Wnts are secreted lipid-modified signaling proteins that influence cell proliferation, differentiation, and survival (1–3). Wnt proteins are divided into two classes. The Wnt1 class activates the canonical Wnt signaling pathway, which involves the formation of a complex between Wnt proteins, Frizzled, and LRPS \(^2\) or LRPS receptors (4, 5). This complex in turn leads to phosphorylation and inactivation of GSK-3\(\beta\), inhibition of \(\beta\)-catenin degradation, and subsequent accumulation of \(\beta\)-catenin in the nucleus (6, 7). Nuclear \(\beta\)-catenin binds the TCF/LEF family of transcription factors and induces target gene expression (8). The noncanonical Wnt5a class binds Frizzled proteins, activates heterotrimeric G proteins, and increases intracellular calcium via protein kinase C-dependent mechanisms or induces Rho- or c-Jun N-terminal kinase (INK)-dependent changes in the actin cytoskeleton (9).

Genetic studies in humans and mice have determined that LRPS (LRP6)/Wnt signaling plays a major role in the control of bone mass. Mutations in LRPS or LRPS6 lead to disorders associated with either low (10) or high bone mass (11–13). In agreement with observations in humans, LRPS6-deficient mice show decreased bone formation and osteoblast proliferation (14), whereas transgenic mice that express the LRPS G171V mutation or inactivation of bone morphogenetic protein 2 (BMP-2) in the induction of osteoblast differentiation (17, 18). However, the osteogenic effects of Wnts in human mesenchymal stem cells remain controversial (19–21). On the other hand, overexpression of the LRPS G171V mutation or inactivation of SFRP-1 in mice reduces osteoblast and osteocyte apoptosis, suggesting that the increased functional life span of osteoblasts is responsible, at least in part, for the favorable effects of Wnt signaling in bone (15, 16).

In the studies described herein we examined the effects of Wnts on osteoblast apoptosis and the molecular signaling pathways that link the two. We found that Wnt signaling prolongs the survival of uncommitted osteoblast progenitors and osteoblastic cells via activation of both the canonical pathway and the Src/ERK and PI3K/Akt cascades, with GSK-3\(\beta\) representing at least one convergence point between the two signaling cascades. \(\beta\)-Catenin-mediated transcription by itself is not sufficient to mediate the anti-apoptotic effects of canonical Wnts and is dispensable for the anti-apoptotic activity of Wnt5a.

EXPERIMENTAL PROCEDURES

Materials—PD98059, wortmannin, etoposide, cycloheximide, and LiCl were purchased from Sigma-Aldrich. Wnt3a, DKK1, and tumour necrosis factor- \(\alpha\)-recombinant proteins were purchased from R&D Systems (Minneapolis, MN).

signal-regulated kinase kinase; GSK, glycogen synthase kinase; dn, dominant negative; PTH, parathyroid hormone; ANOVA, analysis of variance; GFP, green fluorescent protein; EYFP, enhanced yellow fluorescent protein; nRFP, nuclear red fluorescent protein; MEM, minimum essential medium.
**Wnt Signaling in Osteoblasts**

Plasmids—pcDNA, Wnt1, and pCaspase3-EYFP sensor vector were purchased from Invitrogen (Carlsbad, CA), Upstate Biotechnology (Lake Placid, NY), and Clontech (Palo Alto, CA), respectively. *Xenopus* Wnt5a and murine DKK1 were provided by S. Sokol (Department of Microbiology and Molecular Genetics, Harvard Medical School and Molecular Medicine Unit, Boston, MA) (22) and C. Niehrs (Division of Molecular Embryology, Deutsches Krebsforschungszentrum, Germany) (23), respectively. A reporter plasmid carrying three TCF binding sites upstream of a minimal c-fos promoter driving the firefly luciferase gene (TOPFLASH), the plasmid carrying a minimal c-fos promoter driving luciferase expression (FOPFLASH) (24), and an expression construct of constitutively active β-catenin containing a missense mutation of tyrosine for serine at codon 33 (S33Y) (25) were provided by B. Vogelstein (Johns Hopkins University Medical Institutions, Baltimore, MD). The cDNAs for dnMEK, dnAkt, and SrcK295M (SrcK<sup>−</sup>) were provided by N. G. Ahn (University of Colorado, Boulder), M. E. Greenberg (Harvard Medical School, Boston, MA), and W. C. Horne (Yale University, New Haven, CT), respectively. The dnMEK is a catalytically inactive mitogen-activated protein kinase kinase (MAPKK) with a K97M substitution, which can be phosphorylated but not activated (26). dnAkt is a catalytically inactive mutant with a K179M substitution (26). dnAkt is a catalytically inactive mutant with a K97M substitution, which can be phosphorylated but not activated (26). dnAkt is a catalytically inactive mutant with a K179M substitution (26). SrcK295M is a kinase-dead Src with a single amino acid mutation at residue 295 (28). dpnPI3K is a mutated catalytic PI3K subunit with a deletion of amino acids 478–513 and was provided by J. Downward (Imperial Cancer Research Fund, London, United Kingdom) (27).

