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LYN, a key mediator in estrogen-dependent suppression of osteoclast differentiation, survival, and function

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ABSTRACT

Estrogen insufficiency at menopause cause accelerated bone loss due to unwarranted differentiation and function of osteoclasts. Unraveling the underlying mechanism/s may identify mediators of estrogen action which can be targeted for improved management of osteoporosis. Towards this, we analyzed the effect of 17β-estradiol on the proteomes of differentiating human osteoclasts. The major proteomic changes observed included upregulation of LYN by estrogen. We, therefore, investigated the effect of estrogen on osteoclast differentiation, survival, and function in control and LYN knockdown conditions. In control condition, estrogen treatment increased the apoptosis rate and suppressed the calcium signaling by reducing the intracellular Ca^{2+} levels as well as expression and activation of NFATc1 and c-Src during differentiation, resulting in reduced osteoclastogenesis. These osteoclasts were smaller in size with reduced extent of multinuclearity and produced significantly low levels of bone resorbing enzymes. They also exhibited disrupted sealing zone formation with low podosome density, impaired cell polarization and reduced resorption of dentine slices. Interestingly, in LYN knockdown condition, estrogen failed to induce apoptosis and inhibit activation of NFATc1 and c-Src. Compared to effect of estrogen on osteoclast in control condition, LYN knockdown osteoclasts did not show reduction in production of bone resorbing enzymes and had defined sealing zone formation with high podosome density with no impairment in cell polarization. They resorbed significant area on dentine slices. Thus, the inhibitory action of estrogen on osteoclast was severely restrained in LYN knockdown condition, demonstrating the importance of LYN as a key mediator of the effect of estrogen on osteoclastogenesis.

1. Introduction

Osteoclasts are giant multinucleated bone resorbing cells of hematopoietic origin [1]. The controlled differentiation and activity of these cells is essential for the maintenance of bone homeostasis [2]. Estrogen plays a crucial role in maintaining bone homeostasis majorly by negatively affecting osteoclast differentiation and function [3,4]. Thus, at menopause, estrogen insufficiency triggers unwarranted differentiation and activity of osteoclasts, which causes decreased bone mass leading to an increased risk of osteoporosis [4–7]. First proposed by Fuller Albright in the year 1941, many studies have subsequently affirmed this association, yet the underlying molecular mechanism/s remains enigmatic [8–10]. Investigation of the underlying molecular mechanism/s would, therefore, be useful for the understanding of the pathophysiology of postmenopausal osteoporosis.

Estrogen receptors are expressed by osteoclast lineage throughout the differentiation - from monocytes, the osteoclast precursors (OCPs) to the mature multinucleated bone resorbing osteoclasts [11]. This suggests the occurrence of estrogen-mediated downstream proteomic changes during the differentiation process. The aim of the present study was to identify estrogen-regulated key mediators in the proteome of human osteoclasts during different stages of osteoclastogenesis using differential proteomics approach. Among the estrogen-regulated proteins identified, LYN, a tyrosine kinase having anti-osteoclastogenic property was found to be upregulated in response to estrogen [12,13]. LYN is a member of the Src family of intracellular membrane-associated

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protein tyrosine kinases (SFK) which acts as important signaling intermediaries in processes such as cell survival, proliferation, and differentiation [14–16]. Role of LYN in bone metabolism has been appreciated previously in couple of studies. LYN knockout mice exhibit no change in bone volume and osteoclast number. However, upon stimulation with elevated levels of receptor activator of nuclear factor kappa-B ligand (RANKL), enhanced osteoclast recruitment and subsequently decreased bone density is observed [12]. Further, LYN is reported to be downregulated by RANKL [13]. LYN knockdown in osteoclast precursors, *in vivo*, has a pro-osteoclastogenic effect [13]. A recent study by Arteaga *et al* reported a novel somatic mutation in LYN (D189Y) in estrogen receptor-positive (ER^+) breast cancer [17]. It is an activating mutation which mediates antiestrogen resistance in ER^+ breast cancer. Though the role of LYN in osteoclast biology and various cancers is reported, ours is the first study demonstrating the critical involvement of LYN as a key mediator for estrogen-dependent inhibition of human osteoclast differentiation, survival, and function.

2. Materials and methods

2.1. Osteoclast differentiation

Human peripheral blood $CD14^+$ monocytes (#PCS-800-010™, ATCC®, USA) were seeded in phenol red free minimum essential medium-α (MEM α, #4101029, Gibco™, USA) supplemented with 10% charcoal stripped fetal bovine serum (FBS, #12676029, Gibco™, USA) and 1% penicillin-streptomycin (#15140122, Gibco™, USA) at a cell density of 1.6×10^5 cells/well in a 24 well plate (#CLS3527-100EA, Corning® Costar®, USA). The differentiation was carried out for 14 days using 30 ng/mL of macrophage colony stimulating factor (M-CSF, #PHC9504, Gibco™, USA) and 100 ng/mL of receptor activator of nuclear factor kappa-B ligand (RANKL, #PHP0034, Gibco™, USA) as osteoclast differentiation factors. Fresh medium was replenished every third day.

2.2. Protein preparation, iTRAQ labeling, and protein identification

Osteoclast differentiation was carried out in the presence and absence of 1 nM of 17β-estradiol (E2) (#E2758, Sigma). Differentiating cells at time points of day 1, 5, 10, and 14 were harvested and then lysed in 0.5% SDS. The cell lysate was sonicated at 20% amplitude for 4 pulses of 30 s each. The lysate was then centrifuged at 14,000 ×*g* for 20 min at 4 °C to pellet down cell debris. A total of 8 protein sample groups (supernatants) were generated from the four time points carried out in the presence and absence of E2. From each of 8 sample groups, 50 μg of protein (estimated using Pierce™ BCA protein assay kit, Thermo Scientific™, USA) was used for iTRAQ labeling. Prior to labeling, the samples were reduced using tris (2-carboxyethyl) phosphine (TCEP) and cysteine blocked using methyl methanethiosulfonate (MMTS). The samples were then digested with 1:20 (w/w) sequencing grade trypsin (Promega, USA) at 37 °C for 16 h. These peptides were subjected to 8plex-iTRAQ labeling as per manufacturer's instructions (iTRAQ Reagents Multiplex kit; Applied Biosystems/MDS Sciex, USA). Labeling tag details were as follows: day 1 in absence of E2 (–E2) with 113, day 5 –E2 with 114, day 10 –E2 with 115, day 14 –E2 with 116, day 1 in presence of E2 (+E2) with 117, day 5 +E2 with 118, day 10 +E2 with 119, and day 14 +E2 with 121. All the 8 labeled peptide samples were pooled, lyophilized and pre-fractionated into 10 fractions on Strong Cation Exchange (SCX) chromatography column using Agilent's 1200 Series HPLC system. Technical replicate was generated by labeling and fractionating the same samples twice. The fractions obtained were desalted with C18 ZipTip and subjected to LC-MS/MS analysis. The raw files generated by the Orbitrap Fusion Mass spectrometer (Thermo Scientific™) were analyzed using Proteome Discoverer software version 1.4 (Thermo Scientific™) using Sequest as the search algorithm using human NCBI RefSeq database [18]. Search parameters included trypsin as the enzyme with 1 missed cleavage; precursor and fragment mass tolerance were set to 20 ppm and 0.1 Da, respectively; Methionine oxidation was set as a dynamic modification whereas methylthio modification at cysteine and iTRAQ modification at N-terminus of the peptide and lysines were set as static modifications. High confidence peptide identifications were based on FDR threshold of 1% at the peptide level. Relative quantitation of proteins was generated based on the intensities of reporter ions released during MS/MS fragmentation of peptides.

