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DNA–PKcs–SIN1 complexation mediates low-dose X-ray irradiation (LDI)-induced Akt activation and osteoblast differentiation

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ABSTRACT

Low-dose irradiation (LDI) induces osteoblast differentiation, however the underlying mechanisms are not fully understood. In this study, we explored the potential role of DNA-dependent protein kinase catalytic subunit (DNA–PKcs)–Akt signaling in LDI-induced osteoblast differentiation. We confirmed that LDI promoted mouse calvarial osteoblast differentiation, which was detected by increased alkaline phosphatase (ALP) activity as well as mRNA expression of type I collagen (Col I) and runt-related transcription factor 2 (Runx2). In mouse osteoblasts, LDI (1 Gy) induced phosphorylation of DNA–PKcs and Akt (mainly at Ser-473). The kinase inhibitors against DNA–PKcs (NU-7026 and NU-7441) or Akt (LY294002, perifosine and MK-2206), as well as partial depletion of DNA–PKcs or Akt1 by targeted-shRNA, dramatically inhibited LDI-induced Akt activation and mouse osteoblast differentiation. Further, siRNA-knockdown of SIN1, a key component of mTOR complex 2 (mTORC2), also inhibited LDI-induced Akt Ser-473 phosphorylation as well as ALP activity increase and Col I/Runx2 expression in mouse osteoblasts. Co-immunoprecipitation (Co-IP) assay results demonstrated that LDI-induced DNA–PKcs–SIN1 complexation, which was inhibited by NU-7441 or SIN1 siRNA-knockdown in mouse osteoblasts. In summary, our data suggest that DNA–PKcs–SIN1 complexation-mediated Akt activation (Ser-473 phosphorylation) is required for mouse osteoblast differentiation.

1. Introduction

High-/moderate-dose radiotherapy mainly induces detrimental effects in bone cells, causing bone loss and fracture union delay [1,2]. Further, irradiation at high doses leads to single or double-strand DNA breaks in bone cells to block cell cycle progression [1,2]. Interestingly, our groups [3] and others have shown that low-dose irradiation (LDI) could promote bone differentiation, which is associated with increased level of bone formation specific markers including alkaline phosphatase (ALP), and runt-related transcription factor 2 (Runx2) [3–6]. LDI induces differentiation of primary mouse calvarial osteoblasts or osteoblastic-like cells [3–6]. However, the underlying mechanisms are not fully understood.

DNA-dependent protein kinase (DNA–PK) complex is composed of three proteins including DNA–PK catalytic subunit (DNA–PKcs) and Ku hetero-dimer (Ku-70 and Ku-80) [7,8]. DNA–PKcs is a member of phosphatidylinositol 3-kinase (PI3K)-like protein kinases (PIKK). This 460-kDa serine/threonine protein kinase can be activated by irradiation (at high-/moderate-dose), which then activates non-homologous end joining (NHEJ) pathway to repair DNA double-strand breaks [7,8]. However, whether LDI could affect the activity of DNA–PKcs is not well-studied.

Numerous studies have shown that irradiation activate Akt in various cells. Whether LDI could similarly influence Akt activity in osteoblasts, and if so, its potential roles in LDI-induced osteoblast differentiation have not been fully studied. The full activation of Akt requires phosphorylation on both Thr-308 and Ser-473. PDK1 (or 3-phosphoinositide-dependent kinase-1) phosphorylates Akt at Thr-308. The signal mechanism that phosphorylates Akt at Ser-473 is not fully established. Mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is one main Akt Ser-473 kinase [9], which contains following components: mTOR, Rictor, mLST8, Protor, Deptor, and stress-activated protein kinase interacting protein 1 (SIN1) [10]. Significantly, different groups have shown

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that DNA–PKcs could also phosphorylate Akt at Ser-473 under certain stimuli [11–13].

In this study, we investigated the potential role of DNA–PKcs and mTORC2 in LDI-induced Akt activation and osteoblast differentiation. Our results showed that DNA–PKcs–SIN1 (the key component of mTORC2) complexation is important for LDI-induced Akt activation and differentiation in mouse calvarial osteoblasts.

