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Sp7 and Runx2 Molecular Complex Synergistically Regulate Expression of Target Genes

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Abstract

Runx2 and Sp7 transcription factors are essential for skeletogenesis. Targeted deletion of either gene results in failure of osteoblast differentiation and bone formation. Loss of bone-matrix gene expression is surprisingly similar in Sp7 and Runx2 null mice. The molecular mechanisms responsible for similar transcriptional regulation of target genes remain largely unknown. Here, we demonstrate that Runx2 and Sp7 interact physically and functionally. Both proteins are co-expressed in osteoblastic cells. We first characterized a panel of Sp7 antibodies and demonstrate that majority of the published antibodies do not recognize Sp7 protein. Coimmunoprecipitation studies revealed that endogenous Runx2 protein physically interacts with Sp7 protein. We identified that runt homology domain (RHD) of Runx2 protein is involved in physical association with Sp7. Functional consequences of Runx2-Sp7 physical interaction was then assessed by promoter-reporter assays. We selected promoters of osteocalcin (OC), a marker of mature osteoblast and fibroblast growth factor 3 (FGF3), a signaling molecule that determine the fate of embryonic ecto-mesenchyme. Runx2 and Sp7 stimulate OC-promoter activity by 3-folds in epithelial cells. However, when both proteins were co-expressed, a dose-dependent synergistic activation of 22-folds was noted. Similar pattern of synergistic activation of OC-promoter was noted in mesenchymal cell. FGF3 promoter was activated by 25 - and 30-folds with Runx2 and Sp7 respectively. Again a dose-dependent synergistic activation of 130-folds was evident when Runx2 and Sp7 were co-expressed in epithelial cells. Synergistic activation of FGF3 promoter was also noted in mesenchymal cells. Together, our data demonstrated that Runx2–Sp7 molecular complex functionally cooperate for maximal induction of cell-phenotype-restricted genes.

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Keywords

Bone development; gene expression; osteoblast; osterix; transcriptional regulation

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Introduction

Commitment and differentiation of mesenchymal cells to the chondrocytes in cartilage and the osteoblasts in bone require Runx2 and Sp7/Osterix transcription factors (1–4). Runx2 regulates both chondrogenesis and osteogenesis (1,2). Global deletion of Runx2 gene results in complete lack of mineralized tissue development and lethality at birth. Runx2 mutation in human is associated with cleidocranial dysplasia characterized by skeletal abnormalities (5). Specificity protein-7 (Sp7), a member of zinc finger containing Kruppel like family of transcription factors is expressed in both chondrocytes and osteoblasts (3,4). Like Runx2, Sp7 is critical for osteoblast differentiation and targeted gene ablation causes failed ossification and perinatal lethality (3,4).

It is generally believed that commitment of mesenchymal progenitor cells to the pre-osteoblasts require Runx2 and subsequent differentiation to mature osteoblast is regulated by a concerted action of Runx2 and Sp7. Moreover, posttranslational modifications regulate Runx2 and Sp7 function, protein stability, and interaction with coregulatory proteins (6–10). In osteoblasts, levels of Runx2 and Sp7 proteins are also regulated by ubiquitination-mediated degradation. Both Runx2 and Sp7 proteins are phosphorylated by MAPK/ERK signaling (6–8). Phospho-modifications are necessary for Sp7 interaction with the p300, Brg1 and SWI/SNF co-regulatory complexes and regulation of target genes (10). Thus, presence of both transcription factors is considered obligatory for osteoblast differentiation during skeletogenesis.

Stages of osteoblastogenesis are identified by expression of stage-specific marker genes. For example, ALP and type-1 collagen are expressed in preosteoblasts, BSP and Osteocalcin (OC) in mature osteoblasts and sclerostin and Phex in osteocytes (7). Sp7 a downstream target of Runx2 is not expressed in Runx2 null mice (3). Thus, Runx2 null mice represent a compound phenotype due to loss of both Runx2 and Sp7 proteins. However, Runx2 mRNA is expressed at normal level in Sp7 null mice. Interestingly, expression of osteogenic genes is absent in both Runx2 and Sp7 null mice (1–4). The underlying molecular reasons for the surprisingly similar loss of osteogenic genes expression from the two unrelated proteins remain unknown. Here, we demonstrate both proteins are required for each other function. Runx2 and Sp7 physically associate and interact functionally to regulate expression of target genes.

Methods and Materials

Cells culture, transient transfection and luciferase assays

HEK293T mesenchymal cells were cultured and maintained in DMEM medium with high glucose. GH329 epithelial cells were maintained in Modified Eagle's medium. Both culture media were supplemented with 10% fetal bovine serum, and 1% penicillin–streptomycin.