2.3. LYN knockdown by oligonucleotide siRNA

The 21-nucleotide small interfering Ambion® Silencer® Select siRNA for LYN (#4390824, siRNA ID: s8356) and Ambion® Silencer® Select® Negative Control no. 1 (#4390843) siRNA (non-targeting siRNA) as negative control were used for transfection. For transfection experiments, cells were cultured in phenol red free Opti-MEM™ I medium (#11058021, Gibco™, USA) supplemented with 10% FBS and differentiation factors. For LYN knockdown, cells were transfected with 50 pmoles of LYN siRNA for 6 h using Lipofectamine™ RNAiMAX® (#13778100, Invitrogen, USA) as a transfection reagent. Differentiation was initiated after achieving at least ~75% knockdown in LYN expression, estimated by analyzing LYN protein levels (Supplementary Fig. 1). The transfection was repeated on day 8 of osteoclastogenesis to keep the expression silenced throughout the differentiation.

2.4. RNA isolation, cDNA synthesis, and Real-Time Polymerase Chain reaction

Total RNA was extracted using TRIzol™ (#15596, Invitrogen, USA) and cDNA was synthesized using Superscript IV reverse transcriptase enzyme (#18091050, Invitrogen, USA) from DNase I (#18068015, Invitrogen, USA) treated 1 μg of RNA. Real-Time PCR was performed in CFX96™ Real-Time PCR Detection System (Bio-Rad, USA) using iTaq™ Universal SYBR® Green Supermix (#1725124, Bio-Rad, USA). The amplification was performed for 40 cycles, with the denaturation temperature of 95 °C for 10 s, primer-specific annealing temperature for 20 s and extension at 70 °C for 20 s followed by melt curve analysis to verify primer specificity. Each reaction was prepared in duplicates and 'no template control' was included with each run. Expression values were normalized to GAPDH. The primer sequences, annealing temperature and product size for LYN, cathepsin K (CathK), carbonic anhydrase II (CAII), and GAPDH are as shown in Table 1.

Table 1
Primer sequences used for Real-Time Polymerase Chain reaction.

Primer	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	Annealing temperature	Product size
LYN	GGAAGGTGCTAAGTTCCCTATT	GTCATCACGTCGGCATTAGT	60 °C	162 bp
CathK	AATCAGGGTCAGTGTGGTTC	CTCAGACACACAATCCACTAGG	59 °C	126 bp
CAII	GCCAGTCCCCTGTTGACATC	CTCCCTTGAGCACTGCTTTGT	58 °C	165 bp
GAPDH	GTCAACGGATTTGGTCGTATTG	TGTAGTTGAGGTCAATGAAGGG	60 °C	106 bp

2.5. Immunoblot analysis

Total protein was extracted in Pierce™ RIPA buffer (#89900, Thermo Scientific™, USA) containing a protease/phosphatase inhibitor cocktail (#5872, Cell Signaling Technology®, USA). The samples were sonicated and protein content was estimated using Pierce™ BCA protein assay kit (#23227, Thermo Scientific™, USA). Samples, 20 μg from each were resolved on 10% SDS-PAGE and transferred to Amersham™ Hybond™ Blotting nitrocellulose membrane (#RPN203D, GE Healthcare, USA). Blots were washed in phosphate buffered saline (PBS) and stained with Ponceau S dye (0.1%) to visualize the protein profile. After washing out Ponceau S dye with PBS, the membrane was blocked with 5% skimmed milk for 1 h at room temperature with gentle agitation. In case of probing the blot with antibodies against phosphoproteins, 3% bovine serum albumin (BSA) was used instead of 5% skimmed milk as a blocking agent. Post blocking, the blots were incubated at 4 °C overnight with the primary antibody diluted in 1% skimmed milk (1% BSA for phosphoproteins). The blots were washed with PBS containing 0.1% Tween 20 and incubated with respective secondary antibody diluted in 1% skimmed milk for 1 h at room temperature with gentle agitation. Primary antibody dilutions are detailed in Table 2. Polyclonal rabbit anti-mouse immunoglobulin/HRP (#P0260, Dako, Denmark) and polyclonal goat anti-rabbit immunoglobulins/HRP (#P0448, Dako, Denmark) were used as secondary antibodies at 1:1500 dilution. Amersham™ ECL Prime western blotting detection reagent (#RPN2232, GE Healthcare, USA) was used as a substrate and Amersham™ Hyperfilm ECL (#28906835, GE Healthcare, USA) was used to detect the chemiluminescent signal. The band intensity was quantified by densitometry using Gene Tools (version 3.6.4.0).

2.6. F-actin staining

Osteoclasts, on day 14, were fixed with 4% paraformaldehyde for 15 min at room temperature followed by permeabilization with 0.1% TritonX-100 for 5 min at room temperature. F-actin in fixed cells was labeled using F-actin staining kit-Red Fluorescence-Cytopainter (#ab112127, Abcam, UK). The cells were incubated with 1× Red Fluorescent Phalloidin Conjugate working solution for 1 h at room temperature in dark. Nuclei were stained with 300 nM of 4′, 6-diamidino-2-phenylindole (DAPI) (#D1306, Invitrogen, USA) for 30 min at room temperature in dark. ProLong® Gold Antifade Mountant (#P10144, Molecular Probes, USA) was used to mount the slides and images were captured using a Confocal Laser Scanning Microscope (Carl Zeiss). Osteoclast height was measured as the distance between the basolateral membrane (top of the cell) and the ruffled border (bottom of the cell, facing matrix) using Z stack spacing during image capture. ImageJ tool (version 1.52e) was used to analyze the images [19].

2.7. Tartrate-resistant acid phosphatase (TRAP) staining

Cells were stained for TRAP on day 14 of osteoclastogenesis using Acid phosphatase, leukocyte (TRAP) kit (#387A, Sigma, USA) as per manufacturer's instructions. TRAP positive cells with ≥3 nuclei were considered as osteoclasts. The images captured were used to count the number of nuclei per cell and cell size was measured using ImageJ tool.

Table 2
Primary antibody list with dilutions.

Antibody	Dilution	Catalog no.	Company
Anti-LYN	1:500	ab1890	Abcam
Anti-LYN(Phospho Y507)	1:500	ab33914	Abcam
Anti-NFATc1	1:500	MA3-024	ThermoFisher
Anti-NFATc1(Phospho S172)	1:250	MAB5640	R&D Systems
Anti-c-Src	1:1000	04-772	Merck
Anti-c-Src(Phospho Y416)	1:500	2101S	Cell Signaling Technology
Anti-GAPDH	1:2000	CB1001	Merck

2.8. TRAP estimation

TRAP activity in osteoclast on day 14 was quantified using TRACP and ALP assay kit (#MK301, Takara, Japan) and acid phosphatase (#P1146, Sigma, USA) was used to prepare a standard curve. The assay was performed as per manufacturer's instructions.

2.9. Estimation of intracellular Ca^{2+} levels

The relative levels of intracellular Ca^{2+} across different culture conditions were measured using Fluo-4NW calcium assay kit (#F36206, Molecular Probes™, USA). The assay was performed as per manufacturer's instructions. Values are expressed as fluorescence units (%) relative to control for each independent assay performed.