2. Material and methods

2.1. Chemicals and reagents

NU-7026 and NU-7441 were purchased from Calbiochem (San Diego, CA). LY294002 was purchased from Sigma Chemical Co. (St. Louis, MO). Perifosine and MK-2206 were from Selleck Chemicals LLC (Houston, TX). Antibodies against p-DNA–PKcs (Thr 2609), Erk1/2, SIN1 and Akt1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho(p)-Akt (Ser-473), p-Akt (Thr-308) and DNA–PKcs were purchased from Cell Signaling Technology (Denver, MA). Anti-p-DNA–PKcs (Thr 2647) antibody was purchased from Abcam (Cambridge, MA).

2.2. Primary culture of mouse calvarial osteoblasts

As previously described [14,15], the calvariae of 2- or 3-day-old neonatal mice were removed and bathed in α-MEM. The trimmed calvariae were transferred to a 50-ml Erlenmeyer flask and washed with PBS. Calvariae were digested with 0.1% collagenase (Wako, Japan) and 0.2% dispase (Dojindo, Tokyo, Japan) for 7 min at 37 °C. Cell suspensions were then neutralized with α-MEM, pooled, and filtered. The filtrate was centrifuged for 6 min at 1500 rpm, the supernatant was removed, and cells were resuspended in 3–5 ml α-MEM containing 15% FBS. Calvarial osteoblasts were cultured for 7–9 days, with medium was switched every 2–3 days. All animals were maintained in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996). The protocol was approved by Animal Care and Use Committee of all authors' institutions.

2.3. Irradiation of cells

Mouse calvarial osteoblasts were irradiated respectively with 0–4 Gy X-irradiation (at a dose rate of 200 cGy/min) by a medical linear accelerator with a 6 MV radiation source (Siemens Primus, Concord, CA) [3].

2.4. Cell viability assay

The cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay as described [14].

2.5. Western-blot analysis

Western blots and data quantification were performed as previously described [3].

2.6. Co-immunoprecipitation (Co-IP)

Aliquots of 1000 μg of protein samples in 1 ml of lysis buffer from each treatment were pre-cleared by incubation with 30 μl of protein A/G Sepharose (Sigma) for 2 h at 4 °C rotation. The pre-cleared samples were incubated with the specific antibody (1 μg/ml) overnight at 4 °C rotation. 20–30 μl of protein A/G Sepharose were added to the samples 2 h at 4 °C rotation. The beads were washed and boiled, followed by Western blot assay.

2.7. Assay of alkaline phosphatase (ALP) activity assay

Mouse calvarial osteoblasts were plated at a density of 2×10^4 cells/well into 24-well plates. Osteoblasts were cultured for indicated time after irradiation, ALP activity in these osteoblasts was determined by detecting the formation of *p*-nitrophenol, a product of *p*-nitrophenyl phosphate catalyzed by ALP, according to a previously described colorimetric procedure provided by a Sigma Diagnostics Alkaline, Acid, and Prostatic Acid Phosphatase kit (Sigma) see our previous publication [3]. Value was expressed as *p*-nitrophenol produced in nanomoles per minute per microgram of protein.

2.8. Real-time PCR

Total RNA was extracted through the SV total RNA isolation kit (Promega, Shanghai, China), and reverse transcription was performed using TOYOBO ReverTra Ace-a RT-PCR kit (TOYOBO, Japan) with manufacturer's instructions. We mixed the cDNA (7.5 ng total RNA equivalent) with SYBR Green PCR Master Mix and analyzed it by real-time PCR using the ABI7700 (Applied Biosystems). Primers used for amplification were as follows: mouse *Col I* (forward primer, 5′-GCAACAGTCGCTTCACCTACA-3′; reverse primer, 5′-CAATGTCCAAGGGAGCCACAT-3′) [16]; mouse Runx2: (forward primer, 5′-CCGCACGACAACCGCACCAT-3′; reverse primer, 5′-CGCTC CGGCCCACAAATCTC-3′) [17]. Primers for mouse *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were purchased from Invitrogen (Shanghai, China). After amplification, melt curve analysis was performed to calculate product melting temperature. For normalization, GAPDH was tested as the reference gene. The mean ΔΔCT was converted to relative expression value by the equation $2^{-\Delta\Delta Ct}$, and the range was calculated by the equation $2^{-(\Delta\Delta Ct\ +\ StdevAACt)}$.

