Multiple microRNAs (miRNAs) that target the osteogenic Runx-related transcription factor 2 (RUNX2) define an interrelated network of miRNAs that control osteoblastogenesis. We addressed whether these miRNAs have functional targets beyond RUNX2 that coregulate skeletal development. Here, we find that seven RUNX2-targeting miRNAs (miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-205, and miR-217) also regulate the chondrogenic GATA transcription factor tricho-rhino-phalangeal syndrome 1 (TRPS1). Although the efficacy of each miRNA to target RUNX2 or TRPS1 differs in osteoblasts and chondrocytes, each effectively blocks maturation of pre-committed osteoblasts and chondrocytes. Furthermore, these miRNAs can redirect mesenchymal stem cells into adipogenic cell fate with concomitant up-regulation of key lineage-specific transcription factors. Thus, a program of multiple miRNAs controls mesenchymal lineage progression by selectively blocking differentiation of osteoblasts and chondrocytes to control skeletal development.

Genetic mechanisms regulating chondrogenesis and osteogenesis during skeletal development control differentiation of mesenchymal stem cells (1–3). Mesenchymal stem cells can differentiate into osteoblasts to support intramembranous bone formation or into chondrocytes that form cartilage during the initial stages of endochondral ossification. Cell fate determination and lineage progression of mesenchymal stem cells into the osteochondrogenic lineage is controlled by multiple signaling pathways that are activated by different ligands (e.g. Wnt, TGF-β/BMP7), FGF, and IGF) and control the activity of several principal transcriptional factors (e.g. RUNX2, TRPS1, and SOX9) (1,2). Furthermore, recent findings indicate that miRNAs are critical regulators of bone formation and may attenuate signaling pathways and transcription factors that control osteoblast differentiation and function (4–11).

Our group has shown previously that conditional ablation of the Dicer gene in the osteoblast lineage, which prevents formation of mature miRNAs, causes a high-bone mass phenotype (5). Furthermore, miRNA expression is critical for cartilage development because Dicer deficiency in chondrocytes results in severe skeletal growth defects (12). Initial evidence indicates that specific miRNAs control either osteoblast or chondrocyte differentiation in part by regulating master transcription factors and signaling pathways linked to the respective lineages. For instance, at least ten different miRNAs inhibit osteogenesis by targeting the bone-specific transcription factor RUNX2 (4, 8, 13). MicroRNAs that target Hox genes (e.g. miR-196) have a major impact on skeletal patterning (14, 15). Modulation of Wnt signaling by miR-27 and miR-29 promotes human osteoblast differentiation (16, 17). In addition, miR-22 negatively regulates peroxisome proliferator-activated receptor α and BMP7 expression (18), whereas miR-9, miR-98, and miR-146 decrease TGF-α production and MMP-13 secretion in chondrocytes (19). Here, we show that miRNAs that are known to target RUNX2 also control the chondrogenic transcription factor TRPS1. Our data demonstrate that these miRNAs have a key biological function in control of both osteoblast and chondrocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—C3H10T1/2, C2C12, NIH3T3, and 3T3-L1 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM L-glutamine, and penicillin/streptomycin. MC3T3-E1 cells were maintained in minimum essential medium α (without ascorbic acid) (Invitrogen) supplemented with 10% FBS. ATDC5 cells were grown in Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen) supplemented with 2% FBS. 32D cells were maintained in RPMI 1640 medium with 10% heat-inactivated FBS and 10% mouse interleukin-3 culture supplement. For osteoblastic/chondrogenic differentiation experiments, MC3T3-E1 and ATDC5 cells were cultured in osteogenic medium (growth medium supple-
ment with 280 μM ascorbic acid and 5 mM β-glycerophosphate) for 28 days, as described previously (8, 20). To promote C3H10T1/2 cells differentiation, growth media were supplemented with 100 ng/ml BMP2 (generously provided by John Wozney (Wyeth Research, Women’s Health and Musculoskeletal Biology, Cambridge, MA)). All cells were obtained from the ATCC and maintained at 37 °C in a humidified 5% CO₂ environment. Media were replaced every 2 days.

