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The PI3K/Akt/FOXO3a/p27^{Kip1} signaling contributes to anti-inflammatory drug-suppressed proliferation of human osteoblasts

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ABSTRACT

Akt has been reported to suppress p27^{Kip1} promoter activity through Forkhead box O (FOXO) in different kinds of cells. Previous studies indicated that anti-inflammatory drugs up-regulated p27^{Kip1}, and this effect might play an important role in anti-inflammatory drug-induced cell cycle arrest of human osteoblasts (hOBs). In this study, we hypothesized that these drugs might increase p27^{Kip1} expression in hOBs by altering the Akt/FOXO signaling. We tested this hypothesis by examining the influences of three anti-inflammatory drugs on the levels and/or activities of Akt, FOXO and p27^{Kip1} as well as the relationship between these factors and proliferation of hOBs. We tested the effects of indomethacin (10^{-5} and 10^{-4} M), celecoxib (10^{-6} and 10^{-5} M), and dexamethasone (10^{-7} and 10^{-6} M) using PI3K inhibitor, LY294002 (10^{-5} M) as the basis of comparison. The three drugs suppressed the canonical level of phosphorylated Akt in hOBs. This was accompanied by elevated FOXO3a level and increased promoter activity, mRNA expression and protein level of p27^{Kip1}. Furthermore, the anti-inflammatory drugs suppressed the EGF-induced increases in proliferation, phosphorylation, and nucleus translocation of Akt. Simultaneously, they suppressed EGF-induced decreases of FOXO3a nucleus accumulation and p27^{Kip1} mRNA expression. On the other hand, FOXO silencing significantly attenuated the drug-induced up-regulation of p27^{Kip1} and suppression of proliferation in hOBs. To the best of our knowledge, this study represents the first to demonstrate that Akt/FOXO3a/p27^{Kip1} pathway contributes to suppression of hOB proliferation by anti-inflammatory drugs. We suggest that anti-inflammatory drugs suppress hOB proliferation, at least partly, through inactivating Akt, activating FOXO3a, and eventually up-regulating p27^{Kip1} expression.

1. Introduction

Anti-inflammatory drugs are widely used to relieve pain and inflammation in orthopaedic patients. However, reports have suggested that these drugs, including glucocorticoids (GCs), non-selective non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors have adverse effects on bone repair [1–8]. Anti-inflammatory drugs have been further reported to suppress proliferation and/or induce apoptosis in different kinds of cells via affecting cell cycle and pro-apoptotic factors [9–16]. Our previous studies also found that NSAIDs inhibited proliferation and arrested cell cycle at G0/G1 phase in both human bone marrow stem cells (hBMSCs) and osteoblasts (hOBs) [17–20]. Moreover, we found that dexamethasone, non-selective NSAIDs and COX-2 selective inhibitors caused the p27^{Kip1}, a cyclin-dependent kinase (cdk) inhibitor, expression increase and accompanied with cell cycle arrest in both hBMSCs and hOBs, and these effects were independent from anti-inflammatory drug-induced PG insufficiency [19,20]. The p27^{Kip1} is an important factor to regulate cell cycle progression and thus suppressed osteoblast proliferation, and enhanced differentiation by controlling proliferation-related events both in osteoblasts and bone marrow stem cells [21,22]. Base on these previous studies, we hypothesized that the up-regulation of p27^{Kip1} may contribute to an important common mechanism of anti-inflammatory drug-induced suppression of proliferation in osteogenic cells.

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The serine/threonine kinase Akt plays an important regulatory role in phosphatidylinositol-3-kinase (PI3K)/Akt signal transduction. Activated Akt regulates the activities of transcription factors such as Forkhead box class O (FOXO), mTOR, NFkB, and MDM2, and subsequently controls cell proliferation, apoptosis, and differentiation [23–25]. Celecoxib, glucocorticoids, and indomethacin have been reported to inhibit PI3K/Akt signaling in several somatic and cancer cell lines [26–36]. Although the effects of dexamethasone on Akt phosphorylation were examined using mouse osteoblastic cells (MC3T3E-1) [37], no studies reported whether GCs, non-selective NSAIDs, and COX-2 selective inhibitors suppress hOB Akt signaling.

PI3K/Akt signaling has been reported to suppress $p27^{Kip1}$ and thus proceed cell cycle [23–25,38,39]. Celecoxib has been reported to arrest cell cycle of human umbilical vein endothelial cells through its inhibition of Akt signaling [40]. In previous studies, we found three classes of anti-inflammatory drugs, GCs, non-selective NSAIDs, and COX-2 selective inhibitors, to increase the expression of $p27^{Kip1}$ mRNA in hOBs (as well as hBMSCs) [19,20]. Based upon these findings, we hypothesized that these drugs might up-regulate the expression of $p27^{Kip1}$ by inhibiting Akt activity in hOBs. FOXOs, are Akt down-regulated transcription factors reported to mediate cell cycle arrest, DNA repair, and apoptosis [41]. These transcription factors, which belong to the 'O' subgroup of winged-helix/forkhead transcription-factor family, consist principally of four members FOXO1, FOXO2, FOXO3a, and FOXO4 [42–44]. FOXO3a has been reported to induce the transcription of $p27^{Kip1}$ in many cell lines [43,45–51], suggesting that it may be a key regulator of anti-inflammatory drug-induced up-regulation of $p27^{Kip1}$. Therefore, we further hypothesized that anti-inflammatory drug-induced $p27^{Kip1}$ up-regulation may occur through the alteration of the Akt/FOXO3a signaling in hOBs. To test these hypotheses, we studied the influences of the anti-inflammatory drugs, celecoxib, indomethacin and dexamethasone, on changes in Akt, FOXOs and $p27^{Kip1}$, and relationship between these changes and the proliferation in hOBs.

2. Materials and methods

2.1. Materials

Dulbeco's Modified Eagle's Medium (DMEM), ascorbic acid, nonessential amino acid, penicillin/streptomycin, fetal bovine serum (FBS), and trypsin/EDTA were purchased from Gibco-BRL (Grand Island, New York, NY, USA). LY294002 (PI3K inhibitor), recombinant human EGF, DMSO, indomethacin and dexamethasone were obtained from Sigma (Saint Louis, MO, USA). Celecoxib was obtained from Pfizer (New York, NY, USA).

2.2. Normal human osteoblasts (hOBs)

Primary hOBs were isolated from bone chips of twelve 40–60-year-old donors (5 men and 7 women) who were generally healthy with no other bone disorders than hip dysplasia for which they received hip arthroplasty at Kaohsiung Medical University Hospital. The protocol for this study was approved by the Institutional Review Board (IRB) at Kaohsiung Medical University and the informed consent was obtained from each donor. The hOBs were cultured in DMEM containing 100 mg/ml of ascorbic acid, non-essential amino acids, penicillin/streptomycin and 10% FBS. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The doubling time of hOBs was 22–24 h under these experimental conditions. To synchronize cell cycle, hOBs were cultured in medium containing 2% FBS for 24 h before being treated with one of the agents according to procedures described previously [19,52–54].

2.3. Drug treatment

The drugs used to treat the hOBs in this study were indomethacin (10^{-5} to 10^{-4} M), celecoxib (10^{-6} to 10^{-5} M), dexamethasone (10^{-7} to 10^{-6} M), LY294002 (10^{-5} M) (Sigma, St. Louis, MO, USA), and recombinant human EGF (20 ng/ml) (Sigma, St. Louis, MO, USA). The therapeutic concentrations of indomethacin, celecoxib and dexamethasone were approximately 10^{-5}, 10^{-6} and 10^{-7} M, respectively [55–57]. Indomethacin, celecoxib, dexamethasone and LY294002 (Sigma, St. Louis, MO, USA) were dissolved in DMSO as stock solutions, and recombinant human EGF (Sigma, St. Louis, MO, USA) was dissolved in 10 mM acetic acid containing 0.1% BSA. All the drugs were diluted with a medium containing 2% FBS immediately before treatment began. DMSO was diluted to 0.1% or less to reduce the possibility of its influence on the process [18,19]. Because we found no significant cytotoxicity in hOBs incubated in a medium containing 0.1% DMSO, control cultures were cultivated in a medium containing neither anti-inflammatory drugs nor DMSO.

