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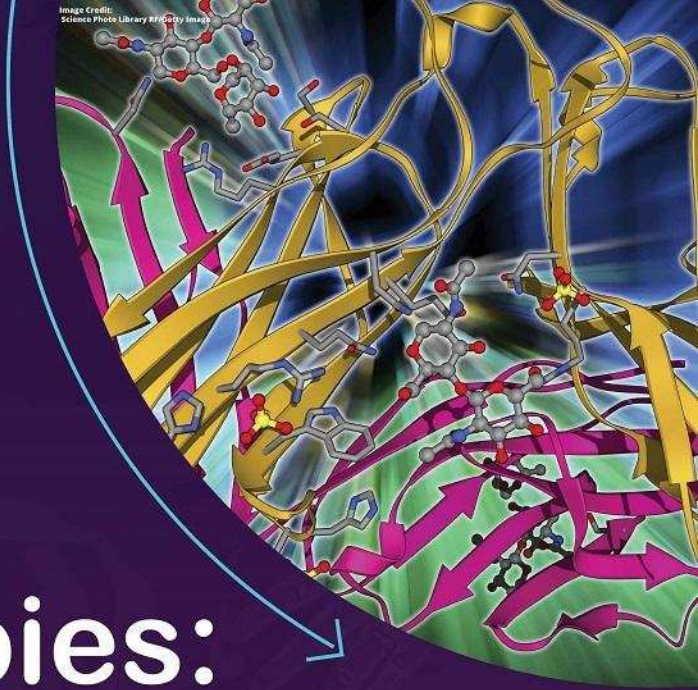Project   Targeting the bone marrow microenvironment in AML: mechanisms mediated by CXCL12 and osteoblasts View project

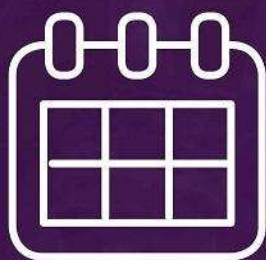Project   MSC-derived EV in chronic kidney diseases View project

Wnt/β-Catenin Signaling Activates Expression of the Bone-Related Transcription Factor RUNX2 in Select Human Osteosarcoma Cell Types

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ABSTRACT

Osteosarcoma is the most common malignant bone tumor in children and adolescents. Metastasis and poor responsiveness to chemotherapy in osteosarcoma correlates with over-expression of the runt-related transcription factor RUNX2, which normally plays a key role in osteogenic lineage commitment, osteoblast differentiation, and bone formation. Furthermore, WNT/β-catenin signaling is over-activated in osteosarcoma and promotes tumor progression. Importantly, the WNT/β-catenin pathway normally activates RUNX2 gene expression during osteogenic lineage commitment. Therefore, we examined whether the WNT/β-catenin pathway controls the tumor-related elevation of RUNX2 expression in osteosarcoma. We analyzed protein levels and nuclear localization of β-catenin and RUNX2 in a panel of human osteosarcoma cell lines (SAOS, MG63, U2OS, HOS, G292, and 143B). In all six cell lines, β-catenin and RUNX2 are expressed to different degrees and localized in the nucleus and/or cytoplasm. SAOS cells have the highest levels of RUNX2 protein that is localized in the nucleus, while MG63 cells have the lowest RUNX2 levels which is mostly localized in the cytoplasm. Levels of β-catenin and RUNX2 protein are enhanced in HOS, G292, and 143B cells after treatment with the GSK3β inhibitor SB216763. Furthermore, small interfering RNA (siRNA)-mediated depletion of β-catenin inhibits RUNX2 expression in G292 cells.

Thus, WNT/β-catenin activation is required for RUNX2 expression in at least some osteosarcoma cell types, where RUNX2 is known to promote expression of metastasis related genes. J. Cell. Biochem. 118: 3662–3674, 2017. © 2017 Wiley Periodicals, Inc.

KEY WORDS: CANCER; OSTEOSARCOMA; Wnt/β-CATENIN; RUNX2 TRANSCRIPTION FACTOR; ONCOGENE

Osteosarcoma is the most frequent primary malignant bone tumor in children and adolescents [Young and Miller, 1975]. The highest incidence of osteosarcoma is during the second decade of life, which suggests a relationship between bone growth and tumor development [Fraumeni et al., 1967; Cotterill et al., 2004], as well as dysregulation of osteoblast differentiation [Wagner et al., 2011]. Multiple studies suggest that RUNX2 is a key pathological factor in osteosarcoma [Martin et al., 2011, 2014]. Importantly, over-expression of RUNX2 in transgenic mice within the osteoblast lineage inhibits osteoblast maturation [Liu et al., 2001; Geoffroy et al., 2002]. Hence, aberrant expression of RUNX2 may contribute to compromised osteoblast growth and differentiation relationships that are evident in osteosarcoma [Stein et al., 1994].

Upregulation of the WNT/β-catenin pathway is also associated with development of osteosarcoma [Hoang et al., 2004a; Wunder et al., 2007; Kansara et al., 2014; Martins-Neves et al., 2016a,b; Techavichit et al., 2016; Zanotti and Canalis, 2016]. Consequently, several attractive molecular strategies for therapeutic intervention are being considered that target WNT signaling in osteosarcoma [Papa et al., 2015; Bravo et al., 2017; Goldstein et al., 2016; Horne et al., 2016; Shen et al., 2016]. WNT signaling normally stimulates osteoblast maturation by activating RUNX2 expression, albeit that part of this mechanism may occur independent of the WNT responsive transcription factor LEF1 [Gaur et al., 2005; Mbalaviele et al., 2005; Kahler et al., 2006; Galindo et al., 2007; Ling et al., 2010; Schoeman et al., 2015]. These findings collectively suggest that the developmental interplay between RUNX2 and WNT is deregulated in osteosarcoma, thus motivating studies to examine the extent to which RUNX2 expression depends on WNT signaling in bone cancer cells.

Analysis of genomic DNA from osteosarcoma patients with amplification of the 6p12-p21 chromosomal interval, which spans the RUNX2 locus, increases RUNX2 gene copy number and aberrantly elevates RUNX2 expression [Lau et al., 2004; Lu et al., 2008; Sadikovic et al., 2009]. RUNX2 protein and mRNA levels are frequently elevated in clinical osteosarcoma specimens and osteosarcoma cell culture models, although there are major variations in expression [Thomas et al., 2004; Andela et al., 2005; Lu et al., 2008; Luo et al., 2008; Nathan et al., 2009; Pereira et al., 2009; Sadikovic et al., 2009; Won et al., 2009; Kurek et al., 2010; Shapovalov et al., 2010; Lucero et al., 2013]. Loss of p53 that is frequently observed in osteosarcoma increases RUNX2 gene expression by relieving repression by miR-34 [van der Deen et al., 2012, 2013]. Increased expression of RUNX2 in osteosarcoma has been associated with increased tumorigenicity, tumor progression, metastases, poor prognosis, and lower survival [Won et al., 2009; Kurek et al., 2010; Sadikovic et al., 2010; van der Deen et al., 2012, 2013]. RUNX2 levels are normally modulated during cell cycle and up-regulated with respect to cessation of cell growth in pre-osteoblastic cells [Pratap et al., 2003; Galindo et al., 2005], but this

mechanism is dysregulated in osteosarcoma [Galindo et al., 2005; Young et al., 2007a; San Martin et al., 2009; Lucero et al., 2013]. In osteosarcoma cells, RUNX2 controls expression of a number of cancer-related genes [van der Deen et al., 2012, 2013] and interacts with several subnuclear factors involved in the DNA damage response [Yang et al., 2015]. The later findings may be linked to the observation that increased expression of RUNX2 is associated with unfavorable prognosis for chemotherapy in pediatric osteosarcoma patients [Martin et al., 2014].

The canonical WNT signaling pathway and its protein mediator β-catenin are essential for development, stem cell maintenance and tissue regeneration [Beachy et al., 2004; Gordon and Nusse, 2006]. In the absence of WNT signaling, inactivation of the canonical WNT signaling pathway is achieved by targeting β-catenin into a multi-protein complex which is formed by Axin, the tumor suppressor APC (adenomatous poliposis coli protein) and the kinases CK1α and GSK3β. Ultimately, GSK3β phosphorylates β-catenin and thereby targets it for proteasomal degradation [Polakis, 2007]. When WNT ligands bind to Frizzled and LRP5/LRP6 receptors in the cell surface, cytoplasmic Dvl (dishevelled) antagonizes GSK3β-dependent phosphorylation of β-catenin. Once the phosphorylation of β-catenin is reduced, it dissociates from the Axin/APC/CK1α/GSK3β complex, stabilizes and accumulates in the cytoplasm [Vielhaber and Virshup, 2001; Knippschild et al., 2005]. Increased cytoplasmic β-catenin levels favor the subsequent β-catenin nuclear accumulation, allowing association with TCF/LEF transcription factors and subsequent activation of genes that contribute to cancer progression and metastasis [Polakis, 2007]. Thus, nuclear β-catenin accumulation is crucial for cancer progression, which explains the increased expression of genes involved in progression and survival of tumor cells [Bienz and Clevers, 2000; Brembeck et al., 2006].

Alterations within the WNT/β-catenin signaling pathway have been described in human osteosarcoma cell lines and clinical osteosarcoma samples, which strongly support a possible role for WNT/β-catenin signaling in the pathobiology and progression of human osteosarcoma. Specifically, the WNT/β-catenin pathway is upregulated in metastatic osteosarcoma cell lines and higher grade osteosarcoma as well as hyper-activated in bone tumors and clinical samples [Haydon et al., 2002; Srivastava et al., 2006; Daino et al., 2009; Flores et al., 2012; Ma et al., 2013]. Moreover, elevated WNT/β-catenin signaling in osteosarcoma cell lines and patient specimens is associated with chemotherapy resistance and tumor cell survival [Rajkumar and Yamuna, 2008; Zhang et al., 2011a; Ma et al., 2013; Wu et al., 2014], tumorigenesis and metastasis [Hoang et al., 2004a; Guo et al., 2008; Kansara et al., 2009; Rubin et al., 2010], tumor progression [Hoang et al., 2004b; Leow et al., 2009], and decreased overall survival [Hoang et al., 2004a; Chen et al., 2008].