**Reporter Constructs**—For luciferase-based miRNA expression reporter assays, the pMIR-REPORT plasmid (Ambion/Applied Biosystems, Foster City, CA) was used. pMIR-REPORT contains a CMV promoter that drives expression of the firefly luciferase gene and permits insertion of gene-specific 3’ UTR sequences upstream of the SV40-derived polyadenylation site. Synthetic 86-bp oligonucleotides encompassing tandemly duplicated 40-nucleotide segments of the corresponding 3’ UTR regions of the *mus musculus Trps1* gene were inserted into the SpeI/Mul site of pMIR-REPORT. Reporters were generated that contain the respective miRNA seed motifs for seven distinct miRNAs (TRPS1/RUNX2 targeting miRNAs: miR-23a, 30c, 34c, 133a, 135a, 205, 217). Reporters with mutations of the same seed sequences were generated to establish specificity of miRNA effects. Nucleotide sequences of oligonucleotides for cloning of TRPS1–3′-UTR reporter constructs are shown in supplemental Table S1.

**Transfection and Luciferase Assays**—MicroRNAs, anti-miRNAs, and miRNA/anti-miRNA negative-control oligonucleotides (Ambion) were transfected into subconfluent (~50%) cells at a concentration of 50 nM with Oligofectamine (Invitrogen). Cells were harvested 72 h after transfection for protein and mRNA analysis. For luciferase (Luc) reporter assays, 50 nM miRNA, and 100 ng of pMIR-REPORT plasmid were cotransfected into cells with Lipofectamine 2000 (Invitrogen). At 48 h posttransfection, cells were scraped into PBS, collected by centrifugation, and lysed with passive lysis buffer (Promega, Madison, WI). In each experiment, cells were also cotransfected with phRL-null *Renilla* luciferase plasmids for normalization. Firefly and *Renilla* luciferase activities were assayed using the dual luciferase reporter assay system according to the instructions of the manufacturer (Promega).

**RNA Isolation and Analysis**—Total RNA was isolated from cultures of cells using TRIzol reagent (Invitrogen) according to the protocol of the manufacturer. Purified RNA was further treated with DNaseI and recovered by DNA-free RNA column purification kits (Zymo Research, Orange, CA). RNA was reverse-transcribed using the SuperScript First Strand synthesis kit (Invitrogen) or the QuantiMir RT kit (System Biosciences, Mountain View, CA) according to the instructions of the manufacturers. The relative gene expression was determined by quantitative real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) and gene- or miRNA-specific primers (supplemental Tables S2 and S3) in an ABI Prism 7000 thermocycler. The primer specificity of each primer pair was verified by dissociation curve analysis. All transcript levels were normalized to GAPDH or U6 snRNA (for miRNAs expression) transcript levels.

**RT-qPCR Array Analysis**—The RT-qPCR array was designed using 91 genes representing specific stages of bone development, morphogenesis, maturation, and/or mineralization as well as five internal controls. Primer sets were evaluated for specificity and efficiency (full sequences and annotation available by request) and aliquoted into 96-well plates. Gene expression was determined by quantitative real-time PCRs using Power SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7000 thermocycler. For each gene, expression levels were normalized to a combination of Hprt1, Mt-cyto, GAPDH, or Actb using the GeNorm algorithm (21). Experiments were performed in triplicate, and results are displayed as mean values ± S.E.

**Immunoblotting**—Cells were lysed in direct lysis buffer (2% SDS, 10 mM dithiothreitol, 10% glycerol, 12% urea, 10 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 1 μl protease inhibitor mixture (Roche), 25 μM MG132 (Calbiochem, La Jolla, CA)) and boiled for 10 min. Equal amounts of total protein were loaded and resolved by SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes. Membranes were blocked and subjected to immunoblotting with the appropriate primary antibodies, followed by incubation with HRP-conjugated secondary antibodies. Immunoreactive proteins were detected using Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences). All antibodies used in this study (anti-Trps1, anti-Sox9, and anti-β-actin) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) with the exception of the anti-Runx2 mouse monoclonal antibody (MBL).

**Statistical Analysis**—Data for both the control and experimental groups were presented as means ± S.E. Statistical significance was assessed using Student’s *t* test, and *p* values < 0.05 were taken as statistically significant.

