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The Cancer-Related Transcription Factor Runx2 Modulates Cell Proliferation in Human Osteosarcoma Cell Lines

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Abstract

Runx2 regulates osteogenic differentiation and bone formation, but also suppresses pre-osteoblast proliferation by affecting cell cycle progression in the G₁ phase. The growth suppressive potential of Runx2 is normally inactivated in part by protein destabilization, which permits cell cycle progression beyond the G₁/S phase transition, and Runx2 is again up-regulated after mitosis. Runx2 expression also correlates with metastasis and poor chemotherapy response in osteosarcoma. Here we show that six human osteosarcoma cell lines (SaOS, MG63, U2OS, HOS, G292, and 143B) have different growth rates, which is consistent with differences in the lengths of the cell cycle. Runx2 protein levels are cell cycle-regulated with respect to the G₁/S phase transition in U2OS, HOS, G292, and 143B cells. In contrast, Runx2 protein levels are constitutively expressed during the cell cycle in SaOS and MG63 cells. Forced expression of Runx2 suppresses growth in all cell lines indicating that accumulation of Runx2 in excess of its pre-established levels in a given cell type triggers one or more anti-proliferative pathways in osteosarcoma cells. Thus, regulatory mechanisms controlling Runx2 expression in osteosarcoma cells must balance Runx2 protein levels to promote its putative oncogenic functions, while avoiding suppression of bone tumor growth.

Osteosarcoma is the most common bone tumor in children and adolescents (Young and Miller, 1975). The highest incidence of osteosarcoma is in the second decade of life, which suggests a relationship between bone growth and tumor development (Fraumeni, 1967; Cotterill et al., 2004). One of the critical steps for normal skeletal development and bone formation is the proliferative expansion of mesenchymal cells, osteoprogenitors, and immature osteoblasts. Cell growth and differentiation of normal osteoprogenitors and pre-osteoblasts is tightly regulated by Runx2, which favors a quiescent state (Pratap et al., 2003; Galindo et al., 2005). The growth suppressive potential of Runx2 is controlled by modulation of its protein levels during the cell cycle (Galindo et al., 2005, 2007). Cell cycle dependent changes of Runx2 levels occur with respect to G₁ progression at a cell cycle stage when normal osteoblasts monitor extra-cellular cues for competency to initiate cell cycle

progression beyond the G₁/S phase transition. Accordingly, transient Runx2 overexpression in synchronized cells delays cell cycle entry into S phase and significantly decreases cell proliferation in the MC3T3 pre-osteoblasts, Runx2 null calvarian osteoprogenitors, C2C12 pluripotent mesenchymal, and IMR-90 fibroblasts cell lines (Pratap et al., 2003; Galindo et al., 2005; Young et al., 2007a; Teplyuk et al., 2008, 2009a). The function of Runx2 as a negative regulator of cell proliferation is also reflected by linkage of Runx2 deficiency to cell immortalization and tumorigenesis (Kilbey et al., 2007; Zaidi et al., 2007a).

Apart from the growth suppressive potential that is evident during late G₁ in osteoblasts (Pratap et al., 2003; Galindo et al., 2005), Runx2 may have mitogenic potential in early G₁ (Teplyuk et al., 2008). Several studies indicate that Runx2-dependent control of proliferation is cell type-specific. Runx2 inhibits proliferation of osteoprogenitors and committed osteoblasts (Pratap et al., 2003; Galindo et al., 2005), but it may have distinct biological roles in chondrocytes (Galindo et al., 2005; Hinoi et al., 2006; Komori, 2008) and endothelial cells (Inman and Shore, 2003; Qiao et al., 2006). While immature osteoblasts from mice with Runx2 null mutations show accelerated proliferative potential, chondrocyte proliferation seems to be decreased in Runx2 null mice (Pratap et al., 2003; Yoshida et al., 2004), suggesting that Runx2 would also have opposite roles in different bone cell types. Moreover, ectopic expression of Runx2 in aortic endothelial cells increases cell proliferation (Sun et al., 2004), whereas Runx2 depletion inhibits cell proliferation in human marrow endothelial cells (Qiao et al., 2006). These findings support the concept that Runx2 protein can function as either a bona fide tumor suppressor or a classical oncoprotein depending on the cellular context (Blyth et al., 2005).

Current evidence indicates that Runx2 expression is a key pathological factor in osteosarcoma (Martin et al., 2011) by controlling a number of cancer-related genes (van der Deen et al., 2012). Moreover, osteosarcoma development may be associated with Runx2 overexpression and defects in osteogenic differentiation (Wagner et al., 2011). Overexpression of Runx2 in transgenic mice within the osteoblast lineage inhibits osteoblast maturation, increases bone resorption, and causes osteopenia with multiple fractures (Liu et al., 2001; Geoffroy et al., 2002). Runx2 is also clearly detected in clinical osteosarcoma samples (Andela et al., 2005; Lu et al., 2008; Sadikovic et al., 2009; Won et al., 2009; Kurek et al., 2010). Analysis of genomic DNA from osteosarcoma patients with amplification of the 6p12#x02013;p21 chromosomal interval, which spans the Runx2 locus, increases the Runx2 gene copy number and aberrantly elevates Runx2 expression (Lau et al., 2004; Lu et al., 2008; Sadikovic et al., 2009). Increased expression of Runx2 in osteosarcoma biopsies has been associated to increased tumorigenicity, tumor progression, metastases, lower survival, and poor prognosis (Won et al., 2009; Kurek et al., 2010; Sadikovic et al., 2010). Interestingly, osteosarcoma cell culture models may exhibit a similar variability of Runx2 gene expression, because Runx2 is expressed at different levels in a number of human osteosarcoma cell lines (Thomas et al., 2004; Lu et al., 2008; Luo et al., 2008; Kurek et al., 2010; Shapovalov et al., 2010). A subset of patient-derived osteosarcoma cell lines exhibit high levels of Runx2, whereas others show decreased Runx2 expression in accordance with the findings of Thomas and colleagues who suggested that Runx2 protein levels are negatively regulated in some types of osteosarcoma (Thomas et al., 2004; Nathan et al., 2009; Pereira et al., 2009; San Martin et al., 2009; Won et al., 2009; Kurek et al., 2010; Sadikovic et al., 2010).

Recently, we presented data indicating that cell cycle control of Runx2, which is readily observed in osteoblasts, is deregulated in osteosarcoma cells (Galindo et al., 2005; San Martin et al., 2009). Runx2 is constitutively expressed throughout the cell cycle in at least two osteosarcoma (human SaOS and rat ROS) cell lines (Young et al., 2007b; San Martin et al., 2009). Hence, the transcriptional and post-transcriptional mechanisms that mediate cell

cycle control of Runx2 gene expression in osteoblasts could be compromised in osteosarcoma cells. The latter may occur in conjunction with abrogation of other molecular mechanisms cells that mediate normal osteoblast proliferation and that may bypass the growth suppressive properties of Runx2 in bone cancer cells (Nathan et al., 2009). In this article, we systematically examined human osteosarcoma cell lines with respect to Runx2 gene expression and cell cycle regulation to understand the biological functions of Runx2 in osteosarcoma cell proliferation. Our main finding is that forced expression of Runx2 suppresses growth in all cell lines, indicating that stimulation of Runx2 beyond its preestablished levels in osteosarcoma cells remains capable of triggering an anti-proliferative response. We propose that osteosarcoma cells in which Runx2 is present must balance prooncogenic functions of Runx2 with the requirement to maintain Runx2 at levels that avoid tumor suppression.

