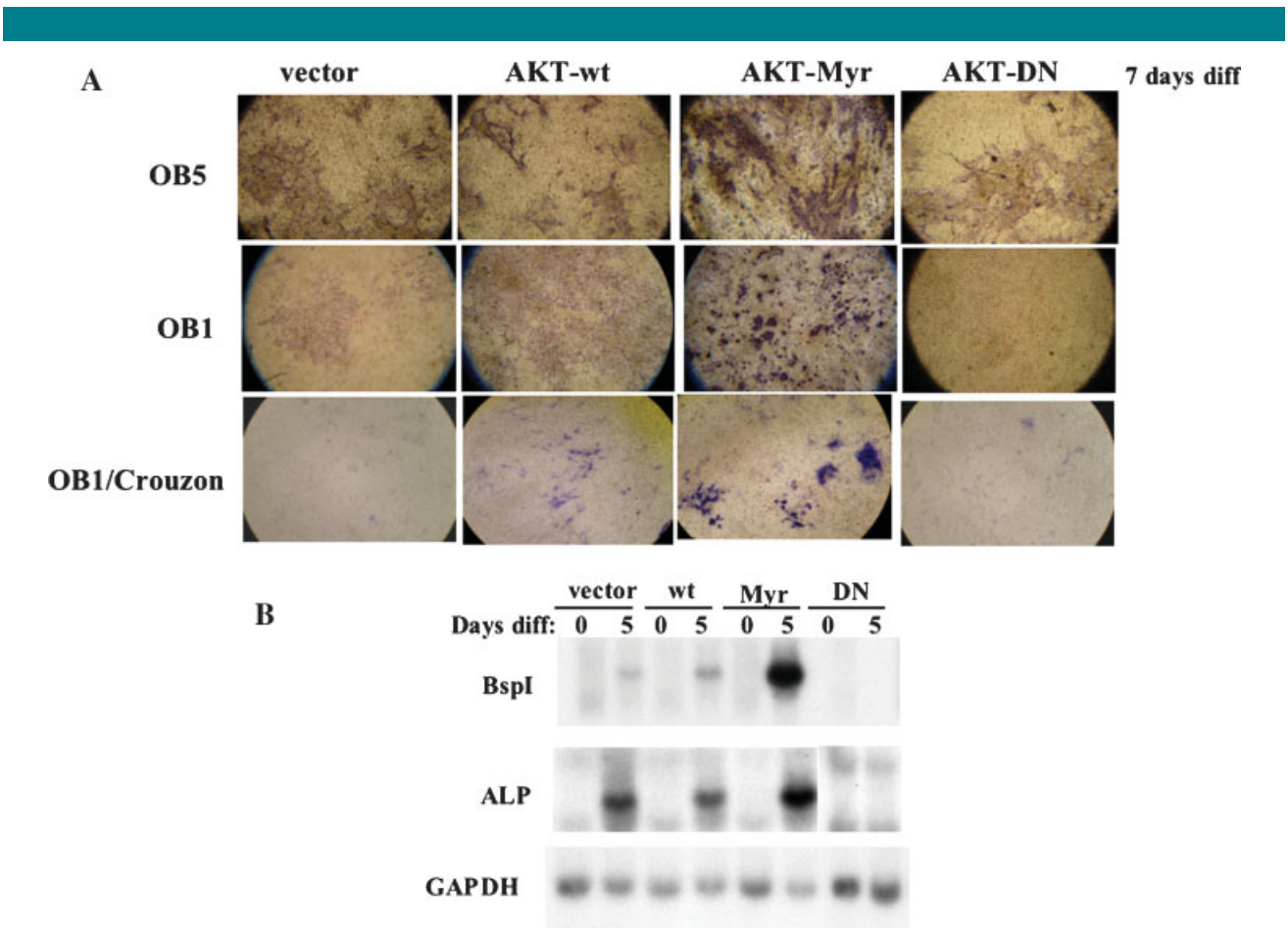


Fig. 4. Increased AKT signaling enhances osteoblast differentiation. A: OB5, OB1, or OB1/Crouzon cells were infected with virus carrying the pBabe vector, pBabe/AKT-wt, pBabe/AKT-Myr, or pBAbe/AKT-dominant negative (DN). After 48 h, at confluence, the cells were maintained in differentiation medium for 7 days, fixed and stained for ALP expression (purple stain). B: Expression of differentiation marker bone sialoprotein (Bsp1) and ALP by northern analysis of OB5 cells infected with the indicated constructs at day 0 or 5 of differentiation. C: OB5 cells were stably infected with AKT-Myr or AKT-DN construct and a pool (AKT-Myr or AKT-DN) or clone (AKT-Myr # 7) was selected in puromycin. Equal numbers of cells were plated for differentiation and stained for ALP on the indicated days (purple stain). D: Quantitative RT-PCR analysis of cbfa1 and osterix mRNA levels from stably infected OB5 cells as indicated at days 0 and 5 of differentiation. Actin was used as an internal control to normalize for RNA levels within each sample. Data are presented as fold change over the level in OB5 cells which was set at 1. Each time point is the average of 2–3 samples ± SD. Statistical differences between treatments were measured by ANOVA. *P*-values at or below 0.05 were considered significant.

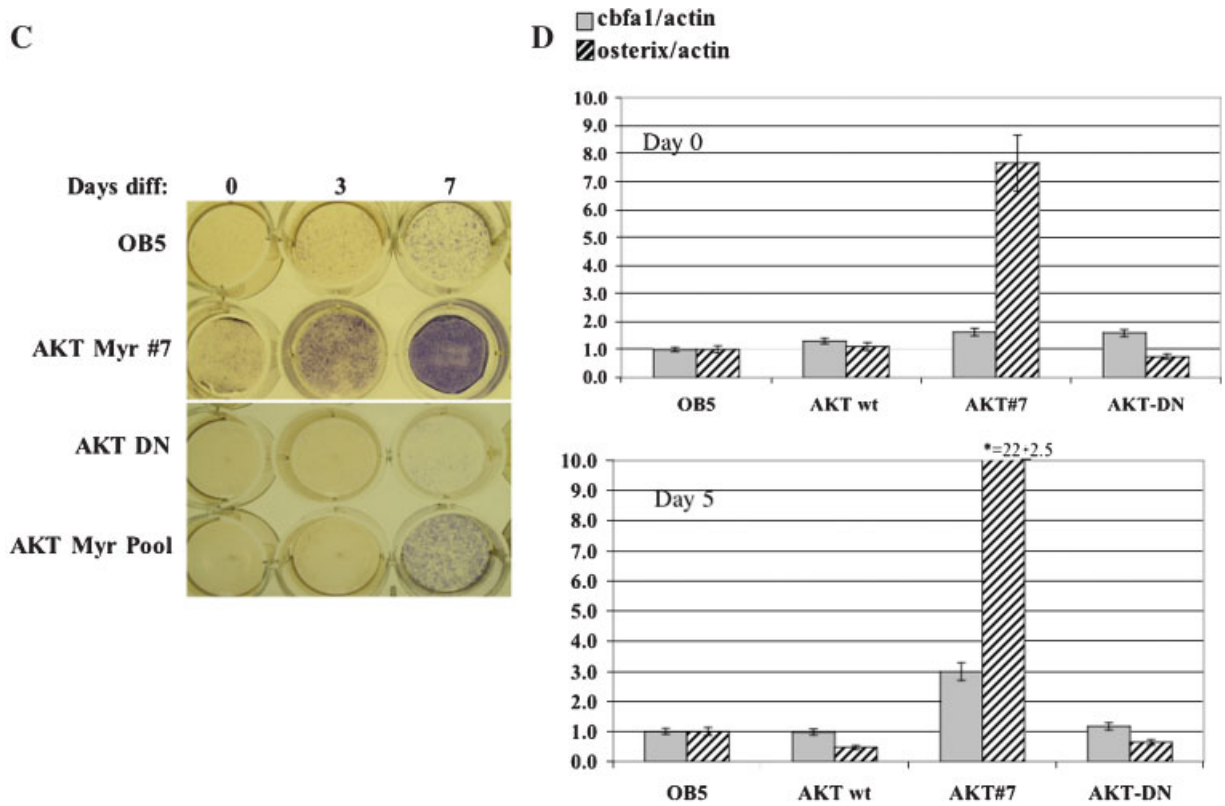


Fig. 4. (*Continued*)