The cell culture was maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. Transient transfections were performed using PolyJet™ transfection reagent (SignaGen Laboratories Rockville, MD). For promoter reporter assays, cells were plated in 12-well dishes, transfected at 80% confluency with varying concentrations of Runx2 and Sp7 expression vectors and OC and fibroblast growth factor 3 (FGF3) promoter-reporter plasmid. Transfections were carried as per manufacturer protocol. Briefly, DNA and Polyjet complex were formed at 1:3 ratios for 15 min at 22°C and then overlaid on cells. After 24 h, cells were washed with ice cold PBS and lysed in 1× mild cell lysis buffer (Promega, Madison, WI). Luciferase activity was determined using a Luciferase Reporter Assay kit (Promega, Madison, WI). Results were obtained from at least three independent experiments with four replicates.

Expression plasmids and antibodies

Flag-tagged Runx1, Runx2 and Runx3, full-length and deletion mutant of Sp7 were used in this study. The 208 bp rat OC and 1.0 kb of mouse FGF3 promoters were cloned upstream of the enhanced green fluorescent protein-luciferase reporter. Four different Sp7 antibodies screened were; rabbit polyclonal (Santa Cruz, CA), goat polyclonal (Santa Cruz, CA), mouse monoclonal (LifeSpan BioSciences Inc., Seattle, WA) and rabbit polyclonal (Abcam, Cambridge, MA). To qualitatively compare and assess the specificity of these antibodies, cell lysates from over expressed Sp7 protein were used. To ensure an optimum range for each antibody, at least three different dilutions were tested in Western blots (WB). In brief, after overnight blocking in 5% non-fat milk, the blots with primary antibody were incubated for 1 h in 0.5–1% nonfat milk at 22°C. Then the blots were incubated with species matched HRP-conjugated secondary antibody (Santa Cruz, CA) for 1 h at 22°C. The blots were washed four times with PBST and one time with PBS. Enhanced chemiluminescence detection was then used and signal captured using Alpha Innotech FlourChem system (Alpha Innotech, San Leandro, CA). Other antibodies used in this study were rabbit polyclonal Runx2 (M70, Santa Cruz, CA), mouse monoclonal Runx2 (Clone-8G5; MBL International, Woburn, MA), mouse monoclonal Flag (Sigma Aldrich, St. Louis, MO) and mouse monoclonal β -tubulin (Sigma Aldrich, St. Louis, MO).

Immuno-precipitation and Western blot analysis

HEK293T cells were co-transfected with 5 μ g each expression plasmids. Cells were harvested after 24 h and lysed with 0.2% NP-40 lysis buffer (150mM NaCl, 10mM Tris-HCl, 0.2% NP-40, 1mM MgCl₂, 1mM DTT, and 1mM EDTA supplemented with 25 μ M MG132, 1mM PMSF and 1× Complete protease inhibitors) and brief sonication. Cell lysate was incubated with 2 μ g of Sp7 or Runx2 M70 antibody at 4°C for 1 h. Then 40 μ l of protein-A/G magnetic beads (Thermo Scientific, Rockford, IL) was added and incubated at 4°C. Beads were washed four times with 0.2% NP-40 buffer and proteins lysed with 50 μ l of 2× SDS lysis buffer. Proteins samples were separated by SDS-PAGE and transferred to Immobilon-Fl PVDF membrane (Millipore Corporation, Billerica MA). Blot was then probed with primary antibody for overnight at 22°C. Finally, blots were probed with secondary antibody conjugated with Infrared dye 700 (IRDye700) or IRDye800 (LI-COR, Lincoln, NB) for 1h at 22°C and washed four times with PBST. The blots were then scanned by Odyssey Imaging System (LI-COR, Lincoln, NB).

Results

Majority of the published Sp7/Osterix antibodies are nonspecific and fail to recognize Sp7 protein