Materials and Methods

Cell culture

Human osteosarcoma cell lines were maintained in culture medium (Invitrogen) supplemented with 10–15% fetal bovine serum (FBS) plus 2 mM L-glutamine and a penicillin–streptomycin cocktail at 37°C and 5% CO₂ according to ATCC recommendations. SaOS cells were cultured in McCoy's medium supplemented with 15% FBS. U2OS and G292 cells were cultured in McCoy's medium with 10% FBS. MG-63 and HOS cells were grown in DMEM medium with 10% FBS. 143B cells were maintained in DMEM medium, 1 mM sodium pyruvate, 100 µg/ml of bromodeoxyuridine and 10% FBS.

Cell growth analysis

Cells were plated in six-well plates (8×10^4 cells/well) and grown in supplemented medium at 37°C and 5% CO₂. Alternatively, after 24 h, cells were infected with Adenovirus Runx2 or Adenovirus Vector as indicated below, and cultured for 6 days. The growth medium was changed every 2 days. Cell numbers were daily counted to determine growth curves.

Cell synchronization

Cells were seeded in 100-mm plates at 0.5×10^6 cells/plate and grown up to 60% of confluence. After that, cell cultures were treated for 24 h with 400 µM mimosine (Sigma–Aldrich, St. Louis, MO) to arrest cells in G₁ phase (Galindo et al., 2005). Cells arrested in G₁ were released by three washes in serum-free medium and stimulated to progress to S phase by the addition of fresh medium without drug containing FBS plus 2 mM L-glutamine and antibiotics (San Martin et al., 2009). After serum stimulation, cells were harvested at selected time points for Western blot and RT-PCR, as well as fluorescence-activated cell sorting (FACS) analysis.

Flow cytometric analysis

Distribution of cells at specific cell cycle stages was evaluated by assessment of DNA content by flow cytometry as previously described (Teplyuk et al., 2008). Cells were trypsinized, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol at –20°C overnight. Cells were then treated with RNase A (10 µg/ml) at 37°C for 15 min. Subsequently, cells were stained with propidium iodide and subjected to FACS analysis based on DNA content. Samples (1×10^6 cells) were analyzed using the FACStar cell sorter and Consort 30 software (Becton–Dickinson, San Jose, CA).

Western blot analysis

Runx2, cyclin D, and β -actin were analyzed by Western blot analysis as described previously (Galindo et al., 2005). Briefly, equal amounts of total cellular proteins collected in the presence of the proteasome inhibitor MG132 (Calbiochem) and Complete[®] cocktail of protease inhibitor (Roche) were resolved in 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Life Science, Buckinghamshire, UK). Blots were incubated with a 1:2,000 dilution of each primary antibody for 1 h. Runx2-specific mouse monoclonal antibody was the generous gift of Dr. Yoshiaki Ito (Cancer Science Institute, Singapore). Mouse monoclonal antibody (cyclin D1) and goat polyclonal antibody (actin) were acquired commercially (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) for 1 h. Immunoreactive protein bands were visualized on a BioMax Light film (Kodak, Rochester, NY) using a chemiluminescence detection kit (PerkinElmer Life Sciences, Waltham, MA), and signal intensities were quantified by densitometry.

cDNA synthesis and PCR

Total RNA was isolated from osteosarcoma cells using TRIzol reagents (Invitrogen) according to the manufacturer's specifications. Total RNA was separated in a 1% agarose-formaldehyde gel. Ethidium bromide staining of the gels was used to assess RNA quality of samples. Purified RNA was treated with RQ1RNase-Free DNase (Promega Corporation, Madison, WI) and subject to reverse transcription using random hexamer primers (Invitrogen Life Technologies, Carlsbad, CA) with M-MLV reverse transcriptase (Promega Corporation) according to the manufacturer's recommendations. cDNA was amplified by PCR using PCR buffer 1 \times (Promega), 0.2 mM dNTPs (Promega), 1.5 mM MgCl₂ (Promega), 0.06 U/ μ l of Taq polymerase (Promega) with the following set of specific primers to human gene: Runx2 (forward primer: 5'-CCAGATGGGACTGTGGTTACC-3', reverse primer: 5'-ACTTGGTGACAGATTCAGGG-3'), cyclin A (forward primer: 5'-GAAGACGACGGGTTGCAC-3', reverse primer: 5'-GCAGTGCCACAAGCTGAAG-3'), cyclin B (forward primer: 5'-GCTCCGAGT CACCAGGA ACT-3', reverse primer: 5'-TCCATTGGGCTTGGAGAGGC-3'), cyclin D1 (forward primer: 5'-CAGAAGAGCGCGAGGGAGCG-3', reverse primer: 5'-CTTCTCGGCCGTCAGGGG A-3'), cyclin E (forward primer: 5'-GGACAAGACCCTGGCCTCAG-3', reverse primer: 5'-TCAGG TGTGGGGATCAGGGA-3'), and GADPH (forward primer: 5'-CCTTCATTGACCTCAACTA-3', reverse primer: 5'-GGCCATCCACAGTCTTCT-3'). Aliquots of the resulting product (5 μ l) were visualized in 1% agarose gels by ethidium bromide staining.

Adenovirus infections

Adenoviral delivery of vector containing cDNA of Runx2-IRES-GFP under the control of the CMV5 promoter was used as previously described (Pratap et al., 2003). Preparation and purification of virus were performed according to the manufacturer's protocols (Promega). For control of infection, the same Adenovirus vector carrying GFP was used. The Adenovirus Runx2 (Adv-Runx2) contains both the GFP cassette and the Runx2 cDNA in forward orientation (+) and Adenovirus Vector (Adv-Vector) contains the GFP cassette in the forward orientation (+) and the Runx2 cDNA in reverse orientation (-) (Pratap et al., 2003).

Cells were plated for infections in 60 mm plates at a density of 30×10^4 cells/plate and cultured in DMEM with 10% FBS. After 24 h, cells were infected at 60–70% of confluence with 30×10^{10} OPU/ml (optical particle unit) of each virus in 900 μ l of DMEM supplemented

with 1% FBS for 4 h. Upon addition of 600 μ l of media containing 1% FBS, cells were incubated for an additional 10 h. After adenoviral infection, cells were grown for 24–72 h. Infection efficiencies were assessed by expression analysis of a green fluorescent protein (GFP) under the control of an IRES signal, and images of GFP-expressing cells were taken using a fluorescence microscope with a CCD camera.

