ARTICLE

Journal of Cellular Biochemistry 115:2208–2217 (2014)

Journal of Cellular Biochemistry

Role of Runx2 in Crosstalk Between Mek/Erk and PI3K/Akt Signaling in MCF-10A Cells

Manish Tandon, Zujian Chen, and Jitesh Pratap*

Department of Anatomy and Cell Biology, Rush University Medical Center, Chicago, Illinois

ABSTRACT

Crosstalk among mitogen-activated protein kinase (MAPK) and phosphatidyl inositol 3′ kinase (PI3K) signaling pathways integrates extracellular cues to regulate mammary epithelial cell growth, proliferation, differentiation, and survival. The runt-related transcription factor, Runx2, is expressed in normal mammary epithelium and promotes differentiation, however, its function in regulation of the MAPK and PI3K signaling crosstalk is not known. We determined the function of Runx2 expression in growth factor-mediated phosphorylation of Erk1/2 and Akt, key downstream kinases in MAPK and PI3K pathway crosstalk in MCF-10A mammary epithelial cells. The Runx2-mediated alterations in cell signaling and associated changes in phenotype were determined by real-time quantitative PCR, Western blotting, immunofluorescence, and flow cytometry approaches. The results revealed that ectopic Runx2 expression differentially downregulates the growth factor (EGF vs. IGF or insulin)-induced pErk1/2 and pAkt levels. Additionally, the ectopic Runx2 expression increases FOXO1 levels, cell cycle G1 stage and promotes survival of MCF-10A cells. Furthermore, we demonstrate that Runx2 expression increases EGF-induced phosphorylation of epidermal growth factor receptor (pEGFR) and relieves Mek/Erk-mediated negative regulation of pEGFR and pAkt levels. Altogether, our results identify functions of Runx2 in MAPK and PI3K signaling crosstalk in MCF-10A cells that could be critical in understanding the mammary epithelial cell growth and survival. J. Cell. Biochem. 115: 2208–2217, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: RUNX2; SIGNALING CROSSTALK; EGFR; MAPK; ERK; PI3K; AKT

The growth factor-induced cell signaling pathways in tissue microenvironment are critical for regulation of normal growth, motility, differentiation, and apoptosis of mammary epithelium [Collins et al., 2005; Worster et al., 2012]. In response to growth factors (EGF, IGF, or insulin) stimulation, receptor tyrosine kinases (EGFR/IGF-1R/IR) activates signaling pathways including MAPK (Mek/Erk) and PI3K (PI3K/Akt) to regulate gene expression [Lemmon and Schlessinger, 2010; Aksamitiene et al., 2012]. Several studies in mammary epithelial cells show critical biological functions of growth factor-mediated Erk and Akt activation in driving mammary gland morphogenesis and phenotypic effects [Debnath et al., 2003; Grassian et al., 2011; Tarcic et al., 2012]. Furthermore, a dynamic crosstalk between these two pathways, as indicated by pharmacological Erk inhibition potently inducing pAkt and pEGFR levels, suggests integrating mechanisms in growth factor-induced cell signaling response [Gopal et al., 2010; Ebi et al., 2011; Turke et al., 2012]. The extent and duration of signaling is also affected by combination of feedback regulatory and transcriptional mechanisms, however, the integration of the master transcription factors in cell signaling crosstalk remains unclear. In this study, we examined the role of the runt-related transcription factor (Runx2) in growth factor-induced MAPK and PI3K signaling crosstalk in non-tumorigenic mammary epithelial MCF-10A cells.

The Runx2 is indispensable for normal bone development [Choi et al., 2001] and expressed in normal mammary epithelium [Shore, 2005]. The studies in animal models or three dimensional mammary epithelial culture indicate regulatory function of Runx2 in mammary epithelial cell differentiation [Shore, 2005; Ferrari et al., 2013]. Recent studies in animal models showed that the ectopic Runx2 expression in mammary epithelium restricts the development of pubertal glands, compromises alveolar development and causes age-related pre-cancerous alterations [McDonald et al., 2014]. In three dimensional culture model, ectopic Runx2 expression disrupts acinar structures [Pratap et al., 2009]. In invasive mammary epithelium, Runx2 is critical for activating PI3K/Akt signaling [Tandon et al., 2014], however, its function in the Mek/Erk and PI3K/Akt signaling crosstalk in normal mammary epithelium is not known.

In this study, we determined the Runx2-dependent crosstalk of EGFR and downstream Mek/Erk or PI3K/Akt signaling pathways.

Grant sponsor: Avon Foundation; Grant number: 02-2010-037; Grant sponsor: Bears Care Foundation.

*Correspondence to: Jitesh Pratap, Department of Anatomy and Cell Biology, Rush University Medical Center, Armour Academic Center, 600 South Paulina Street, Suite 507, Chicago, IL 60612. E-mail: jitesh_pratap@rush.edu

Manuscript Received: 27 June 2014; Manuscript Accepted: 15 August 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 21 August 2014

DOI 10.1002/jcb.24939 • © 2014 Wiley Periodicals, Inc.

Our results show that ectopic Runx2 expression downregulates pErk and pAkt levels in response to growth factor signaling in normal mammary epithelial MCF-10A cells. Furthermore, Runx2 promotes EGFR phosphorylation upon Mek inhibition in response to EGF stimulation.

MATERIALS AND METHODS

CELL CULTURE, VECTORS AND ANTIBODIES

The non-tumorigenic immortalized human mammary epithelial MCF-10A cell line (ER-, PR-, Her2+, WT-p53) was cultured in DMEM/F12 media (Cellgro, Mediatech, Manassas, VA) supplemented with 5% horse serum (Lonza, Walkersville, MD), insulin (10 μg/ml) (USP Inc., Rockville, MD), EGF (20 ng/ml) (BD Biosciences, Bedford, MA), cholera toxin (100 ng/ml) (EMD Chemicals, Gibbstown, NJ) and hydrocortisone (0.5 μg/ml) (MP Biomedical, Santa Ana, CA), and 50 U/ml penicillin and 50 μg/ml streptomycin (pen-strep) (Cellgro, Mediatech) at 37°C in humidified incubator with 5% CO_2. The 3D culture of MCF-10A cells in Matrigel (BD Biosciences) was performed as previously described [Debnath and Brugge, 2005]. For EGF, IGF, and Insulin treatment, the cells were deprived of serum (final 0.25% serum) and growth factors for 16 h. The cells were then treated with EGF (100 ng/ml), IGF (100 ng/ml), and Insulin (1 μg/ml) in serum-deprived media for various time points (10 min to 6 h). In experiments requiring, Mek inhibitor PD184161 (Cayman Chemicals, Ann Arbor, MI), PI3K inhibitor LY294002 (Cayman Chemical), EGFR inhibitor Gefitinib (Cayman Chemicals), or IGF1R inhibitor OSI-906 (Selleck Chemicals, Houston, TX) treatment, the serum-deprived cells were pretreated with inhibitors for 10 min before treatment with growth factors.

The overexpression studies of Runx2 were performed by transducing Runx2 via Adenovirus-mediated delivery of WT-Runx2 on day 7 old acini structures [Pratap et al., 2009]. We selected day 7 acini structures based on previous reports of induction of pAkt levels in acini and to circumvent Runx2-mediated growth effects [Debnath et al., 2003; Pratap et al., 2009]. The Adenovirus vectors for expressing Runx2 or control green fluorescent protein, the lentivirus vectors expressing Runx2 or empty vector control were generated similar to previously described [Pratap et al., 2003, 2009].