We initiated biochemical experiments to better understand why Runx2 and Sp7 deficiency lead to a similar loss of osteoblast gene expression. Numerous studies published have shown expression of Sp7 mRNA but less than 10% of study has data of Sp7 protein. Studies differ not only by the immunological technique, but also the type of antibodies used. We have tested number of commercially advertised monoclonal and polyclonal antibodies. To quantitatively compare and assess the specificity of these antibodies, lysates from transiently transfected cells with Sp7 were used. We failed to detect any specific signal for three antibodies by WB analysis (Figure 1A). Only a non-specific band ~26KDa range was seen with two antibodies. Published reports that have used these antibodies only show either an individual strip or closely trimmed blot, without a molecular weight marker making it impossible to judge if the immunoreactive band shown is of expected size. These antibodies also failed to detect Sp7 protein in its native conformation as assessed by *in situ* immunofluorescence (data not shown). In our analysis, only one antibody showed Sp7-specific immunoreactivity of the expected size (Figure 1A). This antibody specifically detected both full length and the mutant missing first eight residues of Sp7 protein (E1). The lack of detection of mutant Sp7 (383–288) is consistent with the C-terminal epitope against which this antibody was raised. The failure of Sp7 detection by all other antibodies was not related to exposure time, suboptimal antibody dose or protein concentration. As exposure time beyond the linear range of detection (>30 minutes), use of SNAP ID system with 2- and 5- fold more antibody, and with 2-fold increased cell lysates, all failed to show Sp7-specific immunoreactivity. Thus, the sensitivity of our technique is not responsible for the differences between our findings and published results. Since all Sp7 proteins were Flag tagged, the blot was stripped and reprobed with Flag antibody. Both wild type and all of the mutant Sp7 proteins were detected (Figure 1A). These results clearly demonstrate that majority of the available antibodies are unable to recognize Sp7 protein.

Physical interaction between Runx2 and Sp7 proteins is mediated through runt homology domain

To assess if Runx2 and Sp7 proteins physically interact, we performed coimmunoprecipitation experiment. Runx2 and Sp7 expression plasmids were co-expressed in both epithelial and mesenchymal cells. Cell lysates were immunoprecipitated with Sp7 antibody and we found Co-IP of Runx2 protein with Sp7. These results indicate that Runx2 protein physically interact with Sp7 protein (Figure 1C). The specificity of Runx2-Sp7 complex was further confirmed with reciprocal Co-IP experiments. Cell lysates were immunoprecipitated with Runx2 antibody and we find Co-IP of Sp7 (Figure 1C, right panel). Together these data demonstrate that Runx2 and Sp7 proteins form a stable molecular complex in both cell types. It is important to note that endogenous Runx2 and Sp7 are not expressed in epithelial cells. Formation of molecular complex between Runx2 and Sp7 in epithelial cells suggested that endogenous milieu and factor within mesenchymal cells are not essential for Runx2-Sp7 physical interaction. We next defined the domain of Runx2 protein involved in this molecular interaction. Deletion mutagenesis revealed that N-

terminal 230 amino acids of Runx2 protein were sufficient for interaction with Sp7 (data not shown). The three member of runt related transcription factor family are characterized by the presence of highly conserved RHD. To test if RHD is involved in molecular interaction with Sp7, we performed Co-IP by transfecting Runx1, Runx2 and Runx3 with Sp7. All three Runx proteins were co-immunoprecipitated with Sp7 (Figure 1D). This data suggest that RHD is involved in physical interaction with Sp7.

Sp7-Runx2 molecular complex synergistically induces promoter of target genes

Runx2 function as a master switch for inducing skeletal cell differentiation by regulating target genes expression at multiple stages of osteoblast development. Having established that Runx2 form a stable molecular complex with Sp7; we next tested their functional interaction. For this, promoter segment from OC; a marker of mature osteoblast, and FGF3; a signaling molecule required for odontogenesis and bone development were used (Figure 2). These promoter segments contain conserved Runx and Sp binding motif. Runx2 and Sp7 stimulate OC promoter activity by 3-folds in epithelial cells (Figure 2A). However, co-expression of Runx2 and Sp7 results in a dose-dependent synergistic activation of 22-folds.

The zero background of Runx2 and Sp7 in epithelial cells allowed better quantification of synergistic activation of promoters. Runx2 and Sp7 also showed synergistic activation of OC promoter in mesenchymal cells (Figure 2B). FGF3 promoter was induced by 25- and 30-fold with Runx2 and Sp7, respectively. When both proteins were co-expressed, a dose-dependent synergistic activation of 130-fold was observed in epithelial cells (Figure 2C). Similar synergistic activation of FGF3 promoter was noted in mesenchymal cells (Figure 2D). Taken together, these results provide evidence that Runx2 and Sp7 form stable molecular complex and synergistically induce activity of target genes promoter.

Discussion

Runx2 and Sp7 transcription factors play a pivotal role in skeletogenesis. Expression of osteogenic genes is absent in Sp7 and Runx2 null model (1–4). Here, we show Runx2 and Sp7 physically interact and synergistically activate osteogenic genes. To better understand functional role of Runx2 and Sp7, we initially screened a panel of commercially available monoclonal and polyclonal antibodies. Antibodies that specifically detect Runx2 protein were well characterized.