2.4. Enzyme-linked immunoassay (ELISA)

The levels of canonical phosphorylated Akt and total Akt were measured in indomethacin-, celecoxib-, dexamethasone-treated cultures and control cultures. The hOBs were seeded in a 6-well plate (2×10^4/well) and cultured to 80% confluence. After 24-h treatment with indomethacin, celecoxib or dexamethasone, the cells were collected for assay. We measured phosphorylated serine residue 473 and total Akt levels using BioSource AKT [pS473] ELISA and BioSource AKT ELISA, respectively (BioSource, Camarillo, CA, USA). We calculated phosphorylated Akt and total Akt level based on standard curves. All assays were performed in triplicate.

2.5. Luciferase assays

Cells were cultured in 10 cm dish to 80% confluence, and then harvested for plasmid transfection. The promoter region of human $p27^{Kip1}$ gene was subcloned into the XhoI site of the pGL2 basic vector (Promega, Madison, WI, USA) to create the p27PF luciferase reporter plasmid. Deletion constructs of p27PF including p27KpnI, p27ApaI, p27MB-435, and p27SacII were generated as described previously [58,59] and were kindly provided by Dr. Sakai. Cells were transfected with 2 μg of control plasmid, p27PF plasmid, or deleted p27 plasmids using a MicroPorator (Digital Bio Technology, Seoul, Korea) [60–65]. Cells were then seeded into 12-well plates and incubated in the absence or presence of indomethacin, celecoxib, or dexamethasone for 24 h. Luciferase activity was measured using TopCount Microplate Scintillation and Luminescence Counters (Packard, Meriden, CT, USA). The luciferase activity was normalized with total protein. Experiments were repeated in triplicate.

2.6. Western blot analysis

Cells were treated with indomethacin, celecoxib or dexamethasone for 24 h and lysed in the PhosphoSafe™ Reagent (Novagen, Darmstadt, Germany). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Cell lysates containing 40 μg of protein were analyzed using 10% SDS-PAGE. Transferred membranes were blocked using 5% skim milk and incubated overnight with antibodies against $p27^{Kip}$ (BD, San Jose, CA, USA), p-Akt (upstate, Charlottesville, VA, USA), FOXO1 (Santa Cruz, Santa Cruz, CA, USA), and FOXO3a (Cell Signaling, Danvers, MA, USA). These membranes were also probed with anti-actin (Sigma, St. Louis, MO, USA) or Akt (Santa Cruz, Santa Cruz, CA, USA) for house-keeping purposes. Membranes were developed

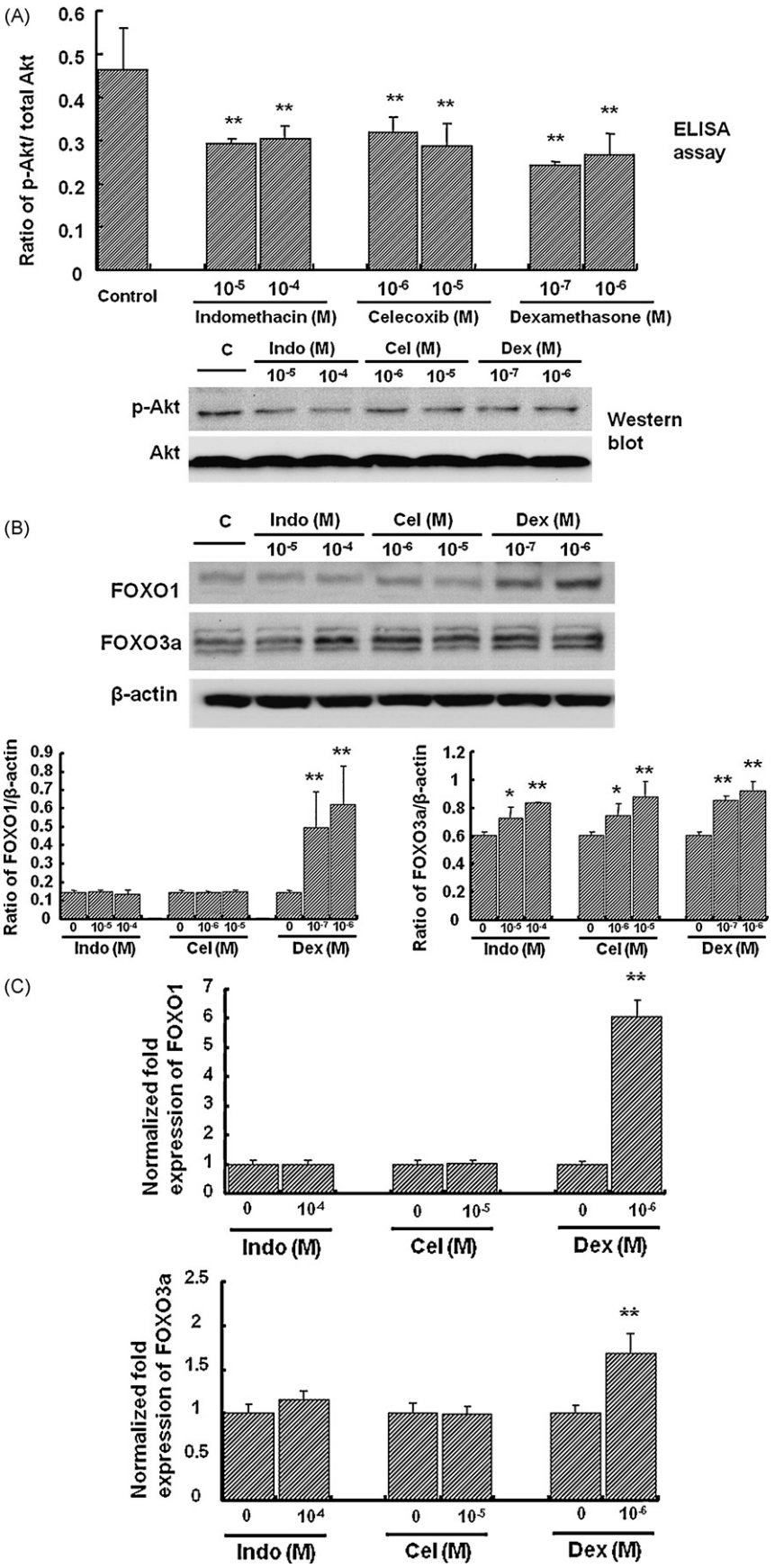

Fig. 1. The effects of anti-inflammatory drugs on canonical Akt phosphorylation, on the expression of FOXO1 and FOXO3a, on $p27^{Kip1}$ promoter activity, and $p27^{Kip1}$ protein level in human osteoblasts (hOBs). The synchronized HOBs were treated with celecoxib (10^{-6} and 10^{-5} M), dexamethasone (10^{-7} and 10^{-6} M) or indomethacin (10^{-5} and 10^{-4} M) for 24 h and analyzed for canonical Akt phosphorylation by ELISA (A), mRNA expressions of FOXO1 and FOXO3a (C) and $p27^{Kip1}$ (E) using real-time PCR, and protein

using Immobilon Western HRP Substrate (Millipore, Billerica, MA, USA). Each blot was digitally detected and analyzed using the UVP AutoChemi™ Image and Analysis System (UVP, Upland, CA, USA).