OB5 cells and reduced in OB5 cells expressing the dominant negative, AKT-DN. We used an antibody that specifically recognizes an epitope containing the unphosphorylated serine residues in the N-terminus of β-catenin, that is the target of GSK3 phosphorylation, and thus identifies active β-catenin (van Noort et al., 2002). In OB5 cells, active β-catenin is detectable in untreated cells, is increased by Wnt3A treatment, and decreased in FGF-treated cells. In OB5/AKT–Myr cells, the levels of active B-catenin were higher in untreated and Wnt-treated cells (Fig. 6B). Figure 6C shows that in transiently transfected OB1 or OB1/Crouzon cells, GSK3 phosphorylation is clearly increased in cells expressing Myr-AKT, in which AKT is constitutively phosphorylated. Active β-catenin, which is essentially undetectable in untransfected cells, is visible in cells expressing Myr-AKT. These data suggest that increasing the amplitude of AKT signaling may increase differentiation of osteoblasts by contributing to the activation of Wnt signaling pathway via activation of β-catenin. We also found that constitutive activation of AKT results in decreased phosphorylation of ERK1/2 in osteoblasts expressing AKT-Myr (Fig. 6C), in line with the notion that these pathways have opposing effects.

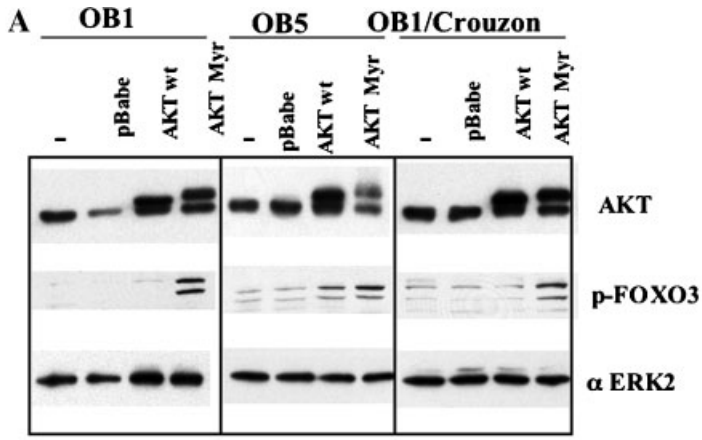
Wnt signaling has been reported to promote osteogenesis by stimulating Runx2 expression (Gaur et al., 2005). To confirm whether Runx2 protein expression was elevated in osteoblasts expressing Myr-AKT in the transient assay, we examined protein expression and found that Runx2/Cbfa1 protein is indeed increased in these cells (Fig. 6D). Thus the positive effect of AKT on differentiation is likely to involve increased Runx2 expression as well.

Discussion

In this report we have examined the contribution of the ERK1/2 and AKT (PKB) signaling pathways to the proliferation, differentiation, and apoptosis of osteoblasts. This complex process is controlled by a network of temporally and spatially regulated signals, that when disrupted result in abnormal bone formation and craniofacial deformities.

While it is known that several transcription factors, such as Runx2 and Osterix are essential for osteoblast commitment and differentiation/function, the process by which signaling molecules and specific signaling pathways regulate osteoblast function are still unclear (Nakashima and de Crombrugghe, 2003). FGFs, IGFs and Wnt signaling are known to regulate osteoblast differentiation. In vitro, FGF signaling increases osteoblast proliferation and inhibits differentiation, while IGF and Wnt promote differentiation both in vitro and in vivo (Yeh et al., 1997; Zhang et al., 2002; Krishnan et al., 2006). FGF signaling in vitro antagonizes the Wnt effects (Mansukhani et al., 2005).