The nuclear red fluorescent protein (nRFP) construct was obtained by attaching the SV40 large T antigen nuclear localization sequence (30) to the amino terminus of the cDNA construct encoding red fluorescent protein (pDsRed1-N1, Clontech). Wild type ERK2 fused to green fluorescent protein (ERK2-GFP) was kindly provided by R. Seger (Department of Biological Regulation, The Weizmann Institute of Sciences, Rehovot, Israel) (31). DsTCF, which is a deletion mutant lacking the NH₂-terminal 30 amino acids of TCF4, and Axin (32) were provided by G. Rawadi (Proskelia, Paris, France) and F. Costantini (Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York), respectively.

**Cell Culture—**MC3T3-E1 cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum to avoid differentiation of the cells. OB-6 and MC3T3-E1 cells were cultured in α-MEM (Invitrogen) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 1% each penicillin, streptomycin, and glutamine. C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% each penicillin, streptomycin, and glutamine, and 1% sodium pyruvate. Murine subcutaneous adipose tissue-derived L-fibroblasts permanently transfected either with Wnt3a (L-Wnt3a) or an empty vector (L) were obtained from Clontech Laboratories, Houston, TX.

**Plasmid Transfections—**To assay Wnt/β-catenin mediated transcription C2C12 cells were transfected with 0.1 μg of TOPFLASH or FOPFLASH, 0.1 μg of each appropriate Wnt expression construct, and 0.1 μg of each constitutively active β-catenin or dominant negative mutants using Lipofectamine Plus (Invitrogen). TCF-luciferase activity was determined 24 h later using the Dual-Luciferase Reporter<sup>®</sup> assay system (Promega), according to the manufacturer’s instructions. Light intensity was measured with a luminometer, and luciferase activity was divided by the *Renilla* activity (control reporter) to normalize for transfection efficiency. To determine caspase3 activity, MC3T3-E1 cells were transfected as described above with 0.1 μg of pCaspase3-EYFP sensor vector and 0.1 μg of each dn kinase and were either treated with Wnt3a protein or co-transfected with 0.1 μg of Wnt5a expression construct.

**Quantification of Apoptotic Cells—** Apoptotic cells were quantified by measuring caspase3 activity (Figs. 1, A and C, and 6C) as described previously (33). In some experiments apoptosis was assayed by monitoring caspase3 activity in individual cells (Figs. 1B, 2B, 3D and F, and 5, A and C) following transfection into the cells of a vector that contains a caspase3 sensor protein fused to a yellow fluorescent protein (Clontech). Data are presented either as caspase3 activity units or as percentage of serum withdrawal-induced apoptosis in the absence of Wnt3a (100%).

**Subcellular Localization of ERK2—** MC3T3-E1 cells were transiently transfected using Lipofectamine Plus (Invitrogen) with wild type MEK along with ERK2-GFP to allow the visualization of ERK and nrFP to allow the localization of the cell nuclei. Following transfection, cells were cultured for 24 h, serum-starved for 40 min, and subsequently treated with vehicle (phosphate-buffered saline) or Wnt3a to a final concentration of 50 ng/ml for 5–120 min. Cells were fixed in neutral buffered formalin for 8 min. The percentage of cells showing nuclear accumulation of ERK2 was quantified by enumerating those cells exhibiting increased GFP in the nucleus compared with the cytoplasm, using a fluorescent microscope. At least 250 cells from random fields were examined for each experimental condition.

