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Impaired cell cycle regulation of osteoblast-related transcription factor Runx2/Cbfa1 in osteosarcoma cells

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Abstract

In mammals, bone differentiation requires the functional expression of the Runx2/Cbfβ heterodimeric complex. Our previous results indicate that Runx2 is also a suppressor of pre-osteoblast proliferation by affecting cell cycle progression at G_1. Runx2 levels are cell cycle regulated, oscillating from a maximum during early G_1 to a minimum during late G_1, S and mitosis phases in proliferating pre-osteoblasts Nevertheless, there is no information concerning Cbfβ gene expression during the cell cycle nor on Runx2 cell cycle expression in bone cancer cells. We analyzed Runx2 and Cbfβ gene expression during cell cycle progression in the pre-osteoblast MC3T3 and osteosarcoma ROS and SaOS cell lines. The expected reduction of Runx2 protein level was observed in MC3T3 cells arrested in late G_1 or M phase using mimosine or nocodazole, respectively. However, this reduction was not observed in the cell cycle arrested osteosarcoma cells. Cbfβ protein levels were not regulated during the cell cycle in pre-osteoblasts and osteosarcoma cells. Using cells synchronized in late G_1 and mitosis we found that Runx2 levels, but not Cbfβ levels, were cell cycle regulated in MC3T3 osteoblasts. Interestingly, both factors showed a constitutively elevated expression throughout the cell cycle in osteosarcoma cells. Proteasome inhibition by MG132 prevented cell cycle-dependent downregulation of Runx2 protein levels in osteoblasts, but not in osteosarcoma. We propose that Runx2 is involved in tumoral osteosarcoma progression. Altogether, deregulated Runx2 expression throughout the cell cycle seems to constitute a central mechanism in the pathogenesis of osteosarcoma.

INTRODUCTION

The transcription factor Runx2 (run-related transcription factor 2) forms a functional heterodimeric complex with Cbfβ (core-binding factor β) (Meyers et al., 1993; Speck and Stacy, 1995). Cbfβ is a cotranscription factor that does not bind directly to DNA but enhances Runx2 binding to DNA (Ogawa et al., 1993; Speck and Stacy, 1995). Osteogenic differentiation induced by Runx2 is dramatically increased by the co-expression of Cbfβ,

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even though this factor fails to induce osteogenesis on its own (Lien et al., 2007). Furthermore, Runx2 protein is stabilized by its interaction with Cbfβ, hence protecting it from ubiquitination and its proteasomal degradation (Huang et al., 2001; Zhao et al., 2003). Thereby, association between Runx2 and Cbfβ is central in the process of bone differentiation *in vivo*, leading to an efficient binding of Runx2 to DNA and the concomitant Runx2 mediated bone phenotypic gene activation(Kundu et al., 2002; Yoshida et al., 2002).

One of the critical steps for normal bone formation is the adequate and strict control of the proliferative expansion of mesenchymal, osteoprogenitor and immature osteoblast cells in response to mitogenic signals such as growth factors and cytokines (Liu et al., 2002). Runx2 has been found to control osteoblast proliferation, promoting a transition from a proliferative stage to a post proliferative stage prior to osteoblast differentiation. This could be achieved in part through epigenetic mechanisms and partly through the ability of Runx2 to regulate the expression of cell growth related genes and promote a non- proliferative state (e.g., G_0/G_1 phase transition and senescence) (Pratap et al., 2003; Galindo et al., 2005; Young et al., 2007a; Young et al., 2007b; Teplyuk et al., 2008; Teplyuk et al., 2009; Zaidi et al., 2007; Kilbey et al., 2007). Accordingly, transient Runx2 overexpression in synchronized cells delays cell cycle entry into S phase and significantly decreases cell proliferation in the MC3T3 preosteoblast, Runx2 null calvaria 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; Teplyuk et al., 2009).

Consequently, Runx2 protein levels are strictly regulated during cell cycle in osteoblast cells. Runx2 protein levels are dramatically elevated in quiescence (G_0) or proliferative arrest induced by serum deprivation or by contact inhibition. On the contrary, 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; Galindo et al., 2007). More specifically, Runx2 protein levels decrease at the G_1/S transition and remain low during mitosis, regaining higher levels postmitotically in early G_1 (Galindo et al., 2005; Galindo et al., 2007).

Runx2′s proliferative function could be related to the interaction between its carboxyl terminal domain and several cofactors that integrate some of the signaling pathways related to cell proliferation, such as Smad, Groucho, MAPK, FGF, pRB, c-Jun, c-Fos and TWIST (Nuthall et al., 2002; Thomas et al., 2001; Xiao et al., 2000; Yousfi et al., 2002; Zaidi et al., 2002). Consequently, Runx2 mutants defective for DNA binding or C-terminal gene repression/activation functions do not block proliferation (Teplyuk et al., 2008).

The proposed function of Runx2 as a regulator of cell cycle proliferation in bone cells is consistent with the regulatory function described for other members of the Runx protein family (Pratap et al., 2003; Bae and Choi, 2004; Cameron and Neil, 2004; Miyazono et al., 2004). Runx proteins may function as tumor suppressors or promoters depending on the biological context (Blyth et al., 2005). Also, Runx2 deficiency has been related to cell immortalization and tumorigenesis (Zaidi et al., 2007). This is in agreement with earlier studies involving Runx1 and Runx3, in which inactivating mutations or epigenetic silencing were associated with hematologic or gastric malignancies. On the other hand, Runx2 has been implicated in cancer progression and bone metastasis in breast and prostate cancer (Blyth et al., 2005). Transient inactivation of Runx2 could promote tumorigenesis, and its subsequent reactivation could sustain its metastatic phenotype (Khalid et al., 2008; Baniwal et al., 2009).