2.10. Estimation of apoptosis rate

The live cells were detached from the culture vessel by incubating the cells with Accutase® cell detachment solution for 10 min at 37 °C. Approximately 1×10^5 cells were stained with Hoechst 33342 and propidium iodide (PI) using Chromatin condensation/dead cell apoptosis kit (#V13244, Molecular Probes™, USA), as per manufacturer's instructions. DAPI and Yellow/Green laser were used for Hoechst 33342 and PI detection respectively. The cells were run on BD FACSARIA III (BD Biosciences, USA) and FACSDiva (version 6.1.3) was used for analysis.

2.11. Pit formation assay

Monocytes (2.5×10^4 cells/well) were seeded on dentine slice placed in 96 well plate. On day 14, the osteoclasts adhered to dentine slice were removed by sonicating the slices in distilled water for 5 min at 20% amplitude. The slices were dried and stained with 1% toluidine blue prepared in 1% sodium borate for 5 min. The excess stain was removed by washing the slices with distilled water. The dried dentine slices were examined under a light microscope. The area of the stained resorbed pit was measured using ImageJ tool.

2.12. Statistical analysis

Each data point represents mean ± SD from three independent cultures. Statistical significance was determined using 1-way or 2-way analysis of variance (ANOVA) performed using GraphPad Prism software (version 8). The *p*-value < 0.05 was taken as a level of significance.

3. Results

3.1. Protein identification and gene ontology analysis

Estrogen-regulated proteins were identified at different stages of osteoclast differentiation (day 1, 5, 10 and 14) using iTRAQ coupled to LC-MS/MS, in replicate runs. A total of 1639 proteins in replicate 1 and 1624 proteins in replicate 2 with ≥2 unique peptides were considered as reliable identifications. A total of 1358 proteins were common in both the replicates. The differential regulation of these proteins across different days of osteoclastogenesis in response to estrogen is summarized in the form of a Heatmap created using the Graphical Proteomics Data Explorer, GProX (version 1.1.13) (Fig. 1A). Expression of bone resorbing enzymes TRAP (isoform 5; having one unique peptide) and cathepsins (B, D, K, S, and Z; having ≥2 unique peptides), which are hallmarks of osteoclast differentiation, were detected and found to be downregulated on day 14 under estrogen effect. Of the 1358 common proteins, a total of 521 non-redundant proteins had ≥1.5 fold change, at any one or multiple stages of osteoclast differentiation (shown in Supplementary Table 1). Among these, we observed a decrease in expression of V-type proton ATPase subunit d 2, ATP6V0D2, on day 10 and 14 under estrogen effect. ATP6V0D2 is required for extracellular acidification for bone resorption by mature osteoclasts [20]. Further, estrogen decreased the expression of a set of eukaryotic translation initiation factor subunits (EIF2S3, EIF3I, EIF3J, and EIF3K) on day 10 and 14 of osteoclast differentiation. Osteoclast differentiation is accompanied by increased cytoplasmic growth, which requires an increase in translational activity within a cell. When differentially regulated proteins were classified according to their biological function using PANTHER Gene Ontology classification system, cellular process class of proteins comprised 30.7% of the differentially regulated proteins. In cellular processes subclass, 51.4% belonged to cell communication class, of which, 85.7% were involved in signal transduction (Fig. 1B). Among these proteins, LYN, a tyrosine-protein kinase was observed to be significantly upregulated (1.6 fold) in presence of estrogen on day 1 of osteoclastogenesis (Fig. 1C).

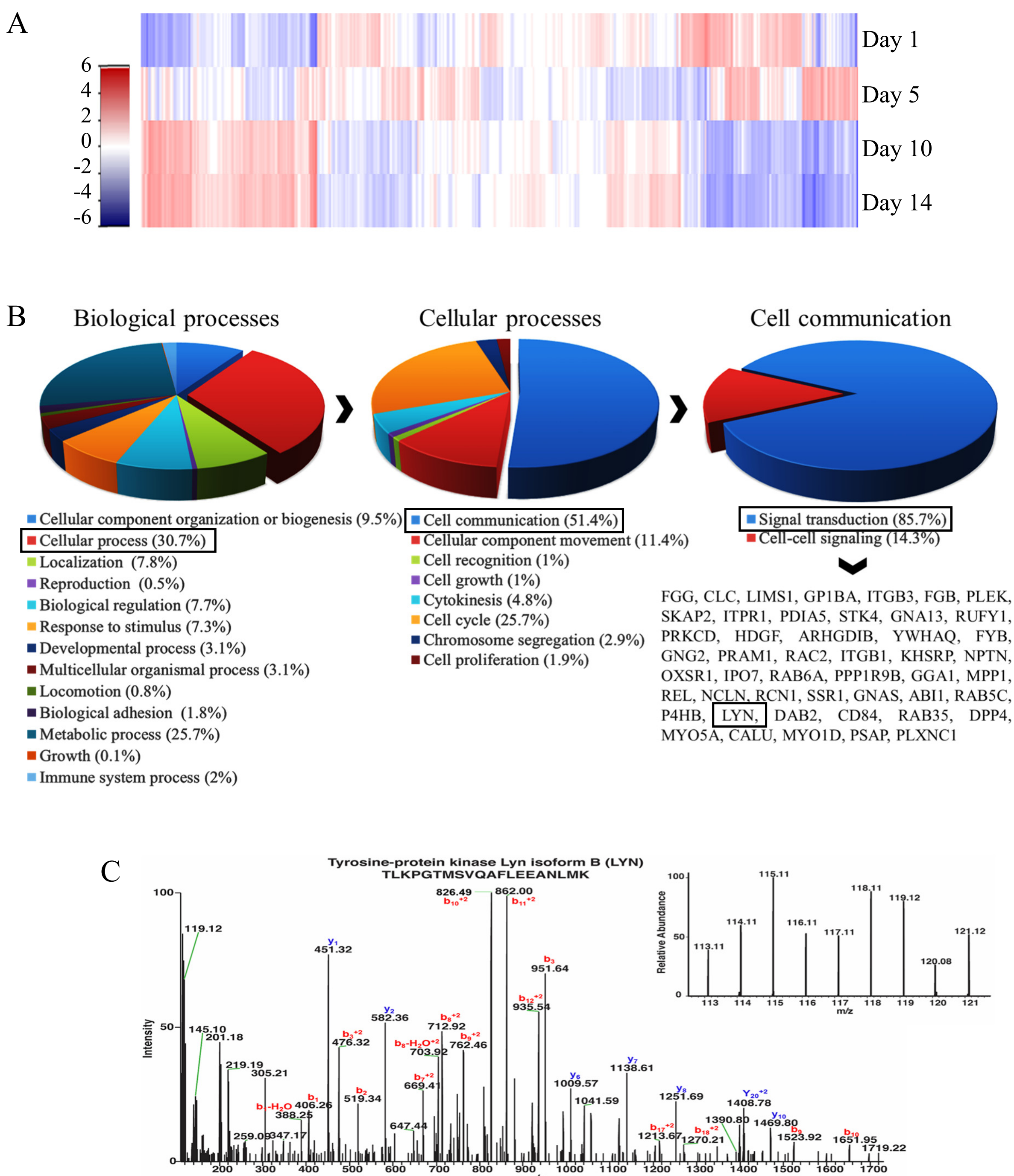

Fig. 1. (A) Graphical overview in the form of a Heatmap of proteins (1358 proteins) identified to visualize their differential expression using GProX. (B) Differentially regulated proteins (521 proteins) classified using PANTHER classification system according to biological processes. (C) Representative MS/MS spectrum of a representative unique peptide of LYN. Inset shows iTRAQ reporter ion intensities for the shown peptide.