**Western Blot Analysis—** The phosphorylation status of Src, ERK1/2, and GSK-3β was analyzed by immunoblotting. The antibodies used were: a rabbit polyclonal antibody recognizing Tyr<sup>416</sup> phosphorylated Src (Cell Signaling); a mouse monoclonal antibody recognizing tyrosine phosphorylated ERK1/2 and a rabbit polyclonal antibody recognizing total ERK1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA); a rabbit monoclonal antibody recognizing Ser<sup>273</sup> phosphorylated Akt (Cell Signaling); a rabbit polyclonal antibody recognizing Ser<sup>21</sup> phosphorylated GSK-3β (Cell Signaling); and a mouse monoclonal antibody recognizing total GSK-3β and its phosphorylated form (BD Biosciences). Protein levels of β-catenin, Bcl-2, and β-actin were analyzed using a mouse monoclonal antibody recognizing β-catenin (BD Biosciences), a rabbit polyclonal antibody recognizing Bcl-2 (Santa Cruz Biotechnology), and a mouse monoclonal antibody recognizing β-actin (Sigma-Aldrich).

**Real-time PCR—** Total RNA was extracted using Ultraspec RNA (Biotec Laboratories, Houston, TX) and reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Primers and probes were manufactured by the Assay-by-Design service (Applied Biosystems Inc.) and were as follows: Bcl-2 probe, 5′-acctggtcctactca-3′, forward primer, ggacgctggcgtat, and reverse primer, cacccagctgggaagc; choB probe, 5′-tccagcagctgcc-3′, forward primer, cccagcatctccagctgct, and reverse primer, cccagcagctgcc-3′. PCR was carried out with 20-μl reaction volumes of Gene Expression Assay Mix, TaqMan Universal Master Mix, and 60–80 ng of cDNA template. The PCR reaction was performed in an ABI 7300 Prism. The -fold change in expression was calculated using the ΔΔCt comparative threshold cycle method.

**Statistical Analysis—** The data were analyzed by ANOVA; the Student-Newman-Keuls method was used to estimate the level of significance of differences between means. All experiments shown were repeated at least three times.

**RESULTS**

Wnts Prevent Apoptosis in C2C12, OB-6, and MC3T3-E1 Cells—Wnt3a abrogated serum withdrawal-induced apoptosis in C2C12
uncommitted osteoblast precursors and OB-6 or MC3T3-E1 osteoblastic cells as measured by active caspase3 levels (Fig. 1A). Transfection of MC3T3-E1 cells with a plasmid expressing Wnt1, which, similar to Wnt3a, activates the canonical Wnt signaling pathway, also prevented apoptosis induced by serum deprivation (Fig. 1B). Further, Wnt3a prevented apoptosis of C2C12 cells when induced by other proapoptotic stimuli, the topoisomerase inhibitor etoposide and tumor necrosis factor-α (Fig. 1C).

Activation of the Canonical Wnt Signaling Pathway Is Not Sufficient to Mediate the Anti-apoptotic Effects of Wnt3a—To determine whether the canonical Wnt signaling pathway is required for the pro-survival effects of Wnt3a we used the following constructs: (a) DKK1, the secreted antagonist of LRP5 or LRP6; (b) Axin, an intracellular inhibitor of canonical Wnt signaling; (c) dnTCF; or (d) S33Y, a β-catenin mutant that accumulates in the nucleus and constitutively activates TCF-mediated transcription. First we confirmed that DKK1, Axin, and dnTCF could inhibit Wnt3a-induced TCF-mediated transcription, whereas constitutively active β-catenin could by itself activate TCF-mediated transcription. For this purpose each one of the mutated constructs was transfected along with a TCF-luciferase reporter plasmid (TOPFLASH) in C2C12 cells (Fig. 2A). C2C12 cells transfected with constitutively active β-catenin were co-transfected either with TOPFLASH or with a negative control plasmid carrying all of the regulatory sequences of TOPFLASH except from the three TCF binding sites (TOPFLASH). As shown in Fig. 2A, in C2C12 cells transfected with an empty vector control plasmid (pcDNA) Wnt3a stimulated luciferase activity from the TCF promoter by 18-fold. The effect of Wnt3a was attenuated in cells transfected with dnTCF, Axin, or DKK1. Moreover, the S33Y constitutive active β-catenin construct stimulated TCF-mediated transcription by 23-fold as measured with the TOPFLASH reporter plasmid but had no effect in cells transfected with TOPFLASH.

Having confirmed the inhibitory action of DKK1, Axin, and dnTCF constructs on canonical Wnt signaling, we examined their effect on the anti-apoptotic actions of Wnt3a. Wnt3a protein protected MC3T3-E1 cells that had been transfected with pcDNA negative control from serum withdrawal-induced apoptosis (Fig. 2B). Transfection of dnTCF, Axin, or DKK1 inhibited the anti-apoptotic effect of Wnt3a in MC3T3-E1 cells. However, transfection of the S33Y constitutive active
**FIGURE 3.** Activation of Src/ERKs and PI3K/Akt is required for the anti-apoptotic effects of Wnt3a.