The observation of elevated levels of Runx2 in OS1 osteosarcoma cells is in contradiction with the findings of Thomas et al, who suggest that Runx2 protein is negatively regulated in

some types of osteosarcoma (Thomas et al., 2004). Previous studies have shown that ROS 17/ 2.8 rat osteosarcoma cells and SaOS-2 human osteosarcoma cells express elevated Runx2 levels (Galindo et al., 2005). However, there is no systematic study regarding Runx2 and Cbfβ expression in osteosarcoma cells nor on their regulation during the cell cycle.

MATERIALS AND METHODS

Cell Culture and Synchronization

Experiments were performed with the mouse pre-osteoblast cell line MC3T3-E1 and osteosarcoma cells (rat ROS17/2.8 and human SaOS-2 cells). MC3T3-E1 cells were maintained in αMEM supplemented with 10% fetal bovine serum (FBS) plus 2mM L-glutamine and a penicillin-streptomycin cocktail. ROS17/2.8 cell were cultured in F12 supplemented with 5% FBS and SaOS-2 cells in McCoy's supplemented with 15% FBS, plus L-glutamine and antibiotics. Cells were seeded in either 6-well or 100-mm plates at 0.08×10^6 cells/well or 0.4×10^6 cells/plate, respectively, and grown in a sub-confluent state for 24 h until the onset of exponential growth. Cell cultures grown in supplemented medium were treated with the indicated cell cycle inhibitors to arrest them at different cell cycle stages (Galindo et al., 2005). Cells were treated for 24 h with 400 μM mimosine (Sigma-Aldrich) to arrest cells in the late G_1 phase. Cell cycle arrest in mitosis was achieved by nocodazole treatment. Cells grown in medium plus FBS were treated with 100 ng/ml nocodazole (Sigma-Aldrich) for 16 h., followed by shake-off of mitotic cells. Cells arrested in mitosis (nocodazole) or at late G_1 (mimosine) were released by three washes in serum-free medium and stimulated to progress, respectively, to G_1 or S phase by the addition of fresh medium without drug containing FBS plus 2mM L-glutamine and antibiotics. After serum stimulation, cells were harvested at selected time points for Western blot analysis, Northern blot analysis and fluorescence-activated cell sorting (FACS) analysis. The proteasome inhibitor MG132 (Calbiochem) was used to inhibit cell cycle specific degradation of proteins. MG132 was prepared as a stock solution of 25 mM in dimethyl sulfoxide and administered to the cells after a 1:1,000 dilution (25 μM).

Flow Cytometric Analysis

The cell distribution at specific cell cycle stages was evaluated by flow cytometry. Cells were trypsinized, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol at −20 °C overnight. Then they were 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 (Teplyuk et al., 2008). Samples (1×10^6 cells) were analyzed using the FACStar cell sorter and Consort 30 software (Becton Dickinson).

Western Blot Analysis

Runx2, Cbfβ and cell cycle markers were analyzed by Western blot analysis as described previously (Galindo et al., 2005; Galindo et al., 2007). Briefly, equal amounts of total cellular protein 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 (Immobilon-P; Millipore, Billerica, MA.). Blots were incubated with a 1:2,000 dilution of each primary antibody for 1 h. Rabbit polyclonal antibodies (Cbfβ, Cdk1, Cdk2, Cdk4, cyclin A and cyclin E), mouse monoclonal antibody (cyclin D1), and goat polyclonal antibodie (actin) were acquired commercially (Santa Cruz Biotechnology, Inc.). Ubiquitin rabbit polyclonal antibody and ubiquitin purified protein were purchased from Sigma (Sigma Life Science). Runx2-specific mouse monoclonal antibody was the generous gift of Dr. Yoshido Ito (Institute for Molecular and Cellular Biology, Singapore). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) for 1 h. Immunoreactive

protein bands were visualized on a film (BioMax, Kodak) by a chemiluminescence detection kit (PerkinElmer Life Sciences), and signal intensities were quantitated by densitometry.

Northern Blot Analysis

Total RNA was isolated from MC3T3, ROS and SaOS cells by using TRIzol reagents (Invitrogen) according to the manufacturer's specifications. Total RNA (20 μg/lane) was separated in a 1% agarose-formaldehyde gel, transferred onto Hybond Plus membranes (Amersham Biosciences), and hybridized to probes specific for Runx2, Cbfβ and histone H4. Hybridization was performed as described previously (Galindo et al., 2005) in the presence of buffer containing 50% formamide at 42 °C. Then, blots were washed extensively in buffer containing 1X SSC (standard saline citrate) and 0.1% SDS at 55 °C. Data were analyzed after exposure using a Storm 840 PhosphorImager (Molecular Dynamics, Inc.). Ethidium bromide staining of the gels was used to assess equal loading and the RNA quality of samples.