The mouse monoclonal antibody for Runx2 was obtained from MBL International Corporation, Woburn, MA. The antibodies for pErk1/2, Erk1/2 (total), pAkt (Serine 473), Akt (total), pmTOR (Serine 2448 and 2481), mTOR (total), Rictor, Raptor, pEGFR (Y992), pEGFR (Y1045), pEGFR (Y1068), EGFR, and FOXO1 were purchased from Cell Signaling Technology, Danvers, MA. The antibodies for β-Actin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

WESTERN BLOTTING

The whole cell lysates were prepared by washing cells in cold PBS and subsequently lysing in sample buffer containing Tris-Cl (62.5 mM, pH 6.8), SDS (2% w/v), DTT (50 mM), glycerol (10%), and bromophenol blue (0.01% w/v). The whole cell lysates were loaded in SDS-Gel and transferred to PVDF membrane and blotting was performed as previously described [Tandon et al., 2014]. The phosphorylated and total proteins were probed on separate PVDF membranes, while normalizing controls were probed on stripped membranes. The data were quantified in Adobe Photoshop (San Jose, CA) and ImageJ software (NIH, Bethesda, MD). The experiments were repeated at least two to three times.

REAL-TIME PCR

The real time PCR with SYBR chemistry was performed as previously described [Tandon et al., 2014]. The following human primer pairs were used. Runx2: (F) TGC CTG CCT GGG GTC TGT A (R) CGG GCC CTC CCT GAA CTC T; GAPDH: (F) ATG TTC GTC ATG GGT GTG AA (R) TGT GGT CAT GAG TCC TTC CA; 28S: (F) GAA CTT TGA AGG CCG AAG TG (R) ATC TGA ACC CGA CTC CCT TT; p19 (CDKN2D/p19INK4d): (F) GTT TCT TCT GCG CCT CAG GCT GC (R) TCT CTG AGC ACA GGC CGG GCA AG. The experiments were repeated at least two to three times.

FLOW CYTOMETRY

The cell cycle analysis was performed in FACS Canto (BD Biosciences) as previously described [Tandon et al., 2014]. The gating and data analysis was performed in FlowJo software (Tree Star, Ashland, OR). The Dean Jett Fox model was used to set gates for G1, S, and G2 stage cells, while Sub-G1 was manually gated before G1 population. The experiments were repeated at least two to three times.

IMMUNOFLUORESCENCE

The immunofluorescence staining was preformed as previously described [Tandon et al., 2014]. The fluorescently tagged antibody (Alexa Fluor-594) and DAPI were obtained from BD Biosciences. The Rush University Medical Center and University of Illinois core facilities were utilized for imaging in immunofluorescence (Zeiss Axioplan 2) or confocal microscope (Zeiss LSM 510).

RESULTS

ECTOPIC RUNX2 EXPRESSION INHIBITS BASAL AND EGF-INDUCED pERK1/2 LEVELS IN MCF-10A CELLS

The activation of Erk1/2 in response to growth factor signaling is critical for migration and survival of MCF-10A cells [Collins et al., 2005; Tarcic et al., 2012]. To determine the function of Runx2 in regulating growth factor-induced Erk1/2 phosphorylation, MCF-10A cells were deprived of serum and growth factors, and then stimulated with epidermal growth factor (EGF), insulin-like growth factor (IGF), or Insulin. As Runx2 is expressed at low levels in MCF-10A cells [Tandon et al., 2014], we ectopically increased Runx2 levels by Adenovirus-mediated Runx2 transduction (Fig. 1A). The ectopic Runx2 expression significantly reduced the total Erk1/2 levels (Figs. 1A and B). A robust induction of pErk1/2 was observed in response to EGF (100 ng/ml) compared to IGF (100 ng/ml) or insulin (1 μg/ml) treatment in vector control cells (Figs. 1C–E). The ectopic Runx2 expression reduced EGF-induced pErk1/2 levels (Fig. 1C). As the extent of pErk1/2 induction by IGF or insulin was low compared to EGF treatment, we further tested higher doses of IGF (500 ng/ml) or insulin (50 μg/ml).

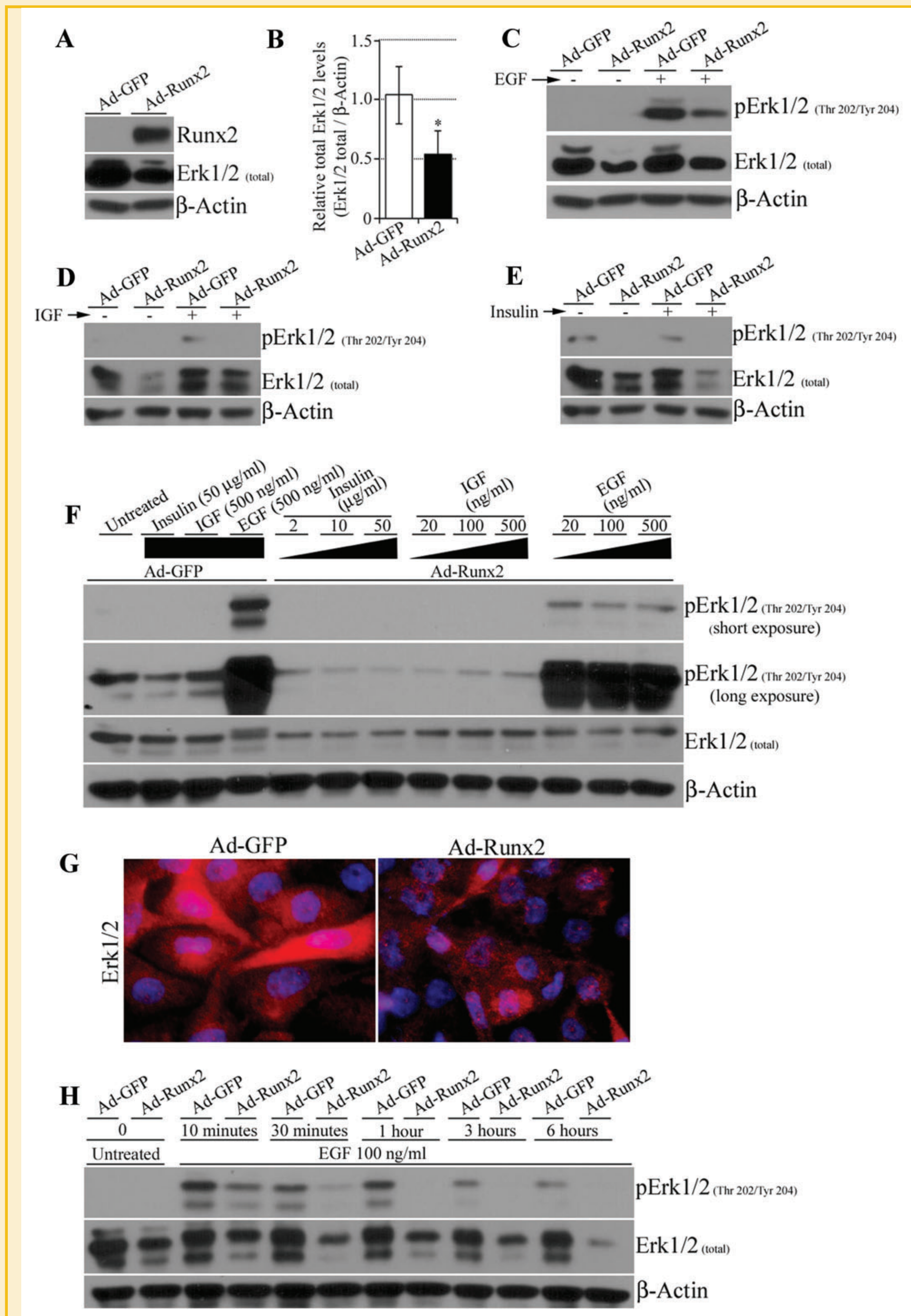

Fig. 1. Ectopically expressed Runx2 blocks EGF-induced pErk1/2 levels. (A) The Western blotting of MCF-10A whole cell lysates show increased Runx2 and reduced Erk1/2 protein levels when transduced with Ad-Runx2 compared to Ad-GFP control. The β-Actin expression is shown as internal loading control. (B) The Erk1/2 expression in Ad-GFP or Ad-Runx2 cells was quantified from five independent Western blots and normalized to β-Action expression (*$P < 0.01$, unpaired student *t*-test). (C–E) The Runx2 overexpressing (Ad-Runx2) or control (Ad-GFP) cells were serum- and growth factor-deprived for 16 h and then stimulated with EGF (100 ng/ml) (C) or IGF1 (100 ng/ml) (D) or insulin (1 μg/ml) (E) for 1 h. The pErk1/2 and total Erk protein levels were examined in whole cell lysates by Western blotting. (F) The control (Ad-GFP) or Runx2 expressing (Ad-Runx2) cells were stimulated with different doses of EGF or IGF (20–500 ng/ml) and insulin (2–50 μg/ml). The pErk1/2 and total Erk levels were determined by Western blotting. (G) The MCF-10A cells transduced with Ad-Runx2 or Ad-GFP were serum and growth factor-deprived and treated with EGF for 1 h. The fixed cells were analyzed for total Erk1/2 expression (Alexa fluor-594/Red) by immunofluorescence. The Erk1/2 positive cells are stained red, while nuclei are stained blue with Dapi. (H) The control (Ad-GFP) or Runx2 expressing (Ad-Runx2) cells were stimulated with EGF (100 ng/ml) for various time points (10 min to 6 h) to determine pErk1/2 and total Erk levels by Western blotting.