**A** and **B**: Western blot analysis showing the time course of Src/ERK phosphorylation in MC3T3-E1 cells treated with vehicle (veh) or Wnt3a (50 ng/ml) for 5–120 min. **C** and **D**: Graphs illustrating the percentage of cells with nuclear ERK2-GFP expression in response to Wnt3a and DKK1 treatments. **E**: Time course of Akt phosphorylation in MC3T3-E1 cells treated with vehicle or Wnt3a (50 ng/ml) for 5–120 min. **F**: Percentage of cells with nuclear ERK2-GFP expression in response to Wnt3a and DKK1 treatments.

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**Beta-catenin construct**, in the absence of Wnt3a, did not protect cells from serum starvation-induced apoptosis, indicating that in addition to the canonical Wnt pathway other signaling cascades are required for the anti-apoptotic effect of Wnt3a. Moreover, in the presence of Wnt3a, the constitutive active beta-catenin mutant S33Y abrogated the anti-apoptotic effect of the protein. S33Y can up-regulate the expression of Axin2, which in turn inhibits canonical Wnt signaling (34, 35). Hence, constitutive active beta-catenin inhibits the anti-apoptotic action of Wnt3a, probably via a negative feedback mechanism that involves up-regulation of Axin2 expression and subsequent inhibition of canonical Wnt signaling.

Wnt3a-induced Activation of Src/ERKs and PI3K/Akt Is Required for Anti-apoptosis—We have previously shown that activation of kinases like Src/ERK and PI3K are required for the pro-survival effect of sex steroids and bisphosphonates on osteoblasts *in vitro* and *in vivo* (33, 36–38). Additionally, there is evidence that Wnts phosphorylate ERKs and Akt (39–42). Prompted by these observations we examined whether activation of the same kinases is also required for the anti-apoptotic effects of Wnt3a in MC3T3-E1 cells. Wnt3a recombinant protein was used to perform a time kinetic analysis of the effects of canonical Wnts on Src, ERK, or Akt phosphorylation. Treatment of MC3T3-E1 cells with Wnt3a increased Src and ERK1/2 phosphorylation within 2 min (Fig. 3, **A** and **B**). However, Src phosphorylation reached a zenith at 15 min and remained high until at least 60 min following treatment with Wnt3a, whereas ERK phosphorylation reached a peak at 10 min and returned to basal levels after 60 min. Identical results were obtained in studies using C2C12 cells (data not shown).

Having established the time kinetics for highest activation of Src and ERKs by Wnt3a, we examined whether LRPS and -6 are involved in these events. Pretreatment of MC3T3-E1 cells with the LRPS/6-secreted inhibitor DKK1 prior to stimulation with Wnt3a increased the basal levels of Src phosphorylation. However, pretreatment with DKK1 did not affect ERK phosphorylation at 10 min following treatment of cells with Wnt3a. The inability of DKK1 to inhibit Wnt3a-stimulated ERK phosphorylation was not because of lack of protein activity. Indeed, DKK1, at a concentration identical to that used for examining its effects on ERK phosphorylation, did abrogate Wnt3a-induced TCF-luciferase activity (Fig. 3C). These results indicated that the co-receptors LRPS and -6 are not required for the Src- or ERK-activating action of Wnt3a.

We have previously found that sex steroids protect osteoblastic cells from apoptosis by promoting rapid and transient translocation of ERKs into the nucleus (33, 38). We examined whether, similar to sex hormones and androgens, Wnt3a also induces nuclear accumulation of ERKs. To this end, MC3T3-E1 cells were transiently co-transfected with constructs containing GFP-ERK2, MEK, and nRFP. Subsequently, cells