**RESULTS**

**RUNX2-targeting miRNAs Have Multiple Predicted Targets**—Osteoblast growth and differentiation is controlled by at least ten distinct miRNAs that posttranscriptionally control expression of the osteogenic master regulator RUNX2 (8). Individual miRNAs are predicted to target a large number of distinct mRNAs, and each mRNA may be regulated by multiple miRNAs (11, 22, 23). Therefore, we investigated whether RUNX2-targeting miRNAs control other regulatory factors that contribute to skeletal development. MiRNA-target prediction programs revealed that RUNX2-targeting miRNAs also recognize multiple other mRNAs (Figs. 1 and supplemental Fig. S1). Strikingly, nine RUNX2-targeting miRNAs are also predicted to target the GATA-related Zn finger transcription factor TRPS1, which promotes chondrogenesis (24, 25). In addition, five RUNX2-targeting miRNAs potentially target the Kruppel-like transcription factor KLF12, a principal transcriptional repressor of the activator protein-2 α (AP-2 α) gene, which controls vertebrate development and carcinogenesis (26–28). Because transcription factors RUNX2 and TRPS1, as well as perhaps KLF12, each control aspects of osteogenesis and chondrogenesis, we investigated whether the cognate miRNAs represent a related miRNA network for regulating skeletal development.

**Expression Profiles of Common Targets and RUNX2-targeting miRNAs in Different Lineages**—To assess whether the expression of the three common target genes correlates with the
expression of the cognate miRNAs, we selected six different
cell types within the mesenchymal cell lineage, including
C3H10T1/2 mesenchymal stem cells, MC3T3-E1 osteoblasts,
ATDC5 chondrocytes, C2C12 myoblasts, NIH3T3 fibroblasts,
and 3T3L1 adipocytes (supplemental Fig. S2A). We also
included 32D myeloid cells, which provide an out-group to
assess whether miRNA expression is specific for mesenchymal
cells. RUNX2 and TRPS1 are expressed in each of the six mes-
enchymal cell lines, whereas mRNA expression of KLF12 is only
observed in C3H10T1/2 mesenchymal stem cells (Fig. 1B, left)

FIGURE 1. Multiple miRNAs target RUNX2 and TRPS1 and are differentially expressed in mesenchymal cell types. A, miRNAs known to attenuate Runx2 levels (n = 11) are also predicted to target TRPS1 (n = 9) and KLF12 (n = 5) on the basis of miRNA target prediction tools (i.e., TargetScan, PicTar, and RNA22). B, the mRNA and protein levels of RUNX2, TRPS1, and KLF12 were determined in seven cell lines by RT-qPCR (left panel). Values were normalized relative to GAPDH and calibrated against the Runx2 mRNA levels in MC3T3. Protein levels were analyzed by immunoblotting (center panel). The β-actin profile shows equal loading of protein samples. The right panel shows quantification of Western blot analysis results using the Alphalmager 2200, normalized to the level of β-actin. RUNX2 and TRPS1 levels in MC3T3 were set as 1. C, expression of selected miRNAs as detected by RT-qPCR. Expression was normalized relative to expression of U6 small RNA, and normalized values were then calibrated against the level of miR-133a in MC3T3-E1 cells, which we assigned a value of 1. The values represent the mean ± S.E. of three independent experiments (n = 3).
The mRNAs for these three transcription factors are not robustly expressed in 32D cells, albeit that low levels of RUNX2 and KLF12 mRNA are detectable. Because KLF12 is not expressed in lineage-committed mesenchymal cells, we did not analyze the protein further. RUNX2 and TRPS1 protein expression varies among each of the cell types in a manner that is generally consistent with their lineage-specific expression in mesenchymal cells. The levels of RUNX2 and TRPS1 protein do not strictly correlate with the corresponding mRNA levels (Fig. 1, center and right panels), indicating that both factors may be controlled by protein translation and/or stability.

Because the miRNAs predicted to target RUNX2 and TRPS1 may contribute to the discordance in mRNA and protein levels in the mesenchymal lineage, we also evaluated their expression profiles in the seven cell lines. Each miRNA shows a distinct lineage-specific expression pattern. Three miRNAs (miR-23a, miR-30c, and miR-34c) are constitutively expressed at high levels in all cell types (Fig. 1C), whereas expression of miR-218 is lower in ATDC5 chondrocytes and 32D myeloid cells (supplemental Fig. S2B, left panel). Five miRNAs (miR-133a, miR-135a, miR-204, miR-205, and miR-217) are expressed at levels that are two to three orders of magnitude lower in all cell lines, except that the myogenesis-related miR-133a is highly expressed in C2C12 myoblasts (Fig. 1C and supplemental Fig. S2B, center panel), consistent with previous reports (29, 30). Expression of miR-137 and miR-338 is typically low and only exhibits sporadically elevated expression in selected cell types (e.g. ATDC5 chondrocytes and 32D myeloid cells) (supplemental Fig. S2B, right panel). The generally modest to high expression of most of these miRNAs in the mesenchymal lineage is consistent with their roles in the regulation of RUNX2 and/or TRPS1 expression.