RESULTS

Regulation of Runx2 and Cbfβ protein and mRNA levels in preosteblast and osteosarcoma cells in late G_1 phase and mitosis

In this study, Runx2 and Cbfβ mRNA and protein levels were assessed in the preosteoblast (MC3T3) and osteosarcoma (ROS and SaOS) cell lines arrested in late G1 phase (mimosine blockade) and mitosis (nocodazole blockade). The specific cell cycle markers cyclin D1 protein (G_1 phase), histone H4 mRNA (S phase) and cyclin A protein (S/G2/Mphase) were used to confirm late G_1 phase and mitotic arrest. Cell cycle blockade at specific phases was also corroborated by FACS analysis.

MC3T3 cells arrested in late G_1 phase displayed high cyclin D1 protein levels, while low cyclin A levels were observed (Fig. 1A). Histone H4 mRNA was absent in this cell cycle stage (Fig. 1B). As expected, arresting these cells in mitosis resulted in elevated cyclin D1 and cyclin A protein levels, with the absence of histone H4 mRNA (Fig. 1, A and B). On the other hand, the osteosarcoma cell lines arrested in late G_1 phase and mitosis exhibited some altered patterns of cell cycle markers (Fig. 1, D, E, G and H). However, flow cytometry analysis validated arrest at distinct cell cycle phases in both the preosteoblast and osteosarcoma cell lines (Fig. 1, A, D and G). We confirmed our previous results showing that Runx2 protein levels were markedly decreased in MC3T3 cells, compared to actively proliferating asynchronic control cells, when cell cycle arrest was induced in late G_1 and mitosis (Fig. 1, B and C). Northern blot data also confirmed that Runx2 mRNA levels were decreased during late G_1 arrest, but elevated in mitosis (Fig. 1B). Contrarily, Cbfβ protein and mRNA levels were increased in this phase of the cell cycle (Fig. 1, B and C).

Strikingly, ROS and SaOS osteosarcoma cells did not downregulate Runx2 protein levels during late G_1 phase and mitosis (Fig. 1, E, F, H and I). Runx2 and Cbfβ protein levels were only slightly reduced in late G_1 and mitosis while their mRNA showed minimal variations specific to each cell line.

The results show that while MC3T3 cells display a differential expression of Runx2 and Cbfβ in the different cell cycle stages, as expected in normal cells, osteosarcoma cells exhibit a more constitutive expression of these factors having only minor changes in late G_1 and mitosis.

Cell cycle regulation of Runx2 and Cbfβ expression in preosteoblast and osteosarcoma cells

With the aim of monitoring the expression of Runx2 and its cofactor Cbfβ throughout the cell cycle, we synchronized MC3T3, ROS and SaOS cell lines using mimosine and subsequently released them from late G_1 phase arrest. Cell cycle progression was monitored by flow cytometric analysis (Fig. 2, A, C and E). Cell cultures arrested in late G_1 exhibited a high proportion of cells in G_1 (70-80%). Cyclin D1 protein levels increased progressively during late G_1 (0-9 h) in MC3T3 cells whereas they remained constitutively elevated in osteosarcoma cells (Fig. 2, A, C and E).. Progression beyond the G_1/S phase transition was evident by 9-12 h in preosteoblasts, and it was observed at 0-3 h in ROS cells and at 6-9 h in SaOS cells. The fraction of cells in S phase was dramatically increased in the osteosarcoma cell lines, reflected by increased levels of the cell cycle regulatory proteins cyclins E and A, and by the expression of DNA replication-dependent histone H4 gene (Fig. 2). By 18-24 h, all cell lines had progressed into the G2/M phases and subsequently to the postmitotic G_1 stage (24-30 h), as based on FACS analysis and levels of cell cycle markers, which showed a decline in cyclins E and A, and in H4 gene expression.

Runx2 protein expression was reduced in late G_1 (0 h), while Cbfβ protein levels were elevated at the same time point in MC3T3 cells (Figs. 2B and 3A). As cells progressed into G_1 and S, a transient but discrete increase in Runx2 was observed (3-18 h), followed by a marked increase after the M/G_1 phase transition (24 h). High Runx2 levels were maintained during early G_1 (30 h) reaching a maximum expression at this time. On the other hand, Cbfβ protein levels were constantly elevated displaying only minor variations throughout the cell cycle (Figs. 2B and 3A). On the contrary, Runx2 and Cbfβ protein levels in osteosarcoma cells showed much greater stability. In ROS cells Runx2 and Cbfβ protein levels were stably elevated throughout the cell cycle (Figs. 2D and 3C). Similarly, these two proteins were elevated in SaOS cells, with a slight increase at the end of S and during the G2/M/G_1 transition (Figs. 2F and 3E).

Runx2 mRNA levels in synchronized MC3T3 cells were elevated in late G_1 (0-6 h), decreasing during the final stages of G_1 (6-9 h), reaching their lowest level at the onset of S (12 h) (Figs. 2B and 3B). After this point, Runx2 mRNA levels increased progressively throughout the S and G2/M phases (15-24 h) reaching a maximum in early G_1 (30 h). Cbfβ mRNA levels behaved in a similar way to Runx2 throughout the cell cycle in MC3T3 cells (Figs. 2B and 3B).