2.9. SiRNA

Two siRNA sequences of mouse SIN1 (5′-GAUUAGAACGACU CCGAAA-3′, SIN1-siRNA-1 [18] and 5′-AGACUCAGGGCUAUGUA UA-3′, SIN1-siRNA-2 [19]) and a negative control scramble siRNA were purchased from Dharmacon Research Inc. (Lafayette, CO, USA). SiRNA sequences for human DNA–PKcs were 5′-GAUCGC ACCUUACUCUGUUdTdT-3′, (DNA–PKcs siRNA-1) [20] and 5′-AGG GCCAAGCUGUCACUCU-3′ (DNA–PKcs siRNA-2), and were synthesized by Kaiji Biotech (Shanghai, China). SiRNA (200 nM each) transfection was performed through Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the procedure [15]. The transfection took 48 h, and expression of targeted protein was tested by Western blots.

2.10. ShRNA knockdown

For shRNA experiments, lentiviral particles were produced by constructing a lentiviral GV248 expression vector (Kaiji Biotech, Shanghai, China) containing a puromycin resistance gene and either a scramble control shRNA (5′-TTCTCCGAACGTGTCACGT), a shRNA to DNA–PKcs (5′-GGAGCTTACATGCTAATGTAT), or a shRNA to Akt1 (5′-TGACCATGAACGAGTTTGA) (Kaiji Biotech). For infection, mouse calvarial osteoblasts were grown in 6-well culture plates in the presence of 5 μg/ml polybrene (Sigma) to 30–40% confluence, 10 μl/ml of lentiviral particles were added to the cells. Virus-containing medium was replaced with fresh medium the next day. Stable clones were selected by puromycin (0.35 μg/ml) for 4 days, expression of targeted protein in the resistant colonies was tested by Western blots.

2.11. Statistical analyses

All experiments were repeated at least three times, and similar results were obtained. Data were expressed as mean ± standard deviation (SD). Statistical analyses were analyzed by one-way analysis of variance using GraphPad InStat version 3. Multiple comparisons were performed using Tukey's honestly significant difference procedure. A p value of <0.05 was considered statistically significant.

3. Results

3.1. LDI increases ALP activity and promotes type I collagen (Col I)/Runx2 mRNA expression in mouse calvarial osteoblasts

In this study, we explored how low-doses of X-ray irradiation (LDI) influenced differentiation of mouse calvarial osteoblasts. ALP is the early phenotypic marker of osteoblast differentiation [21]. We first tested the effect of LDI on ALP activity. Results showed that LDI (X-ray irradiation at doses of 0.5–2 Gy) significantly increased ALP activity in mouse calvarial osteoblasts, while X-ray irradiation at a high dose (4 Gy) failed to do so (Fig. 1A). Meanwhile, LDI (X-ray irradiation at 0.5–2 Gy) induced mRNA expression of type I collagen (Col I) (Fig. 1B) and Runx2 (Fig. 1C) in mouse calvarial osteoblasts, Col I [6,22] and Runx2 [16,17,23] are both markers of osteoblast differentiation. Results from MTT assay demonstrated that only 4 Gy X-ray irradiation reduced cell viability of mouse calvarial osteoblasts, LDI showed no significant effect on osteoblast survival (Fig. 1D). Thus, LDI increased ALP activity and up-regulated differentiation-associated genes (Runx2/Col I) in mouse calvarial osteoblasts, suggesting that LDI induced osteoblast differentiation. Since LDI at 1 Gy showed most significant effect on osteoblast differentiation, this dose was chosen for further experiments.