3.2. LYN expression and activation is upregulated by estrogen

The observed increase in expression of LYN in response to estrogen during osteoclastogenesis was verified by analyzing its transcript and protein levels on day 0, 1, 5, 10, and 14 of differentiation carried out in the presence and absence of 1 nM of 17β-estradiol. Day 0 represents human CD14^{+} monocytes, the osteoclast precursors, cultured in presence of M-CSF (30 ng/mL) alone. LYN transcript (Fig. 2A) and protein levels (Fig. 2B, C) reduced in the control cells undergoing osteoclastogenesis as compared to levels observed on day 0. Consistent with the mass spectrometry data, we observed that estrogen significantly upregulated LYN protein levels (Fig. 2B, C) when compared to the levels in control cells on respective days. Further, LYN transcript levels also increased during osteoclastogenesis in presence of estrogen (Fig. 2A). As dephosphorylation of LYN at Y507 is known to activate LYN by releasing its kinase domain from the inhibitory configuration, we checked the phosphorylation status of LYN at C-terminal tyrosine (Y507) site [21]. Phosphorylation of LYN at Y507 increased significantly in the control cells during differentiation, whereas estrogen significantly reduced it (Fig. 2B, D) indicating an increase in catalytic activation of LYN in presence of estrogen. Further, analysis of LYN promoter sequence (given in Supplementary Fig. 2) using TFBIND tool showed the presence of estrogen response element (ERE) 5′-NNARGNNANNNTG-ACCYNN-3′ (TRANSFAC matrix ID: V$ER_Q6), which matches annotation for ERα [22]. The similarity score obtained was 0.730751 (cutoff = 0.73) and is positioned at 159 on positive strand of the promoter sequence. Estrogen is reported to negatively affect osteoclast differentiation and function through ERα [11,23]. This suggests a probable direct genomic regulation of LYN expression by 17β-estradiol through binding of ERα to ERE present in LYN promoter sequence.

3.3. Estrogen negatively affects calcium signaling in osteoclasts via LYN

Intracellular calcium signaling is crucial for osteoclast differentiation and function [24,25]. Therefore, we investigated the functional significance of LYN in calcium signaling during osteoclastogenesis by LYN knockdown. The possible role of LYN as a mediator of the effect of estrogen on osteoclastogenesis was inspected by carrying out osteoclast differentiation in four different culture conditions, Condition I (C-E2): monocytes transfected with non-targeting siRNA and cultured in the absence of E2, Condition II (C + E2): monocytes transfected with non-targeting siRNA and cultured in the presence of E2, Condition III (siLYN-E2): monocytes transfected with LYN siRNA and cultured in the absence of E2, Condition IV (siLYN + E2): monocytes transfected with LYN siRNA and cultured in the presence of E2.

Total intracellular Ca^{2+} levels were measured in terms of relative fluorescence using Fluo-4NW dye on day 1, 5, 10, and 14 of osteoclastogenesis (Fig. 3A). Across the differentiation process, estrogen treatment significantly reduced the levels in ‘C + E2’ and ‘siLYN + E2’ cells relative to the levels observed in ‘C-E2’ cells. However, levels in ‘siLYN + E2’ cells were significantly high relative to levels in ‘C + E2’ cells. In ‘siLYN-E2’ cells, a significant rise in levels was observed relative to levels in ‘C-E2’ cells.

Further, expression and activation status of NFATc1 was analyzed as it is a key player involved in calcium signaling and osteoclast differentiation [26,27]. During osteoclast differentiation, the expression and activation (observed as dephosphorylation at S172) of NFATc1 was significantly reduced in ‘C + E2’ and ‘siLYN + E2’ cells when compared to ‘C-E2’ cells (Fig. 3B, C). However, the activated levels of NFATc1 were significantly high in ‘siLYN + E2’ cells as compared to ‘C + E2’ cells. The ‘siLYN-E2’ cells showed no significant change in expression of NFTAc1, yet, its activation levels were significantly high when compared to ‘C-E2’ cells. Further, the expression and activation (observed as phosphorylation at Y416) of c-Src was also analyzed as it is reported to be involved in calcium signaling and is crucial for osteoclast differentiation [28–30]. The expression of c-Src significantly reduced in ‘C + E2’ and ‘siLYN + E2’ cells when compared to ‘C-E2’ cells during differentiation. However, the expression in ‘siLYN + E2’ cells was significantly higher than in ‘C + E2’ cells. The ‘siLYN-E2’ cells showed a significant increase in expression of c-Src as compared to ‘C-E2’ cells. On analyzing the levels of activated c-Src, a significant decrease was observed in ‘C + E2’ cells, however, no reduction was seen in ‘siLYN + E2’ cells when compared ‘C-E2’ cells (Fig. 3B, D).

3.4. LYN participates in estrogen-induced apoptosis during osteoclastogenesis

Estrogen induces apoptosis in mature osteoclasts [3,31]. We, therefore, investigated the role of LYN in mediating the apoptosis-inducing effect of estrogen on osteoclast during differentiation. The ‘C + E2’ and ‘siLYN + E2’ cells showed significant high apoptosis rate as compared to basal apoptosis rate observed in ‘C-E2’ cells during

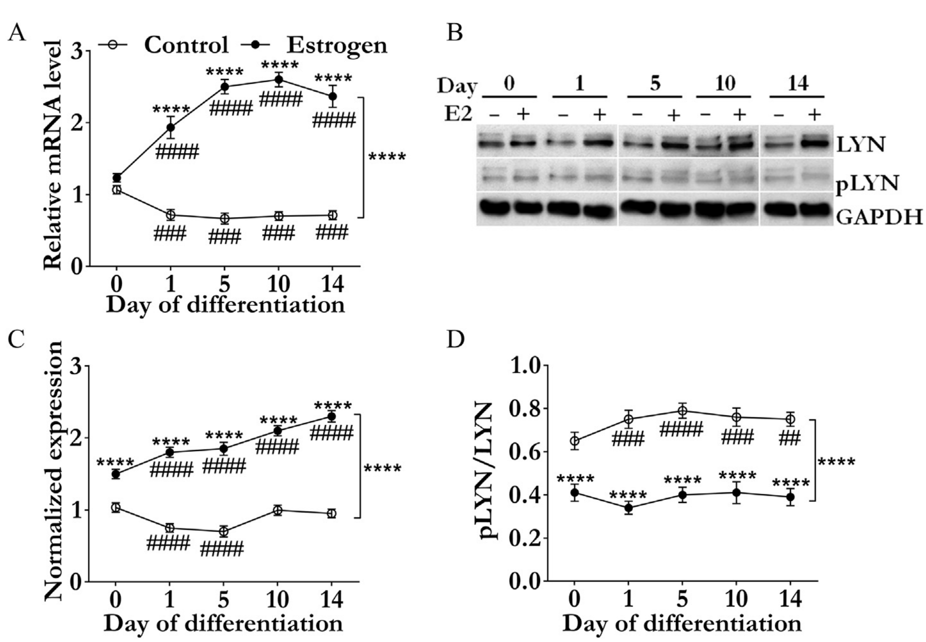

Fig. 2. *Estrogen upregulates LYN expression and activation.* (A) Normalized expression of LYN transcript. (B) Representative western blot image for expression of LYN, phospho LYN-Y507 (pLYN) and GAPDH as housekeeping. (C) Normalized expression of LYN. (D) pLYN/LYN. (Statistical analysis performed with 2-way ANOVA. Dunnett's post-test: ####$p < 0.0001$, ###$p < 0.001$, ##$p < 0.01$ *versus* day 0 of ‘Control’. Sidak's post-test: ****$p < 0.0001$ *versus* ‘Control’ of respective day).