Runx2 and Cbfβ mRNA levels in synchronized osteosarcoma cells displayed a similar pattern to that observed in preosteoblasts. However their fluctuations during the cell cycle were smaller (Figs. 2 D and F). Thus, ROS cells exhibit elevated Runx2 and Cbfβ mRNA levels during late G_1 (0 h) which decreased slightly during the G_1/S phase transition (3-15 h) (Figs. 2D and 3D). Subsequently, Runx2 mRNA levels increased at the end of S and during G2/M (18-24 h) reaching maximum levels in early G_1 (30 h) (Figs.2D and 3D). However, Cbfβ mRNA levels did not show any major fluctuations during the cell cycle (Figs. 2D and 3D). Finally, in SaOS cells, Runx2 and Cbfβ transcript levels fluctuated discretely in parallel during the cell cycle. Both mRNA levels showed a small decrease in late G_1 and early S (0-12 h), increasing progressively during late S, G2/M and early G_1 phases, with Cbfβ displaying only small variations (15-30 h) (Figs. 2F and 3F).

Postmitotic Runx2 and Cbfβ expression in preosteoblast and osteosarcoma cells

Our next step was to assess Runx2 and Cbfβ expression during the M/G_1 phase transition in more detail. To achieve this, osteoblast and osteosarcoma cells were synchronized in mitosis using nocodazole and were then released into G_1. Progression into G_1 was monitored by

FACS analysis and expression of specific cell cycle markers. Cell synchronization was confirmed by measuring DNA content in the three cell lines during the post-mitotic progression in early G_1 phase (Fig. 4, A, C and E). The data show that 85-91% of cells were arrested in mitosis after nocodazole treatment (0 h). After drug withdrawal and stimulation with FBS (2-10 h) the percentage of mitotic cells decreased to 25-39%, while the percentage of cells in G_1 increased to 46-59%, reflecting the synchronized progression into early G_1 phase. As expected, cell cycle marker modulations were consistent with this cell cycle progression. After mitosis, cyclin A protein level decreased abruptly, whereas cyclin D protein levels increased progressively, indicating the onset of early G_1 (0-10 h) (Fig. 4, A, C and E). Consistently, cyclin E protein and histone H4 mRNA were absent during this period (Fig. 4).

Reduced Runx2 protein levels observed in MC3T3 cells arrested in mitosis (0 h), increased acutely at the onset of G_1 (2 h). (Fig. 4B and 5A). Subsequently, Runx2 levels remained high, with some fluctuations, as the cells progressed to early G_1 (2-10 h). On the other hand, Cbfβ protein levels which were elevated in mitosis decreased slightly post-mitotically (2 h), maintaining constitutively high levels thereafter through early G_1 (2-10 h). Interestingly, in both osteosarcoma cell lines Runx2 and Cbfβ protein levels were highly expressed in mitosis (0 h) (Fig. 4, D and F) In these bone cancer cells, we observed a slight reduction in the levels of both factors at the onset of G_1 (2 h), remaining at constitutively high levels during progression to early G_1 (2-10 h) (Fig. 5, C and E).

Elevated Runx2 and Cbfβ mRNA levels in mitotic MC3T3 cells (0 h) decreased when cells progressed to early G_1 (2-4 h) (Figs. 4B and 5B). Runx2 mRNA levels began to increase again after 6 h, decreasing slightly by 10 h, while Cbfβ mRNA levels remained consistently low during early G_1 (4-10 h). In osteosarcoma cells, these mRNAs had a similar temporal expression pattern during post-mitotic progression in early G_1 (Figs. 4, D and F; see also Fig. 5, D and F). Runx2 and Cbfβ mRNA levels were elevated during mitosis in osteosarcoma cells, similarly to their expression pattern in preosteoblasts. However, their levels had a slight decrease during early G_1 (2-6 h) and then increased at the end of this stage (8-10 h) (Fig. 4, D and F; see also Fig. 5, D and F).

Runx2 protein modulation by a ubiquitin proteasome-dependent mechanism during the cell cycle in preosteoblast and osteosarcoma cells

One major mechanism for rapidly modulating protein levels of cell growth regulators at specific cell cycle stages involves the ubiquitination-dependent degradation of proteins through proteasomal pathways (55-57). Previously, we showed that proteolytic degradation of Runx2 is cell cycle- and proteasome-dependent in MC3T3 cells, and intracellular concentrations of Runx2 are enhanced by inhibition of the 26 S proteasome (Galindo et al., 2005). To analyze the global activity of the ubiquitin proteasome system in preosteoblast and osteosarcoma cell lines, MG132 induced proteasome inhibition was used. In the presence of MG132, the levels of endogenous ubiquitinated proteins were significantly increased in preosteoblasts, but only modestly in osteosarcoma cells (Fig. 6A). We also observed a reduced level of ubiquitinated proteins during late G_1 phase arrest induced by mimosine treatment in osteosarcoma cells, as compared to preosteoblasts (Fig. 6B). However, similar level of ubiquitinated proteins were observed during S phase arrest imposed by the cell cycle blocker hydroxyurea, in both preosteoblast and osteosarcoma cells (Fig. 6C). These results suggest reduced protein ubiquitination and/or proteasomal degradation activity in osteosarcoma cells.

To determine whether Runx2 is sensitive to proteasomal degradation in a cycle dependent manner in preosteoblasts and osteosarcoma cells, we examined its protein level at specific cell cycle stages. In fact, MG132 treatment prevented the observed late G_1 phase- and S

phase-related decrease of Runx2 protein levels in preosteoblasts (Fig. 6D). Consistently, the cell cycle protein cyclin D1 was elevated in preosteoblasts treated with the proteasomal inhibitor. On the other hand, as expected, we observed relatively constant Runx2 protein levels in osteosarcoma cells (Fig. 6, E and F). More importantly, MG132 treatment minimally increased Runx2 protein levels in ROS and SaOS cells arrested in late G_1, with no change in those arrested in S. Altogether these results suggest a non operative or attenuated cell cycle related proteasome-dependent degradation of Runx2 in osteosarcoma.