3.2. DNA–PKcs activation is required for LDI-induced ALP activity increase and Col I/Runx2 mRNA expression in mouse calvarial osteoblasts

High-/moderate-dose of irradiation induces a rapid DNA–PKcs activation through phosphorylation [7,8]. Next, we examined the effect of LDI on DNA–PKcs activation in osteoblasts. Results from Western blot assay demonstrated that LDI (at 1 Gy) induced significant DNA–PKcs activation in mouse calvarial osteoblasts, which was detected by DNA–PKcs phosphorylation at both Thr 2647 and Thr 2609 (Fig. 2A). DNA–PKcs kinase inhibitors NU-7026 [24,25] and NU-7441 [26] prevented LDI-induced DNA–PKcs phosphorylation/activation (Fig. 2A). Significantly, NU-7026 and NU-7441 dramatically inhibited LDI-induced ALP activity increase (Fig. 2B) and Col I/Runx2 mRNA expression (Fig. 2C and D) in mouse calvarial osteoblasts. These results suggest that DNA–PKcs activation is important for LDI-induced osteoblast differentiation.

3.3. Akt activation is involved in LDI-induced osteoblast differentiation

Studies have shown that Akt activation could promote osteoblast differentiation and bone development [27,28]. As discussed, DNA–PKcs could activate Akt depending on stimuli [13,29,30].

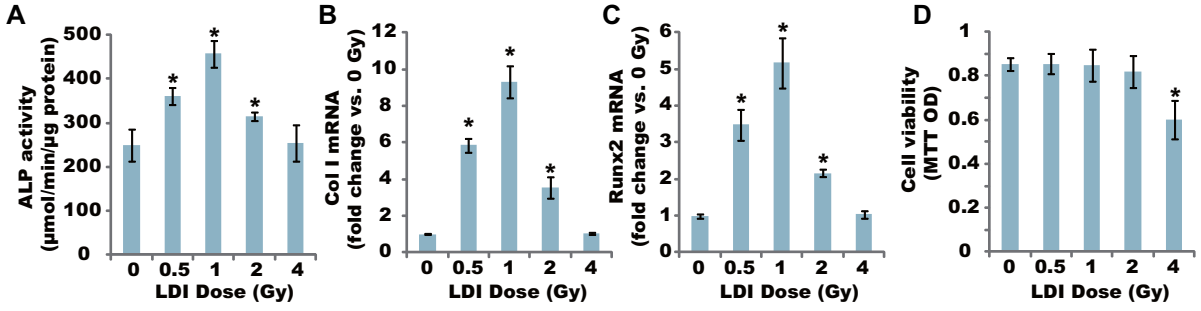

Fig. 1. LDI increases ALP activity and promotes type I collagen (Col I)/Runx2 mRNA expression in mouse calvarial osteoblasts. Mouse calvarial osteoblasts were irradiated with indicated dose of X-ray (0.5–4 Gy) for 3 days, ALP activity, Col I/Runx2 mRNA expression and cell viability were analyzed by ALP activity assay (A), real-time PCR assay (B and C) and MTT assay (D), respectively. For all real-time PCR assay, GAPDH was utilized as an internal control (see Section 2). The values in the figures were means ± SD (same for all figures). Experiments were repeated 3 times, similar results were obtained (same for all figures). *p < 0.05 vs. 0 Gy control group.