In this study, we systematically examined the relationship between WNT/β-catenin signaling and RUNX2 gene expression in a panel of six human osteosarcoma cell lines. Our results indicate

that activation of the WNT/β-catenin pathway enhances RUNX2 expression in osteosarcoma and thus may regulate expression of RUNX2 dependent genes that support tumor progression or metastasis.

MATERIALS AND METHODS

CELL CULTURE

Human osteosarcoma cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Osteosarcoma cells were maintained in culture medium supplemented with 10–15% fetal bovine serum (FBS) (HyClone Laboratories Inc, Logan, UT) plus 2 mM L-glutamine and a penicillin-streptomycin cocktail at 37°C and 5% CO_2 according to the recommendations of ATCC. The growth medium was changed every 2 days. SAOS (alias SaoS-2) cells were cultured in McCoy's medium (Sigma–Aldrich, St Louis, MO) supplemented with 15% FBS. U2OS and G292 cells were cultured in McCoy's medium with 10% FBS. MG63 and HOS cells were grown in DMEM medium (Gibco, Life Technologies, Grand Island, NY) with 10% FBS. 143B cells were maintained in DMEM medium, 1 mM sodium pyruvate, 100 μg/ml of bromodeoxyuridine and 10% FBS. Alternatively, after 72 h cells were treated with 10 μM of SB216367 (Sigma–Aldrich), and cultured for 24 h.

WESTERN BLOT ANALYSIS

RUNX2, β-catenin 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, San Diego, CA) and Complete® cocktail of protease inhibitor (Roche Diagnostics, Mannhein, Germany) were resolved in 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Perkin Elmer, Boston, MA). Blots were incubated with a 1:2,000 dilution of each primary antibody for 1 h. RUNX2-specific mouse monoclonal antibody 8G5 (MBL International, Woburn, MA), β-catenin mouse monoclonal antibody 610154 (BD Transduction Lab, Bedford, MA) and actin goat polyclonal antibody sc-1615 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were acquired commercially. Membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibody (sc-2005 anti-mouse or sc-2020 anti-goat) (Santa Cruz Biotechnology Inc.) for 1 h. Immuno-reactive protein bands were visualized on a Kodak BioMax Light film (Carestream Healt Inc, Rochester, NY) using a chemiluminescence detection kit (Thermo Scientific, Rockford, IL). Signal intensities were quantified by densitometry.

SEMI–QUANTIATIVE PCR AND RNA–seq ANALYSIS

Gene expression analyses were performed using semi-quantitative PCR as described previously [Varela et al., 2016] as a reproducible method that permits direct visualization of amplified cDNAs. Quantitation of mRNA expression detected by PCR was subsequently validated with next generation RNA sequencing (RNA-seq) data that were concurrently generated for SAOS, MG63 and U2OS cell lines using procedures we previously described [Dudakovic et al., 2014]. For RT-PCR analysis, total RNA was isolated from osteosarcoma cells using TRIzol reagents (Invitrogen, Carlsbad, CA) 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 (3 μg) was treated with RQ1RNase-Free DNase (Promega, Madison, WI) and subjected to reverse transcription using random hexamer primers (Promega) with M-MLV reverse transcriptase (Promega) according to the manufacturer's recommendations. Gene expression was assessed by PCR using the following specific human gene primers (0.5 pmol/μl): *RUNX2* (forward primer: 5′-CCGCACGACAACCGCACCAT-3′, reverse primer: 5′-CGCTCCGGCCCACAAATCTC-3′), β-catenin/*CTNNB1* (forward primer: 5′-TGGTGCCCAGGGAGAACCCC-3′, reverse primer: 5′-CCCACCCCTCGAGCCCTCTC-3′), *DKK1* (forward primer: 5′-AACCGGCACGGTTTCGTGGG-3′, reverse primer: 5′-CGCACGGGTACGGCTGGTAG-3′), *TCF4* (forward primer: 5′-TGGCCCTGAGAGGCAGCCAT-3′, reverse primer: 5′-GGTCCTCATCGTCATTATTGCTAGAT-3′), *LEF1* (forward primer: 5′-CCAGCTATTGTAACACCTCA-3′, reverse primer: 5′-TTCAGATGTAGGCAGCTGTC-3′), *LRP5* (forward primer: 5′-CAGCCTG AC GCACCCCTTCG-3′, reverse primer: 5′-CACCTCCT CGG CTCCTGCCT-3′), *LRP6* (forward primer: 5′-GCTGGAATG-GATGGTTCAAGTCG-3′, reverse primer: 5′-CAGAATGGATTTCA CGCAGACCC-3′), *WNT3A* (forward primer: 5′-GTTGG GCCACAG-TATTCCTC-3′, reverse primer: 5′-ATCCCACCAAACTCGATGTC-3′), *WNT10B* (forward primer: 5′-GAATGCGAATCCACAACAACAG-3′, reverse primer: 5′-TTGCGG TTGTGGGT ATCAATGAA-3′), Alkaline Phosphatase/*ALPL* (forward primer: 5′-AGTGCTCTGCG-CAGGATTGGA-3′, reverse primer: 5′-CACACAGGTAG GCG GTGGCG-3′), OC/*BGLAP* (forward primer: 5′-CTGGCCAGG-CAGGTGCGAAG-3′, reverse primer: 5′-CAGGCCAGCAGAGCGA-CACC-3′), and *GADPH* (forward primer: 5′-CCTT CATT GACCT CAACTA-3′, reverse primer: 5′-GGCCATCCACAGTCTTCT-3′). PCR amplification of cDNAs was carried out using 1× PCR buffer (Promega) containing 0.2 mM dNTPs (Promega), 1.5 mM MgCL (Promega), 0.06 U/μl of Taq polymerase (Invitrogen) by incubation for 5 min at 94°C and 20–30 amplification cycles of synthesis were applied to avoid product saturation (1 min at 94°C, 1 min at 52–62°C, and 1 min at 72°C), followed by a final extension step at 72°C for 6 min. Aliquots of the resulting products (5 μl) were visualized in 1% agarose gels by ethidium bromide staining.