DISCUSSION

Our results provide novel information on Runx2 and Cbfβ expression during the cell cycle in preosteoblast and osteosarcoma cell lines. Cbfβ protein was found to be constitutively up-regulated throughout the cell cycle in MC3T3, ROS and SaOS cells. Runx2 was negatively regulated during late G_1 phase, G_1/S and G_2/M phase transitions in MC3T3 cells and recovery of its protein level was observed postmitotically during early G_1, as previously described. Regarding this negative regulation, previous reports have described a role for the G_1 phase progression complex Cdk4/cyclinD1 in Runx2 phosphorylation and its subsequent ubiquitination followed by proteasomal degradation (Shen et al., 2006). Additionally, a stabilizing effect on Runx2 has been attributed to Cbfβ, since Runx2/Cbfβ heterodimerization prevents the ubiquitination of the lysine residues in the Runx2 RHD domain (Zhao et al., 2003; Huang et al., 2001). Furthermore, there is evidence that the Cbfβ promoter possesses motifs that bind cell cycle regulatory proteins, such as Sp1, Ets and Myc (Hajra and Collins, 1996). It is known that c-myc indirectly stabilizes p53 allowing for the transcription of p21, which in turn antagonizes the Cdk4/cyclinD1 complex, which controls cell cycle progression through G_1. Thus, Runx2 protein stabilization would be coordinated by the expression of factors that modulate the G_1/S transition and also Cbfβ expression. However, we observed Runx2 protein destabilization during progression beyond G_1, when Cbfβ protein levels are still high. This finding suggests that degradation of Runx2 by the proteasome required destabilization of the Runx2/Cbfβ interaction is responsible for the down-regulation of Runx2, rather than Cbfβ degradation during cell cycle progression.

On the contrary, the expression of Runx2 was notoriously stable in the osteosarcoma cell lines ROS and SaOS, similarly to the expression of Cbfβ. Thus, we observed a constitutive and elevated expression of Runx2 protein throughout the cell cycle in osteosarcoma cells. An interesting finding was the disturbed expression of the cell cycle marker cyclin D1 in osteosarcoma cells. In the same way, ROS and SaOS cells showed a disturbance in the cell cycle with accelerated progression to the G_1/S transition and a predominant S phase, as compared to preosteoblasts. Interestingly, we did not observe Runx2 destabilization in osteosarcoma cells although Cdk4/cyclinD1 was expressed at high levels in those cells. Thereby, our data suggest a non operative or attenuated protein ubiquitination and/or proteasomal degradation activity in osteosarcoma cells, although increased Runx2 expression rate can not be discarded.

With regards to the Runx2 cell proliferation control properties observed in osteoblasts, it has been hypothesized that Runx2 expression could be frequently silenced in osteosarcoma cells, as proposed for other growth suppressing proteins such as pRB and p53 (Nathan et al., 2008). However, these authors reported that elevated Runx2 protein levels were found in the osteosarcoma cell line OS1. In previous studies we have shown that SaOS and ROS cells express clearly detectable levels of Runx2 (Galindo et al., 2005). Furthermore, we have previously demonstrated that after mitosis Runx2 protein was distributed equivalently in the progeny nuclei in ROS cells, which provides a mechanism for the maintenance of Runx2 cellular levels and activity after mitosis (Zaidi et al., 2003). In the same way, we have shown that Runx2 protein is metabolically stable during cell division in SaOS cells, which remains

associated with mitotic chromosomes through sequence-specific DNA binding (Young et al., 2007).

These findings concur with our present study where we describe constitutively elevated Runx2 levels throughout the ROS and SaOS osteosarcoma cell cycle. Constitutively elevated Runx2 expression in osteosarcoma is in agreement with recent studies that reveal several chromosomal regions that are recurrently amplified in osteosarcoma patients and several osteosarcoma cell lines. Specifically, the amplified region 6p12-p21 that contains known candidate genes including *RUNX2*, appears as an early marker of genetic changes in osteosarcoma, which in turn is associated with progression and poor prognosis in patients with this type of cancer. Consequently, these studies show amplification-related overexpression of Runx2 in clinical samples (Lu et al., 2008; Sadikovic et al., 2009). On the other hand, no genetic defects have been described for *CBFβ* in osteosarcoma yet. However, there is a report of multiple *CBFβ* gene copies detected by FISH in a single case of granulocytic sarcoma associated with myeloid leukemia (Mallo et al., 2007).

Ectopic overexpression of Runx2 revealed an antiproliferative effect of this factor in osteosarcoma cells either in cell culture or in vivo, as well as a proapoptotic function (Thomas et al., 2004; Luo et al., 2008; Eliseev et al., 2008). However, in the current study we did not observe any growth effect in Runx2 knock down osteosarcoma cells by siRNA (data no shown), which suggests that physiologically elevated levels of Runx2 in these cells do not have a role as tumor suppressors. On the other hand, there is evidence that the canonical wnt pathway is upregulated in osteosarcoma (Hoang et al., 2004; Kansara et al., 2009). This pathway promotes the expression of Runx2 at the onset of osteoblast differentiation, but inhibits terminal osseous differentiation that is mediated by this factor (Kehler et al., 2006). These observations are in agreement with the immature bone phenotype described in osteosarcoma cells, which suggests that terminal bone differentiation *per se* is in opposition to tumorigenesis, as proposed by Thomas and Kansara (2006). These previous observations, together with a recently described role for Runx2 in breast and prostate cancer cell migration and metastasis (Pratap et al., 2005; Pratap et al., 2006; Pratap et al., 2008), place Runx2 and its overexpression as a central area of investigation in bone cancer research.