2.7. Thymidine incorporation

Cells (1000 cell/well) cultured in 96-well plates were treated with the anti-inflammatory drugs for 24 h. Four hours before harvesting, [H^3] thymidine (4 μCi/well) was added to the cells. Incubations were terminated by washing with phosphate buffered solution (PBS). Cells were detached using 1% trypsin/EDTA and collected in a 96-well UniFilter (Packard, Meriden, CT, USA) using a FilterMate Harvester (Packard, Meriden, CT, USA). The Unifilter was rinsed using 95% ethanol and maintained in a chemical hood for 30 min until completely dry. After sealing with TopSeal-A (Packard, Meriden, CT, USA), liquid scintillate was added to the sealed and dried UniFilter. [^{3}H] thymidine content was then measured by the TopCount Microplate Scintillation and Luminescence Counters (Packard, Meriden, CT, USA).

2.8. Real-time PCR

After the hOBs had been treated with indomethacin, celecoxib or dexamethasone for 24 h, we isolated total mRNA using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed with a Bio-Rad iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using the iQ™ SYBR® green supermix (Bio-Rad, Hercules, CA, USA). Reactions were performed in a 25-μl mixture containing cDNA, specific primers of each gene and the iQ™ SYBR® green supermix. The cycling conditions were 95 °C for 30 s and 95 °C for 4 min, followed by 35 cycles of 95 °C for 10 s, 61.5 °C for 15 s and 72 °C for 15 s. The primer sequences of $p27^{Kip1}$ and GAPDH were as follows: $p27^{Kip1}$ (forward: GACACCACTGGAGGGTGACT, reverse: CAGGTCCACATGGTCTTCCT), FOXO1 (forward: CAGCCCTGGATCACAGTTTT, reverse: CATCCCCTTCTCCAAGATCA), FOXO3a (forward: CATCATGGCAAGCACAGAGT, reverse: CAGGTCGTCCATGAGGTTTT) and GAPDH (forward: CAATGACCCCTTCATTGACC, reverse: TTGATTTTGGAGGGATCTCG). The specific PCR products were detected by measuring the fluorescence of SYBR Green, a double stranded DNA binding dye [66]. The relative mRNA expression level was calculated using the threshold cycle (Ct) value of each PCR product and normalized with that of GAPDH using the comparative Ct method [67]. The expression of each gene was calculated relative to controls, which were assigned a value of 1. The expression of each gene in drug-treated cells was converted to fold change in relation to base. After PCR reaction, a dissociation (melting) curve was generated to check the specificity of PCR reaction. All the PCR amplifications were performed in triplicate, and experiments were repeated at least three times.

2.9. Immunofluorescence

Cells were grown on sterilized cover glasses placed in a 6-well plate. After being treated with celecoxib, indomethacin, or dexamethasone for 24 h, the cells were treated with and without 20 ng/ml EGF for 30 min. They were then fixed in 3.7% paraformaldehyde and 0.5% Triton X-100, blocked in 3% BSA 3, and incubated simultaneously with both a mouse monoclonal antibody for FOXO3a (Santa Cruz, Santa Cruz, CA, USA) and a rabbit polyclonal antibody for p-Akt (Santa Cruz, Santa Cruz, CA, USA). PE-conjugated anti-mouse and Fluorescein (FITC)-conjugated anti-rabbit secondary antibodies allowed visualization of FOXO3a and p-Akt, respectively. All cells were stained with DAPI for nuclear observation. Cells were then visualized by confocal fluorescence microscopy and photographed.

2.10. The siRNA transfection

Before siRNA transfection, we used the BLOCK-iT™ Alexa Fluor® red fluorescent control (Invitrogen, Carlsbad, CA, USA) as an indicator of the transfection efficiency of Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) on hOBs. Cells were transfected with RNAi negative universal control (as mock control) (Invitrogen, Carlsbad, CA, USA), FOXO1 siRNA (Santa Cruz, Santa Cruz, CA, USA) or FOXO3a siRNA (Santa Cruz, Santa Cruz, CA, USA) using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA). Both siRNA of FOXO1 and FOXO3-a siRNA have three separate strands. The sequences of FOXO1 siRNA were GCAUCCAUGGACAACAACAtt, GAAGGGGAUGUGCAUUCUAtt, and CCACACAGUGUCAAGACAAtt. The sequences of FOXO3a siRNA were GUCAGCCAGUCUAUGCAAAtt, GGAACUUCACUGGUGCUAAtt, and CACAAGACCUACAGAGAAAtt. Cells were cultured in non-serum Opti-MEM medium during siRNA tranfection. After transfection, culture medium was changed from non-serum Opti-MEM medium to culture medium for 24 h, followed by treatment with indomethacin, celecoxib or dexamethasone for another 24 h to measure the mRNA expression of $p27^{Kip1}$ or incorporation of thymidine.

2.11. Statistical analysis

For each study group, data were reported as mean and standard error based on the results of 3 replicated cultures randomly chosen from 12 donors. Cells from each donor were used at least three times in different experiments. All experiments were repeated at least three times. Data were evaluated using one-way ANOVA, and multiple comparisons were performed using Scheffe's method. A $p < 0.05$ was considered significant.

3. Results

3.1. Anti-inflammatory drugs decrease Akt phosphorylation and increase FOXO and $p27^{Kip1}$ in hOBs

To investigate whether Akt phosphorylation and FOXO had an association with anti-inflammatory drug-induced up-regulation of $p27^{Kip1}$, we assessed the influence of the three drugs on phosphorylated Akt, FOXO1, FOXO3a, and $p27^{Kip1}$ levels and $p27^{Kip1}$promoter activity in hOBs. The effects of PI3K inhibitor, LY294002, were also compared. Both ELISA assay and Western blot analysis revealed that phosphorylated Akt levels were significantly suppressed by 24-h treatments with indomethacin (10^{-5} and 10^{-4} M, $p < 0.01$), celecoxib (10^{-6} and 10^{-5} M, $p < 0.01$) or dexamethasone (10^{-7} and 10^{-6} M, $p < 0.01$) in both ELISA assay and Western blotting analysis (Fig. 1A). The protein level of FOXO3a was significantly increased by treatment with indomethacin (10^{-5} M, $p < 0.05$ and 10^{-4} M, $p < 0.01$), celecoxib (10^{-6} M and 10^{-5} M, $p < 0.01$) and dexamethasone (10^{-7} and 10^{-6} M, $p < 0.01$) (Fig. 1B). The protein level of FOXO1 was also increased by treatment with dexamethasone (10^{-7} and 10^{-6} M, $p < 0.01$), but not by treatment with the other drugs (Fig. 1B). However, the mRNA expressions of FOXO3a and FOXO1 were only increased by treatment with dexamethasone (10^{-6} M, $p < 0.01$),

levels of p-Akt (A), and FOXO1 and FOXO3a (B), and $p27^{Kip1}$ (F) by Western blot analysis. The hOBs were transfected with a $p27^{Kip1}$ reporter construct (−3568 $p27^{Kip1}$/−12-pLG2, p27PF) and were harvested to detect luciferase activity (D). Data are reported as mean ± S.E.M. based on four replicated cultures. Comparative control- and drug-treated culture data were tested by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. *Key*: $^{*}p < 0.05$ and $^{**}p < 0.01$, in comparison with the control culture.