Higher doses of IGF or insulin increased the pAkt levels in Runx2-expressing cells (data not shown), but pErk was not induced (Fig. 1F). Additionally, pErk levels did not increase with higher doses of EGF in Runx2 expressing cells. These results indicate that pErk1/2 expression in MCF-10A cells is preferentially induced in response to EGF stimulation and that this stimulation is potently inhibited in Runx2 expressing cells as examined by Western blotting and immunofluorescence studies (Figs. 1F and G). To assess the kinetics of pErk1/2 levels with Runx2 overexpression, cells were treated with EGF for 10 min to 6 h. A robust induction pErk1/2 levels was observed up to 1 h of EGF treatment in control cells (Fig. 1H). The ectopic Runx2 expression associated with reduced pErk1/2 and total levels with all time points examined. These results suggest that Runx2 downregulates basal and EGF-induced pErk levels in MCF-10A cells.

ECTOPIC RUNX2 EXPRESSION DIFFERENTIALLY INHIBITS AKT PHOSPHORYLATION IN RESPONSE TO GROWTH FACTOR STIMULI

The growth factor stimulation also induces PI3K/Akt signaling in addition to Mek/Erk pathway; therefore, we examined pAkt levels upon ectopic Runx2 expression. In contrast to pErk1/2 induction predominantly by EGF, pAkt could also be induced by IGF or insulin in MCF-10A cells (Figs. 2A–C). However, the basal levels of pAkt in growth factor-deprived MCF-10A cells were high due to Adenovirus-mediated pAkt stimulation [Liu et al., 2005]. Nonetheless, the ectopic Runx2 expression downregulated pAkt levels in basal conditions and upon EGF, IGF, or insulin stimulation (Figs. 2A–C). Interestingly, with EGF treatment, Runx2-mediated downregulation of pAkt level was partial compared to IGF or insulin treatment. Next, to confirm that the Runx2-mediated decline in pAkt is relevant in mammary gland morphogenesis, we utilized the MCF-10A three-dimensional (3D) cell culture model [Debnath and Brugge, 2005]. The ectopic Runx2 expression at the beginning of MCF-10A culture disrupts the mammary acinar structures [Pratap et al., 2009], therefore preformed acinar structures were examined for pAkt levels after treatment with control or Ad-Runx2. As indicated by pAkt immunostaining, the ectopic Runx2 expression inhibited pAkt levels (Fig. 2D). Additionally, proteins related to the stimulation of Akt kinase activity (pmTOR, Rictor, Raptor, and PI3K) were reduced in Runx2 expressing cells in both basal as well as EGF-stimulated conditions (Fig. 2E). The levels of PTEN and PHLPP1 phosphatases were not altered suggesting that the decline in pAkt was due to reduced activity of kinases rather than increased phosphatase activity. Taken together, ectopic Runx2 expression downregulates pErk1/2 and pAkt levels in response to growth factor signaling in MCF-10A cells. However, Runx2-mediated decline in pAkt was partially rescued with EGF treatment compared to IGF or insulin treatment suggesting that Runx2 differentially functions in crosstalk of Mek/Erk and PI3K/Akt pathway.

ECTOPIC RUNX2 EXPRESSION INCREASES FOXO1 AND G1 PHASE IN CELL CYCLE PROGRESSION

To determine the downstream events of Runx2-mediated inhibition of Mek/Erk and PI3K/Akt signaling, we examined FOXO1 expression levels. The FOXO1 is negatively regulated by growth factor-induced survival signals, and both Mek/Erk and PI3K/Akt signaling pathways

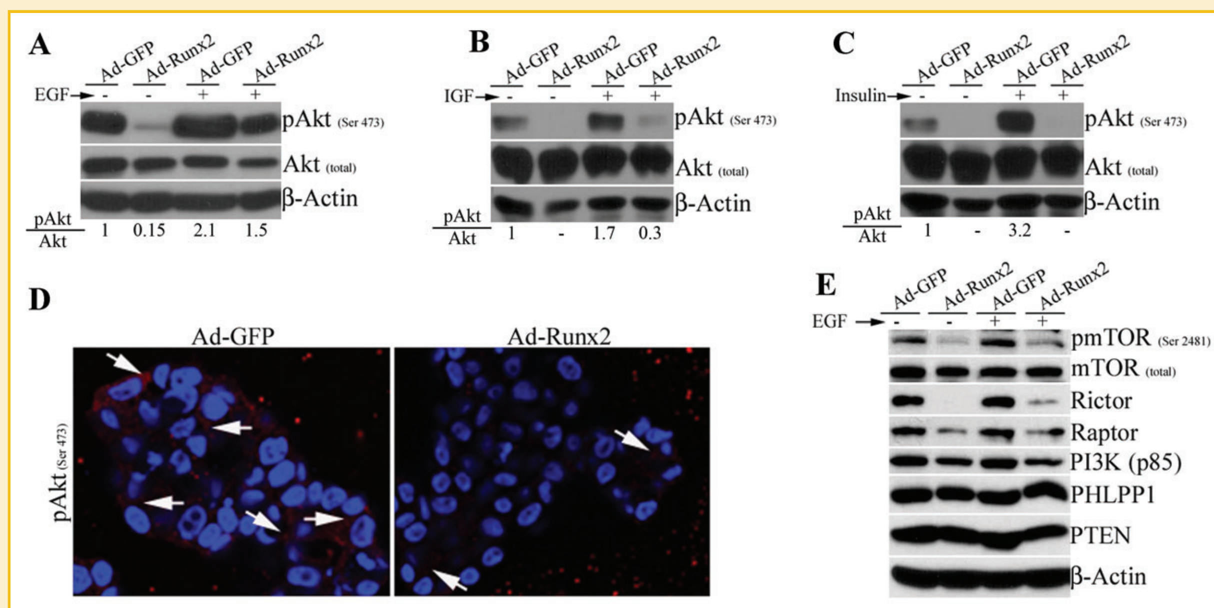

Fig. 2. Ectopically expressed Runx2 blocks growth factor-induced pAkt levels. (A–C) The Runx2 (Ad-Runx2) or control GFP (Ad-GFP) expressing MCF-10A cells were deprived of serum and growth factors, and then treated with EGF (100 ng/ml) (A) or IGF (100 ng/ml) (B) or insulin (1 μg/ml) for 1 h and analyzed for the pAkt (serine 473) and total Akt protein levels in whole cell lysates by Western blotting. The expression of β-Actin protein was utilized as loading control. (D) The acinar structures of MCF-10A cells in Matrigel 3D cultures were transduced with Ad-Runx2 or Ad-GFP, serum- and growth factor-deprived and treated with EGF for 1 h. The fixed cells were analyzed in confocal microscope for pAkt levels (Alexa fluor (AF-594)/Red) by immunofluorescence. The pAkt positive cells are indicated by white arrows, while nuclei are stained blue with Dapi. (E) The Runx2 (Ad-Runx2) or GFP control expressing MCF-10A cells were deprived of serum and growth factors, and then treated with EGF (100 ng/ml) for 1 h. The expression levels of pmTOR (Serine 2481), total mTOR, Rictor, Raptor, PI3K (p85), PHLPP1, and PTEN were determined by Western blotting in whole cell lysates.