Results

Analysis of Runx2 expression and cell proliferation potential in human osteosarcoma cell lines

To better understand Runx2 function in osteosarcoma cell proliferation, we first examined Runx2 protein levels in six human osteosarcoma cell lines (SaOS, MG63, U2OS, HOS, G292, and 143B) (Fig. 1A). Western blot analysis revealed that SaOS cells exhibit the highest Runx2 protein levels compared to the other five osteosarcoma cell lines. Runx2 protein is also detected in HOS, G292, and 143B cells, but it is expressed at significantly lower levels than in SaOS cells. U2OS cells exhibit significantly lower Runx2 protein expression, and Runx2 protein expression is barely detected in MG63. Consistently, relatively high Runx2 mRNA levels are detected by RT-PCR in SaOS cells (Fig. 1B). Runx2 mRNA levels are also detected in U2OS, HOS, G292 and 143B cells, but Runx2 mRNA is only weakly detected in MG63 (Fig. 1B).

To establish a correlation between cell proliferation and Runx2 expression in osteosarcoma, we assessed cell growth profiles. Cell growth was monitored daily by cell counting during a period of 6 days (Fig. 1C). SaOS cells display very slow growth rates compared to the other cell lines. MG63 and G292 cells exhibit moderate growth rates, U2OS and HOS cells have intermediate growth rates, while 143B cells have higher growth rates. Interestingly, SaOS cells show high Runx2 levels and lower growth rates, suggesting that presence of Runx2 may negatively correlated with cell proliferation in osteosarcoma cells.

Characterization of cell cycle profiles in human osteosarcoma cell lines

We characterized the temporal profiles of progression in different phases of the cell cycle (G_1 , S, and G_2/M) in synchronized osteosarcoma cells by assessment of DNA content and cell cycle markers (cyclin D, E, A, and B) using flow cytometry as well as RT-PCR and Western blot analysis, respectively (Fig. 2). We estimated the cell cycle length and length of specific cell cycle phases based on the percentage of cells distributed in G_1 , S and G_2/M at different time points after stimulation of proliferation in mimosine-arrested cells (Figs. 3 and 4). Thus, the apparent duration of the cell cycle is 36 h for SaOS, G292, and MG63 cells; 24 h for U2OS and HOS cells; and 18 h for 143B cells, approximately.

A detailed analysis showed that cell cycle progression was slower in SaOS, G292, and MG63 cells compared to 143B, indicating a dynamic differences in cell cycle kinetics between these cell lines (Figs. 3 and 4). The distinctions in cell cycle lengths observed in SaOS, G292, and MG63 cells is corroborated by a significant change in the lengths of G_1 (from 3 to 6 h in SaOS and MG63, and from 3 to 9 h in G292), S (from 9 to 18 h in MG63, and from 9 to 21 h in G292), and G_2/M (from 6 to 12 h in MG63, and from 6 to 21 h in SaOS) as compared to 143B (Fig. 4). We also observed a progressive reduction in the lengths of specific cell cycle phases in U2OS cells (S: 21 to 6 h and G_2/M : 21 to 12 h) and HOS (S: 21 to 12 h and G_2/M : 21 to 6 h), as compared to G292 and SaOS (Fig. 4). Based on these results, we conclude that changes in cell cycle kinetics are related to major changes in the length of specific cell cycle phases, including extension of the S and G_2/M phases (Fig. 4). Interestingly, the estimated cell cycle lengths and kinetics of progression in different

phases are consistent with the growth rates observed in each of the osteosarcoma cell lines (Figs. 1C and 4).

Cell cycle regulation of Runx2 expression in osteosarcoma

We examined Runx2 expression during cell cycle progression in synchronized osteosarcoma cells (143B, G292, HOS, U2OS, MG63, and SaOS) at multiple time points after stimulation of proliferation (Fig. 5). Runx2 protein levels decrease during G₁/S phase transition in 143B, G292, HOS, and U2OS (Fig. 6B, D–F). Thus, cell cycle dependent changes in Runx2 protein expression observed in normal pre-osteoblasts cells synchronized in the G₁ phase (Galindo et al., 2005) is also evident in 143B, G292, HOS, and U2OS osteosarcoma cells. However, we observed that Runx2 protein levels are rapidly up regulated at the end of S phase in U2OS cells. Furthermore, Runx2 protein levels are retained after mitosis during early G₁. In contrast, Runx2 is constitutively expressed throughout the cell cycle (and beyond the G₁ phase) in SaOS and MG63 cells (Figs. 5A and C and 6A and C). Yet, Runx2 mRNA levels do not exhibit any major fluctuations during the cell cycle in these osteosarcoma cell lines (Fig. 5). These data together indicate that Runx2 protein expression is modulated during the cell cycle in a sub-set of osteosarcoma-derived cells.

Runx2 suppresses cell proliferation in osteosarcoma

We conducted a systematic study on the function of Runx2 gene expression in osteosarcoma cell lines to define the role of Runx2 in osteosarcoma cell proliferation. We achieved 95% infection efficiency for adenoviral vectors based on fluorescence detection of co-expressed GFP proteins, as assessed by fluorescence microscopy (Fig. 7). As expected, exogenous expression of Runx2 into any of the osteosarcoma cell lines inhibits cell proliferation as reflected by a prominent decrease in the slope of the growth curve when compared with control cells expressing GFP alone (Fig. 7).

Discussion

In osteoblast cells, Runx2 protein levels are dramatically upregulated in quiescence (G₀) or proliferative arrest induced by serum deprivation or by contact inhibition. In contrast, Runx2 protein decreases to minimal levels when the cells are stimulated to proliferate, consistent with its function as a negative regulator of cell proliferation (Pratap et al., 2003; Galindo et al., 2005, 2007). More specifically, Runx2 protein levels decreases at G₁/S transition and remains low during G₂ and M phases, regaining higher levels postmitotically in early G₁ (Galindo et al., 2005, 2007). Thus, in normal osteoblastic cells (e.g., MC3T3), Runx2 protein levels are modulated at three different cell cycle stages (i.e., the G₁/G₀, G₁/S, and M/G₁ transitions) (Galindo et al., 2005).

Previously, we showed that Runx2 is constitutively expressed at high levels throughout the cell cycle in osteosarcoma cells (i.e., human SaOS and rat ROS) (Young et al., 2007b; San Martin et al., 2009). However, these studies included only a single human cell line. Our current results advance our understanding of Runx2 expression during the cell cycle in a panel of six osteosarcoma cell lines. In osteosarcoma, Runx2 protein levels are down-regulated at the G₁/S phase transition in 143B, G292, HOS, and U2OS cell lines and it is constitutively expressed through the cell cycle only in MG63 and SaOS cells. Down-regulation of Runx2 in late G₁ (G₁/S transition) in osteosarcoma may be mediated by CDK-related and ubiquitin/proteasome dependent protein degradation as suggested previously (Galindo et al., 2005; Shen et al., 2006; Rajgopal et al., 2007). Moreover, the proteasome inhibitor bortezomib suppresses growth and increases Runx2 protein levels in osteosarcoma cell lines (Shapovalov et al., 2010). The latter finding may be linked to our demonstration that stabilization of Runx2 protein levels by the proteasomal inhibitor MG132 is restricted to

a stage in late G1 in osteosarcoma cells (San Martin et al., 2009). Runx2 gene expression during the cell cycle is also controlled at least in part at the transcriptional level, because Runx2 mRNA levels are also down-regulated in pre-osteoblastic cells progressing towards the G₁/S transition (Galindo et al., 2005). However, we do not observed major variations in Runx2 mRNA levels during progression to S phase in osteosarcoma cells, which suggest that protein destabilization by the proteasome pathway could be one of the principal mechanisms that modulate Runx2 expression during cell cycle progression in osteosarcoma cells.