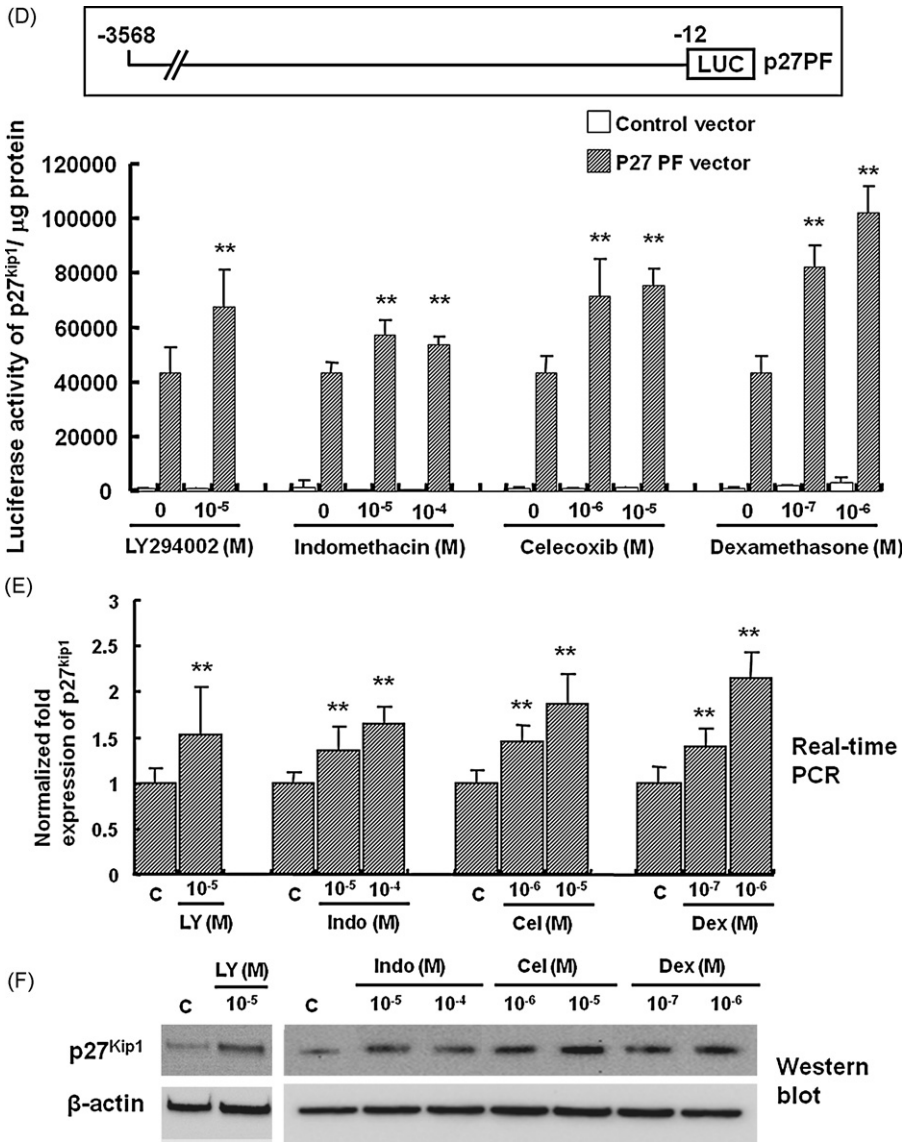

Fig. 1. (*Continued*).

but not with indomethacin and celecoxib (Fig. 1C). Likewise, the promoter activity, mRNA expression, and protein level of $p27^{Kip1}$ were also significantly increased by 24 h treatment with indomethacin (10^{-5} and 10^{-4} M, $p < 0.01$), celecoxib (10^{-6} and 10^{-5} M, $p < 0.01$), or dexamethasone (10^{-7} and 10^{-6} M, $p < 0.01$) (Fig. 1D–F). Treatment with the PI3K inhibitor, LY294002 (10^{-5} M, $p < 0.01$) had a similar effect (Fig. 1D–F). These results indicated that anti-inflammatory drugs decreased Akt phosphorylation and up-regulate FOXO3a and $p27^{Kip1}$ protein levels. Otherwise, dexamethasone also up-regulate transcriptions of FOXO1 and FOXO3a in hOBs.

3.2. Anti-inflammatory drugs have common enhancing effect on the activity of a specific region (−2982 to −2976) of p27PF promoter in hOBs

We wanted to identify which p27PF promoter region (−12 to −3568) may be critically involved in the AID-induced up-regulation of $p27^{Kip1}$. To do this, we determined the promoter activities of $p27^{Kip1}$ in hOBs by luciferase assay using various deletion mutant constructs from p27PF promoter (Fig. 2A). We found that indomethacin (10^{-5} M) significantly enhanced the activity of p27PF promoter ($p < 0.01$), but not the activities of deleted promoters, p27KpnI, p27ApaI, p27MB-435, or p27 SacII (Fig. 2B). Celecoxib (10^{-6} M) increased the activities of p27PF, p27KpnI, and p27ApaI ($p < 0.01$), but not those of p27MB-435 and p27 SacII in hOBs (Fig. 2B). Dexamethasone (10^{-7} M) increased the activities of p27PF, p27KpnI, p27ApaI, and p27MB-435 ($p < 0.01$), but not that of p27 SacII in hOBs (Fig. 2B). Notably, upon treatment with either celecoxib or dexamethasone, there was more than a 60% increase in p27PF promoter activity, compared to that of p27KpnI, p27ApaI, p27MB-435, or p27 SacII in hOBs (Fig. 2B).

3.3. Anti-inflammatory drugs partially reversed EGF-induced phosphorylation of Akt, down-regulation of $p27^{Kip1}$ and proliferation of hOBs

EGF, an activator of PI3K/Akt pathway, was used to increase the phosphorylation of Akt in hOBs ($p < 0.01$) (Fig. 3A). EGF-treated cultures showed a decrease in the mRNA expression of $p27^{Kip1}$ 3 h after treatment ($p < 0.01$) (Fig. 3B) and an increase in proliferation ($p < 0.05$) (Fig. 3C) at 24 h. In hOBs pre-treated with indomethacin, celecoxib, or dexamethasone, EGF-enhanced phosphorylation of Akt was significantly decreased ($p < 0.01$) and $p27^{Kip1}$ mRNA expression suppressed by EGF was partially restored ($p < 0.01$) (Fig. 3A and B). Furthermore, indomethacin, celecoxib, and dexamethasone also

