Expression of Galectin-3 in Skeletal Tissues Is Controlled by Runx2*

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The β-galactoside-specific lectin galectin-3 is expressed in vivo in osteoblasts as well as in epiphyseal cartilage. Here we show that in vitro, galectin-3 expression is up-regulated in the preosteoblastic cell line MC3T3-E1 during the matrix maturation stage of the osteoblast developmental sequence. Expression persists into late differentiation stages when the mature osteoblastic phenotype is established. The similar expression pattern of galectin-3 overlaps at many sites with that of the transcription factor Runx2. Runx2 is a key regulator of osteoblast development and necessary for chondrocyte differentiation in the growth plate. Both human and mouse galectin-3 promoters contain putative Runx-binding sites. The constitutive or inducible forced expression of Runx2 is sufficient for the onset of galectin-3 transcription in the mesenchymal precursor cell line C3H10T1/2. Moreover, Runx2 is able to bind to at least two sites in the galectin-3 promoter region. The crucial role of Runx2 was confirmed in Runx2-deficient mice, which are devoid of galectin-3 expression in skeletal cells. The overlapping expression pattern of galectin-3 with the other two members of the Runt family of transcription factors (Runx1 and Runx3) points to a potential regulation of the galectin-3 gene (LGALS3) by these factors in hematopoietic, skin, and dorsal root ganglial cells.

Galectin-3 (Mac-2, eBP, IgE-binding protein, CBP35, CBP30, L-29, and L-34) is one of ten members of the protein family of β-galactoside specific lectins (1). It was first identified as an antigen on murine thioglycollate-elicited peritoneal macrophages (2). The molecular mass of galectin-3 ranges between 30 and 42 kDa in different species (3). The protein can exhibit intranuclear, cytoplasmatic, or extracellular localization. Lack-}

* 5'ACCPuCPu-3

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(20). The cells were maintained in α-modified minimal essential medium supplemented with 10% fetal calf serum and 2 mM glutamine (Invitrogen). The conditions for osteogenic differentiation were adopted from those reported for differentiation of fetal rat calvaria cells (30). When cells reached confluence, medium supplemented with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid was used (Sigma-Aldrich). The medium was changed every 2 days. Osteogenic differentiation was monitored by alkaline phosphatase staining using Naphthol AS-TR phosphate and Fast Red RC tablets (Sigma-Aldrich), according to the manufacturer’s instructions.

C3H10T1/2 embryonic fibroblasts were obtained from the American Type Culture Collection (Manassas, VA). The C3H10T1/2 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum and 2 mM l-glutamine. C3H10T1/2 cells were subcultured before reaching confluency. For stably transfected C3H10T1/2 cells (C3H10T1/2-Rux2) medium was supplemented with G418 at 600 μg/ml (Sigma-Aldrich). Stably transfected C3H10T1/2 clones with inducible Runx2 expression were grown with the addition of 150 μg/ml zeocin and 200 μg/ml hygromycin (both from Invitrogen).

Cells with inducible expression of Runx2 were generated using the mifepristone-regulated expression system GeneSwitch (all of components were from Invitrogen). For stable transfections, 105 C3H10T1/2 cells were incubated with 2.5 μg of plasmid DNA and 5 μl of FuGENE 6 reagent (Roche Molecular Biochemicals). After transfection with pSswitch, the clones were selected with 200 μg/ml hygromycin. Individual clones expressing the regulatory protein encoded by pSwitch were identified by transient transfection with pGene/V5 His/neo/Z. 24 h after incubation in the presence of 30 nM mifepristone, the cells were stained for β-galactosidase. The cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde for 5 min at room temperature and subsequently stained using X-gal. The cells were washed in 4% FCS/PEN/CN, 5 mM Na3PO4, and 1 mg/ml X-Gal. Two of the clones that proved to be inducible were stably transfected with pGene-Runx2 as described with the addition of 150 μg/ml Zeocin. The clones were screened for inducible expression of Runx2 by Western blotting after induction with 30 nM mifepristone for 24 h. For expression analysis in induced C3H10T1/2 clones, the medium was supplemented with 20 nM mifepristone (dissolved in ethanol), and the cells were harvested after 24–40 h. Control cells were grown for the same period in medium supplemented with the respective volume of ethanol. Pretreatment of inducible C3H10T1/2 cells with trichostatin A (TSA) was performed in standard medium supplemented with 25–200 nM TSA. After 20 h mifepristone was added to a final concentration of 30 nM to induce cells.