We have previously shown that Runx2 deficiency increases the proliferative potential of osteoprogenitors and that reintroduction of wild type Runx2 into primary calvarian cells restores stringent cell growth control (Pratap et al., 2003). Also, ectopic over-expression of Runx2 inhibits proliferation of osteoprogenitors and committed osteoblasts (Galindo et al., 2005; Young et al., 2007a; Teplyuk et al., 2008). Interestingly, exogenous Runx2 suppresses growth of G292 osteosarcoma cells, but other osteosarcoma cell lines, such as SaOS and U2OS, appears to be refractory to Runx2 anti-proliferative functions (Thomas et al., 2004). Our present results suggest that Runx2 may retain its ability to control osteosarcoma cell growth, based on the observation that Runx2 suppresses growth of all osteosarcoma cells when over-expressed. This latter finding complements studies using an osteosarcoma xenograft mouse model in which BMP-promoted tumor growth in MG63 cells is inhibited by exogenous Runx2 expression (Luo et al., 2008).

It has been suggested that lack of functional pRB may account for the inability of Runx2 to attenuate cell proliferation in osteosarcoma (Thomas et al., 2004). However, our results may indicate that Runx2 growth control in osteosarcoma involves other molecular pathway linked to the cell cycle. The growth regulatory functions of Runx2 are mediated in part through epigenetic mechanisms and the ability of Runx2 to regulate the expression of cell growth related genes that promote a nonproliferative state (Pratap et al., 2003; Galindo et al., 2005; Kilbey et al., 2007; Young et al., 2007a,b; Zaidi et al., 2007b, 2010; Teplyuk et al., 2008, 2009a,b). Runx2 target genes linked to cell cycle may contribute to the control of osteosarcoma tumor growth (Manara et al., 2006; Young et al., 2007a,b; Sadikovic et al., 2009; van der Deen et al., 2012).

From a broader perspective, Runx1, Runx2, and Runx3 transcription factors are specific master regulators for hematopoiesis, bone and gastric differentiation, respectively (Stein et al., 2010). Inactivating mutations or epigenetic silencing of Runx1 and Runx3 are associated to leukemia and gastric cancer, respectively (Song et al., 1999; Li et al., 2002; Cameron and Neil, 2004). However, Runx2 mutations have been related mainly to skeletal defects but not to bone cancer, suggesting that this factor is not inactivated in osteosarcoma. Previous studies have reported that Runx2 is expressed in distinct cancer types. Runx2 has been involved in tumor progression and bone metastasis in breast and prostate cancer (Pratap et al., 2005, 2006, 2008), as well as tumorigenesis in lymphomas (Blyth et al., 2005). In normal mammary epithelial cells Runx2 is expressed at low levels, but it is expressed at high levels in metastatic mammary cancer cells (Selvamurugan and Partridge, 2000; Inman and Shore, 2003; Barnes et al., 2004) and promotes bone metastatic properties of breast and prostate cancer cells (Brubaker et al., 2003; Javed et al., 2005; Pratap et al., 2005, 2006, 2008). Runx2 enhanced expression is also associated to lymphoma development where it oncogenic potential has been well established (Blyth et al., 2006). Moreover, Runx2 is strongly expressed in human malignant melanoma, thyroid papillary carcinoma, glioma, and pituitary tumor, which suggests that this cancer gene could promote tumorigenesis in a broad spectrum of tumors (Riminucci et al., 2003; Endo et al., 2008; Vladimirova et al., 2008; Zhang et al., 2009). Regarding to bone cancer, several studies have shown that Runx2 is differentially expressed in osteosarcoma patient specimens where high expression has

been associated with tumor progression, poor response to chemotherapy, metastasis, and lower survival (Won et al., 2009; Kurek et al., 2010; Sadikovic et al., 2010). The findings presented here indicate unambiguously that Runx2 protein controls osteosarcoma cell proliferation when over-expressed beyond its normal pre-established levels in a given osteosarcoma cell type. Therefore, we propose that Runx2 expression is biologically and functionally linked to tumor growth control in human osteosarcoma.

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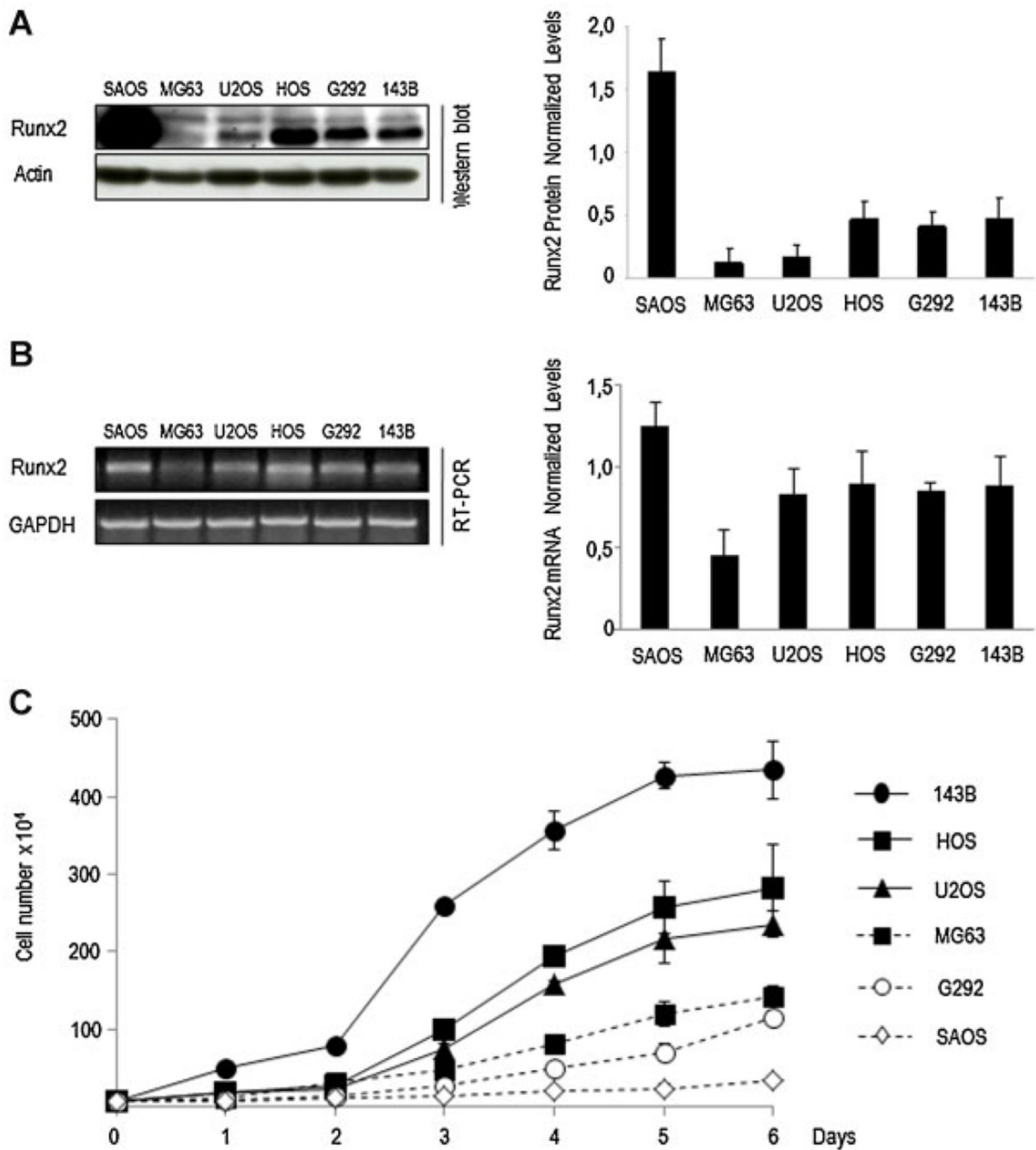