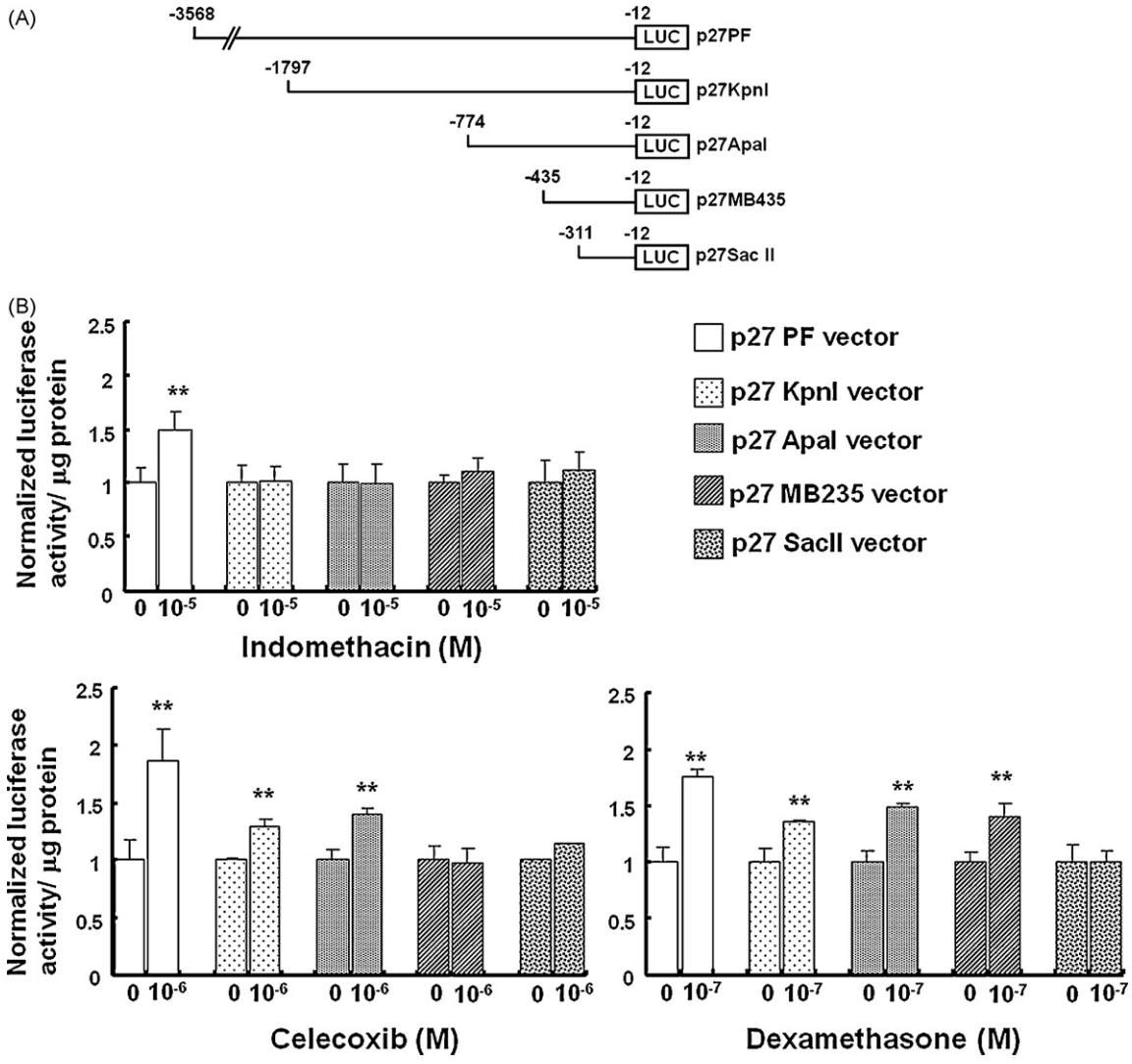

Fig. 2. Regulation of p27^{Kip1} promoter activity by anti-inflammatory drugs is through FOXO transcription factor. HOBs were transfected with a p27^{Kip1} reporter construct and deleted reporter constructs as in (A). After synchronization, cells were then cultured in 2% FBS culture medium with or without celecoxib (10^{-6} M), dexamethasone (10^{-7} M) or indomethacin (10^{-5} M) for 24 h, and luciferase activity was detected and normalized to total protein (B). Data are reported as mean ± S.E.M. based on four replicated cultures. Comparative control- and drug-treated culture data were tested by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. *Key*: $^{*}p < 0.05$ and $^{**}p < 0.01$, in comparison with the control culture.

significantly suppressed EGF-enhanced proliferation of hOBs ($p < 0.01$) (Fig. 3C). Because FOXO has been identified as direct target of Akt, and its activity is known to be highly influenced by their subcellular localization, we investigated whether Akt and FOXO3a were involved in anti-inflammatory-enhanced expression of p27^{Kip1} in hOBs. Examining the effects of these drugs on EGF-evoked nuclear translocation of phosphorylated Akt and FOXO3a in hOBs, we found EGF treatment increased nuclear translocation of p-Akt, but decreased nuclear translocation of FOXO3a (Fig. 3D). Pre-treatment with indomethacin, celecoxib, or dexamethasone attenuated the EGF-increased nuclear translocation of p-Akt and EGF-decreased nuclear translocation of FOXO3a in hOBs (Fig. 3D).

3.4. FOXO3a silencing significantly attenuated the anti-inflammatory drug-induced mRNA expression of p27^{Kip1} and anti-inflammatory drug-suppressed proliferation of hOBs

In this study, we found that the three drugs significantly elevated the protein level of FOXO3a in hOBs. FOXO3 was silenced to verify its influence on anti-inflammatory drug-induced p27^{Kip1} expression in hOBs. We transfected the fluorescent control siRNA into hOBs to measure transfection efficiency, which was found to be around 80% (data were not shown). After transfection with mock or FOXO3 siRNA, we found a significant decrease in mRNA expression and protein level of FOXO3 compared to mock controls ($p < 0.01$) (Fig. 4A). FOXO3a silencing significantly reversed indomethacin- (10^{-4} M), celecoxib- (10^{-5} M), and dexamethasone- (10^{-6} M) induced up-regulation of p27^{Kip1} (Fig. 4B). However, only dexamethasone (10^{-6} M, $p < 0.01$) was found able to elevate the mRNA expression of p27^{Kip1} in FOXO3a-silenced hOBs (Fig. 4B). FOXO3a silencing also significantly reversed indomethacin- (10^{-4} M), celecoxib- (10^{-5} M), and dexamethasone- (10^{-6} M) suppressed incorporation of thymidine (Fig. 4C). In addition, we found that FOXO3a silencing of hOBs partially reversed indomethacin (10^{-4} M, $p < 0.01$), celecoxib (10^{-5} M, $p < 0.01$), and dexamethasone (10^{-6} M, $p < 0.01$) caused suppression of thymidine incorporation (Fig. 4C).

3.5. FOXO1 silencing significantly attenuated the dexamethasone-induced mRNA expression of p27^{Kip1} and dexamethasone-suppressed proliferation of hOBs

Only dexamethasone enhanced the mRNA expression and protein level of FOXO1 in hOBs. We used FOXO1 siRNA to verify the

contribution of FOXO1 to dexamethasone-induced expression of $p27^{Kip1}$. Compared to mock culture, transfection with FOXO1 siRNA significantly reduced mRNA expression and protein level of FOXO1 ($p < 0.01$) (Fig. 5A). FOXO1 silencing significantly lessened the dexamethasone-induced mRNA expression of $p27^{Kip1}$ and decreased the proliferation of hOBs ($p < 0.01$) (Fig. 5B and C). However, effects of dexamethasone on elevating the mRNA expression of $p27^{Kip1}$ and inhibiting the incorporation of thymidine were only partial reversed by FOXO1 ($p < 0.01$) (Fig. 5B and C).