In conclusion, we found constitutively elevated Runx2 protein levels in the ROS and SaOS osteosarcoma cell lines throughout the cell cycle. In addition, we describe constitutive Cbfβ expression in normal and cancer cells, suggesting that the stabilizing effect exerted by this protein on Runx2 could be modulated by other protein complexes. These results, in conjunction with data on the amplification of the Runx2 gene locus in osteosarcoma, suggest a pivotal role for this factor in the pathogenesis of bone cancer.

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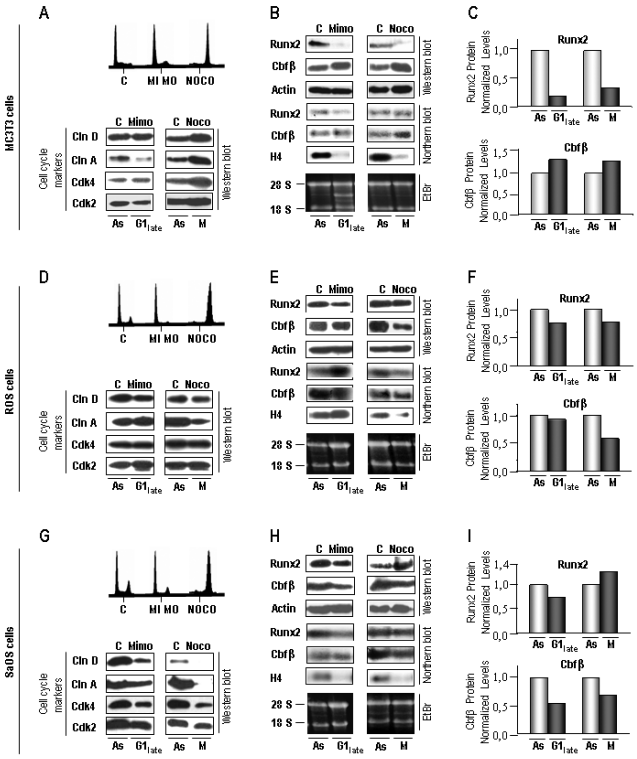

Figure 1. Runx2 and Cbfβ are expressed at high levels in osteosarcoma cells arrested in late G_1 and mitosis

Pre-osteoblast MC3T3 and osteosarcoma ROS and SaOS cells treated for 24 h with 400 μM mimosine (*Mimo*) or 100 ng/ml nocodazole (*Noco*) or untreated controls(*C*) were analyzed by FACS for cell cycle distribution and by western blot for expression of cell cycle markers (cyclins D and A, Cdk2 and Cdk4) (left panels ***A***, ***D*** and ***G***). Cell cultures blocked in late G_1 phase (G_{1late}) and mitosis (M) were compared to proliferating asynchronic cells in culture (As). Middle panels ***B***, ***E*** and ***H***, same as in left panels, but western (*top*) and northern (*center*) blot analysis were performed to assess Runx2 and Cbfβ protein levels, respectively, relative to actin, and Runx2, Cbfβ and histone H4 mRNA levels relative to 28S and 18S rRNA (*bottom*). Right panels ***C***, ***F*** and ***I***, show a graphic representation of cell cycle stage-related changes of data presented in panels B, E and H for Runx2 (*top graph*) and Cbfβ (*bottom graph*) protein levels normalized to actin.

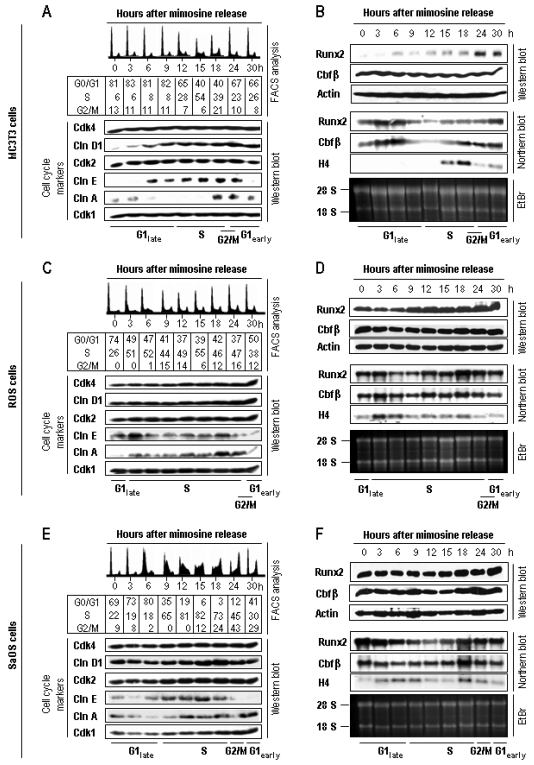

Figure 2. Runx2 and Cbfβ expression are not cell cycle-regulated in osteosarcoma cells