DKK1 recombinant protein (0.5 μg/ml) for 1 h prior to the addition of Wnt3a for another 15 min (**A**) or 10 min (**B** and **E**). Src (**A**), ERK (**B**), or Akt (**E**)) phosphorylation was analyzed by Western blotting in cell lysates. C2C12 cells were transfected with the TCF-luciferase reporter construct TOPFLASH, pretreated with vehicle or DKK1 recombinant protein (0.5 μg/ml) for 1 h, and then treated with 50 ng/ml Wnt3a recombinant protein for an additional 24 h. Bars represent means ± S.D. of the relative luciferase units (RLU) normalized for Renilla activity. D, MC3T3-E1 cells were transfected with ERK2-GFP to allow the visualization of ERK along with wild type MEK and with nRFP to allow the localization of the cell nucleus. 24 h later cells were serum-starved for 40 min and subsequently were treated with vehicle or Wnt3a (50 ng/ml) for 5–120 min. The percentage of cells with nuclear accumulation of ERK2 expressed relative to the total number of transfected cells in each well was determined. F, MC3T3-E1 cells were transiently transfected with pcDNA, dnMEK, ScrK, dnPI3K, or dnAkt plasmid along with a caspase3-EYFP sensor plasmid. Cells were cultured in 10% serum or serum-starved and were then immediately treated with vehicle or Wnt3a recombinant protein (50 ng/ml) for 6 h. Apoptosis was quantified by detecting caspase3 activity in individual cells. Bars indicate means ± S.D. of triplicate determinations: *, p < 0.05 versus vehicle by ANOVA. These experiments were repeated at least three times.
were exposed for different lengths of time to Wnt3a. Using epifluorescence microscopy, we followed the kinetics of the subcellular localization of GFP-ERK2 in response to Wnt3a treatment and determined the percentage of cells exhibiting accumulation of GFP-ERK2 in the nucleus. ERK2 nuclear accumulation occurred rapidly and reached a peak within 5 min (Fig. 3D). Similar to our findings with sex steroids, the increase in ERK2 nuclear accumulation was transient, as it decreased thereafter progressively and returned to basal levels within 120 min following treatment with Wnt3a.

The ability of Wnt3a to stimulate Akt activity was also examined in MC3T3-E1 cells. Treatment of MC3T3-E1 cells with Wnt3a induced phosphorylation of Akt, with a peak at 10 min and a decrease to basal levels after 60 min (Fig. 3E). Treatment of cells with DKK1 protein for 60 min prior to the addition of Wnt3a did not interfere with the stimulatory effect of Wnt3a on Akt phosphorylation. Therefore, as shown for Wnt3a-induced ERK activation, phosphorylation of Akt by Wnt3a does not require a complex formation with LRP5/6.

Finally, we determined whether activation of Src, ERK, and Akt by Wnt3a is required for its anti-apoptotic actions. Because Akt activity is regulated by PI3K, we also examined the involvement of PI3K in the anti-apoptotic effects of Wnt3a. For these experiments we examined whether inhibition of kinase activity by means of dominant negative constructs would affect the anti-apoptotic effects of Wnt3a in MC3T3-E1 cells. In line with the ability of Wnt3a to induce Src, ERK, and Akt phosphorylation, transient transfection of dnMEK (the kinase that phosphorylates ERKs), the kinase-dead Src mutant SrcK-7, dnPI3K, or dnAkt inhibited the protective effect of Wnt3a on serum withdrawal-induced apoptosis of MC3T3-E1 cells (Fig. 3F).

ERKs Are Required for Wnt3a-induced Phosphorylation of GSK-3β and TCF-dependent Transcription—Similar to our observations, others have reported that canonical Wnts stimulate ERK activation. However, it remains unknown whether the stimulatory action of Wnts on ERK activation is required for their ability to activate the canonical signaling pathway. To address this question, we determined whether ERKs interact with components of the canonical Wnt signaling pathway. We specifically examined whether inhibition of ERK phosphorylation affected Wnt3a-induced phosphorylation and inactivation of GSK-3β. LiCl was used as a positive control in these experiments because it phosphorylates and inactivates GSK-3β. As expected, treatment of MC3T3-E1 cells with Wnt3a or LiCl induced GSK-3β phosphorylation (Fig. 4A). However, PD98059, a specific MEK inhibitor, had no effect by itself, but it abrogated Wnt3a-induced phosphorylation of GSK-3β in MC3T3-E1 cells.

The finding that ERKs are required for GSK-3β inactivation by Wnt3a, an event that leads to stabilization and nuclear accumulation of β-catenin, prompted us to examine whether ERKs also mediate the stimulatory action of Wnt3a on β-catenin levels. The phosphorylation of GSK-3β by Wnt3a was correlated with an increase in the levels of β-catenin (Fig. 4B). In contrast to its inhibitory effect on Wnt3a-induced GSK-3β phosphorylation, PD98059 did not attenuate Wnt3a-induced increase in β-catenin levels, although it dramatically decreased the basal levels of β-catenin. These findings suggest that ERKs are required for Wnt3a-mediated phosphorylation of GSK-3β but not for β-catenin nuclear accumulation.