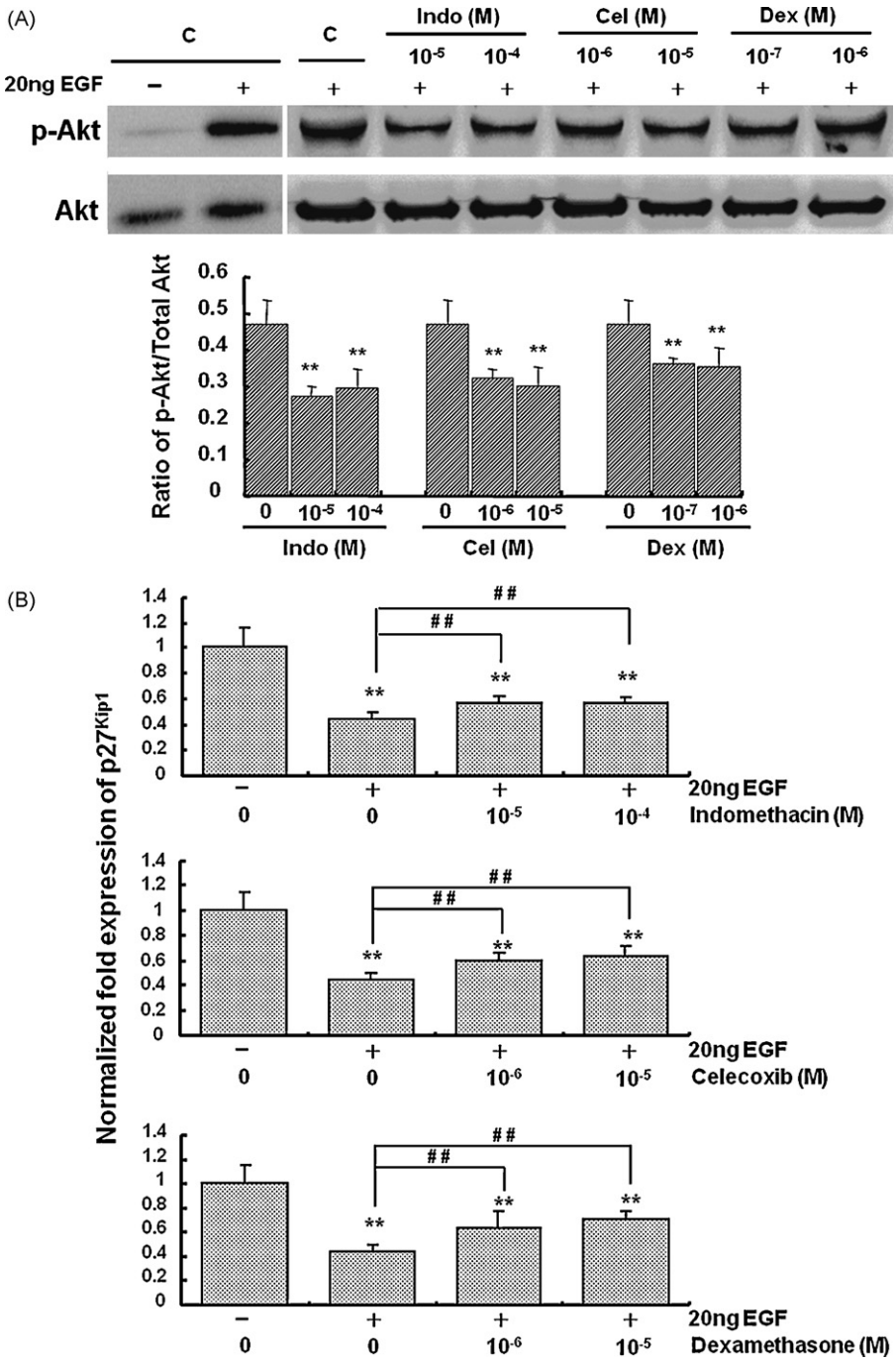

Fig. 3. The effects of anti-inflammatory drugs on Akt phosphorylation, $p27^{Kip1}$ mRNA expression, proliferation, and nucleus translocation of p-Akt and FOXO3a in EGF-treated hOBs. The hOBs were starved in 2% FBS culture medium for 24 h, and then cultured in 2% FBS culture medium with or without celecoxib (10^{-6} and 10^{-5} M), dexamethasone (10^{-7} and 10^{-6} M), or indomethacin (10^{-5} and 10^{-4} M) for 24 h. After anti-inflammatory drug treatment, cells were subsequently treated with 20 ng/ml EGF and then harvested at 30 min to detect Akt phosphorylation by Western blot analysis (A), at 3 h to measure $p27^{Kip1}$ mRNA expression by real-time PCR (B), and at 24 h to detect proliferation by [^{3}H] thymidine incorporation (C). To observe nucleus translocation of p-Akt and FOXO3a, cells were seeded on sterilized cover glass in a 6-well plate. The synchronized HOBs were treated with celecoxib (10^{-6} and 10^{-5} M), dexamethasone (10^{-7} and 10^{-6} M), indomethacin (10^{-5} and 10^{-4} M), or LY294002 (10^{-5} M) for 24 h. Cells were then cultured in 2% FBS culture medium with or without 20 ng EGF and harvested at 60 min to observe p-Akt nuclear translocation (green fluorescence), FOXO3a nuclear accumulation (red fluorescence) and nuclei location (blue fluorescence) using immunofluorescence confocal microscopy (D). Data are reported as mean ± S.E.M. based on four replicated cultures. Comparative control- and drug-treated culture data were tested by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. The comparison of data from each EGF-treated and EGF plus anti-inflammatory drug-treated cultures were evaluated by one-way ANOVA. *Key:* $^{*}p < 0.05$ and $^{**}p < 0.01$, in comparison with the control culture. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, in comparison with the EGF-treated control culture. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

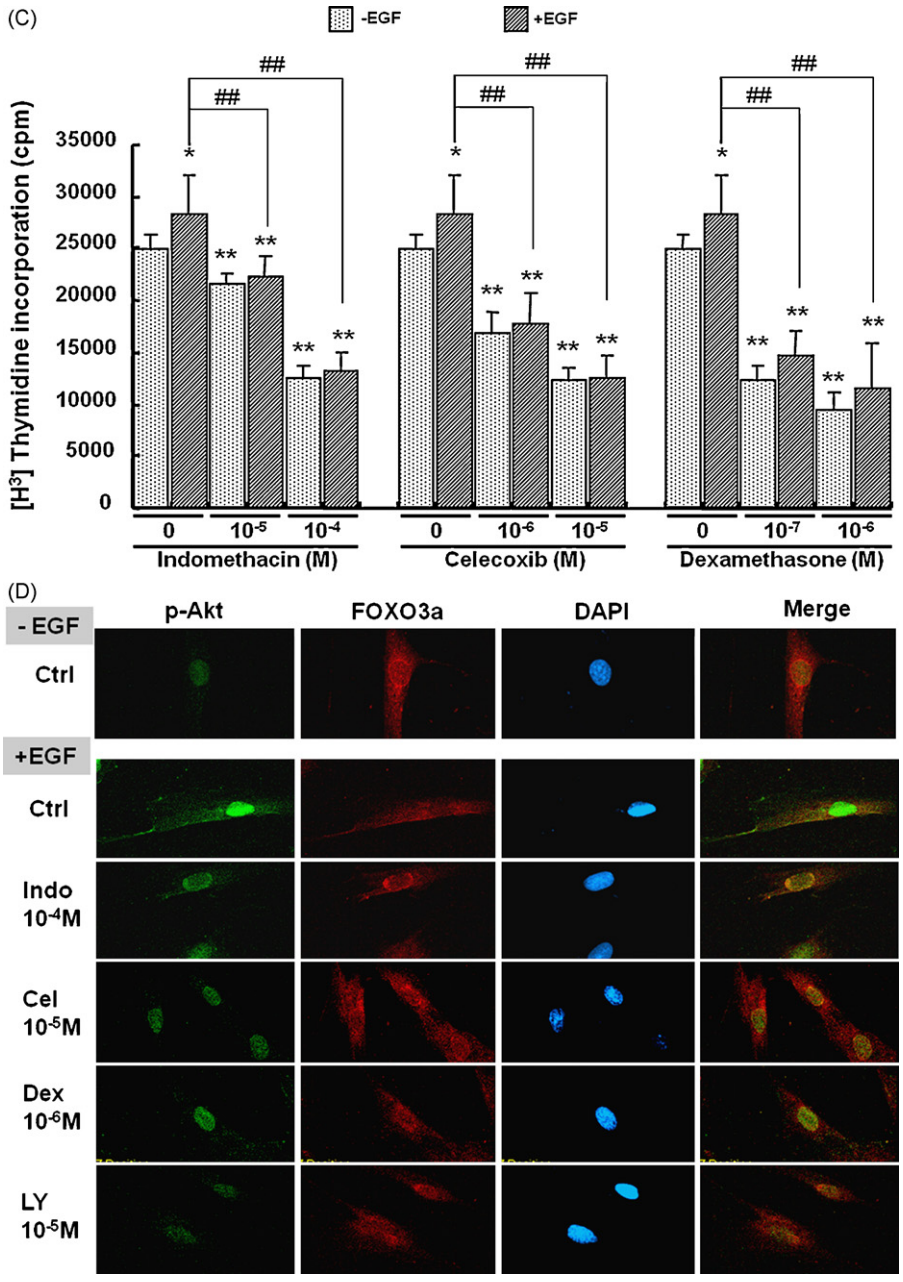

Fig. 3. (*Continued*).