Runx2 and Cbfβ gene expression was assessed during progression through the pre-osteoblast and osteosarcoma cell cycle to determine the specific transition stages when Runx2 and Cbfβ levels are modulated. Cells were synchronized by incubation for 24 h with mimosine to generate a late G_1 phase block. Then, cells were released from late G_1 phase arrest by the addition of fresh culture medium without mimosine and harvested after 0, 3, 6, 9, 12, 15, 18, 24 and 30 h. ***A***, ***C*** and ***E***, progression through successive cell cycle phases (G_{1late}, S, G2/M and G_{1early}) was monitored by flow cytometry (*top*) and expression of cell cycle markers (cyclins D, E and A, and Cdk1, Cdk2 and Cdk4) was evaluated by western blot analysis (*bottom*). ***B***, ***D*** and ***F*** cell cycle-dependent modulations in Runx2 and Cbfβ protein and mRNA levels as well as histone H4 mRNA were assessed as described in figure 1.

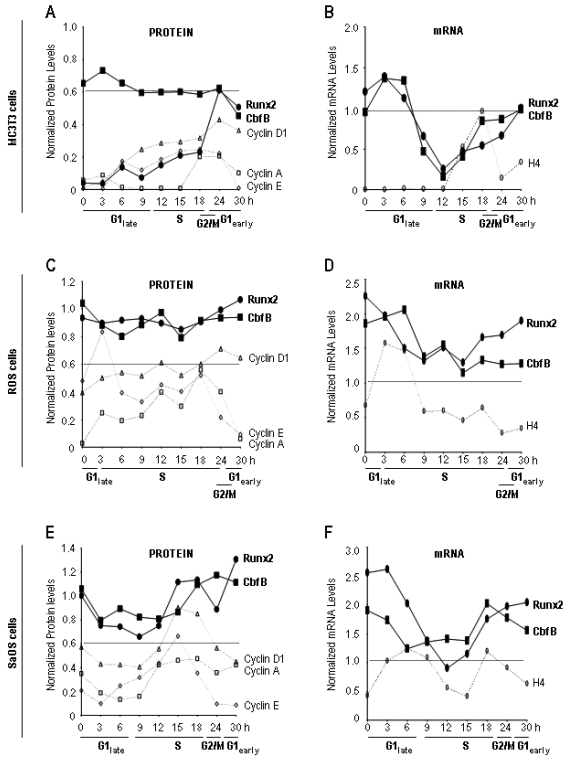

Figure 3. Runx2 and Cbfβ protein levels are maintained at high levels during the cell cycle in osteosarcoma cells

Runx2 and Cbfβ gene expression in pre-osteoblast and osteosarcoma cells were quantified from data shown in figure 2. Graphic representation of cell cycle-related changes in Runx2 (*closed circles*) and Cbfβ (*closed squares*) protein (***A,C and E****, left graphs*) and mRNA (***B, D and F****, right graphs*) levels after serum stimulation were depicted by continuous lines. The cell cycle markers cyclin D1 (*open triangles*), E (*open diamonds*) and A (*open squares*) proteins and histone H4 mRNA (*open circles*) levels are depicted by dotted lines to demarcate progression through G_{1late}, S, G2/M and G_{1early} phases. Protein and mRNA values were normalized to actin and 28S rRNA, respectively. Cell cycle phases as determined by flow cytometry are indicated at the base of the graphs.

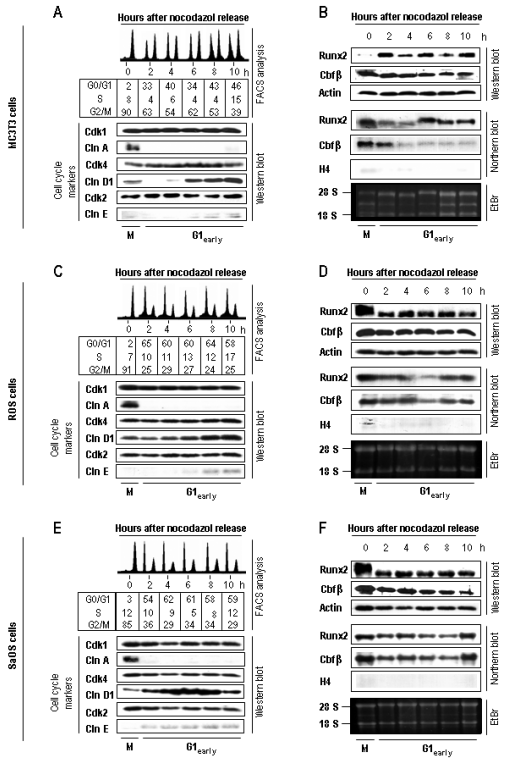

Figure 4. Runx2 and Cbfβ expression are maintained at high levels during post-mitotic progression in G_1 phase in osteosarcoma cells

Pre-osteoblast (MC3T3) and osteosarcoma (ROS and SaOS) cells were synchronized by incubation for 16 h with nocodazole to generate a mitotic block. Mitotic cells were harvested by gentle agitation and then released from mitotic arrest by the addition of culture medium without nocodazole and harvested after 0, 2, 4, 6, 12, 15, 8 and 10 h. ***A***, ***C*** and ***E***, progression through early G_1 phase was monitored by flow cytometry and western blot analysis of cell cycle-related proteins. The M/G_1 transition was defined by monitoring cyclins B1, D1, E and A protein levels. ***B***, ***D*** and ***F***, cell cycle-dependent modulation in Runx2 and Cbfβ protein and mRNA levels were assessed as described in figure 2.