can phosphorylate and induce ubiquitin-mediated degradation of FOXO1 [Asada et al., 2007; Fu et al., 2009; Tzivion et al., 2011]. Previous studies in several cell types have also shown that inhibition of Akt and Erk1/2 signaling upregulates FOXO1 expression levels [Roy et al., 2010; Tandon et al., 2014]. Therefore, we reasoned that inhibition of pErk1/2 and pAkt by ectopic Runx2 expression should prevent FOXO1 degradation upon growth factor stimulation. As expected, FOXO1 protein expression levels robustly increased with ectopic Runx2 expression in basal conditions and modestly changed with growth factor stimulation (Figs. 3A–C). Additionally, the FOXO1 target gene p19 expression increased with Runx2 expression (Fig. 3D). Furthermore, we confirmed the increase in FOXO1 levels in the MCF-10A 3D acinar structures transduced with Ad-Runx2 (Fig. 3E).

Since FOXO1 regulates cell cycle arrest and resistance to apoptosis [Roy et al., 2010; Lv et al., 2013], we determined the alterations in cell cycle progression upon ectopic Runx2 expression. Indeed, the ectopic Runx2 expression increased G1 phase cells compared to control virus treated cells (48% vs. 33%) cultured in normal growth medium (Figs. 3F and G). This effect was pronounced (84% vs. 51%) when the cells were cultured without serum or growth factors. Additionally, ectopic Runx2 expressing cells were resistant to cell

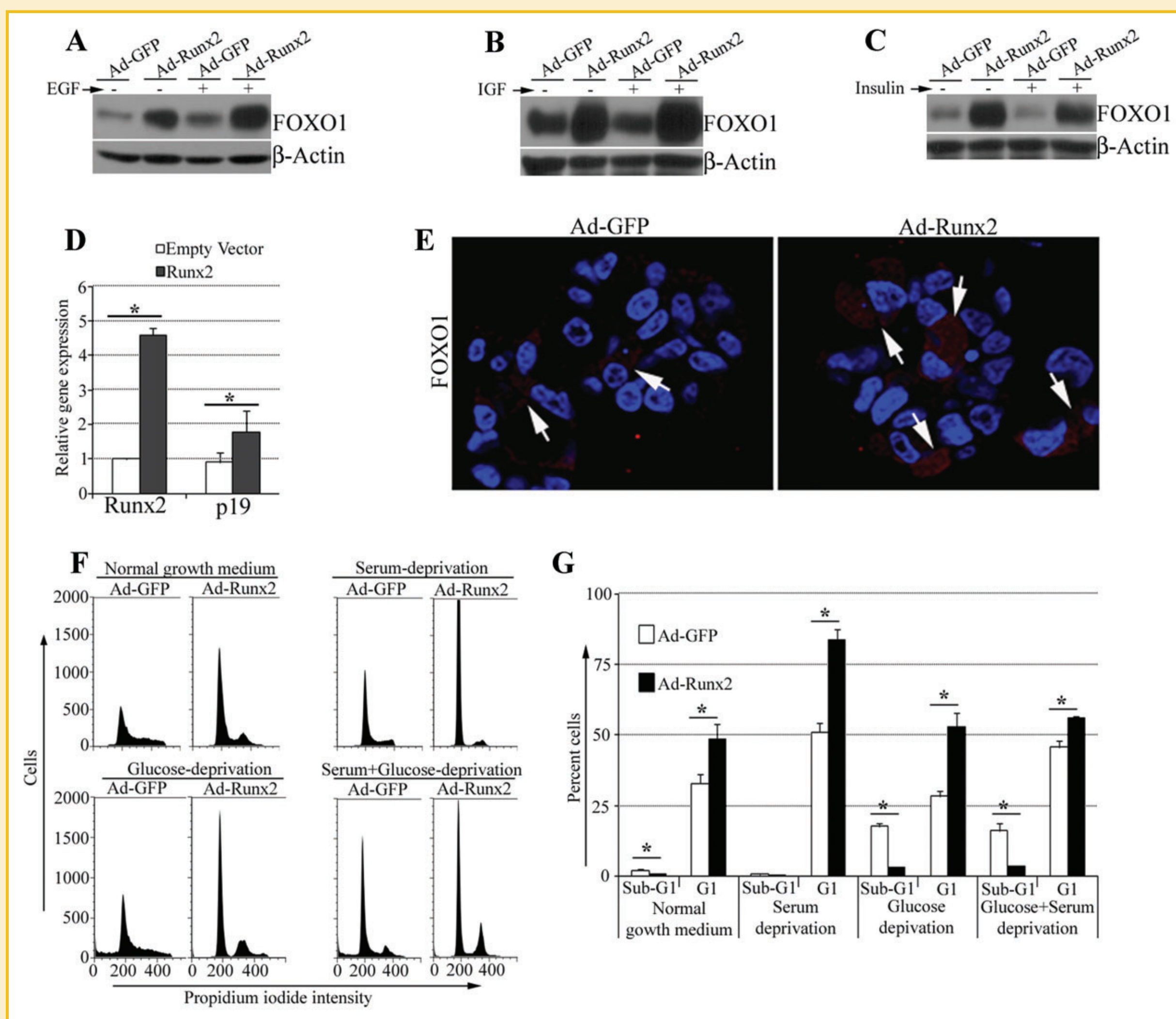

Fig. 3. Runx2 alters FOXO1 expression and cell cycle in MCF-10A cells. (A–C) The MCF-10A cells transiently overexpressing Runx2 (Ad-Runx2) or GFP control were serum- and growth factor-deprived and then treated with EGF (100 ng/ml) (A) or IGF1 (100 ng/ml) or insulin (1 μg/ml) for 1 h. The whole cell lysates were analyzed for FOXO1 expression levels, while β-Actin was used as loading control in Western blotting. (D) The gene expression levels of p19 in stable WT-Runx2 overexpressing MCF-10A cells were determined by real-time quantitative PCR. The expression level of 28S was utilized as internal control. *$P < 0.05$ unpaired student *t*-test. (E) The day 7 acini structures of MCF-10A cells in 3D cultures were transduced with Ad vectors expressing GFP or WT-Runx2 and stimulated with EGF (100 ng/ml). The expression of FOXO1 (AF-594/Red) was analyzed by immunofluorescence in confocal microscopy, while Dapi (blue) was used to stain the nuclei. (F) The MCF-10A cells overexpressing Runx2 (Ad-Runx2) were examined for alterations in cell cycle progression by flow cytometry. The normal growth medium cultured or cells deprived of serum, growth factors, and glucose were collected and stained with propidium iodide (PI). A histogram of representative cell cycle stages is shown. (G) A quantification of average (± standard deviation) sub-G1 and G1 stages is indicated for PI stained cells, *$P < 0.05$ unpaired student *t*-test.

death compared to control cells as indicated by decrease in sub-G1 population (3–4% vs. 16–18%) in serum- and glucose-deprived conditions. These results, together with Runx2-mediated downregulation of pErk1/2 and pAkt levels, suggest that high Runx2 levels promote survival of mammary epithelial cells via G1 arrest during serum and glucose deprivation.