Repression of TRPS1 Expression by miRNAs That Target the 3’ UTR Seed Regions—We investigated whether RUNX2-targeting miRNAs directly control TRPS1 expression through predicted seed regions in the TRPS1 3’ UTR using luciferase reporter assays (Fig. 2) and immunoblot analysis (Fig. 3). We inserted tandemly duplicated 40 nucleotide sequences of the TRPS1 3’ UTR that contain the respective miRNA seed regions for miR-23a, 30c, 34c, 133a, 135a, 205, and 217 at the 3’ end of the firefly luciferase reporter (Fig. 2A and supplemental Fig. S1C). The presence of exogenous miRNAs significantly represses the corresponding luciferase reporter activity (Fig. 2B, left panel). However, mutation of the respective seed regions in luciferase reporters abrogates responsiveness to any of the miRNAs (Fig. 2B, right panel). Furthermore, miRNA inhibitors (anti-miRNAs) increased reporter activity by 1.5- to 2-fold (Fig. 2C). We established that each of these miRNAs also attenuates endogenous TRPS1 protein accumulation in MC3T3 osteoblasts and ATDC5 chondrocytes. However, several miRNAs reproducibly exhibit subtle differences in their potency to repress TRPS1 (Fig. 3A and data not shown) (see “Discussion”). Interestingly, although each miRNA can suppress protein levels of TRPS1 and Runx2 at maximal concentrations (50 nm, Fig. 3A), a combination of two miRNAs (e.g. miR-30c and miR-133) at lower doses appears to be slightly more effective than either miRNA alone (supplemental Fig. S3B). The latter finding suggests that miRNAs may be able to cooperate in suppressing protein levels. Collectively, these data indicate that at least seven RUNX2-targeting miRNAs directly regulate TRPS1 protein expression by targeting the seed regions of the 3’ UTR of TRPS1 mRNA.

We next correlated the levels for RUNX2 and TRPS1 mRNAs with the cognate endogenous miRNAs during lineage progression of phenotype-committed mesenchymal cells. Differentiation of MC3T3 and ATDC5 cells is evident after 28 days, as reflected by increased expression of differentiation marker...
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FIGURE 3. Exogenous miRNAs selectively regulate Trps1 protein expression in a cell type-specific manner. A, Trps1 protein levels were examined in cells treated with miRNA for 72 h. Most of the miRNAs, but not nonspecific miRNA (NS), strongly repress Trps1 expression in both ATDC5 and MC3T3-E1 cells, but the relative efficacy of these miRNAs is different between the two cell types. The β-actin profile shows equal loading of protein samples. Immunoblot data are representative of three independent experiments. B, TRPS1 and RUNX2 mRNA and protein levels were detected in differentiated ATDC5 and MC3T3-E1 cells (day 0 to day 28). Induction of differentiation is evident from increased mRNA expression of selected osteogenic markers (RUNX2, alkaline phosphatase/ALP and osteocalcin/OC) and chondrogenic markers (SOX9, collagen type II/COL2A1, RUNX2). RT-qPCR values were normalized relative to GAPDH and calibrated against the RUNX2 level on day 0 (set as 1). C, TRPS1/RUNX2-targeting miRNAs are down-regulated during ATDC5 chondrocyte differentiation but up-regulated during osteoblast differentiation. The expression level for each miRNA was examined by RT-qPCR, normalized to U6 small RNA, and calibrated against the levels on day 0 in ATDC5 or MC3T3-E1 cells. The values represent the mean ± S.E. (n = 3).

genes (e.g. RUNX2, osteocalcin, alkaline phosphatase, SOX9, TRPS1, and collagen type II) (Fig. 3B). The expression of TRPS1/RUNX2-targeting miRNAs and lineage-specific marker genes is inversely regulated during differentiation of chondrocytes and the late stages of osteoblasts maturation (Fig. 3C and supplemental Fig. S3A). TRPS1 mRNA is sustained at constitutive levels throughout the time course, but its protein expression is barely detectable at day 7 of osteoblast differentiation (Fig. 3B, left panel), consistent with the inhibitory function of TRPS1 during early stages of osteoblast differentiation (31). Conversely, TRPS1/RUNX2-targeting miRNAs are down-regulated by day 28 of chondrogenic differentiation (Fig. 3C and supplemental Fig. S3A), concomitant with a significant increase in the protein levels of RUNX2 and TRPS1 (Fig. 3B, right panel). Reciprocal expression of TRPS1 and RUNX2 proteins with the corresponding miRNAs generally supports the concept that RUNX2 and TRPS1 are controlled by endogenous miRNAs during progression of osteochondrogenesis.