Fig. 1. Runx2 expression and growth profile in human osteosarcoma cell lines. Runx2 expression was assessed in SaOS-2, G292, MG63, U2OS, HOS, and 143B osteosarcoma cells. Runx2 protein (A, right part) and mRNA (B, right part) levels were evaluated by Western blot analysis and RT-PCR, respectively (A). Runx2 protein and mRNA values were normalized to actin (A, left part) and GAPDH (B, left part), respectively. Proliferation was monitored by determining cell number at the indicated days (C). All data are presented as mean \pm SEM.

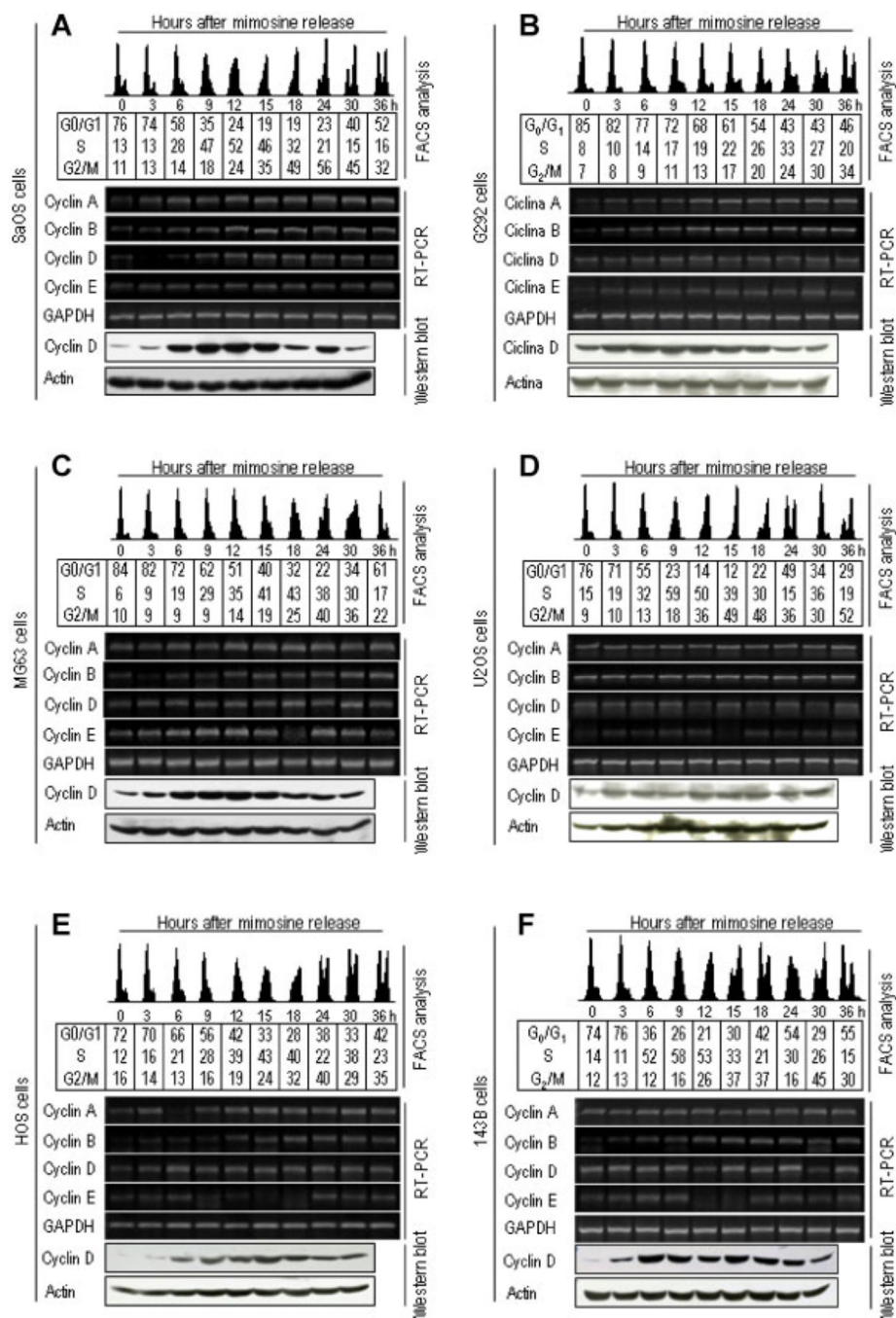


Fig. 2. Human osteosarcoma cell lines exhibit different cell cycle profiles. SaOS-2, G292, MG63, U2OS, HOS, and 143B osteosarcoma cells were synchronized by incubation for 24 h with mimosine to generate a G₁ phase block. Then, cells were released from G₁ phase arrest by the addition of fresh culture medium without mimosine and harvested after 0, 3, 6, 9, 12, 15, 18, 24, 30, and 36 h (A–F). Progression through successive cell cycle phases (G₁, S, and G₂/M) was monitored by flow cytometry (top part). Expression of cell cycle markers cyclins A, B, D, and E mRNA (middle part) and cyclin D1 protein (bottom part) were analyzed by Western blot and RT-PCR, respectively.