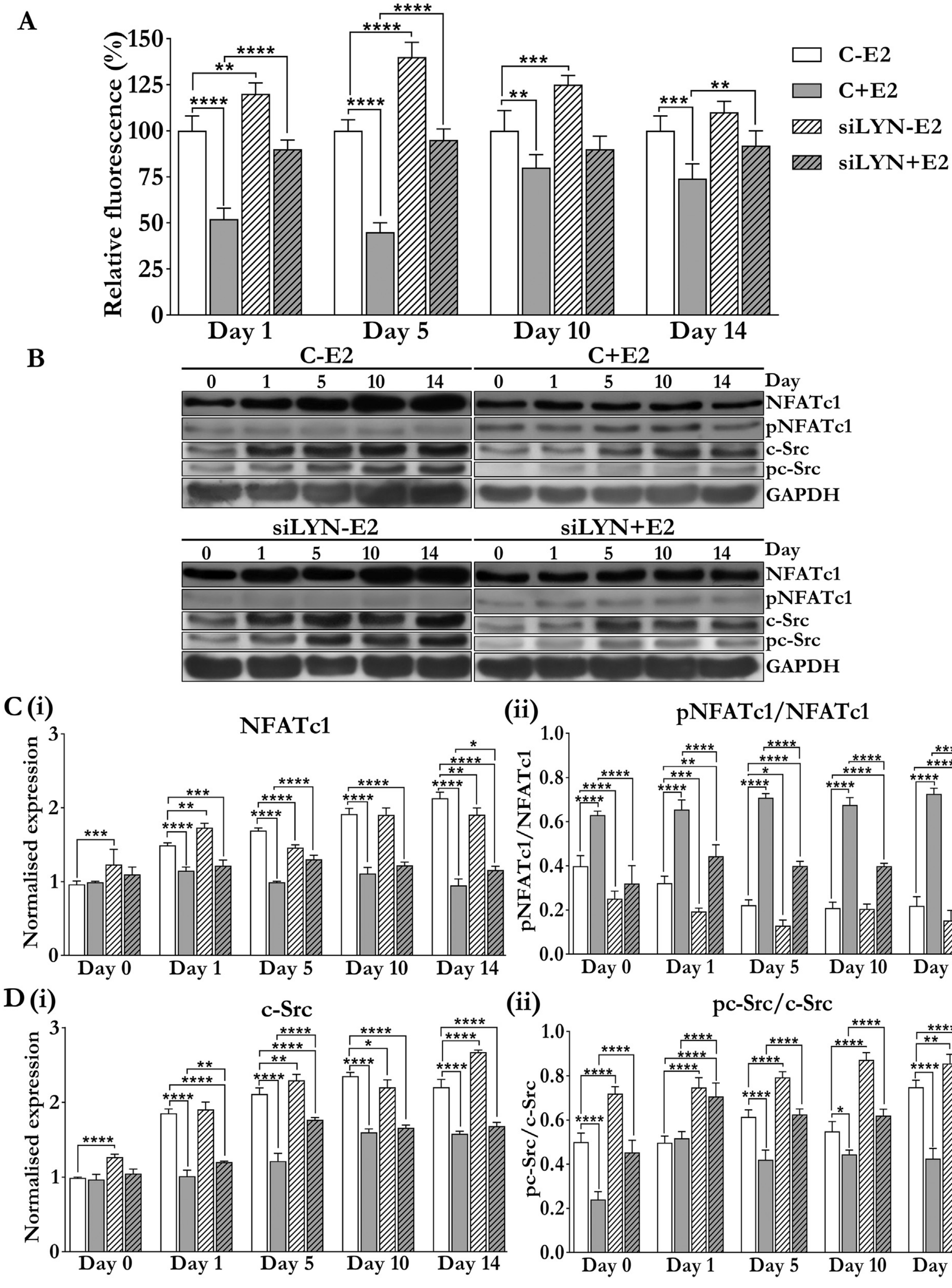

Fig. 3. *Suppressive effect of estrogen on calcium signaling is hampered in LYN knockdown condition during osteoclastogenesis.* (A) Intracellular Ca^{2+} levels measured as relative fluorescence using Fluo-4NW dye. (B) Representative western blot image for expression of NFATc1, phospho NFATc1-S172 (pNFATc1), c-Src, phospho c-Src-Y416 (pc-Src) and GAPDH. (C) Normalized expression of NFATc1 (i) and pNFATc1/NFATc1 ratio (ii). (D) Normalized expression of c-Src (i) and pc-Src/c-Src ratio (ii). (Statistical analysis performed with 2-way ANOVA with Tukey's post-test. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