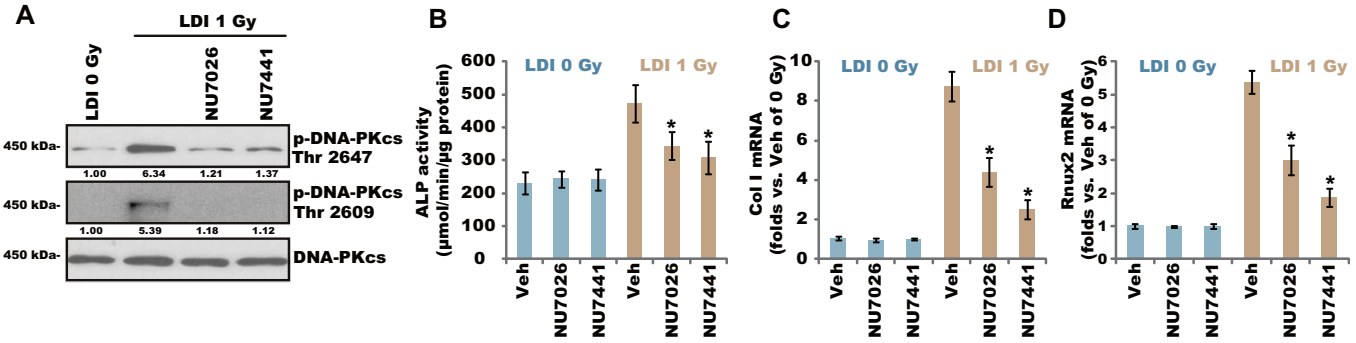

Fig. 2. DNA–PKcs activation is required for LDI-induced ALP activity increase and Col I/Runx2 mRNA expression in mouse calvarial osteoblasts. Mouse calvarial osteoblasts were pretreated with NU-7026 (10 μM) or NU-7441(10 μM) for 1 h, followed by X-ray (1 Gy) irradiation, expression of indicated proteins was tested by Western blots 12 h after irradiation (A), ALP activity and Col I/Runx2 mRNA expression were analyzed by ALP activity assay (B) and real-time PCR assay (C and D) assay 3 days after irradiation, respectively. DNA–PKcs phosphorylation was quantified. *p < 0.05 vs. LDI 1 Gy only group.