IMMUNOHISTOCHEMISTRY

Cell were washed twice with PBS and collected in a 1.5 ml tube using a scraper followed by centrifugation at 380*g* for 2 min. The cell pellets were fixed for 24 h, dehydrated and embedded in paraffin using standard procedures. Paraffin-embedded cells were sectioned (5 μm), adhered to glass slides and rehydrated. Antigens were recovered by treatment with retrieval buffer (1 mM Tris, 0.5 mM EGTA, pH 9.0). Sections were blocked with PBS supplemented with 1% bovine serum albumin. Then sections were incubated with RUNX2 (M70) rabbit polyclonal antibody sc-10758 (Santa Cruz Biotechnology Inc.) or anti-β-catenin antibody, washed, and incubated with the indicated biotylinated secondary antibody. Finally, antibodies bound to specific antigens were detected using a biotin-streptavidin detection system.
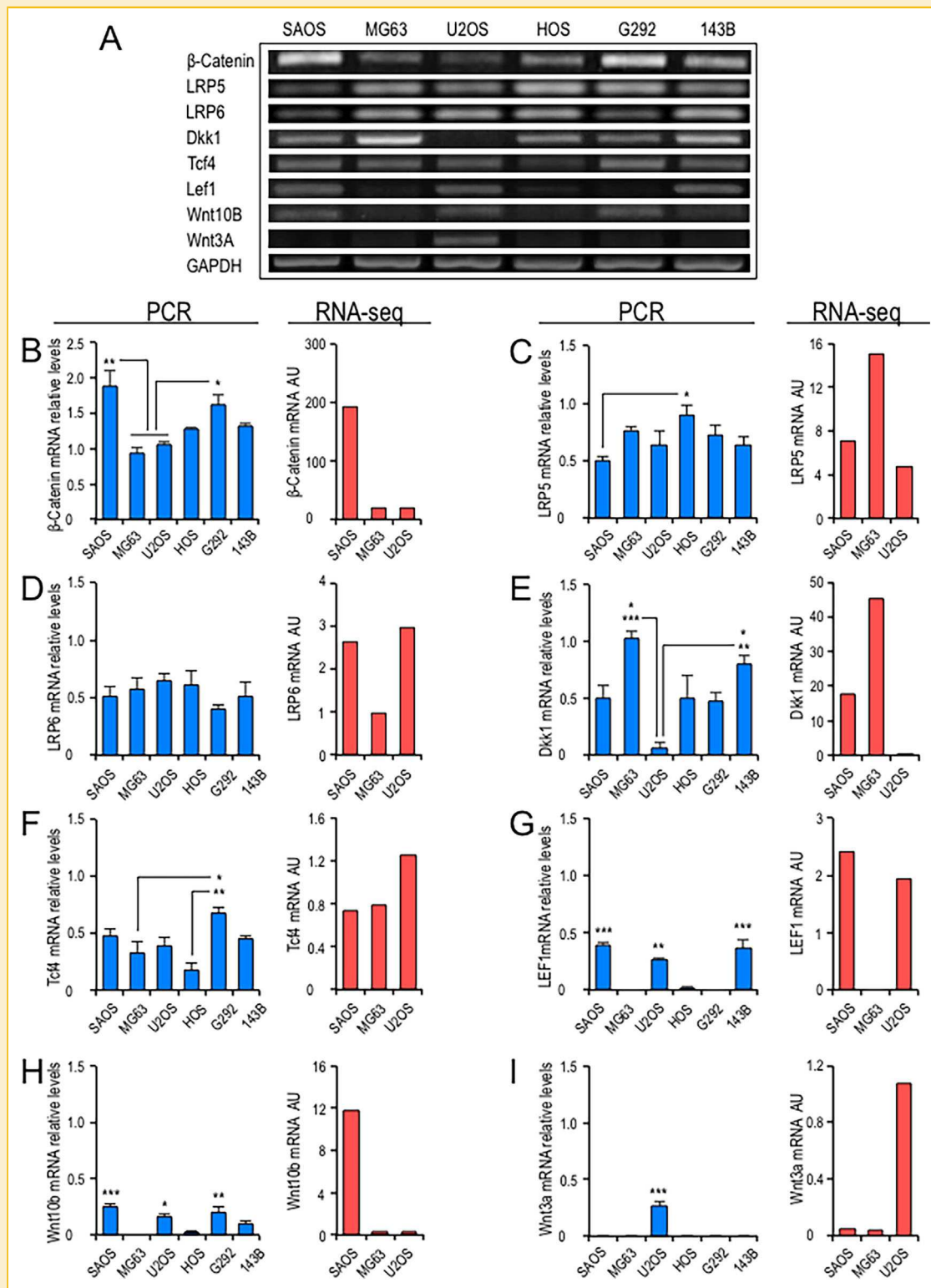
Fig. 1. Expression of Wnt/β–catenin signalling pathway components in human osteosarcoma cell lines. The mRNA levels of β–Catenin/*CTNNB1*, *LRP5*, *LRP6*, *DKK1*, *TCF4*, *LEF1*, *WNT10B*, and *WNT3A* were assessed by RT–PCR and RNA–seq in SAOS, MG63, U2OS, HOS, G292, and 143B osteosarcoma cells (A–I). The graphs below depict quantitation of the RT–PCR data (left graphs) and RNAseq data (right graphs) as indicated upon normalization to *GAPDH* (B–I). All data are presented as mean ± SEM. Statistical significance was determined by Tukey post–hoc tests ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). *DKK1*: MG63 and 143B cells versus the others four osteosarcoma cell lines ($^*P < 0.05$); *LEF1*: SAOS and 143B cells versus MG63, HOS and G292 cells ($^{***}P < 0.001$), U2OS cells versus MG63, HOS, and G292 cells ($^{**}P < 0.01$); *WNT10B*: SAOS cells versus MG63 and HOS cells ($^{***}P < 0.001$), G292 cells versus MG63 and HOS cells ($^{**}P < 0.01$), U2OS cells versus MG63 and HOS cells ($^*P < 0.05$).
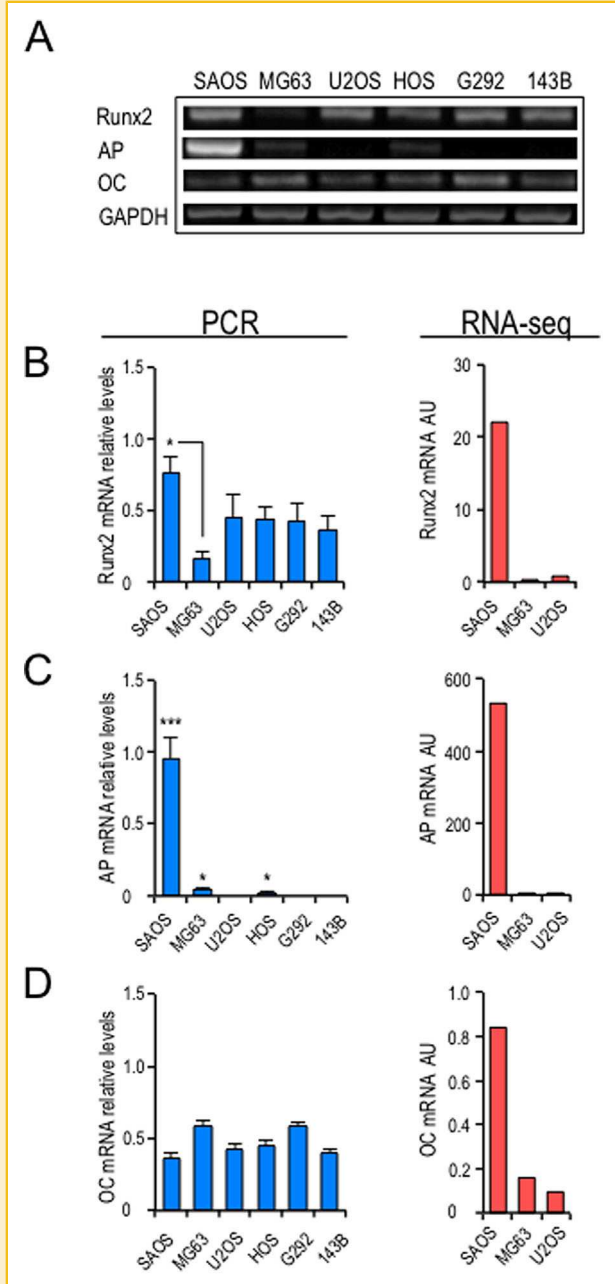
Fig. 2. Expression of *RUNX2* and bone differentiation markers in human osteosarcoma cell lines. Expression of mRNAs for *RUNX2* and the bone differentiation markers alkaline phosphatase (AP, *ALPL*) and osteocalcin (OC, *BGLAP*) were examined by RT–PCR in the human osteosarcoma cell lines SAOS, MG63, U2OS, HOS, G292, and 143B (A). The graphs show quantification of the RT–PCR data (left graphs) and RNAseq data (right graphs) relative to *GAPDH* mRNA (B–D) and are presented as mean ± SEM. Statistical significance was determined by Tukey post–hoc tests ($*P < 0.05$; $***P < 0.001$). AP: SAOS cells versus the others five osteosarcoma cell lines ($***P < 0.001$), MG63 and HOS cells versus U2OS, G292, and 143B cells ($*P < 0.05$).

RNA INTERFERENCE

RNA interference experiments were carried out using commercially available siRNAs (Dharmacon, Lafayette, CO). Specific siRNA (h) for

RUNX2 (ON-TARGET plus, SMARTpool) and β-catenin (ON-TARGET plus, SMARTpool) were designed to inhibit RUNX2 and β-catenin gene expression. Control non-silencing (ns) RNA (ON-TARGET plus, Non-targeting Pool) was used as negative control in our experiments, because this pool of nsRNA has no discernible off-target effects on cell growth parameters examined in our study. Cells were plated in 60 mm plates at a density of 3×10^5 cells/plate and cultured for 24 h in DMEM with 10% FBS in absence of antibiotics at 40–60% of confluence. Subsequently, cells were washed in PBS, and transfected in 2 ml of Opti-MEM medium (Gibco, life Technologies) plus 2,5 to 5 μl of a 20 μM stock solution of each siRNA pool (final concentration of 25–50 nM) and 12 μl of Oligofectamine 2000 (Invitrogen). Concentrated complete DMEM ($3 \times$; 1 ml) was added 4 h after transfection to obtain approximately 3 ml of culture medium with standard final concentrations of penicillin-streptomycin cocktail, 2 mM L-glutamine, and 10% FBS. After siRNA transfection, cells were incubated for 48 h and RUNX2 and β-catenin knock down efficiency was confirmed by Western blot analysis as described above.

STATISTICAL ANALYSIS

The data were represented as mean ± SEM with a minimum of three independent samples and analyzed using one-way ANOVA with Tukey post-hoc tests, or by Student's *t*-test. *P*-value of less than 0.05 was considered statistically significant.