Cloning the Galectin-3 Promoter and cDNAs for Hybridization and Expression—All cDNAs were obtained by reverse transcriptase-PCR using total RNA purified from limbs of newborn mice. Reverse transcription was carried out using 2 μg of total RNA, random hexamer primers, and SuperScript II reverse transcriptase (Invitrogen). Prior to PCR, cDNA was treated with ribonuclease H (Invitrogen). PCR reagents were from Qiagen.

The primers used for reverse transcription-PCR were as follows: Runx2ex1forward, 5′-TACTTACACGACCCAGACAAA-3′; Runx2ex2reverse, 5′-CATATGGAAACAGACACACACCTTT-3′; Runx2hybfor, 5′-AACCCACGCTCCTGCTGACCTTCT-3′; Runx2hybrev, 5′-TGCAGTGCAGTCGCGGGGTTAGGT-3′; Gal3for, 5′-TGGAGAAAGAGAAAGACGTC-3′; and Gal3rev, 5′-GGTTCCACCTCC-TAAGGACAC-3′.

Sequences of β-actin primers were obtained from Gessner et al. (31). glyceraldehyde-3-phosphate dehydrogenase primers from a PCR-Select cDNA library (BD Clontech) from Desbois et al. (32). PCR products were cloned into TA cloning vector pCR2.1 (Invitrogen), and inserts were sequenced by a DyeDeoxy Sequencing system (ABI, Weiterstadt, Germany). Runx2 cDNA was subcloned into expression vector pCMVβ (BD Clontech) replacing the lacZ gene to generate pCMV-Runx2. Likewise, Runx2 cDNA was subcloned into expression vector pGens/V5-His A (GeneSwitch System, Invitrogen). This vector is referred to as pGene-Runx2.

The murine galectin-3 promoter from −1887 to +50 nucleotides relative to the transcription start site was amplified from FvB murine genomic DNA and cloned into pBlue-TOPO (Invitrogen) 5′ to the lacZ gene. The following primers were used: LGAL3sp20000for1, 5′-CTCTGG-GACCTTTGAGGTTCTACTCCTA-3′; and LGAL3srev, 5′-GGCTCAACTT-GATTATGGCTC-3′.

Sequence Analysis of Marine and Human LGALS3 Promoter Fragment—Screening of DNA sequences for putative transcription factor-binding sites was performed using the web-based prediction programs MatInspector (Transfac; transfac.gbf.de) and TFSEARCH (www.cbr.jh.edu/research/db/TFSEARCH.html). The MatInspector thresholds for core similarity and matrix similarity were set to 0.85 and 0.90, respectively. The TFSEARCH minimum score was set to 90.0 points. Putative Runx-binding sites were identified by searching for sequences matching the published consensus motif (21–23) with special respect on structure data (33). Thus the sequence was searched for the motif ACCPuCPu, and positions 2 and 3 (CC) were considered to be most important.

Northern Blot Analysis—Total RNA (15 μg/lane) was resolved on a 1% formaldehyde–agarose gel and transferred onto a Hybond N+ nylon membrane (Amersham Biosciences) using 10× SSC. The probes were labeled with α-32P-dCTP (3000 Ci/mmol; Amersham Biosciences) using a Megaprime DNA labeling system (Amersham Biosciences). The blots were prehybridized and hybridized at 65 °C in Church buffer (500 mM phosphate buffer, pH 7.2, 7% (w/v) SDS, 1 mM EDTA, 10 μg/ml salmon sperm DNA, adapted from Church et al. (34)). The blots were washed in 2× SSC, 0.1% SDS at room temperature for 15 min and twice in 0.1× SSC, 0.1% SDS at 60 °C for 20 min. The blots were exposed to Kodak XAR-5 film at −70 °C with two intensifier screens.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—For Western blot analysis of Runx2 expression, the cells were washed in phosphate-buffered saline and lysed in Laemmli buffer. The protein sample was resolved by SDS-PAGE through denaturing 10% polyacrylamide gels (35). After electrophoresis, the protein was transferred onto Hybond-P membranes (Amersham Biosciences) by semidy electromobbing (36). Afterward the membranes were blocked in 5% skimmed milk in TBST buffer and incubated in 5% skimmed milk in TBST buffer with 1:2000 dilution of secondary antibody goat anti-rabbit IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA). After washing with TBST, the blots were incubated in 15 mM sodium phosphate, pH 8.0) specific protein was visualized by chemiluminiscence using the ECL™ system (Amersham Biosciences) according to the manufacturer’s instructions.