A CROSSTALK BETWEEN MEK/ERK AND PI3K/AKT SIGNALING PATHWAYS IN MCF-10A CELLS

Several reports indicate a crosstalk between Mek/Erk and PI3K/Akt signaling and that abrogation of Mek/Erk activity by pharmacological inhibitors induces Akt activation [Gopal et al., 2010; Ebi et al., 2011; Turke et al., 2012]. Additionally, in invasive MDA-MB-231 mammary epithelial cells, we have reported that treatment with Mek inhibitors U0126 or PD184161 upregulates pAkt levels [Tandon et al., 2014]. In spite of Erk1/2 downregulation in Runx2 overexpressing MCF-10A cells (Fig. 1C); the pAkt levels were not induced to the level of control cells upon EGF treatment (Fig. 2A). These results could be due to the following reasons: (1) the saturating/high levels of pAkt due to Adenovirus treatment in control cells masked the pAkt induction by pErk inhibition; and (2) the incomplete blockage of pErk1/2 by ectopic Runx2 compared to complete abrogation using pharmacological inhibitor (PD184161), suggesting that extent of Erk inhibition may be critical for robust activation of pAkt levels. Therefore, to better understand the combinatorial effect of Runx2 and pharmacological Erk inhibition on pAkt levels, we evaluated pAkt levels upon Mek/Erk inhibition with PD184161 in control or ectopic Runx2 expressing cells. Interestingly, the blocking of pErk1/2 by PD184161 rescued Runx2-mediated downregulation of pAkt basally (Fig. 4A) as well as in response to EGF, IGF, or insulin treatment (Figs. 4B–D). In control virus-treated cells, no further increase in pAkt levels was observed with PD184161 treatment most likely due to Ad-mediated high pAkt levels [Liu et al., 2005]. The inhibition of pAkt via PI3K inhibitor LY294002 did not change pErk1/2 levels (Fig. 4B). Previously, we have also shown that knockdown of Runx2 in MCF-10A cell increases pAkt levels upon EGF stimulation [Tandon et al., 2014]. Together, these results indicate that Runx2 and Erk repress pAkt levels in MCF-10A cells.

RUNX2 PROMOTES THE CROSSTALK BETWEEN MEK/ERK AND PI3K/AKT VIA EGFR

Mek inhibition has been previously shown to increase EGFR autophosphorylation [Li et al., 2008]. Consistent with this, since Runx2 inhibited pErk1/2 predominantly in response to EGF treatment (Fig. 1), we observed that ectopic Runx2 expression was associated with an increase in phosphorylated EGFR levels upon EGF stimulation (Fig. 5A). The EGF treatment decreased total EGFR levels in control but not in Runx2 expressing cells. To further understand the function of increased pEGFR with ectopic Runx2 expression, we utilized pharmacological inhibitors to block the kinase activity of EGFR (gefitinib) in addition to blocking Mek/Erk and PI3K/Akt. The

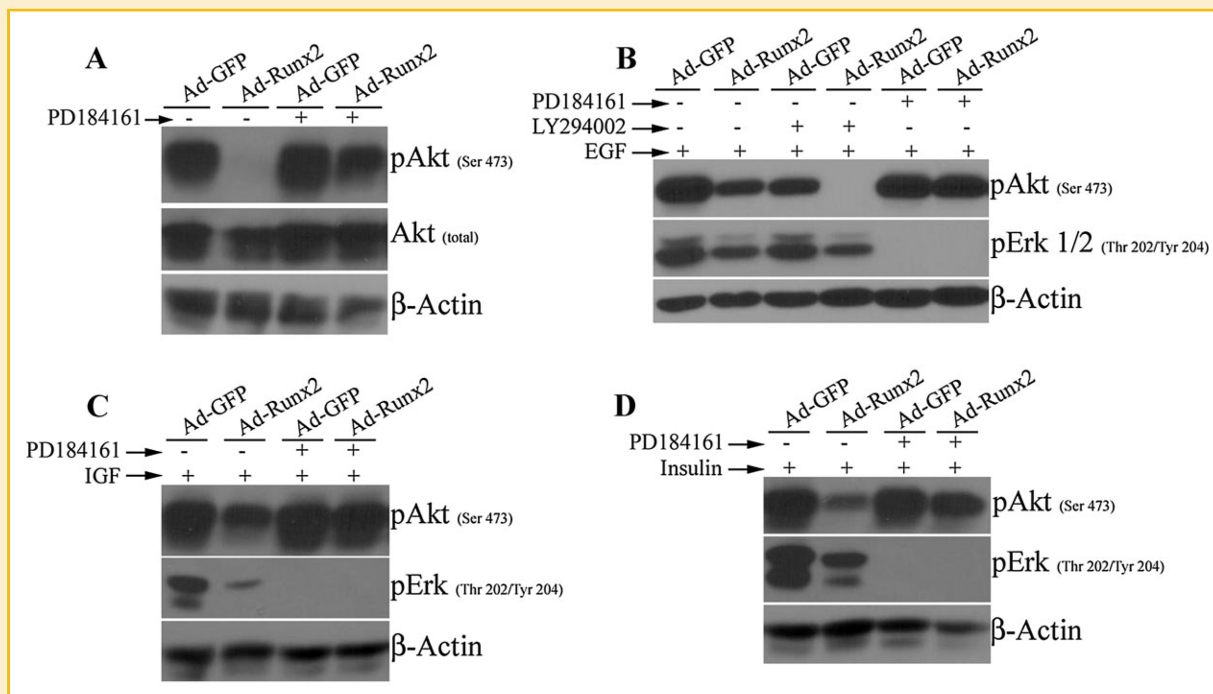

Fig. 4. A crosstalk between pErk1/2 and pAkt in MCF-10A cells. (A) The MCF-10A cells overexpressing Runx2 (Ad-Runx2) or GFP control were serum- and growth factor-deprived, and treated with Mek inhibitor PD184161 (10 μm) for 1 h. The expression level of pAkt (Serine 473) and total Akt proteins were determined in whole cell lysates by Western blotting. The expression of β-Actin was utilized as loading control. (B–D). The MCF-10A cells overexpressing Runx2 (Ad-Runx2) or control (Ad-GFP) were deprived of serum and growth factors and treated with EGF (100 ng/ml) (B), IGF (100 ng/ml) (C) or insulin (1 μg/ml) (D) in the presence or absence or PD184161 or PI3K inhibitor LY294002 (10 um) as indicated for 1 h. The expression levels of pAkt (Serine 473) and pErk (threonine 202 and tyrosine 204) were determined by Western blotting.