We note that correlations between miRNA expression and the levels of miRNA target proteins are clearly non-stoichiometric. For example, Runx2 expression is activated during early MC3T3 osteoblastic differentiation but repressed in late stages of differentiation (Fig. 3B, left panel). Most Runx2-targeting miRNAs are only activated at the late stages of osteoblast differentiation (Fig. 3C and supplemental Fig. S3, upper panel) for physiological down-regulation of Runx2 in mature osteoblasts. Differences in the efficacy of endogenous miRNAs to target Runx2 at distinct temporal stages could be due to cellular factors that control the intrinsic activities of miRNAs or their accessibility to seed-sequences in the 3’ UTR of Runx2.

Attenuation of Chondrocyte Differentiation by TRPS1/RUNX2-targeting miRNAs—Because TRPS1 and RUNX2 are major regulators of chondrogenesis (24, 32–35), we investigated whether these TRPS1/RUNX2-related miRNAs control differentiation of ATDC5 chondrocytes. The up-regulation of chondrogenic marker expression demonstrates progression of ATDC5 cell differentiation (supplemental Fig. S4A). TRPS1/RUNX2-targeting miRNAs still exhibit strong to modest repression of RUNX2 and TRPS1 protein expression at 10 days after transfection of ATDC5 cells (Figs. 4, A and B), although the inhibitory effects are not as striking as those on day 3 after transfection of miRNAs (Fig. 3A). The reduction in the efficacy of repression comparing days 3 and 10 may be due in part to decay of miRNAs. Although miRNA effects may subside and Trps1 levels may partially rebound, our data indicate that the miRNAs still inhibit the initiation of chondrogenic differentiation. Transient repression of Trps1 during early stages of chondrogenesis may delay rather than qualitatively inhibit the expression of mature phenotypic genes that we measured at day 10.

We note that administration of exogenous miRNAs is only efficient in proliferating cell populations (we induce differentiation when transfected cells reach confluence) and not efficient in confluent cells. Thus, it is technically not feasible to perform a longer time course with transiently transfected miRNAs.

Trps1/Runx2-targeting miRNAs, except miR-30c, do not appreciably affect the mRNA levels of RUNX2 or TRPS1 (supplemental Fig. S4B). Analysis of chondrocyte-related marker gene expression reveals that TRPS1/RUNX2-targeting miRNAs (except miR-23a) strongly inhibit mRNA expression of the early differentiation marker collagen type II (Col2A1) by 20–80% (Fig. 4C, left panel). In addition, these miRNAs (except miR-34c and miR-204) show strong repression of alkaline phosphatase (ALP) mRNA (Fig. 4C, center panel). To confirm the biological effects of the miRNAs, we applied three anti-miRNAs (anti-miR-30c, 34c, and 135a) that inhibit the activities of the corresponding highly expressed miRNAs during ATDC5 chondrocyte differentiation. Anti-miR-30c and 135a significantly up-regulate Col2A1 and ALP marker gene expres-
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Differential Regulation of Cell Signaling Mediators during miR-30c-dependent Inhibition of Early Stages of Osteoblastic versus Chondrocytic Differentiation—We examined whether TRPS1/RUNX2-targeting miRNAs control specific gene expression programs associated with differentiation of osteoblasts and chondrocytes using custom-made RT-qPCR arrays (Fig. 4D and supplemental Fig. S5C). MC3T3 osteoblasts and ATDC5 chondrocytes were transfected with miR-30c, which represents the most effective inhibitor of RUNX2 and TRPS1 at both mRNA and protein levels. Gene expression profiles of 91 osteogenic or chondrogenic genes linked to cell signaling or gene regulation were analyzed using RT-qPCR arrays after one week of differentiation in either cell type. Generally, miR-30c down-regulates the majority of genes in differentiated ATDC5 cells, whereas the same genes are up-regulated in differentiated MC3T3 cells (Fig. 4D and supplemental Fig. S5). Three categories of genes that are functionally associated with lineage progression and phenotype maturation exhibit the most pronounced changes in expression in the presence of miR-30c. In differentiated ATDC5 chondrocytes, many extracellular matrix (ECM) genes are down-regulated by miR-30c compared with nonspecific miRNA (Fig. 4D). This result is consistent with the inverse correlation between enhanced expression of chondrocytic markers in ATDC5 cells and decreased levels of endogenous miR-30c during chondrogenic differentiation (Fig. 3C).