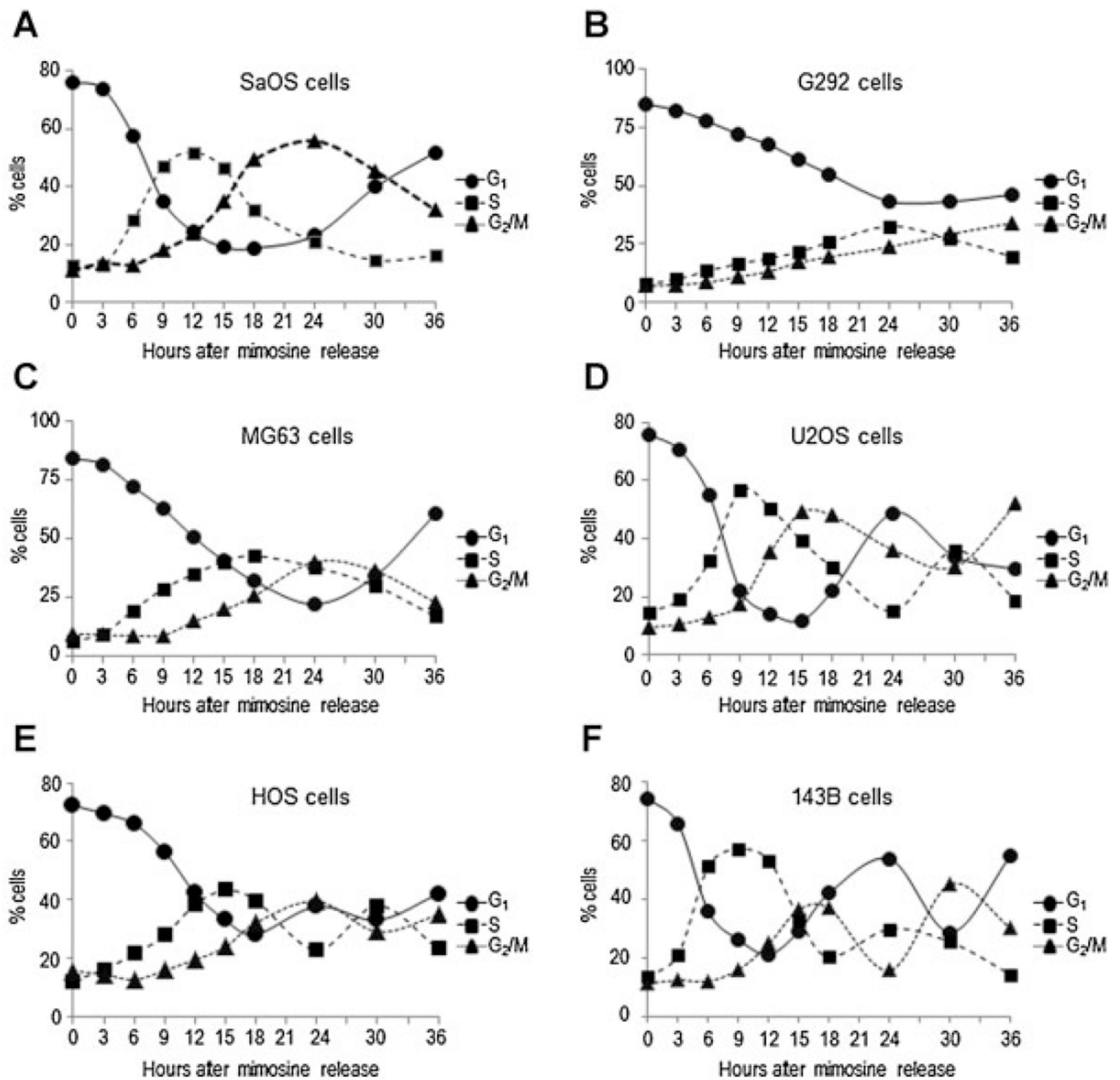
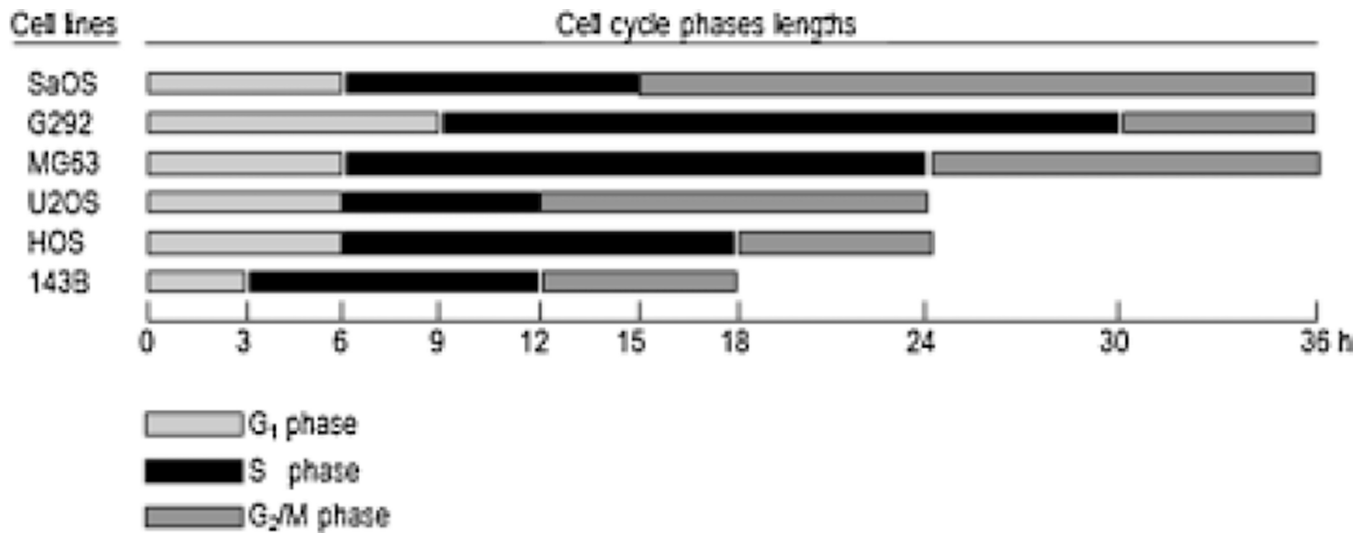


Fig. 3. Human osteosarcoma cell lines show different patterns of progression through the cell cycle. Percentage of osteosarcoma cells in different cell cycle phases (G₁, S and G₂/M) were obtained from data shown in Figure 2. Graphic representation of progression through successive cell cycle phases are showed for each osteosarcoma cell line (A-F).



Estimated duration of specific cell cycle phases in osteosarcoma cell lines.

Cell Line	G ₁ phase	S phase	G ₂ /M phases	S/G ₂ /M phases	Cell Cycle
SaOS	6 h	9 h	21 h	30 h	36 h
G292	9 h	21 h	6 h	30 h	36 h
MG63	6 h	18 h	12 h	30 h	36 h
U2OS	6 h	6 h	12 h	18 h	24 h
HOS	6 h	12 h	6 h	18 h	24 h
143B	3 h	9 h	6 h	15 h	18 h

Fig. 4. Human osteosarcoma cell lines show different cell cycle lengths. Cell cycle lengths and lengths of specific cell cycle phases (G₁, S, and G₂/M) were determined from data shown in Figure 3. Graphic representation of duration of specific cell cycle phases are shown for each osteosarcoma cell line (A–F). The bottom portion shows the estimated duration of specific stages during the cell cycle in hours (h).