Prompted by these observations, we examined whether ERKs are involved in the transcriptional actions of Wnt3a. As shown in Fig. 3E, Wnt3a also induces phosphorylation of Akt. Thus, we investigated whether, in addition to ERKs, the PI3K/Akt signaling cascade could also mediate Wnt3a-induced TCF-mediated transcription. C2C12 cells were transfected with the TCF-luciferase reporter TOPFLASH along with the pcDNA empty vector or dnMEK, dnPI3K, or dnAkt mutants. Wnt3a protein or transfection of a Wnt1 expression plasmid was used to stimulate TCF-mediated transcription. As shown in Fig. 4C, transient transfection of a dnMEK, dnPI3K, or dnAkt mutants in C2C12 cells attenuated, but did not abolish, TCF-luciferase activity induced either
by treatment of transfected cells with Wnt3a protein or by co-transfection of the Wnt1 expression plasmid. Thus, inactivation of GSK-3β by ERKs contributes to the TCF-mediated transcriptional actions of Wnt3a. The partial inhibitory effect of dnMEK on Wnt3a-induced β-catenin/TCF-mediated transcription is consistent with the ability of Wnt3a to increase β-catenin levels even when the action of ERKs is inhibited (Fig. 4B).

The Noncanonical Wnt5a Also Protects Osteoblastic Cells from Apoptosis via Src/ERK and PI3K/Akt Signaling—Our observations that (a) activation of TCF-mediated transcription (with the S33Y constitutive active β-catenin mutant) is not by itself sufficient to protect osteoblastic cells from apoptosis, (b) ERKs and Akt mediate the anti-apoptotic actions of Wnt3a, and (c) the LRP5/6 Wnt co-receptors, which are required for TCF-mediated transcription, are not involved in Wnt3a-induced phosphorylation of Src, ERKs, or Akt prompted us to examine whether members of the noncanonical Wnt family also protect osteoblastic cells from apoptosis. Transfection of Wnt5a in MC3T3-E1 cells prevented apoptosis induced by serum deprivation (Fig. 5A). As shown in Fig. 5B and in agreement with published evidence (9, 43), this effect did not result from activation of the canonical Wnt signaling pathway. Indeed, although transfection of MC3T3-E1 cells with the Wnt1 expression construct or treatment with Wnt3a protein up-regulated TOPFLASH reporter activity, Wnt5a had no effect. Furthermore, similar to our findings with Wnt3a, the anti-apoptotic effect of Wnt5a was abrogated by dnMEK, SrcK−, dnPI3K, and dnAkt (Fig. 5C). However, in line with the contention that the anti-apoptotic effect of Wnt5a did not involve β-catenin-mediated transcription, dnTCF did not affect the pro-survival actions of Wnt5a.

Wnt3a Induces Expression of Anti-apoptotic Bcl-2 in MC3T3-E1—Finally, in the experiments summarized in Fig. 6 we tested the hypothesis that the ability of Wnt3a to protect against apoptosis depends on the induction of one or more anti-apoptotic genes. To this end we examined the effect of Wnt3a on IGF-1, which has been shown previously to be up-regulated by members of canonical Wnt signaling and to mediate the anti-apoptotic effect of Wnt signaling in 3T3-L1 pre-adipocytes (44). Because treatment of osteoblastic cells with Wnt3a induced ERK phosphorylation, we also examined whether the mRNA or protein levels of the ERK-regulated Bcl-2 protein (45) were affected by Wnt3a. MC3T3-E1 cells were cultured in the presence of recombinant Wnt3a protein for 2–6 h, or they were treated with control- or Wnt3a-conditioned media for 1–6 days (Fig. 6A). IGF-1 mRNA levels were not affected by Wnt3a (data not shown). However, Bcl-2 mRNA expression was up-regulated at 4 and 6 h following treatment of cells with Wnt3a protein. Bcl-2 mRNA levels in MC3T3-E1 cells were up-regulated following a 1-day treatment with Wnt3a conditioned media and remained increased thereafter until the end of the experiment 6 days later. Moreover, a 6-h treatment of MC3TE-E1 cells with Wnt3a protein up-regulated Bcl-2 protein levels, an effect that was blocked by the specific MEK inhibitor PD98059 (Fig. 6B). In line with the idea that de novo protein synthesis is required for anti-apoptosis, the protein synthesis inhibitor cycloheximide, at a dosage that inhibits [3H]leucine incorporation (33) without affecting cell viability (data not shown), abrogated the protective effect of Wnt3a on serum withdrawal-induced apoptosis of MC3T3-E1 cells (Fig. 6C).