RESULTS

WNT/β-CATENIN EXPRESSION IN OSTEOSARCOMA CELL LINES

To determine the status of Wnt/β-catenin expression in osteosarcoma, we first analyzed expression of several Wnt/β-catenin related genes in six human osteosarcoma cell lines (SAOS, MG63, U2OS, HOS, G292, and 143B) (Fig. 1). Expression of mRNA levels for β-catenin/*CTNNB1*, *LRP5*, *LRP6*, *DKK1*, *TCF4*, *LEF1*, *WNT10B*, and *WNT3A* was determined by RT-PCR. The mRNA levels for these genes were further validated in three of the lines (SAOS, MG63, U2OS) using RNA-seq data that were generated in parallel. All six osteosarcoma lines express the WNT co-receptors *LRP5* and *LRP6* at comparable mRNAs levels when normalized to *GAPDH* as detected by either PCR or RNA-seq (Fig. 1). However, these cell lines exhibit differences in the expression of other WNT signaling components. For example, SAOS, U2OS, G292, and 143B cells express alternatively high levels of positive WNT signaling components, including β-catenin/*CTNNB1* and the WNT-responsive transcription factors *TCF4* and/or *LEF1*, as well as the ligand *WNT10B* and/or *WNT3A*. In contrast, MG63 and HOS cells express minimal levels of *TCF4*, *LEF1*, *WNT10B*, and *WNT3A*. Yet, MG63 cells express the highest levels of the WNT inhibitor *DKK1*. Interestingly, 143B cells which are a metastatic derivative of HOS cells, express higher levels of *TCF4*, *LEF1*, and *WNT10B* than the parental cells, suggesting that increased expression of these WNT signaling components accompanies metastatic osteosarcoma progression of HOS cells. Taken together, the observed modulations in the mRNA levels of distinct WNT signaling components may reflect intrinsic differences in the potential for WNT signaling, unless other members of these gene
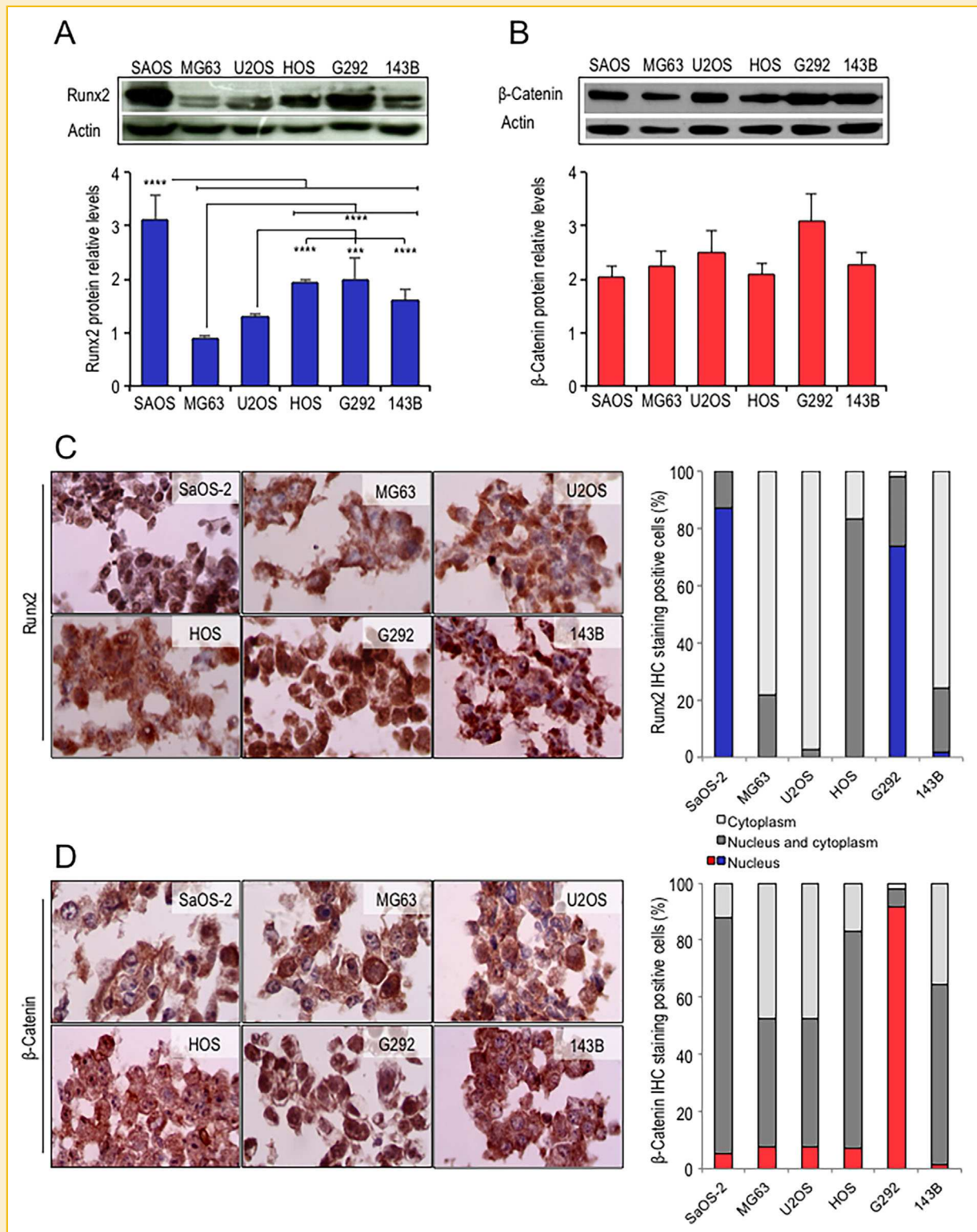
Fig. 3. Human osteosarcoma cell lines exhibit different nuclear expression pattern of β-catenin and RUNX2. Western blot analysis was performed to evaluate protein levels of RUNX2 (A) and β-catenin protein (B) in our panel of osteosarcoma cell lines as indicated. The bar graphs in each case show quantification for both proteins relative to β actin and are presented as mean ± SEM. Statistical significance was determined by Tukey post-hoc tests (*** $P < 0.001$; **** $P < 0.0001$). Each osteosarcoma cell line was grown up to 70% of confluence and fixed in paraformaldehyde for immunohistochemical detection of RUNX2 (C) and β-catenin (D). Magnification 400×. The graphs show the percentage of cell with RUNX2- or β-catenin-immunohistochemical staining in nucleus, nucleus, and cytoplasm or cytoplasm.
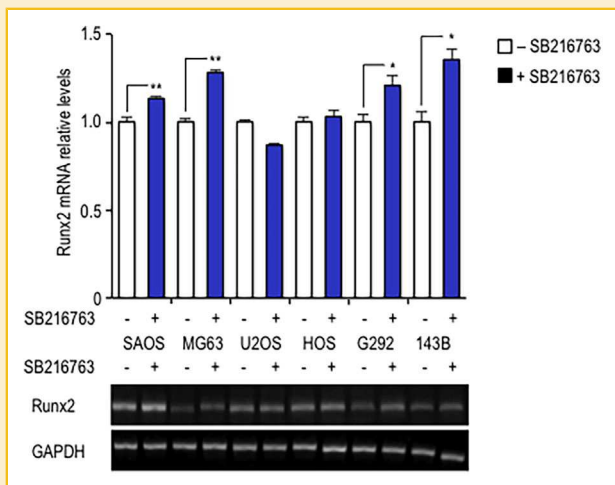
Fig. 4. *RUNX2* mRNA levels are differentially induced after treatment with GSK3β inhibitor in human osteosarcoma cell lines. SAOS, MG63, U2OS, HOS, G292, and 143B osteosarcoma cell lines were grown up to 70% of confluence and then treated with SB216763 for 24 h. RUNX2 mRNA levels were assessed by RT–PCR. The graphs depict data of *RUNX2* mRNA values normalized to *GAPDH* and relative to untreated cells. All data are presented as mean ± SEM. (*P < 0.05 and **P < 0.01).

families or post-transcriptional mechanisms compensate for the observed changes in gene expression in osteosarcoma cells.

EXPRESSION AND CELLULAR LOCALIZATION OF β–CATENIN AND RUNX2 IN OSTEOSARCOMA CELL LINES

Expression analysis indicates that mRNA is expressed for *RUNX2* across all six osteosarcoma cell lines (Fig. 2). SAOS cells exhibit the highest *RUNX2* mRNA levels compared to the others five osteosarcoma cell lines. *RUNX2* mRNA levels are also detected in U2OS, HOS, G292, and 143B cells, but it is barely detected in MG63. These PCR results were confirmed using RNA-seq data for *RUNX2* mRNA in SAOS, MG63, and U2OS cells, although RNA-seq is less effective in detecting *RUNX2* mRNA in U2OS cells (Fig. 2). Expression profiles of the bone markers alkaline phosphatase ("AP"; *ALPL*) and osteocalcin ("OC"; *BGLAP*) show that both are only co-expressed to a significant degree in SAOS cells, and to a lesser extent in U2OS cells. Specifically, SAOS cells, which have the highest relative mRNA levels for *RUNX2* also robustly express AP/*ALPL* and OC/*BGLAP* (Fig. 2). The PCR and RNA-seq data together in most cases show good concordance in expression levels detected in SAOS, MG63 and U2OS cells for the mRNAs of RUNX2, bone phenotypic markers and different WNT signaling components.

Western blot analysis of RUNX2 protein levels in all the osteosarcoma cell lines shows that SAOS cells exhibit the highest expression of RUNX2, consistent with the high mRNA levels observed in this cell type (Fig. 3A). For comparison, total cellular β-catenin protein expression relative to β-actin is very similar in all six cell lines (Fig. 3B). RUNX2 protein is detected in HOS, G292, and 143B cells, but to a lesser extent in U2OS cells and MG63 cells (Fig. 3B). These findings are consistent with data we previously

reported [Lucero et al., 2013]. The reduced levels of RUNX2 protein in MG63 are attributable to low mRNA levels (see Fig. 2), while the discordance between RUNX2 mRNA and protein levels in U2OS cells may be due to expression of the p53 responsive miRNA miR-34 that suppresses RUNX2 protein accumulation [Zhang et al., 2011b; van der Deen et al., 2013].

Because both β-catenin and RUNX2 can exhibit changes in subcellular localization, we analyzed whether these proteins localize to the nucleus (Fig. 3C and D). Nuclear accumulation of both RUNX2 and β-catenin protein is evident to different degrees in nuclei from all six cell lines. Nuclear localization of RUNX2 is most evident in SAOS and G292 cells but is clearly more cytoplasmic in MG63 and U2OS cells (Fig. 3C), which expresses low levels of RUNX2 protein (Fig. 3A). Nuclear accumulation of β-catenin is most pronounced in G292 cells (Fig. 3D), consistent with these cells having an mRNA expression profile for WNT signaling components high levels of β-catenin/*CTNNB1*, *TCF4*, *WNT10B*, and low levels of *DKK1* that is conducive for WNT signaling and β-catenin stabilization (see Fig. 1).

STABILIZATION OF β–CATENIN DIFFERENTIALLY INCREASES RUNX2 PROTEIN LEVELS IN OSTEOSARCOMA CELLS

To determine if WNT/β-catenin pathway modulates RUNX2 expression in osteosarcoma, we treated cells with the GSK3β inhibitor SB216763. *RUNX2* mRNA levels increase modestly in SAOS, MG63, G292, and 143B cells treated with SB216763 compared to control cells (Fig. 4). More importantly, treatment with the GSK3β inhibitor results in up-regulation of both β-catenin and RUNX2 protein levels in HOS, G292, and 143B cells (Fig. 5). Because G292 cells exhibit robust nuclear accumulation of β-catenin, we examined the effect of siRNA-mediated knockdown of β-catenin on RUNX2 expression in this cell type. Control experiments show that β-catenin and RUNX2 proteins are each down-regulated by their respective siRNAs in G292 cells (Fig. 6A and B). As expected, treatment of G292 cells with siRNA against β-catenin (25 or 50 nM), but not with control non-silencing RNA, reduces RUNX2 protein expression by 2–3 fold depending on the dose. For comparison, treatment of cells with RUNX2 siRNA (50 nM) reduces RUNX2 levels by approximately 2 fold, independent of the presence of the β-catenin siRNA (Fig. 6C). Therefore, siRNA depletion of β-catenin is at least as effective in down-regulating RUNX2 as direct depletion of RUNX2 by siRNA for RUNX2.