Although the antichondrogenic function of miR-30c in ATDC5 cells is associated with later stages of chondrocyte differentiation, elevation of miR-30c in MC3T3 osteoblasts appears to be linked to a block in early osteoblastic differentiation. This biological distinction may be in part due to cell typespecific differences in the attenuating activities of miRNAs for different targets (see “Discussion”). For example, miR-30c dramatically up-regulates expression of early-stage ECM genes when administered prior to differentiation of MC3T3 osteoblasts, including three collagen genes (Col11A1, Col1A2, and Col3A1) and matrix metalloproteinases 2 and 9 (Mmp2 and Mmp9) (Fig. 4D, left panel). In addition, late-stage ECM genes, including OP, osteocalcin (OC-BGLAP), and Dmp1 expression, are suppressed by miR-30c (Fig. 4D). Thus, exogenous elevation of miR-30c is permissive for early but not subsequent stages of phenotype maturation in precommitted MC3T3 osteoblasts.

Chondrogenesis is controlled by multiple ligands (e.g., TGF-β, IGF-1, and FGF2) that control both early and late stages of chondrogenesis by activating mitogenic or cartilage-anabolic signaling pathways in chondrocytes. Our RT-qPCR array data clearly show that miR-30c modulates expression of genes encoding ligands and receptors of TGFβ, FGF, and IGF signaling and that this modulation is strikingly different during differentiation in ATDC5 chondrocytes and MC3T3 osteoblasts (Fig. 4D, center and right panels). Specifically, TGFβs, TGFβRs, and SMADs are clearly down-regulated by miR-30c in chondrocytes but up-regulated in osteoblasts. Also, expression of ligands for Fgfr2, Fgfr1, and IGF1r is clearly stimulated in MC3T3 cells, whereas expression of Fgfr1, Fgfr3, Fgfr2, and Igfl control progression of chondrogenic differentiation at least in part by reducing the availability of chondrogenic transcription factors.

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genes is suppressed in ATDC5 cells. The miR-30c mediated reciprocal changes in the expression of cell surface receptors in both cell types suggest that this representative TRPS1/RUNX2-targeting miRNA is a potent modulator of the cellular responsiveness to ligands controlling skeletal development.

TRPS1/RUNX2-targeting miRNAs Control Mesenchymal Stem Cell Fate—Biological modulation of miRNA levels accompanies differentiation of multipotent mesenchymal stem cells. Basal and BMP2-controlled expression of TRPS1/RUNX2-targeting miRNAs and representative marker genes specific for the osteoblastic, chondrocytic, and adipocytic lineages is evident in multipotent C3H10T1/2 mesenchymal progenitor cells (Fig. 5 and supplemental Fig. S5). Therefore, we investigated whether TRPS1/RUNX2-targeting miRNAs modulate mesenchymal cell fate decisions and lineage progression. C3H10T1/2 cells were transfected with each miRNA, including miR-204 (a RUNX2-targeting miRNA that does not regulate TRPS1), and allowed to differentiate with or without BMP2. All miRNAs tested block the osteogenic (e.g. Osterix and ALP) and/or chondrogenic markers (e.g. Col2A1 and Sox9). A number of miRNAs (e.g. miR-30c, 34c, 133a, 135a, and 204) selectively enhance expression of adipogenic markers (aP2 and PPARγ), indicating stimulation of adipogenesis (Fig. 5C and supplemental Fig. S5C). Corroborating these results, selected anti-miRNAs that inhibit three highly expressed miRNAs have opposite effects on lineage marker expression (Fig. 5D).