4. Discussion

Anti-inflammatory drugs have been found to have adverse effects on osteogenic cells, but the molecular mechanism underlying their effect remains vaguely understood [1–5]. We previously demonstrated that NSAIDs suppressed proliferation and arrested cell cycle at G0/G1 phase, and further found increases in the expression of $p27^{Kip1}$ to play a key role in the effects of anti-inflammatory drugs on BMSCs and osteoblasts [17–20]. In this study, we further showed that the anti-inflammatory drug up-regulation of $p27^{Kip1}$ occurred through the Akt/FOXO/$p27^{Kip1}$ signaling. We found that anti-inflammatory drugs (indomethacin, celecoxib and dexamethasone) decreased phosphorylation of Akt, increased protein level of FOXO3a, and then elevated the transcription of $p27^{Kip1}$, subsequently inhibiting the proliferation of hOBs. Treatment with the PI3K inhibitor had a similar effect on hOBs. These results suggest that these drugs may act as PI3K/Akt pathway blockers and contribute to the elevation of $p27^{Kip1}$ and the reduction in proliferation of hOBs. This finding provided insight into the molecular mechanism underlying the common effects of anti-inflammatory drugs on the Akt/FOXO3a/$p27^{Kip1}$ pathway and their effect on the proliferation of hOBs (Fig. 6).

The FOXO family has been reported to be important positive transcription regulators of $p27^{Kip1}$ expression [38,42,46,68]. In this study, we found that anti-inflammatory drugs enhanced the level of FOXO3a and the promoter activity of $p27^{Kip1}$ in hOBs. Furthermore, silence of FOXO3a significantly reversed NSAID-elevated $p27^{Kip1}$ expression. These results verified that FOXO3a plays an important role in NSAID up-regulation of $p27^{Kip1}$ in hOBs. On the other side, this study found that dexamethasone could activate the deleted p27PF promoters that could not be activated by NSAIDs. Either FOXO1 or FOXO3a silencing partially reversed dexamethasone-induced $p27^{Kip1}$ up-regulation in hOBs. This indicated that transcription factors other than FOXOs

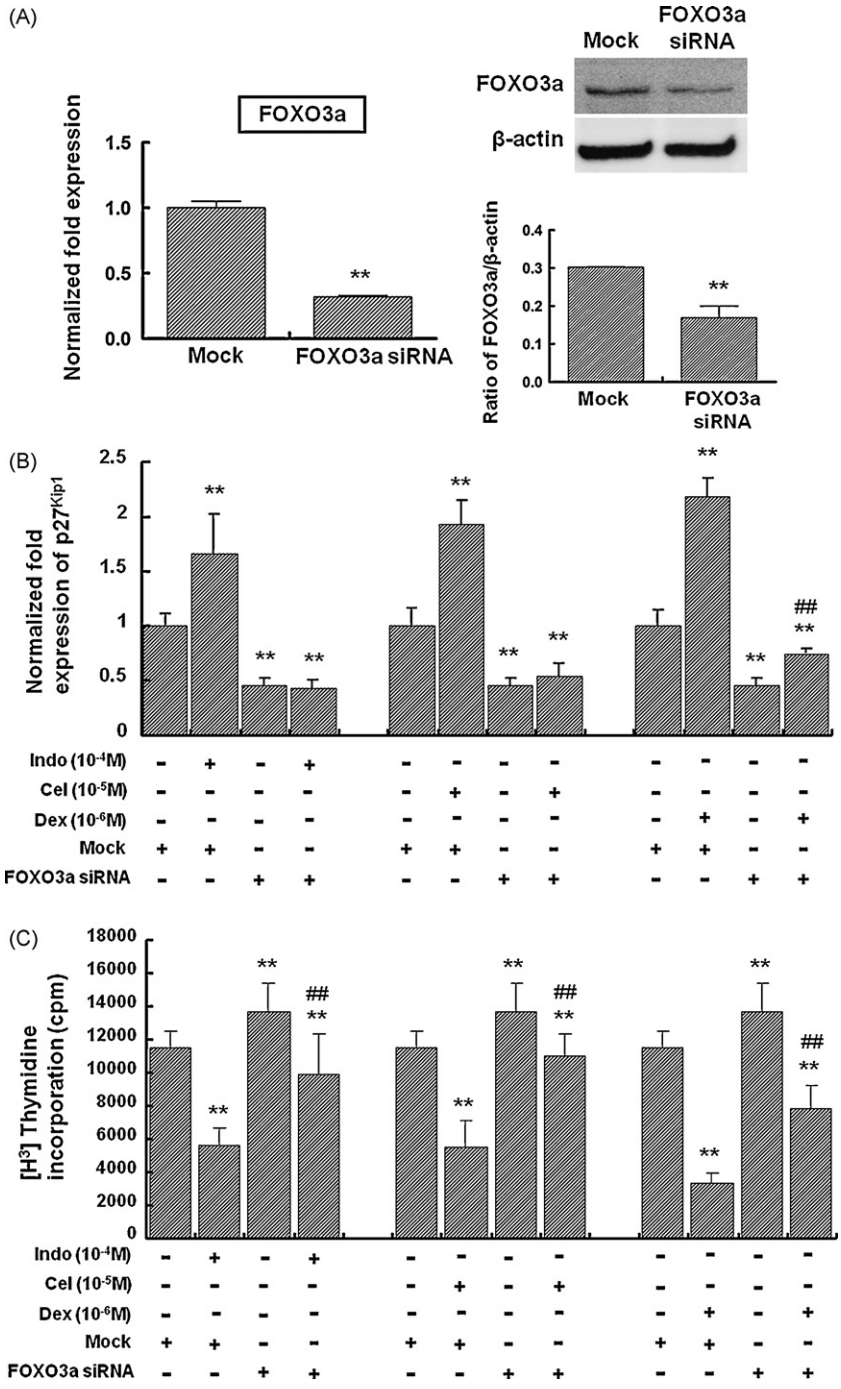

Fig. 4. The effect of anti-inflammatory drugs on $p27^{Kip1}$ mRNA expression and proliferation in FOXO3a-silenced hOBs. Cells were transfected with negative control siRNA (mock) or FOXO3a siRNA, and then FOXO3a mRNA expression and protein level assessed by real-time PCR and Western blot analysis (A). The FOXO3a silenced hOBs were treated with celecoxib (10^{-5} M), dexamethasone (10^{-6} M), or indomethacin (10^{-4} M) for 24 h, and then assessed for $p27^{Kip1}$ mRNA expression (B) and proliferation (C). Data are reported as mean ± S.E.M. based on four replicated cultures. The control and drug-treated culture data were compared by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. The comparison of data from each FOXO3a-silenced and FOXO3a-silenced plus anti-inflammatory drug-treated cultures were evaluated by one-way ANOVA. *Key*: $^{*}p < 0.05$ and $^{**}p < 0.01$, in comparison with the control culture. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, in comparison with the FOXO3a-silenced control culture.

may also involve in dexamethasone-induced $p27^{Kip1}$ up-regulation in hOBs. Studies have indicated that other transcription factors, such as Sp1, CRE and NFkB, regulate $p27^{Kip1}$ promoter activity [69,70]. Dexamethasone also has been found to increase Sp1 binding to DNA probes in rat and human cells [69,71–73]. Present finding suggested that dexamethasone may regulate $p27^{kip1}$ expression not only through FOXO1 or FOXO3a but also through other transcription factors in hOBs. Although celecoxib was also found to activate the deleted p27PF promoters that could not be activated by indomethacin, FOXO3a silencing completely reversed the celecoxib-increased $p27^{Kip1}$ up-regulation. Furthermore, celecoxib significantly increase the p27PF promoter activity 60% higher than those of the other deleted p27 prompters in hOBs. This effect suggested that FOXO3a may be a major positive regulator on indomethacin- and celecoxib-increased $p27^{Kip1}$ mRNA expression in hOBs. Even if the molecular mechanism of glucocorticoid on cells is different from NSAIDs, this study and other reports showed