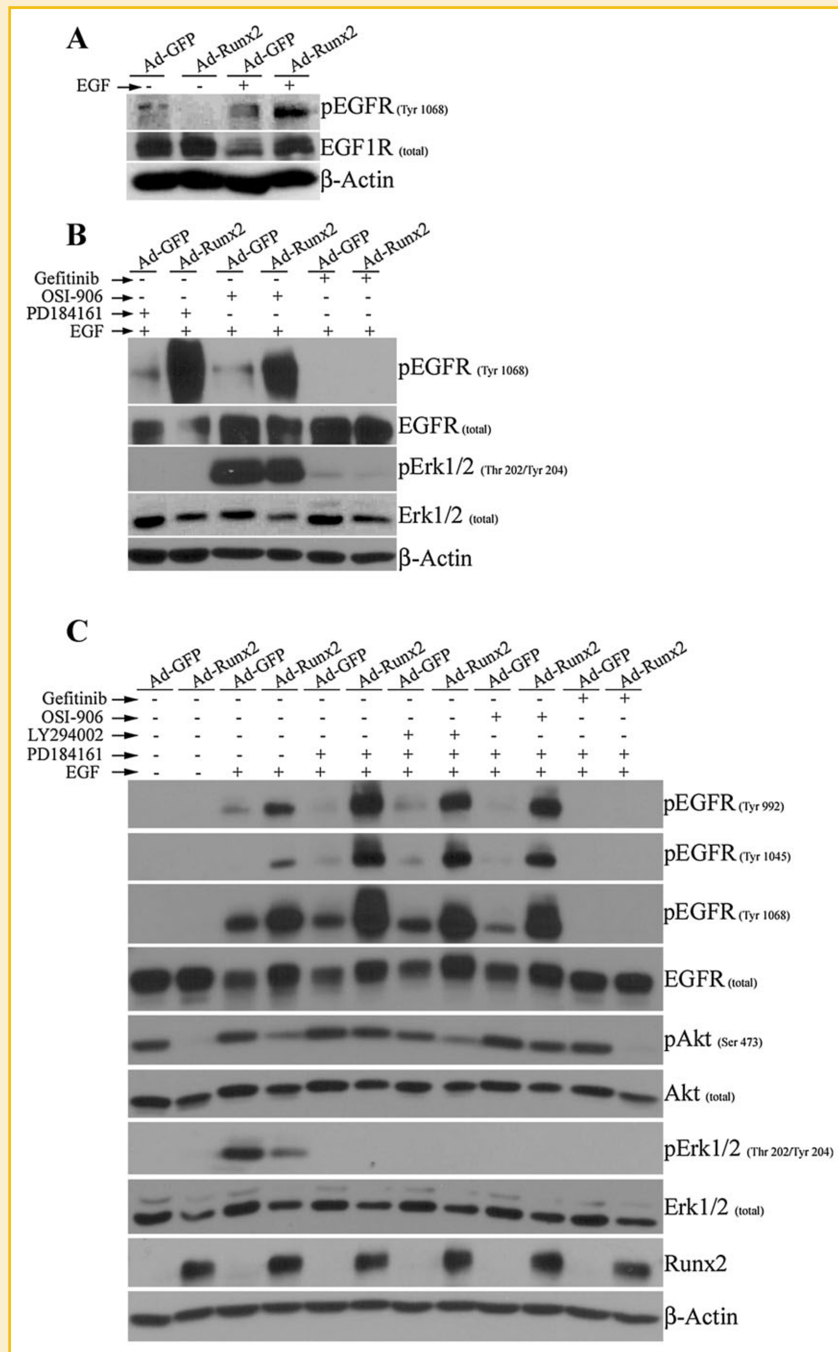

Fig. 5. Runx2-dependent EGFR phosphorylation promotes Mek/Erk and PI3K/Akt crosstalk. (A) The MCF-10A cells overexpressing Runx2 (Ad-Runx2) or control (Ad-GFP) were deprived of serum and growth factors and treated with EGF (100 ng/ml) for 1 h. The expression levels of pEGFR (tyrosine 1068) and total EGFR levels were determined by Western blotting. The expression of β-Actin was utilized as loading control. (B) The MCF-10A cells overexpressing Runx2 (Ad-Runx2) or control (Ad-GFP) were deprived of serum and growth factors and treated with EGF (100 ng/ml) for 1 h in the presence or absence of PD184161, IGF1R inhibitor OSI-906 (10 μM) or EGFR inhibitor gefitinib (10 μM) as indicated in the figure. The expression levels of pEGFR (tyrosine 1068), total EGFR, pErk1/2 (threonine 202 and tyrosine 204), and total Erk1/2 were determined by Western blotting. (C) The MCF-10A cells overexpressing Runx2 (Ad-Runx2) or control (Ad-GFP) were deprived of serum and growth factors and treated with EGF (100 ng/ml) for 1 h in the presence or absence or combinations of PD184161 (10 μM), LY294002 (10 μM), OSI-906 (10 μM), or gefitinib (10 μM) as indicated in the figure. The expression levels of pEGFR (tyrosine 998, 1045, and 1068), total EGFR, pErk1/2 (threonine 202 and tyrosine 204), total Erk1/2 and pAkt (Serine 473), total Akt and Runx2 were determined by Western blotting.

combination of ectopic Runx2 expression with PD184161 robustly enhanced pEGFR compared to treatment alone (Fig. 5B). Furthermore, pErk1/2 levels were downregulated only by blocking EGFR activity with gefitinib treatment (Fig. 5B). These results are consistent with our findings described in Figures 1C–E indicating that Erk1/2 is predominantly activated by EGF–EGFR pathway.

To clarify the role of Runx2 in Mek/Erk and PI3K/Akt crosstalk, we inhibited Mek in combination with PI3K or EGFR inhibitors and

utilized IGF-1R inhibitor OSI-906 to confirm the specificity of pharmacological inhibition. The results indicated that the induction of pAkt in Runx2 overexpressing cells treated with Mek inhibitor is completely lost when combined with EGFR inhibition but not with IGF-1R inhibition (Fig. 5C). The inhibition of pErk and pAkt with their respective inhibitors was also confirmed. Taken together, these results suggest that Runx2 enhances negative feedback regulation of EGFR by Mek/Erk signaling.

DISCUSSION

The crosstalk between Mek/Erk and PI3K/Akt pathways is critical for stringent control of normal cell growth and survival [Aksamitiene et al., 2012]. The positive and negative feedback mechanisms of these pathways determine the extent and duration of growth factor stimulation. Evidence from previous genetic and pharmacologic studies demonstrate that EGFR stimulation signals primarily via MAPK pathway in MCF-10A mammary epithelial cells [Tarcic et al., 2012]. Furthermore, this pathway is regulated by a negative feedback mechanism where the activation of Mek/Erk leads to EGFR phosphorylation at threonine 669 residue and inhibits auto-phosphorylation at tyrosine residues [Li et al., 2008]. We show that Runx2 plays a critical role in crosstalk between the Mek/Erk and PI3K/Akt in EGFR pathway. Our results in MCF-10A cells with ectopic expression of Runx2 in combination with pharmacologic inhibition of EGFR/Erk/Akt signaling revealed that Runx2 relieves Erk-mediated inhibition of EGFR and Akt pathway. The ectopically expressed Runx2 inhibits both pErk and pAkt levels in MCF-10A cells, however the extent of inhibition of pAkt is low in response to EGF stimulation compared to IGF or insulin stimulation (Figs. 2A–C). Furthermore, the ectopic Runx2 expression enhanced G1 phase cell population and resistance to cell death in glucose- or serum-deprivation-induced stress. Our results in MCF-10A cells are similar to those observed in osteoblasts wherein forced expression of Runx2 in MC3T3 cells delays G1 cell cycle progression [Galindo et al., 2005]. Additionally, in MCF-10A cells, it is reported that the cell cycle arrest in G1 can provide resistance from detachment-induced apoptosis [Collins et al., 2005]. Taken together, we demonstrate that Runx2 functions in crosstalk of Mek/Erk and PI3K/Akt signaling in growth factor-induced survival cell signaling.

The non-tumorigenic mammary epithelial cells express low Runx2 levels compared to tumorigenic or invasive mammary epithelial cells [Inman and Shore, 2003; Tandon et al., 2014]. In osteoblastic cells, the Runx2 expression levels are regulated by autoregulatory mechanism and HOX-related transcription factors [Drissi et al., 2000], but the mechanisms for differential Runx2 expression in mammary epithelium and its regulation are currently not entirely understood. Moreover, in addition to its expression levels, the extent of Runx2 phosphorylation as shown in normal osteoblast cells could also be important for EGFR/Erk pathway signaling [Ge et al., 2009; Selvamurugan et al., 2009]. Previous studies in normal (endothelial cells and osteoblasts) and cancer (breast cancer and prostate cancer) cells further show an association between Runx2 with PI3K or MAPK, where growth factor signaling can modulate Runx2 phosphorylation and its binding to downstream target genes [Qiao et al., 2006; Pande et al., 2013]. Altogether in context of these studies, our data suggests differential roles of Runx2 in feedback regulatory loops of Mek/Erk and PI3K/Akt signaling to generate context-dependent responses in growth factor signaling.