DISCUSSION

Gene regulatory pathways governed by WNT/β-catenin and RUNX2 are both synergistically involved in osteoblast growth and differentiation, and are each compromised in osteosarcoma. Therefore, we examined the relationship between expression of WNT signaling components and RUNX2 in osteosarcoma cells. One key findings of this paper is that the mRNA levels of distinct WNT signaling components (e.g., *DKK1*, *TCF4*, *LEF1*, *WNT10B,* and *WNT3A*) are highly variable among six osteosarcoma cell lines, while others (e.g., *LRP5* and *LRP6*) are relatively constant. Interestingly, β-catenin protein levels are very similar in all six
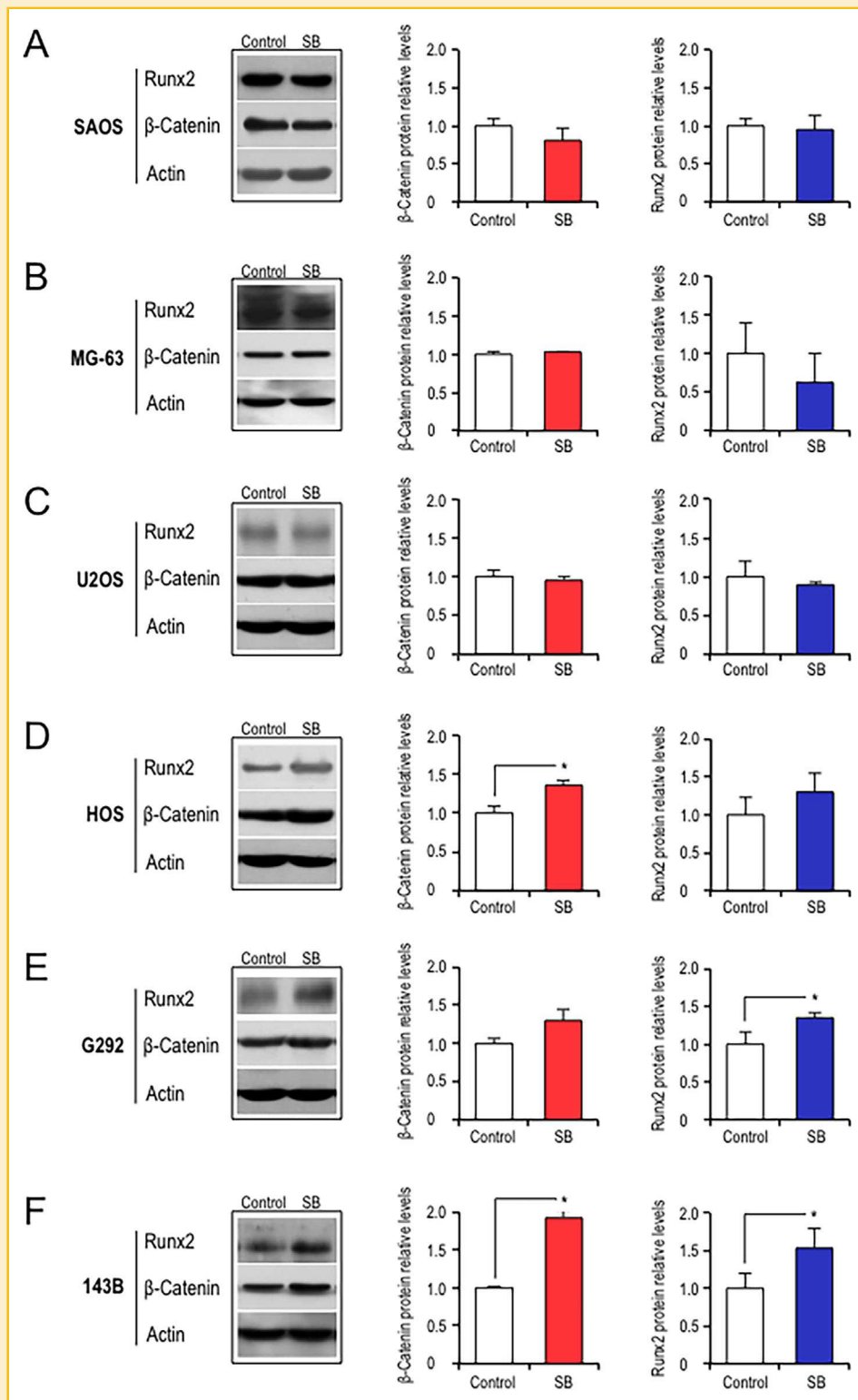
Fig. 5. RUNX2 protein levels are differentially up-regulated after treatment with GSK3β inhibitor in human osteosarcoma cell lines. SAOS, MG63, U2OS, HOS, G292, and 143B osteosarcoma cell lines were grown up to 70% of confluence and then treated with the GSK3β inhibitor SB216763 (SB) for 24 h. RUNX2 and β-catenin protein levels were assessed by Western blot analysis (A–F). Graphs depict data of RUNX2 and β-catenin protein values normalized to β-actin . All data are presented as mean ± SEM. ($^*P < 0.05$).

Fig. 6. β-Catenin deficiency reduces RUNX2 expression in human osteosarcoma cell line. G292 cells were treated with β-catenin siRNA (25–50 nM) (A) or RUNX2 siRNA (50 nM) (B) to assess efficiency of ei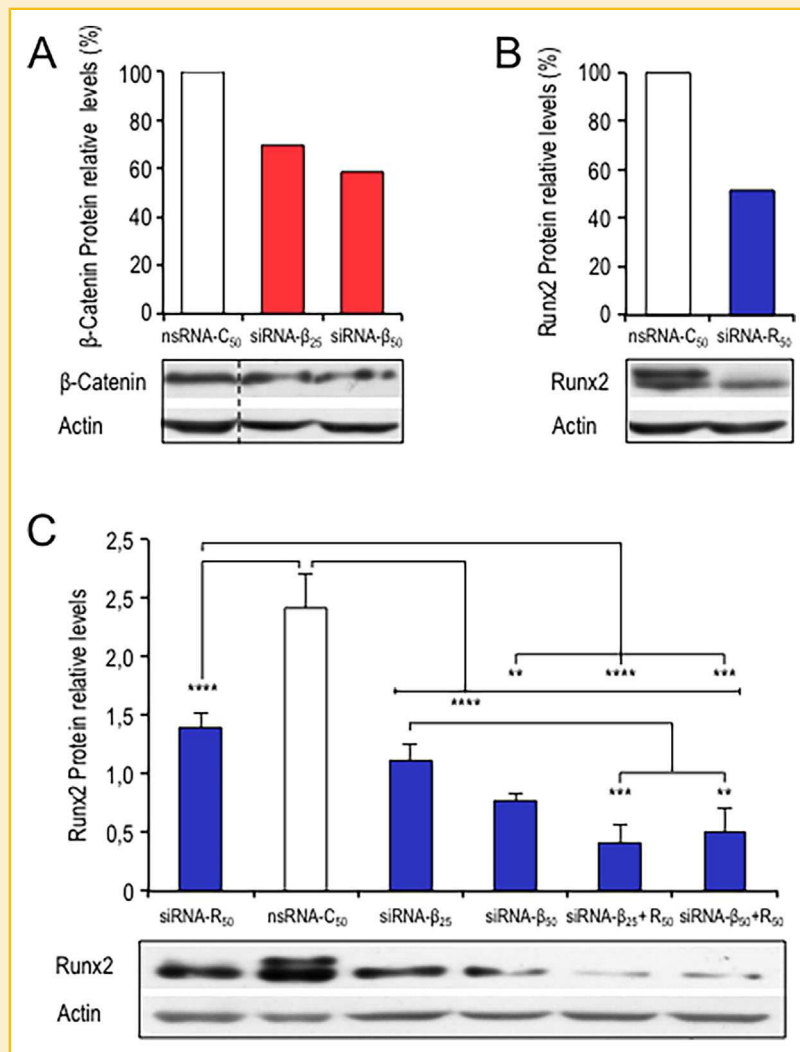ther siRNAs compared to control non-silencing (ns) RNA. Graphs depict data of RUNX2 and β-catenin protein values as a percentage of cells treated with control nsRNA. G292 osteosarcoma cells were transfected with β-catenin siRNA and/or RUNX2 siRNA (C) to determine if β-catenin deficiency reduces RUNX2 expression, as compared to RUNX2 siRNA or control nsRNAs. RUNX2 protein levels were assessed by Western blot analysis at day 2 after transfection. Graphs depict data of RUNX2 protein levels normalized to β-actin. All data are presented as mean ± SEM. ($^{**}P<0.01$ and $^{***}P<0.001$, $^{****}P<0.0001$).

osteosarcoma cell lines. However, β-catenin nuclear accumulation was observed at different degrees in osteosarcoma cells. As expected, *RUNX2* mRNA and protein levels are robustly expressed in most osteosarcoma cell types, except MG63 cells that have low expression of RUNX2 with residual protein levels showing mostly cytoplasmic localization. These expression differences suggest that cross-talk between WNT signaling and RUNX2 activity is cell type dependent among osteosarcomas.

The strong expression of RUNX2 in actively proliferating osteosarcoma cells is remarkable because RUNX2 protein expression is normally increased at the cessation of proliferation and initiation of differentiation of pre-osteoblastic cells, to reinforce a quiescent state [Pratap et al., 2003; Galindo et al., 2005, 2007; Young et al., 2007b]. 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 cell growth [van der Deen et al., 2012; Lucero et al., 2013]. The growth suppressive potential of RUNX2 is normally controlled by modulation of its protein and mRNA levels during the cell cycle [Galindo et al., 2005, 2007]. Cell cycle dependent changes of RUNX2 protein and mRNA levels occur with respect to the G1 phase when osteoblasts monitor extra-cellular cues for competency to initiate cell cycle progression beyond the G1/S phase transition [Galindo et al., 2005, 2007]. Down-regulation of RUNX2 in late G1 phase may be mediated by CDK-related and ubiquitin/proteasome dependent protein degradation [Galindo et al., 2005; Shen et al., 2006; Rajgopal et al., 2007]. Cell cycle control of RUNX2 protein levels is retained in 143B, G292, HOS, and U2OS osteosarcoma cells [Lucero et al., 2013]. In this study we show that the low levels of RUNX2 observed in MG63 and U2OS cells are mostly cytoplasmic, while the

constitutively high levels of RUNX2 proteins in SAOS cells are mostly localized in the nucleus. The remaining cell types show a mixed nuclear and cytoplasmic distribution of RUNX2. Hence, the possibility arises that cell cycle dependent changes in nuclear RUNX2 protein levels may be linked to nuclear/cytoplasmic shuttling in osteosarcoma cells.