Although they utilize similar tyrosine kinase receptors, FGF and IGF-1 have opposing biological effects on osteoblast proliferation and differentiations. We show that while FGF acts preferentially through the ERK pathway and only weakly stimulates AKT activation, IGF-1 treatment of the same cells leads to a strong induction of AKT phosphorylation and a weak ERK response. AKT is thought to be the main effector of IGF-1 signaling and AKT null mice and IGFR-1 null mice have a similar skeletal phenotype of delayed ossification (Peng et al., 2003). More recently, an osteoblast-specific knockout of the AKT inhibitor, PTEN resulted in mice with increased bone mineral



B

OB1/CR % apoptosis	Day 0	Day 3	Day 7
pBabe	3.4 ± 1.2	15.9 ± 2.3	17.5± 2.3
AKT DN	6.2 ± 2.2	17.9 ± 2.7	23.4 ± 4.7
AKTMyr	2.5 ± 2.6	9.1 ± 2.7	9.6 ± 3.6

OB5 % apoptosis	Day 0	Day 3	Day 7
pBabe	2.7 ± 0.2	4.6 ± 0.6	8.1 ± 1.2
AKT DN	2.3 ± 1.4	15.6 ± 4.2	19.5 ± 6.6
AKTMyr	2.3 ± 0.4	3.1 ± 0.8	4.8 ± 3.6

Fig. 5. AKT signaling increases osteoblast survival during differentiation. A: Cellular lysates from OB5, OB1, or OB1/Crouzon, previously infected with the viruses carrying the indicated cDNAs, or uninfected, (−), were analyzed by Western blot using antibodies against AKT, phospho-FOXO3 or ERK2 as a loading control. AKT expressed by the cDNAs migrate slower than the endogenous AKT. B: After infection, OB5 and OB1/Crouzon expressing AKT-DN, AKT-Myr or just the vector pBabe were maintained for 3 and 7 days in differentiation medium. Cells were then fixed and stained for TUNEL and Hoechst. Nuclei from at least ten different fields were counted and the apoptotic nuclei were calculated as percentage of total cells. Data are represented as the mean ± SD.

density (Liu et al., 2007). Thus, AKT activation correlates with osteoblast differentiation while ERK activation correlates with proliferation. Several reports suggest that the ERK and AKT pathways have opposing effects in other cell types. A strikingly similar situation has been observed in myoblasts treated with HGF and IGF-1. In these cells HGF, that inhibits differentiation, induces strong ERK1/2 activation and a weak AKT response, while IGF-1, which promotes differentiation, strongly activates the P13-K/AKT pathway and only weakly the MAP/ERK kinases (Halevy and Cantley, 2004). Furthermore, ERK and AKT are activated downstream of the VEGF receptor in angioblasts-cells, and have opposing roles in artery/vein specification (Hong et al., 2006).

We show that blocking the ERK pathway with chemical inhibitors leads to increased activation of AKT by FGF in OB1 cells, suggesting that active ERK signaling blocks the AKT pathway. While this observation is intriguing, its mechanism is still unclear. ERK1 and 2 exert a negative feedback on the function of FRS2, an essential FGF signaling intermediate, by phosphorylating FRS2 on threonine residues (Lax et al., 2002). FRS2 is also involved in PI3-K activation (Hadari et al., 2001), and it is possible that inhibiting threonine phosphorylation of FRS2 favors activation or recruitment of the regulatory subunit of P13K, or simply potentiates FRS2 activity. Although in the reciprocal experiment, in the presence of AKT pathway inhibitors, we did not observe an appreciable induction of IGF-induced ERK activity, we did find that ERK phosphorylation is suppressed in osteoblasts stably expressing constitutively active AKT (AKT-Myr). These data suggest that these pathways cross-regulate and may oppose each other in osteoblasts.