However, information about validated antibodies that can consistently and specifically detect Sp7 protein was lacking. Published reports that have used these antibodies have not established specific reactivity with Sp7 protein. We failed to detect any specific signal for the three antibodies tested. Only non-specific band ~26kDa and ~56kDa marker range was seen with two antibodies. Parallel blots were probed with either 1:500, 1:1000 or 1:2000 dilution of the same Sp7 antibody. Independent blots were also probed with 2× and 5× antibody for 5 min at room temperature with SNAP ID system. Furthermore, chemiluminescence reactions were carried for 2, 5, 15 and 30 min to improve detection of protein signal. However, none of these modifications resulted in detection of specific signal for overexpressed Sp7 protein. The rabbit polyclonal antibody consistently detected both endogenous and exogenous Sp7 protein. This antibody also detects Sp7 protein by *in situ*

immunochemistry (11). The presence of Sp7 protein in our sample was confirmed by using Flag antibody as all of the over-expressed Sp7 protein carry Flag-epitope. Thus, the sensitivity of our technique is not responsible for the differences between our findings and published results. Recently, another rabbit polyclonal antibody raised against the N-terminal epitope of Sp7 is shown to detect Sp7 protein by *in situ* immunochemistry (12). However, we have not tested this antibody in WB analysis. Our data clearly shows that majority of the commercially available Sp7 antibodies are non-specific and brings into question results of some of the published studies.

Concerted action of Runx2 and Sp7 is required for differentiation of preosteoblasts into mature osteoblasts. The differentiated osteoblasts produce most of the proteins present in the bone ECM such as collagen, BSP and OC. Most of the ECM proteins are absent in Runx2 and Sp7 null models indicating they are regulated by Runx2 and Sp7. Consistent with this notion, we find both Runx2 and Sp7 regulates OC promoter. However, maximal activity of OC promoter requires presence of both Runx2 and Sp7 proteins. The synergistic activation of target gene by Runx2 and Sp7 was further evident in experiment using FGF3 promoter. Runx2 involvement in synergistic activation of target genes is known to require protein-protein interaction. For example, molecular interaction between Runx2 and CEBP β results in synergistic activation of the OC promoter (13). The underlying mechanism for synergistic activation is yet to be fully understood. It is possible that physical association between Runx2 and Sp7 enhances their interaction with target DNA or increase residence time at the target genes promoters.

Conclusion

We demonstrated for the first time that Runx2 and Sp7 interact physically and functionally to synergistically activate cell-phenotype restricted target genes.

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Abbreviations

Runx2	Runt-related transcription factor-2
Sp7	Specificity protein-7
OC	Osteocalcin
ALP	Alkaline phosphatase
BSP	Bone sialo-protein
Phex	Phosphate regulating endopeptidase homolog, X-linked
FGF3	Fibroblast growth factor-3
Co-IP	Coimmunoprecipitation
RHD	Runt homology domain

ECM	Extracellular matrix
CCD	Cleidocranial Dysplasia

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Figure 1. Runx2 physically interacts with Sp7 through Runt homology domain
(A) Assessment of specificity of Sp7 antibodies. GH239 cells were transfected with 5μg expression vectors of full length and deletion mutant of Sp7. Cells were lysed 24 h later for Western blot (WB) analysis. Blots were incubated with primary and secondary antibodies as described in method. Blots were stripped and reprobed with Flag antibody to confirm the presence of Sp7 protein and tubulin antibody as an internal control. (B) Schematic illustration shows epitops of different Sp7 antibodies used in this study. Detail of each antibody epitope is noted in the table. Asterisk indicates only 3 PubMed citations for this antibody at the time of this experiment. (C) Runx2 and Sp7 proteins form molecular complex. Cells were co-transfected with 5μg of Runx2 and Sp7 expression plasmids. Immunoprecipitation were performed with Sp7 and Runx2 antibody. Input represent 10% of

the total lysate. WB was performed with indicated antibody. For reciprocal Co-IP, Runx2 protein was immunoprecipitated by Runx2 antibody (Right panel). (D) RHD is involved in physical association with Sp7. Runx family members were co-transfected with Sp7 in HEK293T cell. Lysates were immunoprecipitated with Sp7 antibody.

Figure 2. Runx2 and Sp7 synergistically activate OC and FGF3 gene promoters in multiple cell types

(A) OC proximal promoter contains both Runx and Sp regulatory motif. OC-promoter-luciferase plasmid was co-transfected with fixed amount (100 ng) of Runx2 and increasing amount (100–300 ng) of Sp7 expression vectors. Normalized luciferase activity from three independent experiments with four replicates is shown in (A) epithelial and (B) mesenchymal cells. (C) FGF3 promoter contains multiple Runx and Sp regulatory motif. FGF3 promoter-luciferase plasmid was co-transfected with fixed amount (100 ng) of Runx2 and increasing amount (100–300 ng) of Sp7 expression vectors. Pooled data from three

independent experiments with four replicates is shown as fold activation in (C) epithelial and (D) mesenchymal cells.