In Vitro Translation of Runx2 Protein and Electromobility Shift Assays (EMSA)—For in vitro translation Runx2 cDNA was subcloned into pGEM-3Zf(-). In vitro translation was carried out using T7-coupled wheat germ Extract system (Promega) according to the supplier’s instructions. In vitro translated protein was analyzed by Western blot. Double-stranded galectin-3 promoter-derived oligonucleotides were designed with 5′-G overhangs for labeling with [α-32P]dCTP, and oligonucleotide Oligo A for binding control was adopted from Tahirov et al. (33). For immunodetection of Runx2 protein, the blots were incubated in 5% skimmed milk in TBST buffer with 1:2000 dilution of secondary antibody goat anti-rabbit IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min. After washing with TBST, the blots were incubated in 15 mM sodium phosphate, pH 8.0) and hybridized using a MegaPrime DNA labeling system (Amersham Biosciences). Labeled oligonucleotides were purified by column chromatography using Sephadex G25 Quickspin columns (Roche Molecular Biochemicals). For binding reactions, 20 μl samples were prepared containing 3 μl in vitro translation reaction, 5 μl of labeled double-stranded oligonucleotide (20000–25000 cpm), 1 μl of respective unlabeled competitor oligonucleotide (resulting in 15– or 60-fold molar excess of competitor, respectively), 1 μl of pepstatin, and 1 μl of poly(dI-dC) (1 A260/ml in 2% glycine, 5 mM Tris-HCl, 0.2 mM EDTA, 0.01% Nonidet-P 40, 0.1 mM dithiothreitol, 17.5 mM NaCl, 10 μg/ml bovine serum albumin, pH 7.5). The binding reactions were incubated at room temperature and resolved on a 10% acrylamide gel in 0.5× TBE. The gels were dried and then exposed to Kodak XAR-5 film at −70 °C using intensifier screens.

RNA in Situ hybridization—In situ hybridizations using 32P-labeled antisense riboprobes were carried out as previously described (37). Briefly, the sections were deparaffinized, rehydrated, pretreated with Runx2 and hybridized at 30°C for 3 h. Sections were washed and dipped in photoemulsion (Kodak), dried, and exposed for 2–8 days at 4°C. The slides were counterstained with Toluidine blue O mounted with entellan.

**RESULTS**

Galectin-3 Gene Expression Is Up-regulated during Osteogenic Differentiation of Mouse Calvaria MC3T3-E1 Cells—MC3T3-E1 is a pre-osteoblastic cell line derived from murine

**The abbreviations used are:** TSA, trichostatin A; EMSA, electromobility shift assay; C/EBP, CCAAT/enhancer-binding protein.
calvaria cells. These cells have been demonstrated to undergo osteogenic differentiation in response to prolonged confluent culture (29). To investigate galectin-3 gene expression during osteogenesis, we performed an in vitro differentiation assay based on MC3T3-E1 cells. The cells were grown in nonsupplemented medium until confluency. (The first day of confluent culture is referred to as day 0.) From day 0, osteogenic differentiation was supported by medium supplemented with β-glycerophosphate and ascorbic acid as described under "Materials and Methods." The cells were harvested at days 0, 4, 8, 12, 16, and 20, and the total RNA was isolated for Northern blot analysis. In parallel, the cells were stained for alkaline phosphatase protein as a marker for the intermediate stage of osteogenic differentiation. Histochemically distinct alkaline phosphatase-positive cells were seen as early as day 4 post-confluence (Fig. 1A). As specific marker for late stages of osteogenic differentiation, osteocalcin mRNA synthesis was assessed by Northern blot. Osteocalcin mRNA became detectable at very low levels already at day 4 post-confluence and is expressed at highest levels on day 20 post-confluence, indicating efficient development of the mature osteoblastic phenotype at this late stage of cultivation (Fig. 1B). Galectin-3 mRNA was expressed in vitro at any time point during the differentiation kinetics in MC3T3-E1 cells. However, galectin-3 mRNA levels increased continuously during ongoing osteogenic differentiation up to day 8 post-confluence when expression levels reached a maximum during central matrix maturation stage of the osteoblast developmental sequence. Thereafter, galectin-3 mRNA expression persists at considerable levels into later stages of osteogenic differentiation (day 20 post-confluence) (Fig. 1B). Thus galectin-3 mRNA expression parallels the transition of the cell line MC3T3-E1 from the fibroblastic to a distinct osteoblastic phenotype as assessed by the expression of markers typical for osteoblastic differentiation in these cells. Although already present at the preosteoblastic stage, galectin-3 expression increases during ongoing osteogenic differentiation and persists at high levels late into the osteoblast developmental sequence in vitro.