DISCUSSION

Wnts prevent apoptosis in a variety of tissues during embryonic development (46–48). Moreover, prevention of osteoblast apoptosis in postnatal bone may be at least one mechanism by which Wnts potently augment bone mass. However, the precise mechanisms by which Wnts exert their pro-survival effects seem to vary among different cell types, and the mechanism via which they prolong bone cell survival is completely unknown. However, activation of the canonical β-catenin/TCF pathway, a synergistic action between kinases and β-catenin-mediated transcription, and activation of PI3K/Akt independently of β-catenin have all been proposed to confer the pro-survival effect of Wnts in cell types other than bone (40, 44, 49–52).

The results of the present report indicate that both canonical and noncanonical Wnts exert their pro-survival effect on uncommitted osteoblast progenitors and osteoblastic cells by a common mechanism that involves activation of the kinase signaling cascades Src/ERK and PI3K/Akt (Fig. 7). These actions are independent of LRPS/6 and lead to downstream up-regulation of the expression of the anti-apoptotic protein Bcl-2. In difference to Wnt5a, Wnt3a-induced phosphorylation of ERKs in turn regulates the canonical Wnt signaling pathway by inacti-
Wnt Signaling in Osteoblasts

Wnt Signaling in Osteoblasts

**FIGURE 6.** Wnt3a induces expression of Bcl-2 and prolongs MC3T3-E1 cell survival via de novo protein synthesis. A, total RNA was isolated from MC3T3-E1 cells treated with vehicle (veh) or Wnt3a protein (50 ng/ml) for 2–6 h in serum-free medium (upper panel). In a separate experiment MC3T3-E1 cells were treated with L- or L-Wnt3a-conditioned medium (CM, 2% serum) for 1, 3, or 6 days (lower panel). Real-time PCR was performed for Bcl-2 and ribosomal RNA. B, MC3T3-E1 cells were incubated for 30 min in serum-free media with vehicle or PD98059 (50 μM). Wnt3a protein (50 ng/ml) was then added to the cells, and cultures were continued for an additional 6 h. Bcl-2 protein level was determined by Western blot analysis of cell lysates. C, MC3T3-E1 cells were cultured in 10% serum or were serum-starved and pretreated with vehicle or cycloheximide (10 μM) for 1 h followed by Wnt3a protein (50 ng/ml) for 6 h. Apoptosis was quantified by determining caspase-3 activity. Bars indicate means ± S.D. of triplicate determinations: *, p < 0.05 versus vehicle by ANOVA. These experiments were repeated at least three times.

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It is possible that noncanonical pathways involving activation of Rho kinases and JNK, protein kinase C, and intracellular calcium release may also contribute to the anti-apoptotic effects of Wnt5a. Moreover, Wnt5a has also been reported to activate the TAK/NLK (NEMO-like kinase) pathway, which in turn can phosphorylate TCF/LEF transcription factors, thereby interfering with their DNA binding ability and suppressing β-catenin signaling (53, 54). However, in studies not shown here, we have found that Wnt5a (at a dose that protected osteoblastic cells from serum withdrawal-induced apoptosis) was not able to antagonize Wnt3a-stimulated activation of TCF-mediated transcription, suggesting that in our cell models Wnt5a does not activate the TAK/NLK pathway. This property of Wnt5a may be more closely related to its inhibitory effects on secondary axis formation in Xenopus embryos (55).

It has previously been reported that Wnt1 phosphorylates ERKs in mammary epithelial cells (39). Growth factors like IGF-1, FGF-2, or epidermal growth factor (EGF) cause ERK-dependent phosphorylation of GSK-3β and activation of TCF/LEF-dependent transcription in various cell types (56–58). However, activation of ERKs by Wnts has not been linked to GSK-3β phosphorylation. Our studies demonstrate that Wnt3a-induced phosphorylation of ERKs leads to phosphorylation and inactivation of GSK-3β and downstream potentiation of TCF-mediated transcription. It has recently been shown that ERKs associate with GSK-3β through a docking motif and prime it for subsequent phosphorylation at Ser9, resulting in up-regulation of β-catenin (59). On the basis of this evidence, we expect that in our bone cell model in response to Wnt3a ERKs directly associate with and inactivate GSK-3β.