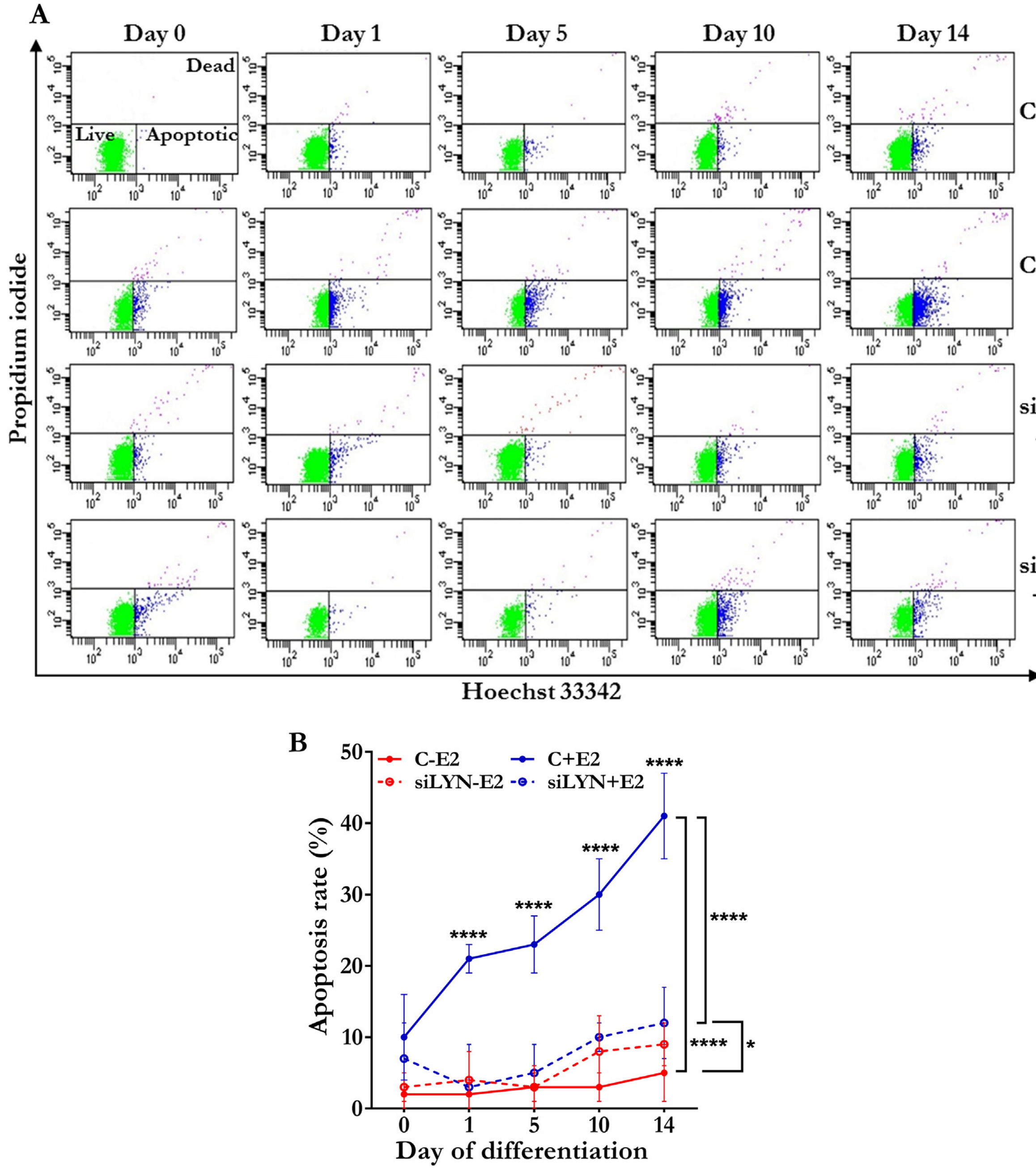

Fig. 4. LYN is involved in estrogen-induced apoptosis during osteoclastogenesis. (A) Representative scatter plots of cells stained with Hoechst 33342 and Propidium iodide. (B) Apoptosis rate (%). (Statistical analysis performed with 2-way ANOVA with Tukey's post-test. ****$p < 0.0001$, *$p < 0.05$).

osteoclastogenesis (Fig. 4A, B). Yet, the apoptosis rate in 'siLYN + E2' cells was significantly low when compared to that observed in 'C + E2' cells.

3.5. LYN is required for the estrogen-dependent decrease in osteoclast formation

After demonstrating the involvement of LYN in calcium signaling and apoptosis induction to mediate the effect of estrogen during osteoclastogenesis, we then analyzed the number, extent of multinuclearity and the size of osteoclasts formed at the end of the differentiation. On day 14 of differentiation, cells were stained for tartrate-resistant acid phosphatase (TRAP) which is a major bone resorbing enzyme produced by osteoclasts and counterstained with hematoxylin to visualize their nuclei (Fig. 5A). TRAP positive multinucleated cells (TRAP$^+$ MNCs) with ≥3 nuclei were considered as osteoclasts. It was observed that the number of osteoclasts (TRAP$^+$ MNCs with ≥3 nuclei per cell) formed per well under 'C + E2' and 'siLYN + E2' conditions were significantly less when compared to the number of osteoclasts formed under 'C-E2' condition. However, the osteoclasts formed per well under 'siLYN + E2' condition were significantly more in number when compared to 'C + E2' condition. Under 'siLYN-E2' conditions, a significantly high number of osteoclasts were observed as compared to 'C-E2' condition (Fig. 5B). Further, the extent of multinuclearity, which is indicative of the extent of osteoclast precursor fusion, was analyzed by scoring the number of osteoclasts with ≥10 nuclei and ≥30 nuclei per cell per well. The extent of multinuclearity was significantly low in both, 'C + E2' and 'siLYN + E2' osteoclasts, when compared to that observed in 'C-E2' osteoclasts. However, 'siLYN + E2' osteoclasts showed significantly higher extent of multinuclearity as compared to 'C + E2'

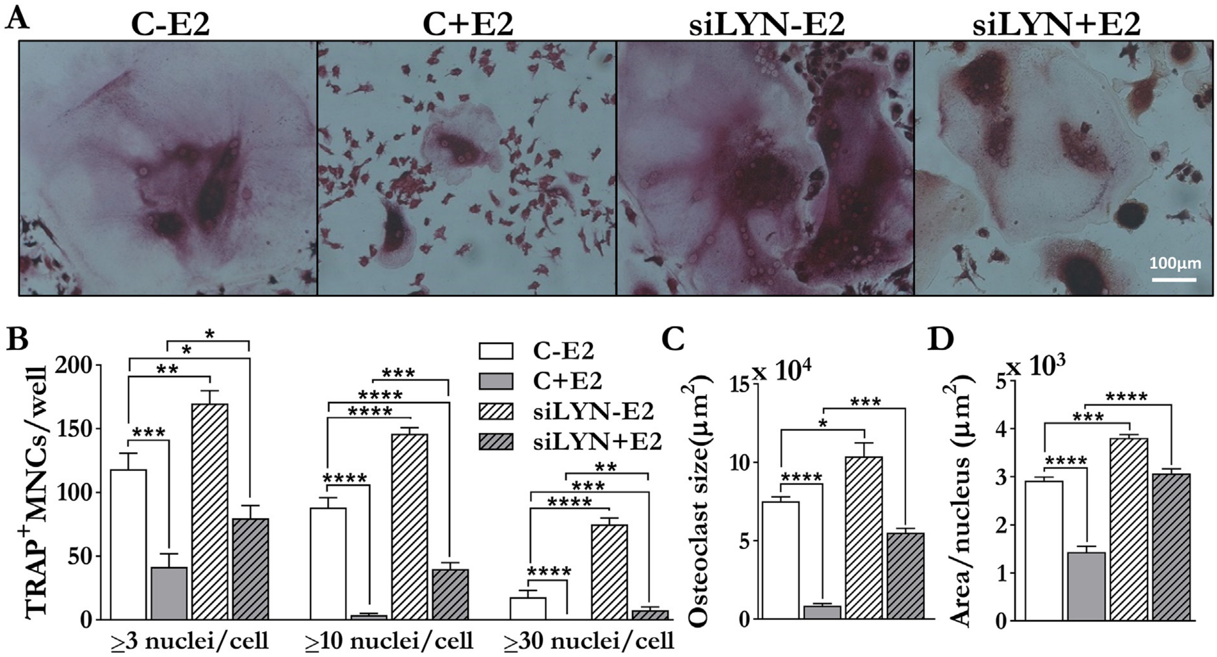

Fig. 5. *LYN is required by estrogen for effective suppression of osteoclastogenesis.* (A) Representative images of cells on day 14 of differentiation stained for TRAP enzyme. (B) Quantitation of TRAP positive multinucleated cells (TRAP+ MNCs) with ≥3 nuclei, ≥10 nuclei and ≥30 nuclei per cell per well. (C) Size of $TRAP^+$ MNCs. (D) Cytoplasmic growth (area/nucleus). (Statistical analysis performed with 1-way ANOVA with Tukey's post-test. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

osteoclasts. The 'siLYN-E2' osteoclasts showed significantly high extent of multinuclearity, with many of the osteoclasts having ≥30 nuclei per cell. On measuring the osteoclast size, we observed a distinct decrease in the size of 'C+E2' osteoclasts as compared to 'C-E2' osteoclasts (Fig. 5C). However, the cell size in 'siLYN+E2' showed no significant reduction when compared to 'C-E2' osteoclasts. The 'siLYN-E2' osteoclasts were significantly larger in size as compared to 'C-E2' osteoclasts. Further, we also analyzed the cytoplasmic growth of the osteoclasts formed, which can be indicated in terms of cell area per nucleus [32]. Estrogen significantly decreased the cytoplasmic growth in 'C+E2' osteoclasts but not in 'siLYN+E2' osteoclasts when compared to 'C-E2' osteoclasts (Fig. 5D). The 'siLYN-E2' osteoclast showed a significantly high cytoplasmic growth when compared to 'C-E2' osteoclasts.