In addition to Runx2, Runx1, and Runx3 are also expressed in normal mammary gland with tightly regulated spatio-temporal expression pattern [Jiang et al., 2008; Blyth et al., 2010; Chimge and Frenkel, 2013]. The alterations in Runx1 and Runx2 disrupt normal mammary acinar structures [Pratap et al., 2009; Wang et al., 2011). Our study in non-tumorigenic MCF-10A cells show that ectopically expressed Runx2 negatively regulates pErk and pAkt, and results in reduced levels of mTOR, Rictor and Raptor proteins. The Mek/Erk pathway positively regulates bone development through Runx2 [Ge et al., 2007; Ge et al., 2009]. The transgenic mouse lines constitutively expressing Mek1 showed increased Runx2 phosphorylation and transcriptional activity in calvarial osteoblasts. The Erk1/2-dependent phosphorylation of Runx2 at serine 319 residue is critical for osteoblast-specific gene expression and differentiation [Franceschi et al., 2007]. In addition to Runx2, Erk1/2 regulates the phosphorylation and transcriptional activity of Runx1 [Tanaka et al., 1996]. The relative strengths of Erk1/2 and Akt pathway may also be important for mammary morphogenesis as shown for osteoblast proliferation or differentiation [Raucci et al., 2008]. Previously, in invasive mammary epithelial cell lines with Ras mutations, we have shown that endogenous Runx2 is required to maintain pAkt levels by regulating mTORC2 complex [Tandon et al., 2014]. The mutations in Ras and constitutive Erk1/2 activation could lead to downregulation of PI3K/Akt signaling [Fukazawa et al., 2002; Hayashi et al., 2008]. Therefore, in such genomic context, high endogenous Runx2 could promote PI3K/Akt signaling via mTORC2 complex [Tandon et al., 2014]. Taken together, the results from non-tumorigenic and invasive mammary epithelial cells suggest cell-type dependent function of Runx2 at multiple regulatory nodes and receptor levels in maintaining growth factor signaling pathways.

Our present study in MCF-10A cells demonstrating that Runx2-mediated regulation of Erk levels could be due to directly suppressing total Erk levels and thereby affecting its phosphorylation and downstream targets. One of the downstream events of Erk activity is phosphorylation of EGFR protein at threonine 669 residue and further blocking its autophosphorylation [Li et al., 2008]. Consistent with this report, our results show that the Runx2-mediated increase in EGFR phosphorylation could be due to relieving negative feedback from Erk pathway. As only modest (30%) increase in EGFR mRNA levels was observed in Runx2 expressing MCF-10A cells (data not shown), suggesting a post-transcriptional regulatory mechanism of EGFR level by Runx2/Erk axis in MCF-10A cells. In addition to the role of Runx2 in EGFR signaling via Erk, negative regulatory crosstalk between Runx2 and EGFR signaling has been reported in differentiating osteoblast [Nakamura et al., 2010; Zhu et al., 2011], and myoblasts [Yu et al., 2013]. The EGFR ligands reduced the expression and transcriptional activity of Runx2 during osteoblast differentiation [Nakamura et al., 2010; Zhu et al., 2011]. Furthermore, in osteogenic differentiation of C2C12 cells, Runx2 has been shown to inhibit EGFR signaling [Yu et al., 2013]. Our results

demonstrating that the upregulation of pAkt upon pharmacological Mek/Erk inhibition could be completely reversed by EGFR but not IGF-1R inhibition in Runx2 expression cells suggest that Runx2–Erk–Akt pathway is dependent upon EGFR signaling. Further mechanistic studies are required to identify direct target genes of Runx2 in EGFR/Erk/Akt pathway. The preferential activation of Erk signaling via EGF stimulation in MCF-10A cells is in accordance to previously published reports [Tarcic et al., 2012]. The lack of pErk stimulation via IGF in Runx2 expressing cells further indicate the specificity of Runx2 function in EGFR-induced Mek/Erk and PI3K/Akt crosstalk. The EGFR which is expressed in 18–35% breast cancers [Foley et al., 2010] has also been shown to be regulated by Erk via extracellular matrix [Grassian et al., 2011], EGFR ligands [Roberts and Der, 2007] and threonine phosphorylation [Gan et al., 2010] in multiple cell types. Altogether, our data on Runx2 function in regulating signaling crosstalk and cell survival highlight the regulatory roles of Runx2 in growth factor-driven cellular phenotype.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. Gary stein, Jane Lian and Janet Stein, University of Vermont, Dr. Andre J van Wijnen, Mayo Clinic, and Drs. Carl Maki, Rick Sumner and Amarjit Virdi, Rush University Medical Center for stimulating discussions throughout the study. We would like to thank confocal microscopy core facilities of Rush University Medical Center and University of Illinois at Chicago.

REFERENCES

Aksamitiene E, Kiyatkin A, Kholodenko BN. 2012. Cross-talk between mitogenic Ras/MAPK and survival PI3K/Akt pathways: A fine balance. Biochem Soc Trans 40:139–146.

Asada S, Daitoku H, Matsuzaki H, Saito T, Sudo T, Mukai H, Iwashita S, Kako K, Kishi T, Kasuya Y, Fukamizu A. 2007. Mitogen-activated protein kinases, Erk and p38, phosphorylate and regulate Foxo1. Cell Signal 19:519–527.

Blyth K, Vaillant F, Jenkins A, McDonald L, Pringle MA, Huser C, Stein T, Neil J, Cameron ER. 2010. Runx2 in normal tissues and cancer cells: A developing story. Blood Cells Mol Dis 45:117–123.

Chimge NO, Frenkel B. 2013. The RUNX family in breast cancer: Relationships with estrogen signaling. Oncogene 32:2121–2130.

Choi JY, Pratap J, Javed A, Zaidi SK, Xing L, Balint E, Dalamangas S, Boyce B, van Wijnen AJ, Lian JB, Stein JL, Jones SN, Stein GS. 2001. Subnuclear targeting of Runx/Cbfa/AML factors is essential for tissue-specific differentiation during embryonic development. Proc Natl Acad Sci USA 98: 8650–8655.

Collins NL, Reginato MJ, Paulus JK, Sgroi DC, Labaer J, Brugge JS. 2005. G1/S cell cycle arrest provides anoikis resistance through Erk-mediated Bim suppression. Mol Cell Biol 25:5282–5291.

Debnath J, Brugge JS. 2005. Modelling glandular epithelial cancers in three-dimensional cultures. Nat Rev Cancer 5:675–688.

Debnath J, Walker SJ, Brugge JS. 2003. Akt activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner. J Cell Biol 163:315–326.

Drissi H, Luc Q, Shakoori R, Chuva De Sousa LS, Choi JY, Terry A, Hu M, Jones S, Neil JC, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2000. Transcriptional autoregulation of the bone related CBFA1/RUNX2 gene. J Cell Physiol 184:341–350.

Ebi H, Corcoran RB, Singh A, Chen Z, Song Y, Lifshits E, Ryan DP, Meyerhardt JA, Benes C, Settleman J, Wong KK, Cantley LC, Engelman JA. 2011. Receptor tyrosine kinases exert dominant control over PI3K signaling in human KRAS mutant colorectal cancers. J Clin Invest 121:4311–4321.

Ferrari N, McDonald L, Morris JS, Cameron ER, Blyth K. 2013. RUNX2 in mammary gland development and breast cancer. J Cell Physiol 228:1137–1142.

Foley J, Nickerson NK, Nam S, Allen KT, Gilmore JL, Nephew KP, Riese DJ. 2010. EGFR signaling in breast cancer: Bad to the bone. Semin Cell Dev Biol 21:951–960.

Franceschi RT, Ge C, Xiao G, Roca H, Jiang D. 2007. Transcriptional regulation of osteoblasts. Ann NY Acad Sci 1116:196–207.

Fu W, Ma Q, Chen L, Li P, Zhang M, Ramamoorthy S, Nawaz Z, Shimojima T, Wang H, Yang Y, Shen Z, Zhang Y, Zhang X, Nicosia SV, Zhang Y, Pledger JW, Chen J, Bai W. 2009. MDM2 acts downstream of p53 as an E3 ligase to promote FOXO ubiquitination and degradation. J Biol Chem 284:13987–14000.

Fukazawa H, Noguchi K, Murakami Y, Uehara Y. 2002. Mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitors restore anoikis sensitivity in human breast cancer cell lines with a constitutively activated extracellular-regulated kinase (ERK) pathway. Mol Cancer Ther 1:303–309.

Galindo M, Pratap J, Young DW, Hovhannisyan H, Im HJ, Choi JY, Lian JB, Stein JL, Stein GS, van Wijnen AJ. 2005. The bone-specific expression of Runx2 oscillates during the cell cycle to support a G1-related antiproliferative function in osteoblasts. J Biol Chem 280:20274–20285.