Because RUNX2 expression is transcriptionally stimulated by WNT/β-catenin signaling [Gaur et al., 2005], increased activation of this pathway could potentially enhance RUNX2 expression in osteosarcoma. In addition, RUNX2 controls components of multiple signaling pathways in osteosarcoma cells, including Wnt ligands [van der Deen et al., 2012], suggesting the potential for cross-talk through self-reinforcing positive feedback loops. However, RUNX2 stimulation of the mouse osteocalcin promoter is suppressed by protein/protein interactions with LEF1 and β-catenin in several osteoblastic cell types and osteosarcoma cells [Kahler and Westendorf, 2003]. Thus, both negative and positive feedback mechanisms may mediate cross-talk between RUNX2 and the WNT/β-catenin pathway to control bone cell differentiation or progression of osteosarcoma.

Previous reports have demonstrated that RUNX2 is expressed in distinct cancer types [Ito et al., 2015]. For example, RUNX2 has been involved in tumor progression and bone metastasis in breast and prostate cancer [Pratap et al., 2005, 2006, 2008; Leong et al., 2010; Ferrari et al., 2013; McDonald et al., 2014], 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]. RUNX2 also promotes bone metastatic properties of breast and prostate cancer cells [Brubaker et al., 2003; Javed et al., 2005; Pratap et al., 2005, 2006, 2008; Leong et al., 2010]. Enhanced expression of RUNX2 is also associated with stimulating the oncogenic potential of cells during lymphoma development [Blyth et al., 2006]. Moreover, RUNX2 is strongly expressed in human malignant melanoma, thyroid papillary carcinoma, gliomas, and pituitary tumors, which suggests that this cancer gene promotes tumorigenesis in a broad spectrum of tumors [Riminucci et al., 2003; Endo et al., 2008; Vladimirova et al., 2008; Zhang et al., 2009]. Interestingly, several studies have shown that the Wnt/β-catenin pathway is also aberrantly expressed in breast and prostate cancer [Geyer et al., 2011; Kypta and Waxman, 2012], melamona [Omholt et al., 2001], lymphoma [Gelebart et al., 2008], gliomas [Zhang et al., 2012], and pituitary tumors [Elston and Clifton-Bligh, 2010], as well as in thyroid papillary carcinoma [Gilbert-Sirieix et al., 2011]. These studies collectively suggest that cross-talk between Wnt/β-Catenin signaling and RUNX2 may be operative in both osseous and non-osseous tumors.

In osteosarcoma specimens from human patients, expression of RUNX2 varies depending on tumor stage and grade, and its levels are prognostic for chemotherapy responsiveness, metastasis, and long term survival [Won et al., 2009; Kurek et al., 2010; Sadikovic et al., 2010; van der Deen et al., 2013]. One key finding of this is that WNT/β-catenin signaling positively modulates RUNX2 protein levels in a subset of osteosarcoma cell types. WNT/β-catenin signaling is most pronounced in G292 cells which exhibit the greatest nuclear accumulation of β-catenin. We propose that the WNT/β-catenin pathway may promote parameters of tumor progression in osteosarcoma at least in part by functional cross-talk including stimulation of RUNX2 gene expression.

ACKNOWLEDGMENTS

REFERENCES

Andela VB, Siddiqui F, Groman A, Rosier RN. 2005. An immunohistochemical analysis to evaluate an inverse correlation between RUNX2/Cbfa1 and NFκB in human osteosarcoma. J Clin Pathol 58:328–330.

Barnes GL, Hebert KE, Kamal M, Javed A, Einhorn TA, Lian JB, Stein GS, Gerstenfeld LC. 2004. Fidelity of RUNX2 activity in breast cancer cells is required for the generation of metastases associated osteolytic disease. Cancer Res 64:4506–4513.

Beachy PA, Karhadkar SS, Berman DM. 2004. Tissue repair and stem cell renewal in carcinogenesis. Nature 432:324–331.

Bienz M, Clevers H. 2000. Linking colorectal cancer to Wnt signaling. Cell 103:311–320.

Blyth K, Cameron ER, Neil JC. 2005. The RUNX genes: Gain or loss of function in cancer. Nat Rev Cancer 5:376–387.

Blyth K, Vaillant F, Hanlon L, Mackay N, Bell M, Jenkins A, Neil JC, Cameron ER. 2006. RUNX2 and MYC collaborate in lymphoma development by suppressing apoptotic and growth arrest pathways in vivo. Cancer Res 66:2195–2201.

Bravo D, Shogren KL, Zuo D, Wagner ER, Sarkar G, Yaszemski MJ, Maran A. 2017. 2-Methoxyestradiol-Mediated induction of Frzb contributes to cell death and autophagy in MG63 osteosarcoma cells. J Cell Biochem 118:1497–1504.

Brembeck FH, Rosario M, Birchmeier W. 2006. Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. Curr Opin Genet Dev 16:51–59.

Brubaker KD, Vessella RL, Brown LG, Corey E. 2003. Prostate cancer expression of runt-domaintranscription factor RUNX2, a key regulator of osteoblast differentiation and function. Prostate 56:13–22.

Chen K, Fallen S, Abaan HO, Hayran M, Gonzalez C, Wodajo F, MacDonald T, Toretsky JA, Uren A. 2008. Wnt10b induces chemotaxis of osteosarcoma and correlates with reduced survival. Pediatr Blood Cancer 51:349–355.

Cotterill SJ, Wright CM, Pearce MS, Craft AW. 2004. Stature of young people with malignant bone tumors. Pediatr Blood Cancer 42:59–63.

Daino K, Ugolin N, Altmeyer-Morel S, Guilly MN, Chevillard S. 2009. Gene expression profiling of alpha-radiation-induced rat osteosarcomas:

Identification of dysregulated genes involved in radiation-induced tumorigenesis of bone. Int J Cancer 125:612–620.

Dudakovic A, Camilleri E, Riester SM, Lewallen EA, Kvasha S, Chen X, Radel DJ, Anderson JM, Nair AA, Evans JM, Krych AJ, Smith J, Deyle DR, Stein JL, Stein GS, Im HJ, Cool SM, Westendorf JJ, Kakar S, Dietz AB, van Wijnen AJ. 2014. High-resolution molecular validation of self-renewal and spontaneous differentiation in clinical-grade adipose-tissue derived human mesenchymal stem cells. J Cell Biochem 115:1816–1828.

Elston MS, Clifton-Bligh RJ. 2010. Identification of Wnt family inhibitors: A pituitary tumor directed whole genome approach. Mol Cell Endocrinol 326:48–54.

Endo T, Ohta K, Kobayashi T. 2008. Expression and function of cbfa-1/RUNX2 in thyroid papillary carcinoma cells. J Clin Endocrinol Metab 93:2409–2412.

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.

Flores RJ, Li Y, Yu A, Shen J, Rao PH, Lau SS, Vannucci M, Lau CC, Man TK. 2012. A systems biology approach reveals common metastatic pathways in osteosarcoma. BMC Syst Biol 6:50.

Fraumeni JF, Jr. 1967. Stature and malignant tumors of bone in childhood and adolescence. Cancer 20:967–973.

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.

Galindo M, Kahler RA, Teplyuk NM, Stein JL, Lian JB, Stein GS, Westendorf JJ, van Wijnen AJ. 2007. Cell cycle related modulations in RUNX2 protein levels are independent of lymphocyte enhancer-binding factor 1 (Lef1) in proliferating osteoblasts. J Mol Histol 38:501–506.

Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2005. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem 280:33132–33140.

Gelebart P, Anand M, Armanious H, Peters AC, Dien Bard J, Amin HM, Lai R. 2008. Constitutive activation of the Wnt canonical pathway in mantle cell lymphoma. Blood 112:5171–5179.

Geoffroy V, Kneissel M, Fournier B, Boyde A, Matthias P. 2002. High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage. Mol Cell Biol 22:6222–6233.

Geyer FC, Lacroix-Triki M, Savage K, Arnedos M, Lambros MB, MacKay A, Natrajan R, Reis-Filho JS. 2011. β-Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with CTNNB1 mutation. Mod Pathol 24:209–231.

Gilbert-Sirieix M, Makoukji J, Kimura S, Talbot M, Caillou B, Massaad C, Massaad-Massade L. 2011. Wnt/β-catenin signaling pathway is a direct enhancer of thyroid transcription factor-1 in human papillary thyroid carcinoma cells. PLoS ONE 6:e22280.

Goldstein SD, Trucco M, Bautista Guzman W, Hayashi M, Loeb DM. 2016. A monoclonal antibody against the Wnt signaling inhibitor dickkopf-1 inhibits osteosarcoma metastasis in a preclinical model. Oncotarget 7:21114–21123.

Gordon MD, Nusse R. 2006. Wnt signaling: Multiple pathways, multiple receptors, and multiple transcription factors. J Biol Chem 281:22429–22433.

Guo Y, Rubin EM, Xie J, Zi X, Hoang BH. 2008. Dominant negative LRP5 decreases tumorigenicity and metastasis of osteosarcoma in an animal model. Clin Orthop Relat Res 466:2039–2045.

Haydon RC, Deyrup A, Ishikawa A, Heck R, Jiang W, Zhou L, Feng T, King D, Cheng H, Breyer B, Peabody T, Simon MA, Montag AG, He TC. 2002. Cytoplasmic and/or nuclear accumulation of the β-Catenin protein is a frequent event in human osteosarcoma. Int J Cancer 102:338–342.