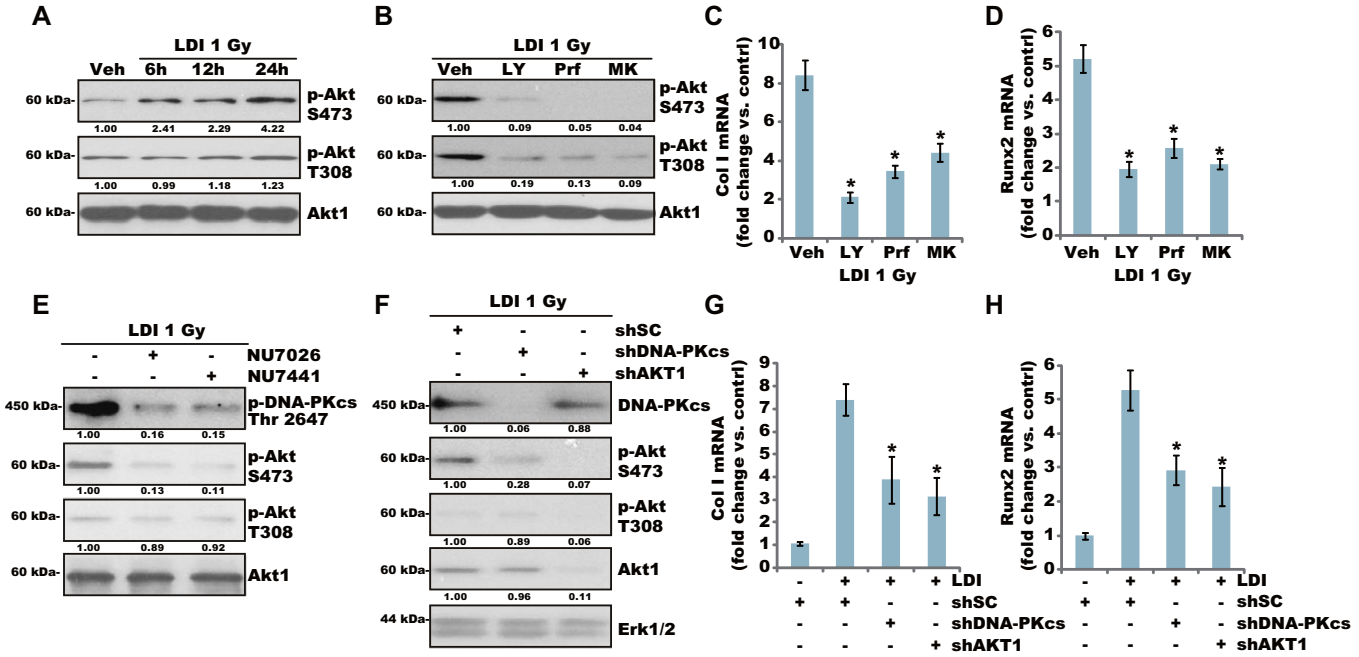

Fig. 3. Akt activation is involved in LDI-induced osteoblast differentiation. Mouse calvarial osteoblasts were irradiated with X-ray (1 Gy), cells were further cultured for indicated time, p-Akt (Ser-473 and Thr-308) and regular Akt1 were tested by Western blots (A). Mouse calvarial osteoblasts were pretreated with LY-294002 (LY, 1 μM), perifosine (Prf, 10 μM) or MK-2206 (MK, 10 μM), followed by X-ray (1 Gy) irradiation, cells were further cultured, p-Akt and regular Akt1 were tested by Western blots 12 h after irradiation (B), Col I/Runx2 mRNA expression (C and D) was analyzed 3 days after irradiation. Mouse calvarial osteoblasts were pretreated with NU-7026 (10 μM) or NU-7441(10 μM) for 1 h, followed by X-ray (1 Gy) irradiation, Western blot assay was performed to test indicated proteins 12 h after irradiation (E). Stable clones of mouse calvarial osteoblasts expressing scramble shRNA (shSC), DNA–PKcs-shRNA (shDNA–PKcs) or Akt1-shRNA (shAkt1) were irradiated with X-ray (1 Gy), cells were further cultured for 12 h, indicated proteins were tested (F), Col I (G) and Runx2 (H) mRNA expression was analyzed 3 days after irradiation. $^{*}p < 0.05$ vs. LDI 1 Gy only group. "Veh" stands for vehicle control (0.1% DMSO) group.

Thus, we tested the potential role of LDI on Akt activation in osteoblasts. The full activation of Akt requires phosphorylation on both Thr-308 and Ser-473 [31]. Western blot assay results in Fig. 3A demonstrated that LDI (at 1 Gy) mainly induced Akt phosphorylation at Ser-473 in mouse calvarial osteoblasts, LDI only slightly upregulated Akt phosphorylation at Thr-308 (Fig. 3A). PI3K/Akt inhibitor LY 294002 (also an inhibitor of DNA–PKcs [32]) as well as two Akt specific inhibitors perifosine [33,34] and MK-2206 [35] blocked LDI-induced Akt activation (Fig. 3B). Significantly, LDI-induced Runx2 and Col I mRNA expression (Fig. 3C and D) was also inhibited by above Akt inhibitors (LY 294002, perifosine and MK-2206). Note that these inhibitors had no significant effect on the viability of mouse calvarial osteoblasts (Data not shown). Results in Fig. 3E showed that DNA–PKcs inhibitors (NU-7026 and NU-7441) dramatically inhibited LDI-induced Akt phosphorylation at Ser-473 in mouse calvarial osteoblasts, suggesting that DNA–PKcs is required for LDI-induced Akt Ser-473 phosphorylation. To further support our hypothesis, shRNA strategy was applied. Stable clones of mouse calvarial osteoblasts were generated through infecting the lentiviral shRNA construct targeting DNA–PKcs or Akt1. The shRNA (shAkt/shDNA–PKcs) caused significant knockdown (over 90%) of the corresponding protein. In consistent with the inhibitor data, DNA–PKcs knockdown inhibited LDI-induced Akt Ser-473 phosphorylation, but not Thr-308 phosphorylation (Fig. 3F), while depletion of Akt1 by shRNA inhibited both Ser-473 and Thr-308 phosphorylation (Fig. 3F). LDI-induced Runx2 and Col I mRNA expression was also inhibited by either DNA–PKcs- or Akt1-knockdown (Fig. 3G and H). These shRNAs alone had no detectable effect on Runx2 and Col I mRNA expression or osteoblast survival (Data not shown). Together, these results suggest that Akt Ser-473 phosphorylation by LDI lies downstream of DNA–PKcs, and DNA–PKcs-Akt1 activation is required for LDI-induced osteoblast differentiation.