Using chemical inhibitors, we show that blocking the ERK pathway impairs osteoblast proliferation while blocking AKT has no effect. We found that the ERK pathway contributes to osteoblast differentiation as well, but has little effect on osteoblast survival. The requirement for ERK activity during differentiation was somewhat unexpected since ERK signals induce proliferation and inhibit the differentiation-inducing effects of BMP (Osyczka and Leboy, 2005). However, ERK activity may induce factors required early in the differentiation program and is induced by differentiation-inducing signals such as Wnt. On the other hand, inhibitors of AKT pathway applied during differentiation resulted in increased apoptosis, highlighting the primary role for the AKT-pathway in survival during osteoblast differentiation. In osteoblasts, AKT activation occurs downstream of strong differentiation inducing signals such as BMP, and it has been shown to be essential for BMP-induced expression of ALP and differentiation (Ghosh-Choudhury et al., 2002). Our results from two osteoblast lines, OB1 and OB5, clearly indicate that constitutive activation of the AKT pathway by introduction of a myristylated form of AKT, enhances osteoblast differentiation. This effect is not only due to prevention of apoptosis, which is not particularly high in untreated cells, but is likely to involve a direct or indirect effect of AKT on the expression of differentiation genes such as cbfa1 and osterix. These findings are also in line with the hypothesis that the opposite effects of