Hoang BH, Kubo T, Healey JH, Sowers R, Mazza BA, Yang R, Huvos AG, Meyers PA, Gorlick R. 2004a. Expression of LDL receptor-Related protein 5 (LRP5) as a novel marker for disease progression in high-Grade osteosarcoma. Int J Cancer 109:106–111.

Hoang BH, Kubo T, Healey JH, Yang R, Nathan SS, Kolb EA, Mazza BA, Meyers PA, Gorlick R. 2004b. Dickkopf 3 inhibits invasion and motility of saos-2 osteosarcoma cells by modulating the wnt-β-Catenin pathway. Cancer Res 64:2734–2739.

Horne L, Avilucea FR, Jin H, Barrott JJ, Smith-Fry K, Wang Y, Hoang BH, Jones KB. 2016. LRP5 signaling in osteosarcomagenesis: A cautionary tale of translation from cell lines to tumors. Transl Oncol 9:438–444.

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.

Ito Y, Bae SC, Chuang LS. 2015. The RUNX family: Developmental regulators in cancer. Nat Rev Cancer 15:81–95.

Javed A, Barnes GL, Pratap J, Antkowiak T, Gerstenfeld LC, van Wijnen AJ, Stein JL, Lian JB, Stein GS. 2005. Impaired intranuclear trafficking of RUNX2 (AML3/CBFA1) transcription factors in breast cancer cells inhibits osteolysis in vivo. Proc Natl Acad Sci USA 102:1454–1459.

Kahler RA, Westendorf JJ. 2003. Lymphoid enhancer factor-1 and β-Catenin inhibit RUNX2-dependent transcriptional activation of the osteocalcin promoter. J Biol Chem 278:11937–11944.

Kahler RA, Galindo M, Lian J, Stein GS, van Wijnen AJ, Westendorf JJ. 2006. Lymphocyte enhancer-binding factor 1 (Lef1) inhibits terminal differentiation of osteoblasts. J Cell Biochem 97:969–983.

Kansara M, Tsang M, Kodjabachian L, Sims NA, Trivett MK, Ehrich M, Dobrovic A, Slavin J, Choong PFM, Simmons PJ, Dawid IB, Thomas DM. 2009. Wnt inhibitory factor 1 is epigenetically silenced in human osteosarcoma, and targeted disruption accelerates osteosarcomagenesis in mice. J Clin Invest 119:837–851.

Kansara M, Teng MW, Smyth MJ, Thomas DM. 2014. Translational biology of osteosarcoma. Nat Rev Cancer 14:722–735.

Knippschild U, Gocht A, Wolff S, Huber N, Lfhler J, Stfter M. 2005. The casein kinase 1 family: Participation in multiple cellular processes in eukaryotes. Cell Signal 17:675–689.

Kurek K, Del Mare S, Salah Z, Abdeen S, Sadiq H, Lee S, Gaudio E, Zanesi N, Jones KB, De Young B, Amir G, Gebhardt M, Warman M, Stein GS, Stein JL, Lian JB, Aqeilan RI. 2010. Frequent attenuation of the WWOX tumor suppressor in osteosarcoma is associated with increased tumorigenicity and aberrant RUNX2 expression. Cancer Res 70:5577–5586.

Kypta RM, Waxman J. 2012. Wnt/β-catenin signalling in prostate cancer. Nat Rev Urol 9:418–428.

Lau CC, Harris CP, Lu XY, Perlaky L, Gogineni S, Chintagumpala M, Hicks J, Johnson ME, Davino NA, Huvos AG, Meyers PA, Healy JH, Gorlick R, Rao PH. 2004. Frequent amplification and rearrangement of chromosomal bands 6p12-p21 and 17p11.2 in osteosarcoma. Genes Chromosomes Cancer 39:11–21.

Leong DT, Lim J, Goh X, Pratap J, Pereira BP, Kwok HS, Nathan SS, Dobson JR, Lian JB, Ito Y, Voorhoeve PM, Stein GS, Salto-Tellez M, Cool SM, van Wijnen AJ. 2010. Cancer-related ectopic expression of the bone-related transcription factor RUNX2 in non-osseous metastatic tumor cells is linked to cell proliferation and motility. Breast Cancer Res 12:R89.

Leow PC, Tian Q, Ong ZY, Yang Z, Ee PLR. 2009. Antitumor activity of natural compounds, curcumin and PKF118-310, as Wnt/β-catenin antagonists against human osteosarcoma cells. Invest New Drugs 28:766–782.

Ling L, Dombrowski C, Foong KM, Haupt LM, Stein GS, Nurcombe V, van Wijnen AJ, Cool SM. 2010. Synergism between Wnt3a and heparin enhances osteogenesis via a phosphoinositide 3-kinase/Akt/RUNX2 pathway. J Biol Chem 285:26233–26244.

Liu W, Toyosawa S, Furuichi T, Kanatani N, Yoshida C, Liu Y, Himeno M, Narai S, Yamaguchi A, Komori T. 2001. Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. J Cell Biol 155:157–166.

Lu XY, Lu Y, Zhao YJ, Jaeweon K, Kang J, Xiao-Nan L, Ge G, Meyer R, Perlaky L, Hicks J, Chintagumpala M, Cai WW, Ladanyi M, Gorlick R, Lau CC, Pati D, Sheldon M, Rao PH. 2008. Cell cycle regulator gene CDC5L, a potential target for 6p12-p21 amplicon in osteosarcoma. Mol Cancer Res 6:937–946.

Lucero CM, Vega OA, Osorio MM, Tapia JC, Antonelli M, Stein GS, van Wijnen AJ, Galindo MA. 2013. The cancer-related transcription factor RUNX2 modulates cell proliferation in human osteosarcoma cell lines. J Cell Physiol 228:714–723.

Luo X, Chen J, Song WX, Ni Tang, Luo J, Deng ZL, Sharff KA, He G, Bi Y, He BC, Bennett E, Huang J, Kang Q, Jiang W, Su Y, Zhu GH, Yin H, He Y, Wang Y, Souris JS, Chen L, Zuo GW, Montag AG, Reid RR, Haydon RC, Luu HH, He TC. 2008. Osteogenic BMPs promote tumor growth of human osteosarcomas that harbor differentiation defects. Lab Invest 88:1264–1277.

Ma Y, Ren R, Han EQ, Li H, Chen D, Jacobs JJ, Gitelis S, O'Keefe RJ, Konttinen YT, Yin G, Li TF. 2013. Inhibition of the Wnt-β-catenin and Notch signaling pathways sensitizes osteosarcoma cells to chemotherapy. Biochem Biophys Res Commun 431:274–279.

Martin JW, Zielenska M, Stein GS, van Wijnen AJ, Squire JA. 2011. The role of RUNX2 in osteosarcoma oncogenesis. Sarcoma 2011:282745.

Martin JW, Chilton-MacNeill S, Koti M, van Wijnen AJ, Squire JA, Zielenska M. 2014. Digital expression profiling identifies RUNX2, CDC5L, MDM2, RECQL4, and CDK4 as potential predictive biomarkers for neo-adjuvant chemotherapy response in paediatric osteosarcoma. PLoS ONE 9:e95843.

Martins-Neves SR, Paiva-Oliveira DI, Wijers-Koster PM, Abrunhosa AJ, Fontes-Ribeiro C, Bovée JV, Cleton-Jansen AM, Gomes CM. 2016a. Chemotherapy induces stemness in osteosarcoma cells through activation of Wnt/β-catenin signaling. Cancer Lett 370:286–295.

Martins-Neves SR, Corver WE, Paiva-Oliveira DI, van den Akker BE, Briaire-de-Bruijn IH, Bovée JV, Gomes CM, Cleton-Jansen AM. 2016b. Osteosarcoma stem cells have active wnt/é-catenin and overexpress SOX2 and KLF4. J Cell Physiol 231:876–886.

Mbalaviele G, Sheikh S, Stains JP, Salazar VS, Cheng SL, Chen D, Civitelli R. 2005. Beta-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. J Cell Biochem 94:403–418.

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.

Nathan SS, Pereira BP, Zhou YF, Gupta A, Dombrowski C, Soong R, Pho RW, Stein GS, Salto-Tellez M, Cool SM, van Wijnen AJ. 2009. Elevated expression of RUNX2 as a key parameter in the etiology of osteosarcoma. Mol Biol Rep 36:153–158.

Omholt K, Platz A, Ringborg U, Hansson J. 2001. Cytoplasmic and nuclear accumulation of beta-catenin is rarely caused by CTNNB1 exon 3 mutations in cutaneous malignant melanoma. Int J Cancer 92:839–842.

Papa V, Bimonte VM, Wannenes F, D'Abusco AS, Fittipaldi S, Scandurra R, Politi L, Crescioli C, Lenzi A, Di Luigi L, Migliaccio S. 2015. The endocrine disruptor cadmium alters human osteoblast-like SAOS cells homeostasis in vitro by alteration of Wnt/β-catenin pathway and activation of caspases. J Endocrinol Invest 38:1345–1356.

Pereira BP, Zhou Y, Gupta A, Leong DT, Aung KZ, Ling L, Pho RW, Galindo M, Salto-Tellez M, Stein GS, Cool SM, van Wijnen AJ, Nathan SS. 2009. RUNX2, p53, and pRB status as diagnostic parameters for deregulation of osteoblast growth and differentiation in a new pre-chemotherapeutic osteosarcoma cell line (OS1). J Cell Physiol 221:778–788.

Polakis P. 2007. The many ways of Wnt in cancer. Curr Opin Genet Dev 17:45–51.

Pratap J, Galindo M, Zaidi SK, Vraidii 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 pre-osteoblasts. Cancer Res 63:5357–5362.

Pratap J, Javed A, Languino LR, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2005. The RUNX2 osteogenic transcription factor regulates matrix metal-loproteinase 9 in bone metastatic cancer cells and controls cell invasion. Mol Cell Biol 25:8581–8591.