3.4. DNA–PKcs–SIN1 complexation is required for LDI-induced Akt Ser-473 phosphorylation and osteoblast differentiation

A recent study by Tu et al. demonstrated that UV radiation induces DNA–PKcs complexation with SIN1, which is required for subsequent Akt Ser-473 phosphorylation [13]. We thus tested this scenario in LDI-treated mouse calvarial osteoblasts. Co-IP assay results showed that LDI induced DNA–PKcs–SIN1 complexation in mouse calvarial osteoblasts, which was inhibited by NU-7441 or by SIN1 siRNA-knockdown (Fig. 4A and B). Knockdown of SIN1 by RNAi blocked LDI-induced Akt phosphorylation at Ser-473, while Akt Thr-308 phosphorylation was un-changed (Fig. 4C). Significantly, LDI-induced ALP activity increase (Fig. 4D) and Col I/Runx2 mRNA expression (Fig. 4E and F) were also inhibited by SIN1 knockdown in mouse calvarial osteoblasts. These results suggest that DNA–PKcs–SIN1 complexation is required for LDI-induced Akt Ser-473 phosphorylation and osteoblast differentiation.

4. Discussion

Moderate- or high-dose of X-irradiation could delay fracture union and causes osteoradionecrosis [1,2], while LDI promotes bone growth through inducing osteoblast differentiation [3–6]. Here, we confirmed pro-differentiation ability of LDI in mouse osteoblasts. LDI increased ALP activity and induced mRNA expression of Col I/Runx2 in mouse calvarial osteoblasts. Runx2, or core binding factor alpha 1, plays a vital role in osteoblast differentiation through transcriptional regulation of bone-specific genes [23]. Col I constitutes 90% of the total organic extracellular matrix in mature bone, and is an important marker of osteoblast differentiation [22]. Our data suggest that LDI-activated Akt signaling is an important mediator of osteoblast differentiation. While Akt