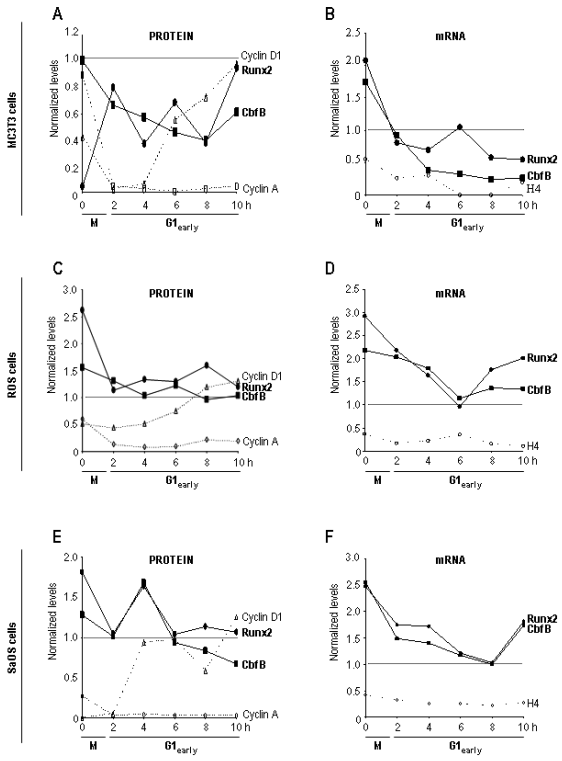

Figure 5. Elevated Runx2 and Cbfβ protein levels observed in mitosis are maintained during G_1 phase in osteosarcoma cells

Runx2 and Cbfβ gene expression in pre-osteoblast and osteosarcoma cells were quantified from data shown in figure 4. Graphic representation of cell cycle-related changes in Runx2 (*closed circles*) and Cbfβ (*closed squares*) protein (***A,C and E****, left graphs*) and mRNA (***B, D and F****, right graphs*) levels after serum stimulation were depicted by continuous lines. The cell cycle markers cyclin D1 (*open triangles*), A (*open squares*) and B (*open diamonds*) proteins and histone H4 mRNA (*open circles*) levels are depicted by dotted lines to demarcate progression through M/G_1 transition, G_{1early} and S phases. Protein and mRNA values were normalized to actin and 28S rRNA, respectively. Cell cycle phases as determined by flow cytometry are indicated at the base of the graphs.

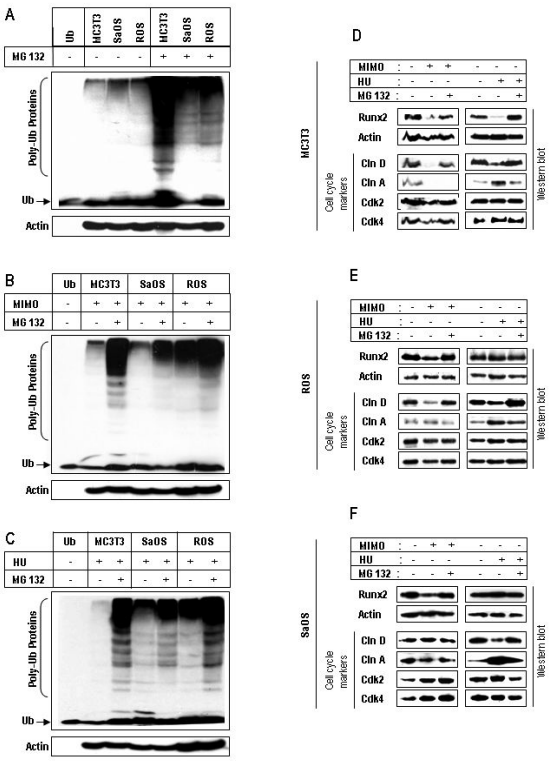

Figure 6. Stabilization of Runx2 protein levels by the proteasome inhibitor MG132 is restricted to later G_1 in osteosarcoma cells which exhibits low amounts of ubiquitin-conjugated proteins

MG132 treatment accumulates high molecular mass forms of ubiquitin-conjugated proteins. Western blot analysis was performed to detect accumulation of ubiquitin-conjugated proteins after treatment of MC3T3 pre-osteoblast and ROS and SaOS osteosarcoma cells with (+) or without (−) 25 μM MG132 for 12 h (***A***). Cell proteins were separated by SDS–PAGE and immunoblotted either with ubiquitin (top panel) or actin specific antibodies (bottom panel). Ubiquitin protein (Ub) was included to monitor endogenous ubiquitin levels. Also, cells treated for 24 h with 400 μM mimosine (*Mimo*) (***B***) or 2 mM hydroxyurea (*HU*) (***C***) and alternatively incubated 12 h after drug imposed cell cycle arrest with (+) or without (−) MG132 (25 μM) were analysed as described in *A*. MC3T3 (***D***), ROS (***E***) and SaOS (***F***) cells treated for 24 h with 400 uM mimosine (*Mimo*) or 2 mM hydroxyurea (*HU*) and alternatively incubated 12 h after drug imposed cell cycle arrest with the proteasome MG132 (25 μM) were analyzed by Western blot analysis for expression of Runx2 relative to actin and cell cycle markers. Levels of cyclin D1 are clearly elevated, indicating that proteasomal degradation is blocked by MG132.