Pratap J, Lian JB, Javed A, Barnes GL, van Wijnen AJ, Stein JL, Stein GS. 2006. Regulatory roles of RUNX2 in metastatic tumor and cancer cell interactions with bone. Cancer Metastasis Rev 25:589–600.

Pratap J, Wixted JJ, Gaur T, Zaidi SK, Dobson J, Gokul KD, Hussain S, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2008. RUNX2 transcriptional activation of Indian Hedgehog and a downstream bone metastatic pathway in breast cancer cells. Cancer Res 68:7795–7802.

Rajgopal A, Young DW, Mujeeb KA, Stein JL, Lian JB, van Wijnen AJ, Stein GS. 2007. Mitotic control of RUNX2 phosphorylation by both CDK1/cyclin B kinase and PP1/PP2A phosphatase in osteoblastic cells. J Cell Biochem 100:1509–1517.

Rajkumar T, Yamuna M. 2008. Multiple pathways are involved in drug resistance to doxorubicin in an osteosarcoma cell line. Anticancer Drugs 19:257–265.

Riminucci M, Corsi A, Peris K, Fisher LW, Chimenti S, Bianco P. 2003. Coexpression of bone sialoprotein (BSP) and the pivotal transcriptional regulator of osteogenesis, Cbfa1/RUNX2, in malignant melanoma. Calcif Tissue Int 73:281–289.

Rubin EM, Guo Y, Tu K, Xie J, Zi X, Hoang BH. 2010. Wnt Inhibitory Factor 1 (WIF-1) decreases tumorigenesis and metastasis in osteosarcoma. Mol Cancer Ther 9:731–741.

Sadikovic B, Yoshimoto M, Chilton-MacNeill S, Thorner P, Squire JA, Zielenska M. 2009. Identification of interactive networks of gene expression associated with osteosarcoma oncogenesis by integrated molecular profiling. Hum Mol Genet 18:1962–1975.

Sadikovic B, Thorner P, Chilton-MacNeill S, Martin JW, Cervigne NK, Squire J, Zielenska M. 2010. Expression analysis of genes associated with human osteosarcoma tumors shows correlation of RUNX2 overexpression with poor response to chemotherapy. BMC Cancer 10:202.

San Martin IA, Varela N, Gaete M, Villegas K, Osorio M, Tapia JC, Antonelli J, Mancilla E, Pereira BP, Nathan SS, Lian JB, Stein JL, Stein GS, van Wijnen AJ, Galindo M. 2009. Impaired cell cycle regulation of the osteoblast-related heterodimeric transcription factor RUNX2-Cbfbeta in osteosarcoma cells. J Cell Physiol 221:560–571.

Schoeman MA, Moester MJ, Oostlander AE, Kaijzel EL, Valstar ER, Nelissen RG, Löwik CW, de Rooij KE. 2015. Inhibition of GSK3β stimulates BMP signaling and decreases SOST expression which results in enhanced osteoblast differentiation. J Cell Biochem 116:2938–2946.

Selvamurugan N, Partridge NC. 2000. Constitutive expression and regulation of collagenase-3 in human breast cancer cells. Mol Cell Biol Res Commun 3:218–223.

Shapovalov Y, Benavidez D, Zuch D, Eliseev RA. 2010. Proteasome inhibition with bortezomib suppresses growth and induces apoptosis in osteosarcoma. Int J Cancer 127:67–76.

Shen J, Meyers CA, Shrestha S, Singh A, LaChaud G, Nguyen V, Asatrian G, Federman N, Bernthal N, Eilber FC, Dry SM, Ting K, Soo C, James AW. 2016. Sclerostin expression in skeletal sarcomas. Hum Pathol 58:24–34.

Shen R, Wang X, Drissi H, Liu F, O'Keefe RJ, Chen D. 2006. Cyclin D1-Cdk4 induce RUNX2 ubiquitination and degradation. J Biol Chem 281:16347–16353.

Srivastava A, Fuchs B, Zhang K, Ruan M, Halder C, Mahlum E, Weber K, Bolander ME, Sarkar G. 2006. High WT1 expression is associated with very poor survival of patients with osteogenic sarcoma metastasis. Clin Cancer Res 12:4237–4243.

Stein GS, van Wijnen AJ, Stein JL, Lian JB, Bidwell JP, Montecino M. 1994. Nuclear architecture supports integration of physiological regulatory signals for transcription of cell growth and tissue-specific genes during osteoblast differentiation. J Cell Biochem 55:4–15.

Techavichit P, Gao Y, Kurenbekova L, Shuck R, Donehower LA, Yustein JT. 2016. Secreted Frizzled-Related Protein 2 (sFRP2) promotes osteosarcoma invasion and metastatic potential. BMC Cancer 16:869.

Thomas D, Johnson S, Sims N, Trivett M, Slavin J, Rubin B, Waring P, McArthur G, Walkley C, Holloway A, Diyagama D, Grim J, Clurman B, Bowtell D, Lee J, Gutierrez G, Piscopo D, Carty S, Hinds P. 2004. Terminal osteoblast differentiation, mediated by RUNX2 and p27KIP1, is disrupted in osteosarcoma. J Cell Biol 167:925–934.

van der Deen M, Akech J, Lapointe D, Gupta S, Young DW, Montecino MA, Galindo M, Lian JB, Stein JL, Stein GS, van Wijnen AJ. 2012. Genomic promoter occupancy of runt-Related transcription factor RUNX2 in osteosarcoma cells identifies genes involved in cell adhesion and motility. J Biol Chem 287:4503–4517.

van der Deen M, Taipaleenmäki H, Zhang Y, Teplyuk NM, Gupta A, Cinghu S, Shogren K, Maran A, Yaszemski MJ, Ling L, Cool SM, Leong DT, Dierkes C, Zustin J, Salto-Tellez M, Ito Y, Bae SC, Zielenska M, Squire JA, Lian JB, Stein JL, Zambetti GP, Jones SN, Galindo M, Hesse E, Stein GS, van Wijnen AJ. 2013. MicroRNA-34c inversely couples the biological functions of the runt-related transcription factor RUNX2 and the tumor suppressor p53 in osteosarcoma. J Biol Chem 288:21307–21319.

Varela N, Aranguiz A, Lizama C, Sepulveda H, Antonelli M, Thaler R, Moreno RD, Montecino M, Stein GS, van Wijnen AJ, Galindo M. 2016. Mitotic inheritance of mRNA facilitates translational activation of the osteogenic-Lineage commitment factor runx2 in progeny of osteoblastic cells. J Cell Physiol 231:1001–1014.

Vielhaber E, Virshup DM. 2001. Casein kinase I: From obscurity to center stage. IUBMB Life 51:73–78.

Vladimirova V, Waha A, Luckerath K, Pesheva P, Probstmeier R. 2008. RUNX2 is expressed in human glioma cells and mediates the expression of galectin-3. J Neurosci Res 86:2450–2461.

Wagner ER, Luther G, Zhu G, Luo Q, Shi Q, Kim SH, Gao JL, Huang E, Gao Y, Yang K, Wang L, Teven C, Luo X, Liu X, Li M, Hu N, Su Y, Bi Y, He BC, Tang N, Luo J, Chen L, Zuo G, Rames R, Haydon RC, Luu HH, He TC. 2011. Defective osteogenic differentiation in the development of osteosarcoma. Sarcoma 2011:325238.

Won KY, Park HR, Park YK. 2009. Prognostic implication of immunohisto-chemical RUNX2 expression in osteosarcoma. Tumori 95:311–316.

Wu J, Liao Q, He H, Zhong D, Yin K. 2014. TWIST interacts with β-catenin signaling on osteosarcoma cell survival against cisplatin. Mol Carcinogenesis 53:440–446.

Wunder JS, Nielsen TO, Maki RG, O'Sullivan B, Alman BA. 2007. Opportunities for improving the therapeutic ratio for patients with sarcoma. Lancet Oncol 8:513–524.

Yang S, Quaresma AJ, Nickerson JA, Green KM, Shaffer SA, Imbalzano AN, Martin-Buley LA, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2015. Subnuclear domain proteins in cancer cells support the functions of RUNX2 in the DNA damage response. J Cell Sci 128:728–740.

Young JL, Jr, Miller RW. 1975. Incidence of malignant tumors in U.S. children. J Pediatr 86:254–258.

Young DW, Hassan MQ, Yang XQ, Galindo M, Javed A, Zaidi SK, Furcinitti P, Lapointe D, Montecino M, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2007a. Mitotic retention of gene expression patterns by the cell fatedetermining transcription factor RUNX2. Proc Natl Acad Sci USA 104:3189–3194.

Young DW, Hassan MQ, Pratap J, Galindo M, Zaidi SK, Lee SH, Yang X, Xie R, Javed A, Underwood JM, Furcinitti P, Imbalzano AN, Penman S, Nickerson JA, Montecino MA, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2007b. Mitotic occupancy and lineage-specific transcriptional control of rRNA genes by Runx2. Nature 445:442–446.

Zanotti S, Canalis E. 2016. Notch signaling and the skeleton. Endocr Rev 37:223–253.

Zhang HY, Jin L, Stilling GA, Ruebel KH, Coonse K, Tanizaki Y, Raz A, Lloyd RV. 2009. RUNX1 and RUNX2 upregulate Galectin-3 expression in human pituitary tumors. Endocrine 35:101–111.

Zhang F, Chen A, Chen J, Yu T, Guo F. 2011a. SiRNA-mediated silencing of β-catenin suppresses invasion and chemosensitivity to doxorubicin in MG-63 osteosarcoma cells. Asian Pacific J Cancer Prev 12:239–245.

Zhang Y, Xie RL, Croce CM, Stein JL, Lian JB, van Wijnen AJ, Stein GS. 2011b. A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. Proc Natl Acad Sci USA 108: 9863–9868.

Zhang K, Zhang J, Han L, Pu P, Kang C. 2012. Wnt/beta-catenin signaling in glioma. J Neuroimmune Pharmacol 7:740–749.