**Analysis of the Galectin-3 Promoter Sequence**—To elucidate the mechanism by which galectin-3 expression is up-regulated during osteogenic differentiation in MC3T3-E1 cells, we directly sequenced 2.0 kb of the promoter region of the murine galectin-3 (Fvb mouse strain; pGal3-2000) and screened for transcription factor-binding sites in the promoter region that might be involved in establishing or maintaining the osteoblastic lineage. To elucidate biologically relevant transcription factor-binding sites, we scanned 900 bp of the 5′ flanking region of the murine and 836 bp of the corresponding 5′ flanking region of the human LGALS3 gene (GenBank™ accession number AF031421.1). The sequences were examined using the predic-
tion programs MatInspector and TFSEARCH and were compared with find binding motifs conserved between both the human and murine promoters. As reported previously, the promoter regions of either species do not contain a TATA box, and CAAT motifs are missing. A GC-rich region harboring an SP1-binding site is located immediately upstream the transcription start site in both murine and human promoters, which is typical for TATA-less promoters (18, 19). We identified additional putative binding sites for AP1, AP4, C/EBP, CDP CR, c-Ets, CP2, c-Rel, GATA proteins, GFI1, HNF3b, Ikaros factors, Lmo2 complex, MyoD, MZF1, NF1, NF-AT, NF-κB, NFY, Nkx-2.5, Runx factors, S8, and USF in the LGALS3 promoter regions of both species (Table I and Fig. 2). Of special interest with respect to osteogenesis are five binding sites for Runx, one for Ets factors, four for C/EBP, and three for SP1 in the murine promoter. In the murine promoter one additional Runx-binding site was identified further upstream at -1477 nucleotides relative to the transcription start. Runx2, Ets-1, and C/EBP are bone-related transcription factors that control the transcription of several bone-specific genes (26, 38, 39). Osterix was recently identified as a zinc finger transcription factor involved in osteogenesis and was shown to bind to the SP1 consensus motif (40). Hence, galectin-3 expression in osteous tissues may be regulated by the transcription factor Runx2 and modulated by Ets-1, C/EBP, and osterix.

**Forced Stable and Inducible Expression of Runx2 in Mesenchymal Progenitor C3H10T1/2 Cells Leads to Osteogenic Differentiation**—To investigate the functional relevance of Runx2-binding sites in the promoter of the LGALS3 gene, the influence of Runx2 on the expression of galectin-3 was determined in cellular differentiation systems in vitro. A recombinant murine C3H10T1/2 cell line was established harboring an expression vector mediating constitutive Runx2 expression (C3H10T1/2-Runx2). The murine cell line C3H10T1/2 has properties of mesenchymal stem cells. These cells differentiate into...
Regulation of Galectin-3 Expression

Fig. 4. Inducible expression of Runx2 in C3H10T1/2 cells leads to an up-regulation of galectin-3 transcription. Cells from one clone 18/17 were treated with 30 nM mifepristone (+) or the respective volume of ethanol (−) for 38 h. A, mifepristone-induced Runx2-transfected C3H10T1/2 cells express Runx2 protein of correct size (57 kDa). Western blot with Runx2 antiserum and staining with Coomassie Brilliant Blue for loading and transfer control is shown. B, Runx2 protein expressed after induction is biologically active. Northern blot analysis from induced and noninduced cells was performed with the probes indicated. Runx2 expression is confirmed after induction with mifepristone. Osteocalcin mRNA levels were measured to investigate the functional activity of recombinant Runx2. Galectin-3 gene expression is up-regulated after induction of Runx2 expression. mRNA sizes are indicated for orientation. Mif, mifepristone.

Fig. 5. Pretreatment with TSA increases the inducible expression of Runx2 and galectin-3 in C3H10T1/2 cells. A, cells were grown as described under “Materials and Methods.” Induction of Runx2 expression by the addition of 30 nM mifepristone after preincubation with different concentrations of TSA. B, Runx2-dependent synthesis of galectin-3 mRNA can be stimulated by pretreatment of cells with TSA. The molecular masses are indicated. Mif, mifepristone.