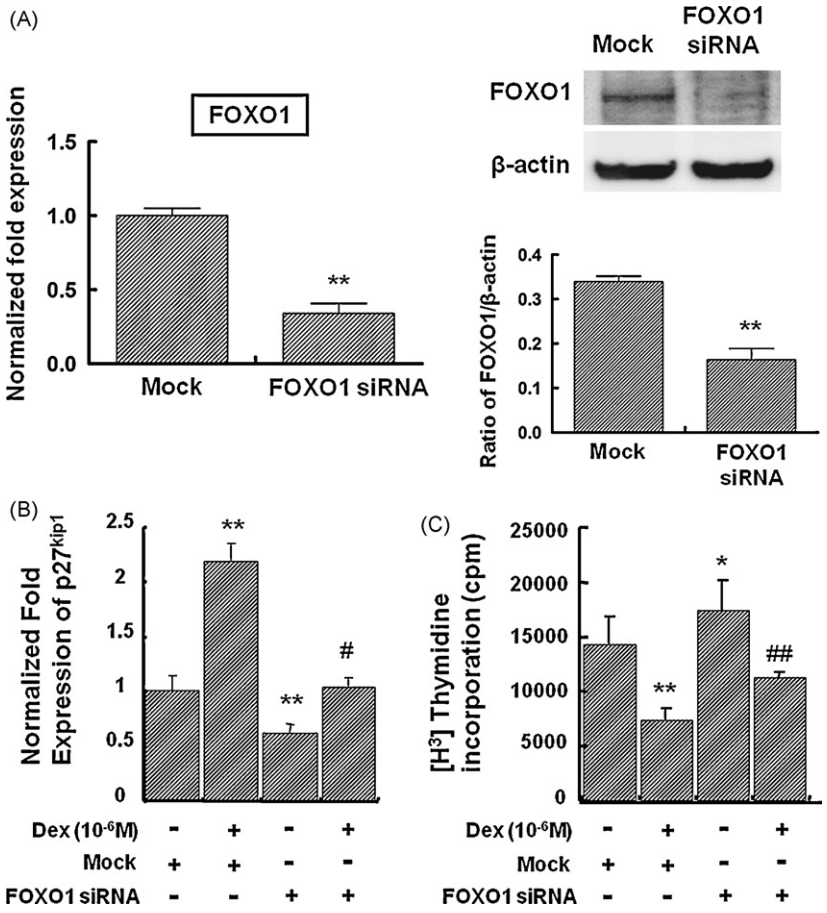

Fig. 5. The effects of dexamethasone on $p27^{Kip1}$ mRNA expression and proliferation in FOXO1-silenced hOBs. After transfection with negative control siRNA (mock) or FOXO1 siRNA, cells were measured mRNA expression and protein level of FOXO1 by real-time PCR and Western blot analysis (A). The FOXO1 silenced hOBs were then treated with dexamethasone (10^{-6} M) for 24 h, and analyzed for $p27^{Kip1}$ mRNA expression (B) and proliferation (C). Data are reported as mean ± S.E.M. based on four replicated cultures. Comparative control- and drug-treated culture data were tested by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. The comparison of data from each FOXO1-silenced and FOXO1-silenced plus dexamethasone-treated cultures were evaluated by one-way ANOVA. *Key*: $^{*}p < 0.05$ and $^{**}p < 0.01$, in comparison with the control culture. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, in comparison with the FOXO1-silenced control culture.

that both glucocorticoid and NSAIDs increase $p27^{Kip1}$ expression [19,74,20]. Notably, upon treatment with indomethacin, celecoxib or dexamethasone, there was a significant increase in p27PF promoter activity comparing to those of the other deleted p27 prompters in hOBs. A FOXO binding domain, GTAAACA, has been founded to locate at sequence location −2982 to −2976 of promoter p27PF, but did not find in location −1791 to −1. Accordingly, we suggest that FOXO3a may be an important common transcription factor involved in both GC- and NSAID-enhanced $p27^{Kip1}$ expressions.

Our results also showed that FOXO3a silencing completely reversed indomethacin- and celecoxib-induced up-regulation of $p27^{Kip1}$. However, we found that FOXO3a silencing reversed 24–35% of the anti-inflammatory drug-suppressed proliferation in hOBs, indicating that anti-inflammatory drug-induced increases in $p27^{Kip1}$ are regulated by FOXO3a, but anti-inflammatory drug-suppressed proliferation can be regulated by other factors besides $p27^{Kip1}$. Our previous study showed that anti-inflammatory drugs not only elevated $p27^{Kip1}$ expression but also suppressed the expression of the cell cycle regulator cyclin D2 and increased protein level of the pro-apoptotic factors Bak or Bad in hOBs [20]. These results confirmed one of our previous studies that anti-inflammatory drug-suppressed proliferation in hOBs involves expression changes of multiple cell cycle regulators. Nevertheless, in the current study we found that the interference of $p27^{Kip1}$ transcription is the common mechanism of anti-inflammatory drug-suppressed proliferation of hOBs.

More importantly, we found that all three tested drugs suppressed Akt phosphorylation and increased expression of FOXO3a and $p27^{Kip1}$ expression, resulting in the inhibition of hOB proliferation. Several studies have reported that anti-inflammatory drugs inhibit PI3K/Akt signaling in various cancer cell lines [26,27,31,35,36]. Therefore, it is good reason to suspect that there may be an important factor involved in anti-inflammatory drug-regulated Akt/FOXO3a/$p27^{Kip1}$ signaling in hOBs. Pharmacologically, NSAIDs and glucocorticoid inhibit the activity and synthesis of cyclooxygenase-2 (COX-2), respectively [75]. COX-2 is reported to be an enzyme induced by tissue injury and inflammation; however, in some organs including the central nervous system, kidneys and the gonads, COX-2 is expressed in a constitutive manner similar to another isoform, cyclooxygenase-1 (COX-1) [75,76]. The physiological role of constitutive expressed COX-2 in different tissues has not been well understood. Whether the actions of anti-inflammatory drugs in inhibiting COX-2 function and affecting PI3K/Akt/FOXO3a/$p27^{Kip1}$ pathway share common route remains a question.

In conclusion, this study represents the first prospect in human osteoblasts to demonstrate that Akt/FOXO3a/$p27^{Kip1}$ signaling contributes to the suppressive effect of anti-inflammatory drugs on proliferation. Our finding provides the molecular mechanism of clinical used anti-inflammatory drugs on delaying bone repair.

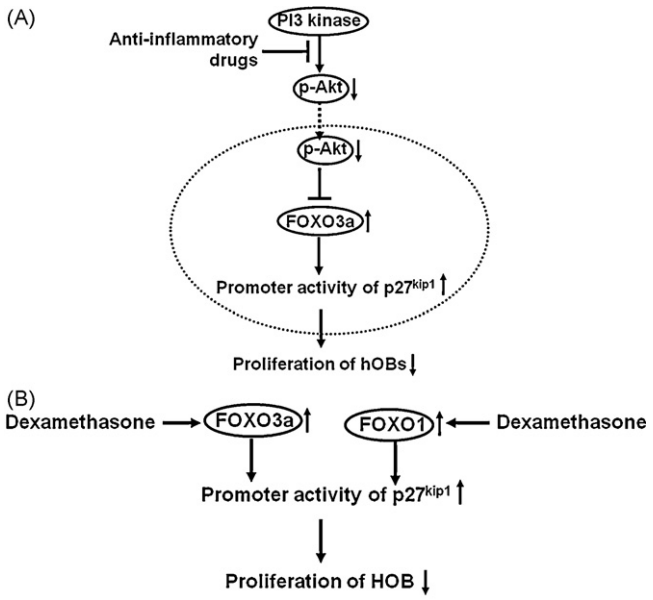

Fig. 6. Diagram illustrating the molecular mechanism proposed to underlie the effects of anti-inflammatory drugs Akt/FOXO/$p27^{Kip1}$ pathway and their regulation of human osteoblastic cell proliferation.

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