Gan Y, Shi C, Inge L, Hibner M, Balducci J, Huang Y. 2010. Differential roles of ERK and Akt pathways in regulation of EGFR-mediated signaling and motility in prostate cancer cells. Oncogene 29:4947–4958.

Ge C, Xiao G, Jiang D, Franceschi RT. 2007. Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. J Cell Biol 176:709–718.

Ge C, Xiao G, Jiang D, Yang Q, Hatch NE, Roca H, Franceschi RT. 2009. Identification and functional characterization of ERK/MAPK phosphorylation sites in the Runx2 transcription factor. J Biol Chem 284:32533–32543.

Gopal YN, Deng W, Woodman SE, Komurov K, Ram P, Smith PD, Davies MA. 2010. Basal and treatment-induced activation of AKT mediates resistance to cell death by AZD6244 (ARRY-142886) in Braf-mutant human cutaneous melanoma cells. Cancer Res 70:8736–8747.

Grassian AR, Schafer ZT, Brugge JS. 2011. ErbB2 stabilizes epidermal growth factor receptor (EGFR) expression via Erk and Sprouty2 in extracellular matrix-detached cells. J Biol Chem 286:79–90.

Hayashi H, Tsuchiya Y, Nakayama K, Satoh T, Nishida E. 2008. Down-regulation of the PI3-kinase/Akt pathway by ERK MAP kinase in growth factor signaling. Genes Cells 13:941–947.

Inman CK, Shore P. 2003. The osteoblast transcription factor Runx2 is expressed in mammary epithelial cells and mediates osteopontin expression. J Biol Chem 278:48684–48689.

Jiang Y, Tong D, Lou G, Zhang Y, Geng J. 2008. Expression of RUNX3 gene, methylation status and clinicopathological significance in breast cancer and breast cancer cell lines. Pathobiology 75:244–251.

Lemmon MA, Schlessinger J. 2010. Cell signaling by receptor tyrosine kinases. Cell 141:1117–1134.

Li X, Huang Y, Jiang J, Frank SJ. 2008. ERK-dependent threonine phosphorylation of EGF receptor modulates receptor downregulation and signaling. Cell Signal 20:2145–2155.

Liu Q, White LR, Clark SA, Heffner DJ, Winston BW, Tibbles LA, Muruve DA. 2005. Akt/protein kinase B activation by adenovirus vectors contributes to NFkappaB-dependent CXCL10 expression. J Virol 79:14507–14515.

Lv Y, Song S, Zhang K, Gao H, Ma R. 2013. CHIP regulates AKT/FoxO/Bim signaling in MCF7 and MCF-10A cells. PLoS One 8:e83312.

McDonald L, Ferrari N, Terry A, Bell M, Mohammed ZM, Orange C, Jenkins A, Muller WJ, Gusterson BA, Neil JC, Edwards J, Morris JS, Cameron ER, Blyth K. 2014. RUNX2 correlates with subtype-specific breast cancer in a human tissue microarray, and ectopic expression of Runx2 perturbs differentiation in the mouse mammary gland. Dis Model Mech 7:525–534.

Nakamura T, Toita H, Yoshimoto A, Nishimura D, Takagi T, Ogawa T, Takeya T, Ishida-Kitagawa N. 2010. Potential involvement of Twist2 and Erk in the regulation of osteoblastogenesis by HB-EGF-EGFR signaling. Cell Struct Funct 35:53–61.

Pande S, Browne G, Padmanabhan S, Zaidi SK, Lian JB, van Wijnen AJ, Stein JL, Stein GS. 2013. Oncogenic cooperation between PI3K/Akt signaling and transcription factor Runx2 promotes the invasive properties of metastatic breast cancer cells. J Cell Physiol 228:1784–1792.

Pratap J, Galindo M, Zaidi SK, Vradii D, Bhat BM, Robinson JA, Choi JY, Komori T, Stein JL, Lian JB, Stein GS, van Wijnen AJ. 2003. Cell growth regulatory role of Runx2 during proliferative expansion of preosteoblasts. Cancer Res 63:5357–5362.

Pratap J, Imbalzano KM, Underwood JM, Cohet N, Gokul K, Akech J, van Wijnen AJ, Stein JL, Imbalzano AN, Nickerson JA, Lian JB, Stein GS. 2009. Ectopic runx2 expression in mammary epithelial cells disrupts formation of normal acini structure: Implications for breast cancer progression. Cancer Res 69:6807–6814.

Qiao M, Shapiro P, Fosbrink M, Rus H, Kumar R, Passaniti A. 2006. Cell cycle-dependent phosphorylation of the RUNX2 transcription factor by cdc2 regulates endothelial cell proliferation. J Biol Chem 281:7118–7128.

Raucci A, Bellosta P, Grassi R, Basilico C, Mansukhani A. 2008. Osteoblast proliferation or differentiation is regulated by relative strengths of opposing signaling pathways. J Cell Physiol 215:442–451.

Roberts PJ, Der CJ. 2007. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 26:3291–3310.

Roy SK, Srivastava RK, Shankar S. 2010. Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer. J Mol Signal 5:10.

Selvamurugan N, Shimizu E, Lee M, Liu T, Li H, Partridge NC. 2009. Identification and characterization of Runx2 phosphorylation sites involved in matrix metalloproteinase-13 promoter activation. FEBS Lett 583:1141–1146.

Shore P. 2005. A role for Runx2 in normal mammary gland and breast cancer bone metastasis. J Cell Biochem 96:484–489.

Tanaka T, Kurokawa M, Ueki K, Tanaka K, Imai Y, Mitani K, Okazaki K, Sagata N, Yazaki Y, Shibata Y, Kadowaki T, Hirai H. 1996. The extracellular signal-regulated kinase pathway phosphorylates AML1, an acute myeloid leukemia gene product, and potentially regulates its transactivation ability. Mol Cell Biol 16:3967–3979.

Tandon M, Chen Z, Pratap J. 2014. Runx2 activates PI3K/Akt signaling via mTORC2 regulation in invasive breast cancer cells. Breast Cancer Res 16: R16.

Tarcic G, Avraham R, Pines G, Amit I, Shay T, Lu Y, Zwang Y, Katz M, Ben-Chetrit N, Jacob-Hirsch J, Virgilio L, Rechavi G, Mavrothalassitis G, Mills GB, Domany E, Yarden Y. 2012. EGR1 and the ERK-ERF axis drive mammary cell migration in response to EGF. FASEB J 26:1582–1592.

Turke AB, Song Y, Costa C, Cook R, Arteaga CL, Asara JM, Engelman JA. 2012. MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors. Cancer Res 72:3228–3237.

Tzivion G, Dobson M, Ramakrishnan G. 2011. FoxO transcription factors; Regulation by AKT and 14–3-3. proteins Biochim Biophys Acta 1813:1938–1945.

Wang L, Brugge JS, Janes KA. 2011. Intersection of FOXO- and RUNX1-mediated gene expression programs in single breast epithelial cells during morphogenesis and tumor progression. Intersection of FOXO- and RUNX1-mediated gene expression programs in single breast epithelial cells during morphogenesis and tumor progression. Proc Natl Acad Sci USA 108:E803–E812.

Worster DT, Schmelzle T, Solimini NL, Lightcap ES, Millard B, Mills GB, Brugge JS, Albeck JG. 2012. Akt and ERK control the proliferative response of mammary epithelial cells to the growth factors IGF-1 and EGF through the cell cycle inhibitor p57Kip2. Sci Signal 5:ra19.

Yu S, Geng Q, Ma J, Sun F, Yu Y, Pan Q, Hong A. 2013. Heparin-binding EGF-like growth factor and miR-1192 exert opposite effect on Runx2-induced osteogenic differentiation. Cell Death Dis 4:e868.

Zhu J, Shimizu E, Zhang X, Partridge NC, Qin L. 2011. EGFR signaling suppresses osteoblast differentiation and inhibits expression of master osteoblastic transcription factors Runx2 and Osterix. J Cell Biochem 112:1749–1760.