Adipocytes, myoblasts, chondrocytes, and osteoblasts dependent on the culture conditions (41–43).

C3H10T1/2 cells have already been used successfully in the characterization of known Runx2 target genes (26). Here, the stable expression of Runx2 in C3H10T1/2-Runx2 cells was confirmed by Northern and Western blot analysis (Fig. 3, A and B). In contrast to untransfected cells, C3H10T1/2-Runx2 cells expressed osteocalcin mRNA as shown by Northern blot, indicating that the biological activity of transgenic Runx2 mediates osteogenic differentiation (Fig. 3B) (26). C3H10T1/2-Runx2 cells grew more slowly than wild type cells and exhibited a similar but more spindle-like morphology (data not shown).

To investigate whether or not galectin-3 is directly regulated by Runx2, we generated C3H10T1/2 cells with an inducible expression system for Runx2 (“Gene Switch” system, Invitrogen; “Materials and Methods”). After selection, the clones were analyzed for the inducible expression of Runx2 with 30 nM mifepristone for 38 h or with the solvent ethanol as a control. Functional analysis of clone 18/17 is shown in Fig. 4. Northern blot analysis showed the presence of Runx2 mRNA in cells stimulated with mifepristone (Fig. 4B). Cells treated with ethanol alone did not express detectable amounts of Runx2 mRNA. To confirm that protein of correct size was being translated, Western blotting was performed. A band at 57 kDa was present in protein lysate of induced cells (Fig. 4A). Osteocalcin gene expression is known to be directly controlled by Runx2 (26). Therefore, Northern blot analysis for osteocalcin expression was used to verify biological activity of transgenic Runx2. The results shown in Fig. 4B reveal high levels of osteocalcin mRNA in induced as compared with noninduced cells. Taken together, these results confirm that the transfectants inducibly express biologically active Runx2. The morphology of these cells did not change after induction with mifepristone up to 5 days (data not shown). Likewise, a change in growth rate could not be observed (data not shown). A stepwise increase in mifepristone concentration from 5 to 80 nM did not lead to a further elevation in Runx2 expression levels when assessed by Western blot (data not shown). After induction Runx2 expression could be maintained for 5 days, with maximum expression levels at 24 h (data not shown). To enhance the expression from the Runx2 transgene in cells treated with mifepristone, we investigated the effect of the histone acetyltransferase inhibitor TSA. TSA has been reported to derepress silenced genes by elevating the overall acetylation status of histones, thus increasing the accessibility of genomic DNA to DNA-binding factors. The pretreatment of cells with TSA led to an elevated Runx2 expression in induced clones. The Runx2 expression level was dependent on the concentration of supplemented TSA (Fig. 5A). Both Runx2 and osteocalcin expression levels were higher in C3H10T1/2 cells constitutively expressing Runx2 than in those with inducible expression (data not shown).

Forced Stable and Inducible Expression of Runx2 in Mesenchymal Progenitors C3H10T1/2 Leads to Up-regulation of Galectin-3 mRNA Synthesis—The two expression systems described above were used to study the influence of Runx2 on galectin-3 gene expression. Galectin-3 expression in C3H10T1/2-Runx2 cells was compared with that in wild type C3H10T1/2 cells. The two cell lines were cultured in parallel experiments for Northern blot analysis. Galectin-3 mRNA levels were found to be dramatically higher in C3H10T1/2-Runx2 cells as compared with wild type cells (Fig. 3B). Hybridization with a Runx2 cDNA probe confirmed that Runx2 was only expressed in transgenic C3H10T1/2-Runx2 cells (Fig. 3B).

To exclude the possibility that the observed increase in galectin-3 expression is a result of differences in differentiation states, galectin-3 expression was also investigated in C3H10T1/2 cells with inducible Runx2 expression. A moderate up-regulation of galectin-3 gene expression was noted in
C3H10T1/2 cells expressing Runx2, 38 h after induction with mifepristone (Fig. 4B). Note that the stronger hybridization signal of noninduced cells (Fig. 4B) compared with that of wild type cells (Fig. 3B) is a result of longer film exposure. As a control for Runx2 expression, the same blot was hybridized to a Runx2 cDNA probe. This showed tight repression of transgenic Runx2 in noninduced cells and potent induction of gene expression after the addition of mifepristone (Fig. 4). These results were confirmed in three individual experiments, using two different cell clones (11/2 and 18/17).