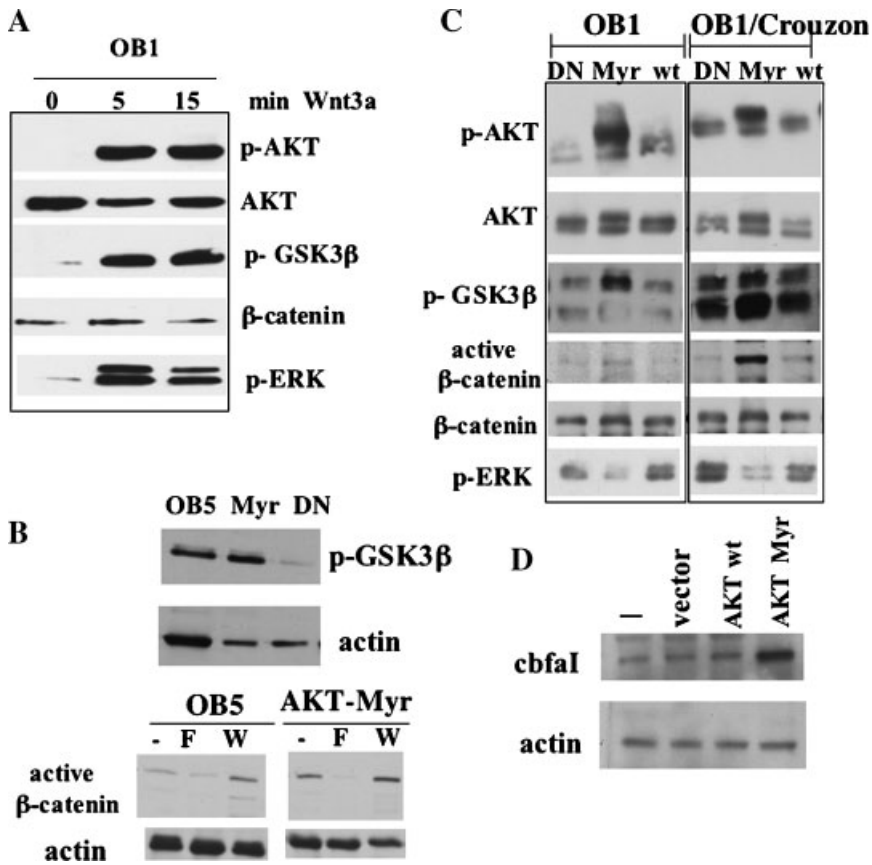


Fig. 6. Signaling molecules in the Wnt-AKT pathway. A: Western analysis of AKT and GSK3β activation in OB1 cells untreated (0) or treated with Wnt3A (100 ng/ml) for 5 or 15 min. B: Steady-state levels of p-GSK3β in OB5 control, OB5 expressing AKT-Myr (Myr) or AKT-DN, (DN) cells (upper part). Active beta catenin in OB5 control or OB5 expressing AKT-Myr in cells left untreated in complete medium (–) or treated with FGF1 (F) or Wnt3A (W) for 24 h. C: p-AKT, p-GSK3, p-ERK and active beta catenin in OB1 or OB1/Crouzon cells expressing AKT-Myr (Myr), AKT-DN (DN), or AKT-wt (wt). D: OB1 osteoblasts expressing the indicated constructs were analyzed by Western blot for Cbfa1 expression.

FGF and IGF could be due to the reciprocal strengths of activation of the ERK and AKT pathways.

We have previously described that FGF can inhibit Wnt signaling and differentiation in osteoblasts, and represses several genes identified as Wnt targets (Mansukhani et al., 2005). It was therefore conceivable that increased AKT activity, that enhances differentiation, could have potentiated the activation of Wnt pathways in osteoblasts, since AKT is known to phosphorylate and inactivate GSK3β, an inhibitor of the canonical Wnt pathway. In line with the hypothesis that AKT can cooperate with the Wnt pathway, we could show that in osteoblasts expressing AKT-Myr, GSK3β is phosphorylated and the amount of active β-catenin enhanced. We also found that in addition to activating the Wnt-β-catenin pathway, Wnt3A treatment of OB1 osteoblasts also activated AKT. Activation of the PI3K-AKT pathway has also been shown to play a role in the anti-apoptotic effect of Wnt3A (Almeida et al., 2005).

To prove more directly that increased AKT activity targeted β-catenin/Wnt target genes, we cultured calvarial osteoblasts from transgenic mice harboring a TOPGAL plasmid, where the β-galactosidase gene is controlled by a β-catenin/TCF responsive promoter (DasGupta and Fuchs, 1999). Cells were infected with a retroviral vector expressing AKT-Myr, or treated with IGF-1 or Wnt3A, and β-gal activity was measured. We could show that cells which had been kept in differentiation medium for 14 days had high β-gal activity (about 15× that of controls) and that IGF-1 and Wnt3A also stimulated β-gal activity about five- to sixfold, but infection with a retrovirus expressing Myr-AKT had no significant effect (data not shown). Two possible explanations for this negative result are that (i) as recently discussed (Barolo, 2006), it has to be considered that a transgenic β-catenin/TCF reporter may not give a complete readout of Wnt target gene activation. (ii) It is possible that in osteoblasts as in other cell types, inactivation of GSK3 by AKT is distinct from inactivation of GSK3 by Wnt (Ding et al., 2000). By quantitative RT-PCR analysis of the mRNA of Wnt-induced secreted protein (WISP-1), a Wnt-responsive gene that plays a role in bone development (French et al., 2004), we found that WISP-1 was elevated in AKT-Myr expressing osteoblasts but WISP-2 was not (data not shown). Further experiments defining Wnt target genes in osteoblasts, that are in progress, will be needed to clarify this issue.

An additional mechanism by which AKT activation leads to differentiation may be via transcription factors such as osterix and runx2 that are essential for osteoblast differentiation and bone development. AKT signaling has been reported to enhance DNA binding of Runx2 and Runx2 upregulates PI3-K subunits suggesting that Runx2 and AKT may be mutually dependent in osteoblasts (Fujita et al., 2004). Runx2 has also been shown to be a direct target of Wnt-beta catenin signaling (Gaur et al., 2005). The increase in Runx2 mRNA and protein

expression that we observe in osteoblasts expressing myristylated AKT, suggests that the AKT pathway may cooperate with Wnt signaling to promote osteoblast differentiation.

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