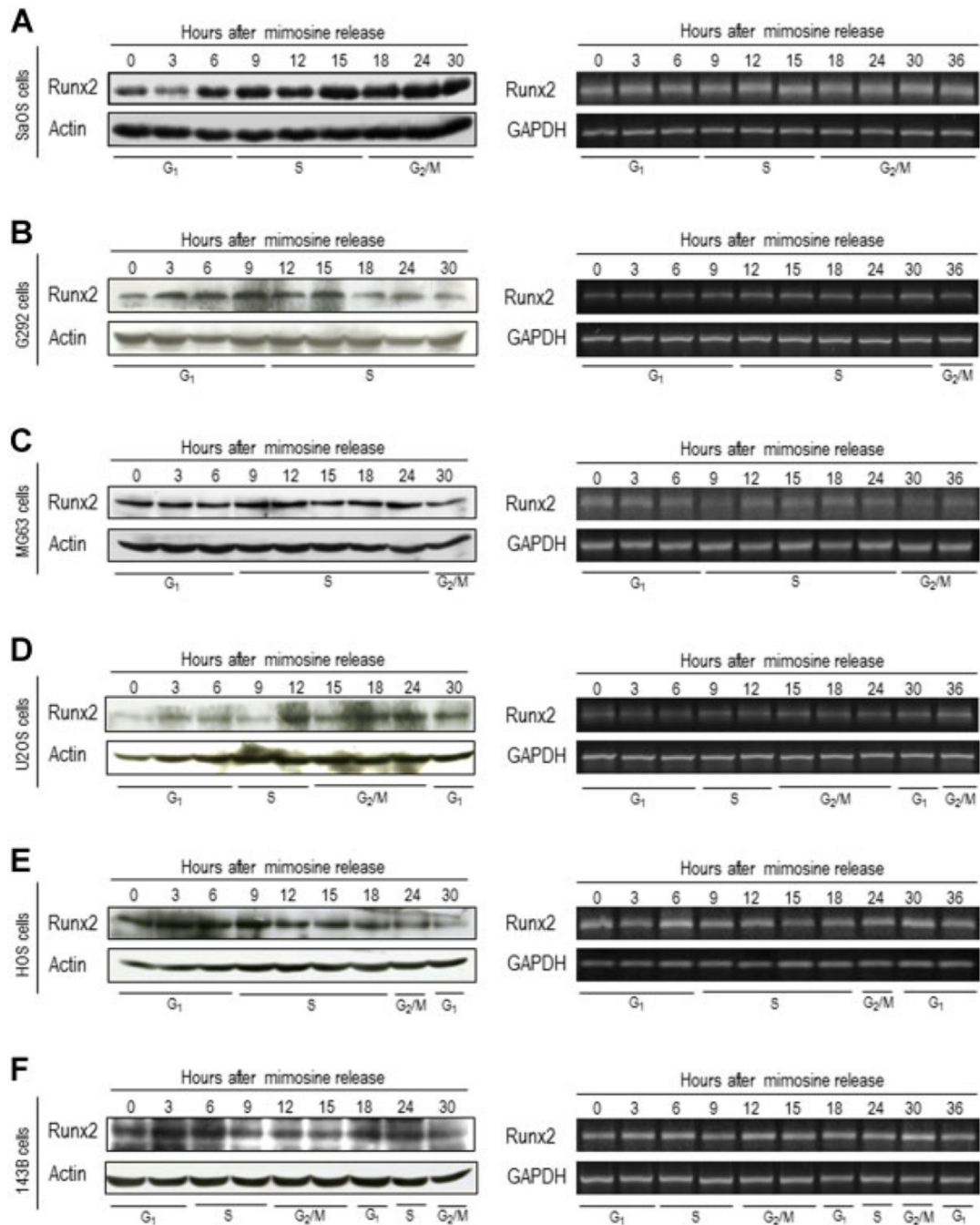


Fig. 5. Runx2 expression is differentially regulated during the cell cycle in different human osteosarcoma cell lines. Runx2 gene expression was assessed during progression through the cell cycle to determine the specific transition stages when Runx2 levels are modulated in SaOS-2, G292, MG63, U2OS, HOS, and 143B osteosarcoma cell lines (A–F). Cells were synchronized at the G₁ phase and stimulated to progress through the cell cycle as described in Figure 2. Cells were harvested after 0, 3, 6, 9, 12, 15, 18, 24, 30 and 36 h stimulation. This set of samples was the same that were used to assess cell cycle markers showed in Figure 2. Cell cycle-dependent modulations in Runx2 protein and mRNA levels were evaluated by Western blot analysis and RT-PCR. Actin and GAPDH showed in Figure 2

were also used as a loading control for Runx2 protein and mRNA, respectively. Cell cycle phases as determined by flow cytometry are indicated at the base of the parts.