However, a more striking difference in galectin-3 expression between induced and noninduced cells had been expected. Therefore, we investigated whether the stimulation of galectin-3 expression in the inducible Runx2 cell system could be increased by pretreatment with TSA. Inducible C3H10T1/2 cells were pretreated with TSA 20 h prior to induction with 30 nM mifepristone. Under these conditions, a further increase in galectin-3 mRNA was monitored in Northern blot analyses.

Stimulation of galectin-3 expression was dependent on the concentration of TSA used (Fig. 5B). This experiment was reproduced three times with different clones and resulted in comparable induction levels. In conclusion, these experiments provide strong evidence for transcriptional control of the galectin-3 gene by Runx2 in C3H10T1/2 cells.

Runx2 Binds to Sequences Identified in the Galectin-3 Promoter—To verify that putative sites in the galectin-3 promoter region identified by in silico analysis are able to interact with the Runx2 protein, electromobility shift assays were performed. Using an in vitro transcription and translation system, Runx2 protein was produced (Fig. 6B). Double-stranded oligonucleotides representing the four putative Runx-binding sites as well as two additional sequences that had not been identified by in silico analysis but are very similar to the Runx consensus sequence were incubated with Runx2 protein after radiolabeling (Fig. 6A). Unlabeled oligonucleotides were used as competitors to show specific binding (Fig. 6C). As control, a mutant Runx2 protein carrying a mutation identified in patients with cleidocranial dysplasia were used (data not shown). In a second set of experiments, a labeled standard oligonucleotide with known Runx2 binding capacity was used. Sequences identified in the galectin-3 promoter were used as competitors (Fig. 6D). Both sets of experiments pointed to sites 2 and 3 as having the strongest Runx2 binding ability.

**Fig. 6.** Runx2 protein binds to sequences in the promoter of the LGALS3 gene. A, oligonucleotides used for EMSA experiments. The putative binding sites are shaded. B, Western blot showing in vitro translated Runx2 protein used in EMSA experiments (pGEM-Runx2). The pGEM vector containing CBF/H9252 was used as control (pGEM). C, EMSA performed using the oligonucleotides derived from the LGALS3 promoter. N.S. denotes a nonspecific band. D, EMSA using a labeled standard oligonucleotide with unlabeled oligonucleotides derived from the LGALS3 promoter as competitors.
Galectin-3 Expression Is Absent in Chondrocytes of Runx2-deficient Mice—The results described above demonstrate the involvement of Runx2 in the transcriptional regulation of galectin-3 in cell culture systems. To determine the relevance of Runx2 for galectin-3 transcription under in vitro conditions, we studied its expression in limbs of wild type and Runx2-deficient embryos. Paraffin sections of 17.5 days post coitum embryos were submitted to RNA in situ hybridization using a 33P labeled antisense RNA probe for galectin-3 (Fig. 7). In wild type animals the antisense probe revealed a strong galectin-3 expression in prehypertrophic chondrocytes and skin. Posthypertrophic cartilage exhibited no galectin-3 expression, whereas moderate hybridization signals could be detected in bone. In Runx2-deficient mice osteoblast development as well as chondrocyte maturation are disturbed. These animals are devoid of osteoblasts, and cartilage differentiation is severely impaired. Some hypertrophic cartilage, however, develops at embryonic day 17.5 in the anlagen of radius and ulna (24). We used sections through embryonic day 17.5 forearms (radius and ulna) to test for galectin-3 expression in Runx2 −/− mice. We were not able to detect galectin-3 expression in chondrocytes of Runx2-deficient animals. Galectin-3 expression levels in the skin of Runx2-deficient mice were similar to wild type animals. These data support the in vitro and cell culture data presented in this study and propose a role for Runx2 in the transcriptional regulation of the LGALS3 gene in skeletal tissues.

DISCUSSION

In this study, we identified LGALS3, the gene coding for the β-galactoside-specific lectin galectin-3, as a transcriptional target of Runx2. Overexpression of Runx2, a transcription factor with a key role in skeletal development, leads to an increase in galectin-3 expression in a mesenchymal progenitor cell line. In contrast, galectin-3 expression is severely reduced in Runx2-deficient mice at sites where both genes are coexpressed in wild type animals.