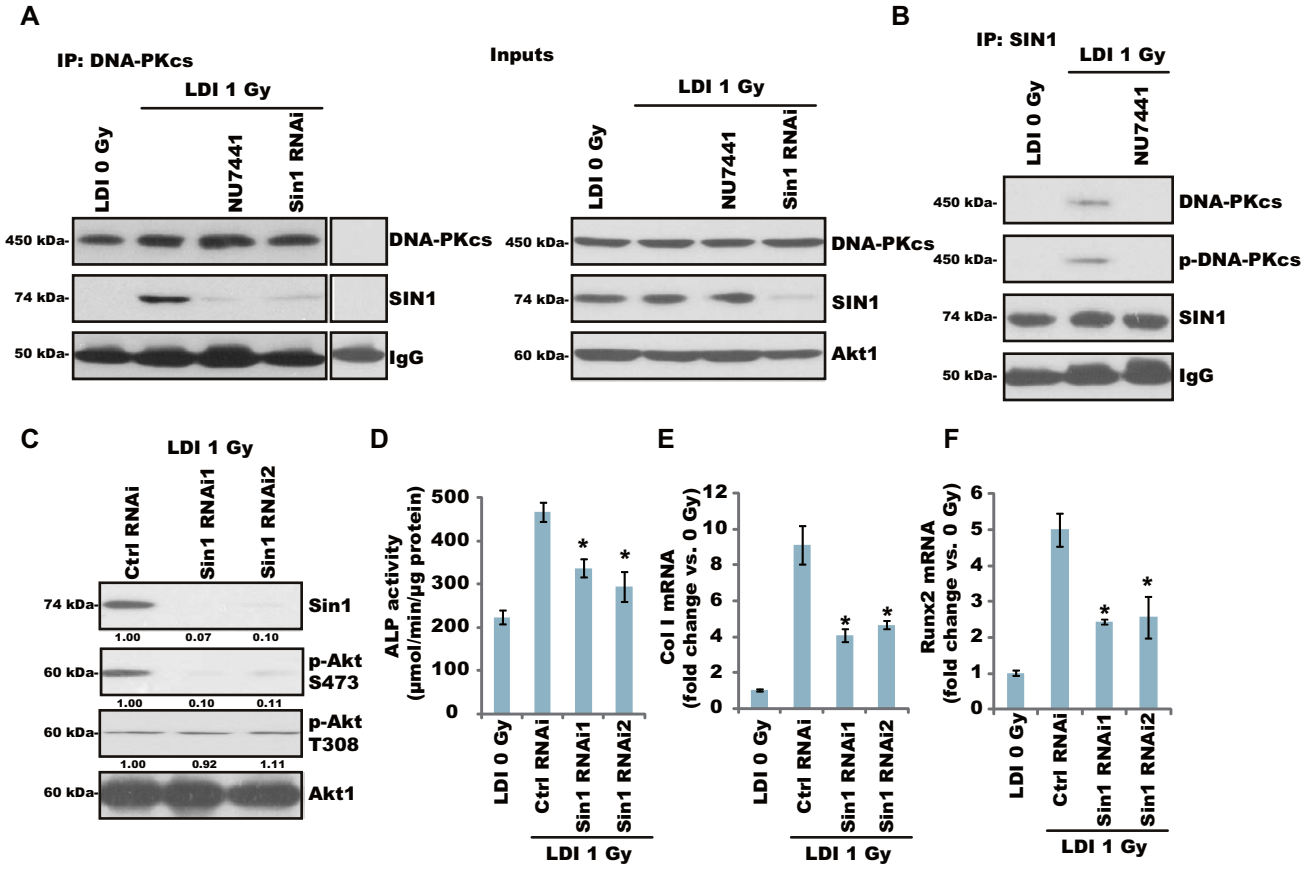

Fig. 4. DNA–PKcs–SIN1 complexation is required for LDI-induced Akt Ser-473 phosphorylation and osteoblast differentiation. Mouse calvarial osteoblasts were pretreated with NU-7441(10 μM, 1 h) or SIN1 siRNA-1 (200 nM, 48 h), followed by X-ray (1 Gy) irradiation for 12 h, complexation between SIN1 and DNA–PKcs was tested by Co-IP (A and B), expression of indicated proteins was also tested ("Inputs"). Mouse calvarial osteoblasts were transfected with scramble siRNA ("Ctrl RNAi") or SIN1 siRNA-1/-2 (200 nM each) for 48 h, followed by X-ray (1 Gy) irradiation, Akt (p- and regular) and SIN1 were tested 12 h after irradiation (C), ALP activity (D), collagen type I/Runx2 mRNA expression(E and F) were also analyzed 3 days after irradiation. $^{*}p < 0.05$ vs. LDI 1 Gy of "Ctrl RNAi" group.

inhibitors as well as Akt1 knockdown alleviated LDI-induced Col I/Runx2 expression in osteoblasts. Interestingly, we found that LDI mainly induced Akt phosphorylation at Ser-473 (but not Thr-308) in mouse calvarial osteoblasts.

Identification of the kinases responsible for phosphorylating Akt at Ser-473 has been a challenge. Akt Ser-473 could be phosphorylated by both mTORC2 and DNA–PKcs [11,12] depending on type of stimuli. Although the main function of DNA–PKcs is to maintain DNA stability, this kinase could also activate targeted downstream events of the PI3K pathway [11,36]. As a matter of fact, DNA–PKcs has long been proposed as a Akt Ser-473 kinase [11,36]. However, the physiological role of DNA–PKcs in the regulation of Akt phosphorylation remains to be established, how DNA–PKcs phosphorylates Akt is not fully understood. It has been showed that DNA–PKcs directly associates and activates Akt in the plasma membrane, causing a 10-fold enhancement of Akt activity [11]. Chu's group, however, demonstrated that DNA–PKcs co-localizes with Akt after CpG-DNA stimulation mainly in the cytosol [12]. A recent study by Tu et al. showed that UV radiation induces DNA–PKcs association with mTORC2 component SIN1 to phosphorylate Akt at Ser-473 [13]. Our results suggest that the latter is likely the case here. We observed that LDI induced DNA–PKcs–SIN1 complexation, inhibition of this complex by the DNA–PKcs inhibitors/knockdown, or by SIN1 knockdown, not only suppressed LDI-induced Akt Ser-473 phosphorylation, but also inhibited osteoblast differentiation. Thus, after LDI, mTORC2 and DNA–PKcs worked together to phosphorylate Akt at Ser-473 kinase, and to promote mouse osteoblast differentiation.

In summary, our results suggest that DNA–PKcs–SIN1 complexation is required for LDI-induced Akt activation and mouse calvarial osteoblast differentiation.

Competing interests

The authors have no conflict of interests.

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