3.6. Inhibitory effect of estrogen on the bone resorption potential of osteoclasts is hampered in LYN knockdown condition

The effect of LYN knockdown on mediating the effect of estrogen on osteoclast function was investigated by analyzing the bone resorption potential of osteoclasts. It was assessed by analyzing the expression levels of bone resorbing enzymes, F-actin ring formation, and area resorbed by osteoclasts when differentiated on dentine slices. TRAP, cathepsin K, and carbonic anhydrase II (CAII) are the major bone resorbing enzyme secreted by osteoclasts. The 'C+E2' osteoclasts showed a significant reduction in expression of TRAP, cathepsin K, and CAII when compared to 'C-E2' osteoclasts (Fig. 6A). In 'siLYN+E2' osteoclasts, no significant reduction in expression of all the three enzymes was observed as compared to 'C-E2' osteoclasts. The 'siLYN-E2' osteoclasts showed a significant increase in the expression of all these enzymes when compared to 'C-E2' osteoclasts.

Next, we examined the F-actin ring formation and podosome density by staining the osteoclasts for F-actin with phalloidin. The 'C+E2' osteoclasts displayed a disrupted F-actin ring formation and a significant reduction in podosome density when compared to 'C-E2' osteoclasts (Fig. 6B, C). Interestingly, 'siLYN+E2' osteoclasts displayed a defined F-actin ring formation with significantly higher podosome density when compared to either 'C-E2' osteoclasts or 'C+E2' osteoclasts. Similarly, 'siLYN-E2' osteoclasts showed defined F-actin ring formation with significantly higher podosome density when compared to 'C-E2' osteoclasts.

Furthermore, osteoclast height, which serves as an index of osteoclast polarization was measured using Z stack spacing during image capture [33]. A significant decrease in cell height was observed in 'C+E2' osteoclasts but not in 'siLYN+E2' osteoclasts when compared to 'C-E2' osteoclasts (Fig. 6D). The 'siLYN-E2' osteoclasts showed a significant increase in cell height when compared to 'C-E2' osteoclasts. Lastly, we measured the area of resorption pits formed due to resorption of the surface of dentine slices by osteoclasts. A significant decrease in area resorbed by 'C+E2' osteoclasts was observed, whereas 'siLYN+E2' osteoclasts showed no significant difference when compared to area resorbed by 'C-E2' osteoclasts (Fig. 6E, F). The 'siLYN-E2' osteoclasts resorbed significantly larger area on dentine slices when compared to 'C-E2' osteoclasts.

4. Discussion

Estrogen is a potent positive regulator of bone mass and its insufficiency elicits unwarranted differentiation and activity of osteoclasts, causing excessive bone resorption and thus leads to increased risk of osteoporosis observed in postmenopausal women [5–7]. However, the precise mechanism through which estrogen regulates osteoclast differentiation and activity is not well understood. Towards this, we differentiated human primary monocytes into osteoclasts in the presence and absence of estrogen and identified LYN, a member of the Src family of non-receptor tyrosine kinase (SFK) to be significantly upregulated on day 1 of osteoclastogenesis in response to estrogen. To the best of our knowledge, this is the first study reporting the use of human primary monocytes for the identification of estrogen-regulated proteins simultaneously during different stages of osteoclastogenesis in a single experimental setup. LYN is expressed in many tissues where it is involved in various signaling pathways regulating differentiation, proliferation, migration, and metabolism [14,34]. Besides, it has an immense potential to influence immune cell signaling and its dysregulation is associated with many leukemia phenotypes including acute

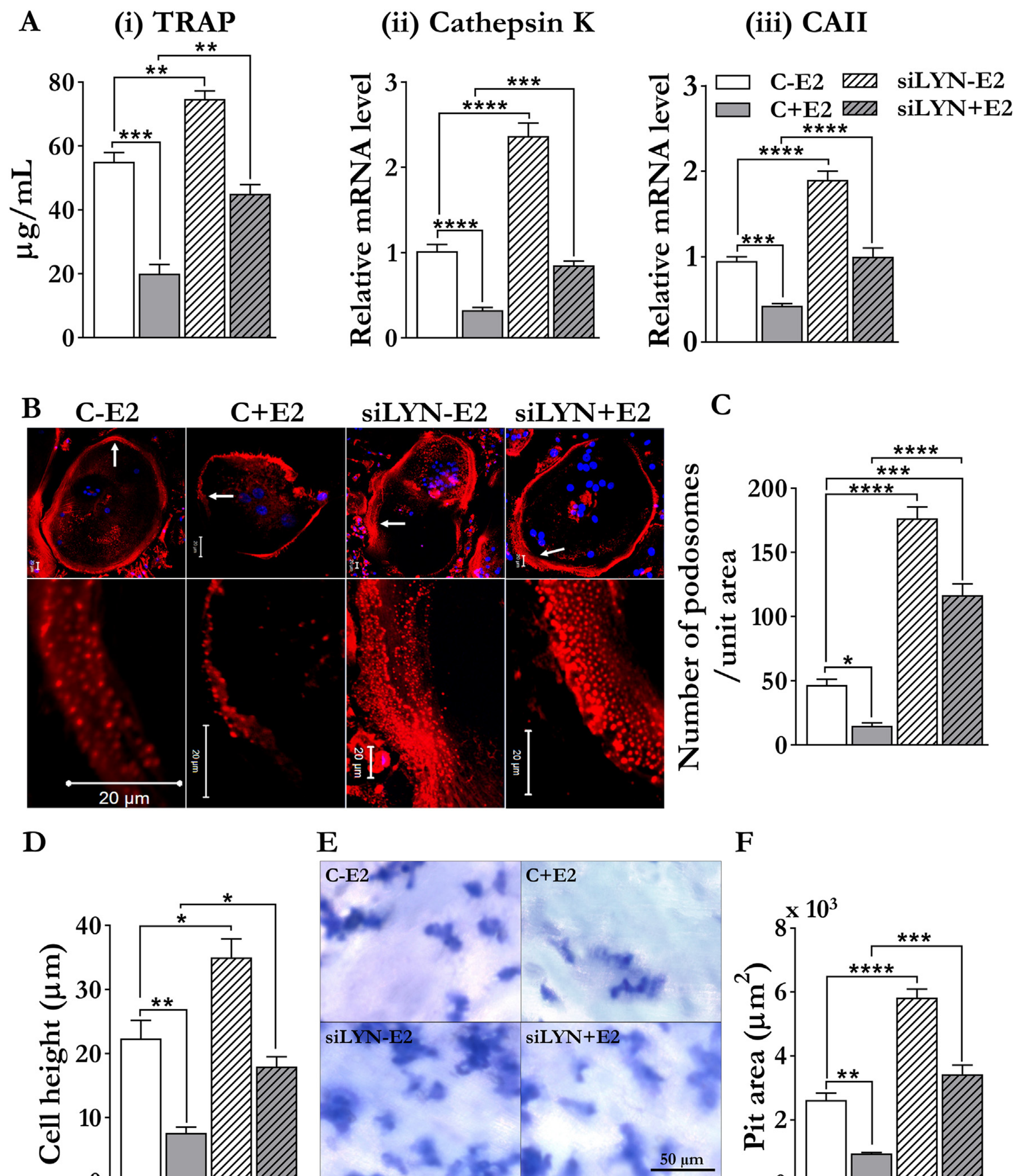

Fig. 6. *Osteoclast differentiation and function is enhanced and osteoclasts are protected from the inhibitory effect of estrogen in LYN knockdown condition.* (A) Expression of bone resorbing enzymes estimated on day 14 of osteoclastogenesis: (i) Quantitation of TRAP (ii) Normalized expression of cathepsin K and (iii) Carbonic anhydrase II. (B) Representative images of osteoclasts stained with phalloidin (for F-actin) and DAPI (for nuclei). White arrow points to the representative area magnified (lower panel) to score the number of podosomes per unit area. (C) Quantification of the number of podosomes per unit area. (D) Osteoclast height. (E) Representative images of the stained resorption pits formed by osteoclasts on dentine slices. (F) Area resorbed. (Statistical analysis performed with 1-way ANOVA with Tukey's post-test. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