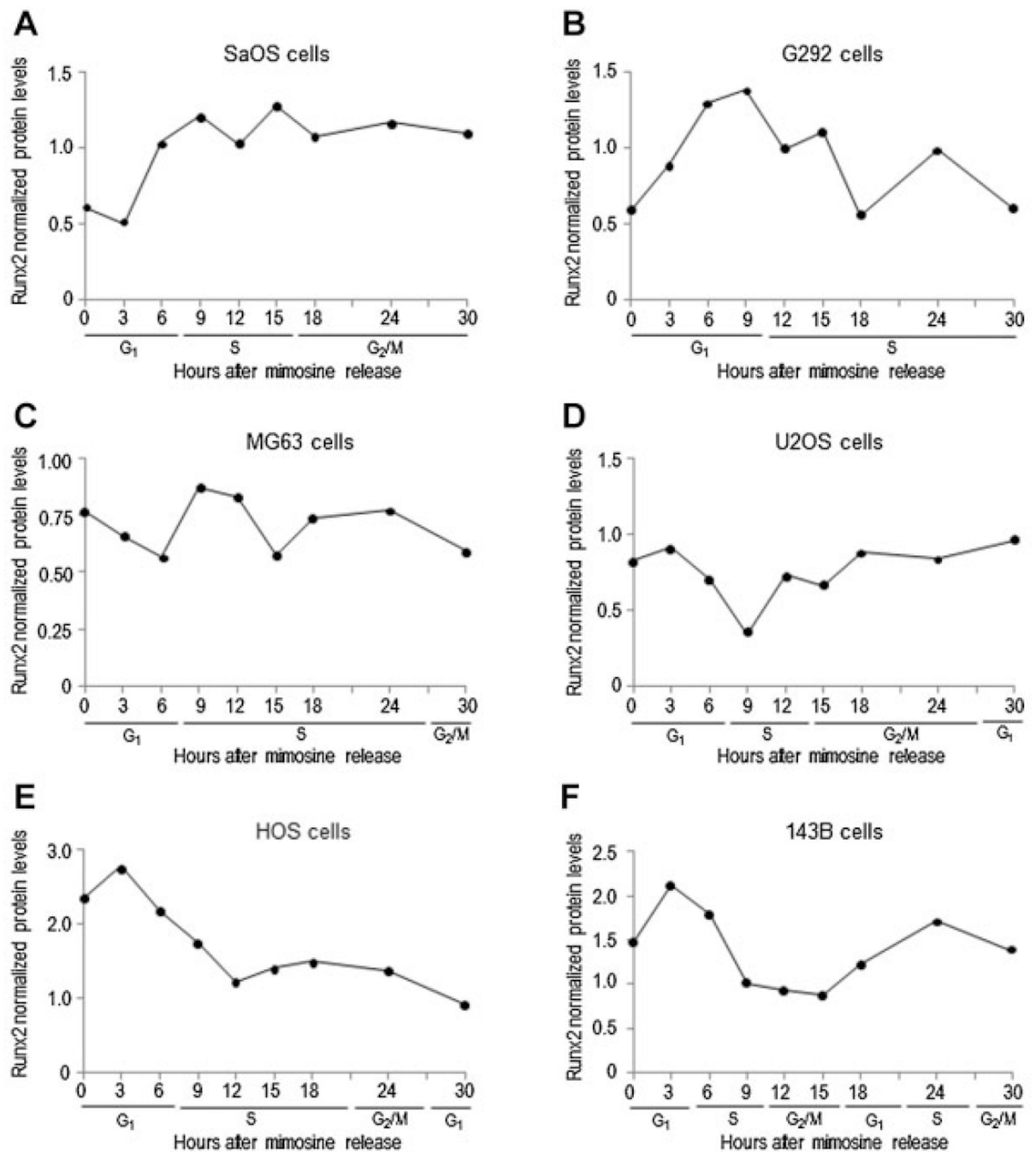


Fig. 6. Runx2 protein levels are differentially modulates during the cell cycle in human osteosarcoma cell lines. Runx2 expression in osteosarcoma cells were quantified from data shown in Figure 5. Cell cycle-related changes in Runx2 protein levels after serum stimulation were depicted in a graphic representation (A–F). Protein values were normalized to actin. Cell cycle phases as determined by flow cytometry are indicated at the base of the graphs.

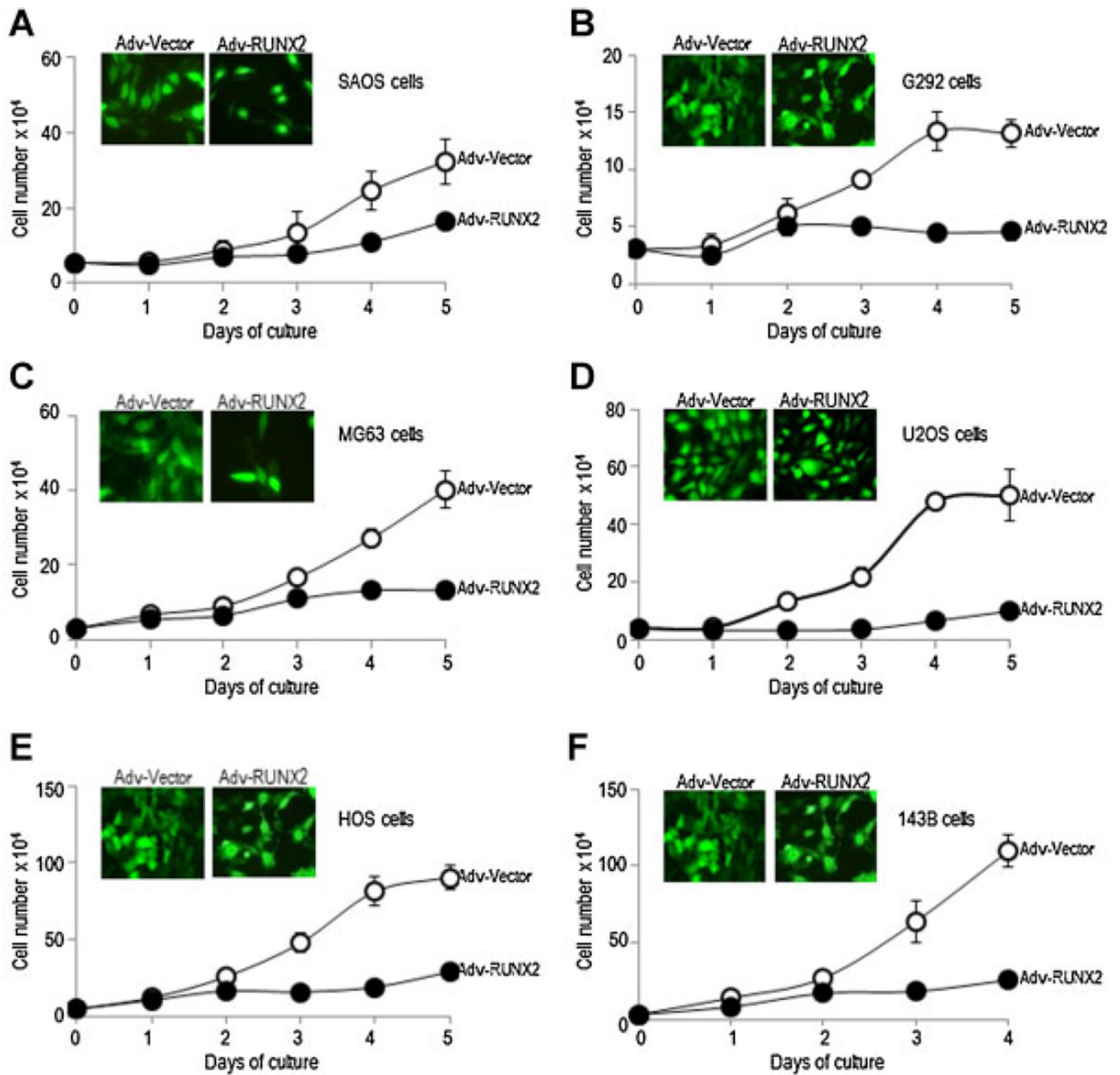


Fig. 7. Runx2 modulates cell proliferation in human osteosarcoma cell lines. SaOS-2, G292, MG63, U2OS, HOS, and 143B osteosarcoma cells were infected with adenoviral vectors expressing Runx2 plus GFP (Adv-Runx2, closed circles) or GFP alone (Adv-Vector, open circles) at comparable efficiencies of infection. After transduction, cell growth was monitored at the indicated days. Growth curves for each osteosarcoma cell lines were obtained by cell counting at daily intervals until 5 days of culture after infection (A–F). Day 0 is the day of infection. Microscopic analysis of GFP expression shows efficient transduction (up to 90%) for each cell line (insert parts, cells infected with Adv-Runx2 or Adv-Vector). All data are presented as mean \pm SEM.