Of all of the putative Runx-binding sites in the galectin-3 promoter, the sequence 5‘-AACCACA-3‘ (site 2) shows the strongest affinity for Runx2 protein in our EMSA experiments. This sequence may indeed represent the preferred element for Runx2 binding, because a number of proven Runx2-binding sites (e.g. in the osteocalcin and Runx2 promoters) exactly match this sequence (38, 44). Interestingly, site 2 is located in close proximity to a C/EBPβ-binding site. A cooperative binding of Runx2 and C/EBPβ transcription factors with physical interaction of the two proteins has been demonstrated for the osteocalcin promoter where the respective binding sites show virtually identical spacing (38).

Chondrocytes in the growth plate of long bones mature in a defined sequence. From the resting zone they enter the proliferation zone and emerge to mature and undergo hypertrophy. Eventually they undergo apoptosis in the posthypertrophic zone to be replaced by osteoblasts. Chondrocytic expression of galectin-3 starts in the proliferation zone and persists through maturation to hypertrophy. In the posthypertrophic zone galectin-3 protein is still present at low levels, but no galectin-3 mRNA is produced by posthypertrophic chondrocytes. The physiological role of galectin-3 in chondrocyte maturation is evident from mice deficient in its expression. These animals are characterized by disturbances in growth plate architecture. This effect is most pronounced in the posthypertrophic zone where an increased number of empty lacunae point to enhanced apoptosis of terminally differentiated chondrocytes (45). The transcription factor Runx2, which was shown in this study to enhance galectin-3 expression, is present in all stages of chondrocyte maturation that are characterized by galectin-3 expression (24). This is consistent with a positive regulatory effect of Runx2 on galectin-3 transcription in these cells. The Runx2 expression domain in the growth plate exceeds that of galectin-3 slightly in addition to comprising the posthypertrophic zone. Members of the TLE family of transcriptional corepressors (e.g. TLE-1 and TLE-3) are expressed in the developing skeleton and have been shown to interact with runt proteins, thereby converting their transactivating properties to active transcriptional repression (46, 47). Thus TLE proteins might interact with Runx2 to down-regulate galectin-3 expression in posthypertrophic chondrocytes.

The two other members of the Runt family of transcription factors, i.e. Runx1 and Runx3, are also expressed in growth plate chondrocytes, and their expression domains overlap that of Runx2 (48). All three mammalian Runt proteins bind to the same consensus sequence in their respective target gene promoters (20–23). Hence it is possible that galectin-3 expression in growth plate cartilage is controlled by all three Runt proteins. The lack of galectin-3 expression in the growth plates of mice deficient in Runx2 expression, however, indicates that there is no redundancy in this respect of Runt factor function. Interestingly, the expression pattern of Runx1 and Runx3 overlaps at several nonskeletal sites with that of galectin-3. In hematopoietic cells, notably including macrophages where galectin-3 was initially identified, both transcription factors are expressed, and Runx1 was shown to be essential for mature hematopoiesis (2, 48, 49). Other sites of overlapping expression are skin and olfactory epithelium (13, 14, 48). Recently dorsal root ganglia neurons have been identified as dependent on Runx3 in their development (48). Dorsal root ganglia cells also express significant amounts of galectin-3 (50). Therefore, it is tempting to speculate that Runt factors control galectin-3 expression at these extraskeletal sites.

Galectin-3 is expressed by cells of the osteoblastic lineage
as shown by us and others. Its expression increases significa-
cantly at the matrix maturation stage. Culture conditions for
MC3T3-E1 cells involving ascorbic acid have been shown to
enhance the binding affinity of Runx2 to its binding site (51).
Runx2 is phosphorylated via the mitogen-activated protein ki-
nase pathway in MC3T3-E1 cells. Phosphorylation of Runx2 in
these cells leads to increased binding to and expression from
the promoter of the known Runx2 target gene osteocalcin (52).
This is in agreement with our finding that galectin-3 expres-
sion increases, whereas Runx2 mRNA and protein levels are
stable during differentiation of MC3T3-E1 cells in the presence
of ascorbic acid.

Further experimental work will have to provide direct evi-
dence for a functional interaction of different transcription-
factors on the galectin-3 promoter in skeletal tissue. Moreover,
an analysis of the transcriptional regulation of the galectin-3
gene in nonoskeletal tissues may show this gene to be a first
example of a common target for the different Runx factors.

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Expression of Galectin-3 in Skeletal Tissues Is Controlled by Runx2
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