The requirement for ERKs for Wnt3a-induced inactivation of GSK-3β was being not correlated with an ERK-dependent increase in β-catenin stabilization by Wnt3a. This discrepancy may be explained by the fact that cytoplasmic and nuclear β-catenin levels are regulated not only by Wnt signaling but also by the interactions of β-catenin with various proteins such as the gap junction protein connexin 43 (60), the cell adhesion protein cadherin (61) or the de-ubiquitinating enzyme Fam (62). Adding a further level of complexity, distinct molecular forms of β-catenin are involved in adhesive versus transcriptional complexes (63). It is possible, therefore, that in the absence of Wnt3a the total amount of β-catenin is comprised mainly by the “adhesive” pool of β-catenin, which is stabilized through ERK-dependent interactions with cadherins. This scenario would explain the potent down-regulation of basal β-catenin levels that ensues upon ERK inhibition with PD98059 (Fig. 4B). However, in the presence of Wnt3a, the total amount of β-catenin may comprise both the “adhesive” and the “transcriptional” pool of molecules, with the latter pool, at least partially, also stabilized via ERK-dependent inactivation of GSK-3β (Fig. 4A). If that were the case, inhibition of ERK activity would result in the inhibition of the adhesive and, at least part, the transcriptional pool of the protein.

Mouse models in which disruption or activation of Wnt signaling occurs at the initial step of the formation of an active complex between Wnts, LRP5, or Frizzled receptors affect osteoblast survival, differenti-
Importantly, Ryk (the mammalian homolog of the Drosophila Derailed), a nontypical member of the receptor tyrosine kinase family, is a Wnt receptor that can either form a complex with Frizzled to activate canonical Wnt signaling or transduce noncanonical Wnt signals via Frizzled-independent pathways that involve downstream activation of ERKs (68, 69). Therefore, Wnts may utilize Ryk or receptors other than Frizzled proteins to activate kinases and promote osteoblast survival. Future studies are needed to explore this possibility.

Several in vitro studies have examined the involvement of Wnt signaling in osteoblast differentiation, and LRPS/6 actions have been related to canonical Wnt signaling. Thus, it is generally assumed that Wnts exert their bone beneficial action via activation of the canonical Wnt signaling pathway. However, the plethora of different responses that Wnts elicit in osteoblasts, as well as the variety of receptors, regulators, and antagonists that interact with Wnt proteins, indicates that Wnts are able to regulate bone formation by more than one mechanism. Indeed, in the LRPS−/− mouse model, diminished bone formation and trabecular bone volume appear to be secondary to a decreased osteoblast proliferation and function, without any significant changes in the number of apoptotic cells (14). On the other hand, increased bone mass and strength in mice overexpressing the LRPS G171V high bone mass mutation is associated with a decrease in osteoblast and osteocyte apoptosis and an increase in the number of functional osteoblasts (15). In support of the latter observations, deletion of SFRP-1 in mice not only reduces osteoblast and osteocyte apoptosis but it also enhances osteoblast proliferation and differentiation (16). On the basis of these lines of evidence, we propose that kinase-mediated prolongation of the survival of uncommitted osteoblast progenitors, as well as mature osteoblasts, may contribute to increased osteoblast numbers, an effect that until now has been attributed solely to the pro-differentiating or proliferative actions of Wnts.

Similar to our findings with Wnts, PTH as well as sex steroids and bisphosphonates control bone mass in part by promoting osteoblast survival via activation of kinases (36, 70–72). Additionally, we have found that activation of nongenotropic actions of the estrogen or androgen receptor with 4-estren-3α,17β-diol (estren), a compound that stimulates kinase-mediated transcription at 3 to 4 orders of magnitude lower concentrations than those required for classical genotropic transcription, can potentiate β-catenin mediated transcription and increase BMP-2 expression and Smad phosphorylation (29, 73, 74). Consistent with these effects, estren, but not classical sex steroids or androgenic metabolites, induces commitment of pluripotent mesenchymal progenitors and promotes differentiation of committed osteoblastic cells toward the osteoblastic lineage in vitro in a Src/ERK-, PI3K-, and JNK-dependent manner. We propose that kinase signaling is crucial for both prolongation of osteoblast survival and induction of osteoblastic lineage commitment and is a significant mechanism via which osteotropic agents such as Wnts, sex steroids, estren, or PTH control osteoblast number and thereby osteogenesis.

![Wnt Signaling in Osteoblasts](image)
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