myeloid leukemia, chronic myeloid leukemia and B-cell lymphocytic leukemia [35–37]. It is also associated with certain autoimmune diseases such as systemic lupus erythematosus and various solid tumors including glioblastoma, prostate and certain aggressive subtypes of breast cancer [38–41]. Therefore, currently LYN is emerging as a potential therapeutic target in many diseases. The inhibitory role of LYN in osteoclast biology is reported recently [12,13]. However, this is the first study reporting the role of LYN as an important signaling intermediary for estrogen in osteoclast biology.

Yoon *et al* reported that LYN transcript and protein levels reduced in mouse bone marrow-derived macrophages (BMMs) 24 h after stimulation with RANKL [13]. They also observed decreased levels of activated LYN in these cells. Similar to their findings, we also observed a decrease in transcript and protein levels with a reduction in its activated levels throughout osteoclastogenesis. Interestingly, monocytes, the osteoclast precursors, cultured in presence of M-CSF showed upregulation of LYN expression and activity in response to estrogen. This shows that estrogen is able to upregulate LYN in osteoclast precursors prior to their commitment to the osteoclast lineage. Further, we found the estrogen response element in LYN promoter sequence, which matches annotation for ERα, suggesting a probable direct genomic regulation of LYN transcription by estrogen.

Calcium signaling is essential for osteoclastogenesis, and NFATc1 and c-Src are key players involved in the same. Yoon *et al* reported increased intracellular Ca^{2+} concentration and expression of NFATc1 in LYN silenced mouse BMMs after incubation with M-CSF and RANKL for 2 days [13]. They also reported an increase in expression and activation of c-Src in LYN silenced BMMs. Corroborative to their findings, we also observed increased intracellular Ca^{2+} levels in LYN knockdown cells throughout osteoclastogenesis and increased activation of NFATc1, and increased expression and activation of c-Src. Estrogen suppressed calcium signaling during osteoclastogenesis by reducing the intracellular Ca^{2+} levels, expression and activation of both, NFATc1 and c-Src in cells transfected with non-targeting siRNA. However, in LYN knockdown cells, estrogen failed to significantly inhibit the activation of NFATc1 and c-Src, thus its suppressive effect on calcium signaling was hampered, suggesting that estrogen may inhibit the expression of NFATc1 and c-Src in LYN knockdown cells, but expression and activation of LYN is required for estrogen-dependent inhibition of their activation.

We further studied the role of LYN in mediating estrogen-induced apoptosis during osteoclastogenesis. We observed that estrogen induced apoptosis not only in mature osteoclasts (day 14) but also during osteoclastogenesis in cells transfected with non-targeting siRNA. Interestingly, LYN knockdown appeared to protect osteoclasts from apoptosis-inducing effect of estrogen.

Osteoclasts are giant multinucleated cells formed by the fusion of monocytes. Osteoclasts with a higher number of nuclei have high resorption potential and are capable to substantially increase in size [42]. These multinucleated osteoclasts can resorb larger area due to their large expanse and intense production of bone resorbing enzymes. We observed that estrogen significantly reduced the number of osteoclasts formed and the osteoclasts thus formed were significantly smaller in size with a lesser number of nuclei and cytoplasmic growth. Whereas, LYN knockdown cells not only differentiated into giant cells with more number of nuclei but also exhibited a significant increase in cytoplasmic growth, suggesting the increased fusion of monocytes during differentiation and also increased transcriptional activity of the nuclei in LYN knockdown osteoclasts. However, the inhibitory effect of estrogen on osteoclast number, size, and extent of multinuclearity was significantly inadequate in LYN knockdown osteoclasts. Further, estrogen significantly reduced the expression of TRAP, cathepsin K, and CAII, which are major bone resorbing enzymes produced by osteoclasts. In LYN knockdown osteoclasts, no reduction in expression of these enzymes was observed in presence of estrogen.

Osteoclasts on attachment to bone surface or culture vessel reorganize their actin cytoskeleton to form a sealing zone, wherein they create an acidic environment and secrete bone resorbing enzymes. They also form integrin-based and F-actin rich unique cell adhesion structures called podosomes which are crucial for cell adhesion, spreading, migration, and thus for bone resorption [43]. Estrogen disrupted the actin ring formation and also decreased the podosome density in osteoclasts transfected with non-targeting siRNA. Moreover, these osteoclasts displayed defective polarization. Kim *et al* reported that $LYN^{-/-}$ osteoclast culture has more number of cells with defined actin ring upon stimulation with elevated levels of RANKL [12]. They also report an increase in the number of resorption pits formed by these osteoclasts on dentine slices. Corroborative to their findings, we also observed LYN knockdown osteoclasts with proper actin ring formation and resorbed larger area on dentine slices. Interestingly, LYN knockdown osteoclasts differentiated in presence of estrogen showed defined actin ring formation with significantly high podosome density and showed no impaired polarization and no reduction in area resorbed on dentine slices as compared to osteoclasts transfected with non-targeting siRNA differentiated in presence of estrogen. This suggests that LYN knockdown has a pro-osteoclastogenic effect and protects osteoclasts from the inhibitory effect of estrogen.

To summarize, we show that expression and activity of LYN, a negative regulator of osteoclastogenesis, is upregulated by estrogen and is required for an estrogen-dependent phenomena which includes decelerating osteoclastogenesis and reducing bone resorption potential of osteoclasts. Further, we have conclusively elucidated that LYN is required for estrogen-dependent effective inhibition of the activation of NFATc1 and c-Src for suppression of calcium signaling during osteoclastogenesis. In addition, we showed that in LYN knockdown osteoclasts, estrogen failed to induce apoptosis and also reduce the production of bone resorbing enzymes. Moreover, these cells were protected from disruption of sealing zone formation and showed high podosome density, enabling them to migrate, spread and attach more efficiently on the bone surface. These osteoclasts resorbed abundant area on dentine slices. Thus, this study provides the first line of evidence of the role of LYN as a significant signaling mediator of the inhibitory effect of estrogen on human osteoclast differentiation, survival, and function. Future *in vivo* studies are required to analyze the effect of estrogen on bone mass in LYN knockout animal models which may produce corroborative findings to support and broaden the understanding of this link. Consequently, it may pave the path towards the development of novel therapeutic strategies for increasing the expression and/or activation of LYN during osteoclastogenesis for improved management of bone resorptive pathology.

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Conflicts of interest

The authors report no conflicts of interest.

Authors' roles

MIK and SG designed the study and drafted the manuscript. SG performed all the experiments and interpreted data. MW trained SG in osteoclast culture. iTRAQ experiments were designed by MIK, SG, RS, MG, BD. MG performed mass spectrometry and helped in iTRAQ data analysis. MIK, RS, MW, MG extensively modified the manuscript. All the authors read and commented on the manuscript.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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