We also investigated the molecular interplay between miRNAs and BMP2. The results show that BMP2 treatment alters miRNA-dependent cell fate selection. BMP2 treatment of C3H10T1/2 cells, in which miRNAs are introduced, results in stimulation of primarily chondrogenic and adipogenic differentiation (Fig. 5C). For example, BMP2 attenuates the inhibition of osteogenic markers (osterix and ALP) by miR-30, 133, 135, 204, and 205. Also, repression of chondrogenic markers (Sox9 and Col2A1) by TRPS1/RUNX2-targeting miRNAs is generally relieved by BMP2. BMP2 decreases miRNA dependent up-regulation of the adipogenic regulator PPARγ (e.g. observed for miR-30, 133a, and 135a) but enhances stimulation of PPARγ by miR-30, 204, and 205. Thus, while TRPS1/RUNX2-targeting miRNAs alter spontaneous cell fates of C3H10T1/2 cells, they promote primarily adipogenic and chondrogenic cell fates upon BMP2 induction.

DISCUSSION

In this study, we show that a subset of the multiple miRNAs that attenuate expression of the osteogenic factor RUNX2 and osteoblast differentiation (4, 8, 9) also control the levels of the chondrogenic GATA transcription factor TRPS1 and chondrogenic differentiation. At least ten Runx2-targeting miRNAs significantly block MC3T3 osteoblast differentiation, and several of the corresponding anti-miRNAs have reverse effects (8). Seven of these ten RUNX2-targeting miRNAs also target TRPS1. On the basis of studies using a representative TRPS1/RUNX2-targeting miRNA (miR-30c), we find that miRNAs broadly affect a series of cell signaling mediators during early stages of osteoblastic and chondrocytic differentiation. Furthermore, each of these TRPS1/RUNX2-targeting miRNAs blocks osteogenic and chondrogenic differentiation, whereas application of several corresponding anti-miRNAs has the opposite biological effect. These findings establish a novel network of miRNAs and the cognate transcription factors that control selection of and progression in the osteochondrogenic lineage (Fig. 6).

Loss of function mutations in the Runx2 gene causes the dominantly inherited skeletal malformation cleidocranial dys-
plasia. Similarly, mutations in the human *Trps1* gene lead to the tricho-rhino-phalangeal syndrome types I and III, which are characterized by craniofacial and skeletal abnormalities as well as disturbed hair development (36–38). TRPS1 mediates transcriptional responses that alter signaling pathways associated with proliferation and differentiation of chondrocytes (39, 40). Genetic interactions between TRPS1 and RUNX2 transcription factors have been established both in vitro and in vivo. TRPS1 was identified as a repressor of osteocalcin, which is transcriptionally activated by RUNX2 (31). TRPS1 inhibits the transcriptional activity of RUNX2 but also the Runx2 promoter (37). Furthermore, mutation of the TRPS1 binding motif in the Runx2 gene is linked to cleidocranial dysplasia (37). The genetic interactions and biological correlations between RUNX2 and TRPS1 are remarkable in view of our finding that common miRNAs regulate the expression of both proteins to block their osteochondrogenic functions. This regulatory interconnection unifies the activities of RUNX2 and TRPS1 into a common function linked to early stages of differentiation in skeletal cells.

The TRPS1/RUNX2-targeting miRNAs characterized here likely have multiple targets beyond RUNX2 and TRPS1 to generate complicated modulatory networks that directly or indirectly regulate lineage commitment. For example, our studies using qPCR arrays that monitor osteochondrogenic markers provide clear evidence in support of this hypothesis. Treatment of osteoblastic or chondrocytic cells with a representative TRPS1/RUNX2-targeting miRNA (miR-30c) resulted in dramatically opposite and coordinate effects on the expression of a number of genes encoding components of signaling pathways (e.g. responding to TGF-β/BMP2, IGF1, or FGF2) that control bone or cartilage development (35, 41, 42). Furthermore, we observed that several of the TRPS1/RUNX2-targeting miRNAs exhibit differences in their potency to repress TRPS1 in osteoblastic versus chondrocytic cells (Fig. 3A). This finding suggests that cell type- and mRNA-specific factors influence the activities of these miRNAs.

Coordinate changes in the expression of signaling components could reflect intricate regulatory feedback networks in which miR-30c and its targets (e.g. TRPS1/RUNX2) respond to and regulate signaling pathways that stimulate skeletal development. For example, TGFβ-induced activation of SMAD3 inhibits the RUNX2-mediated stimulation of osteoblast differentiation and expression of bone-specific genes encoding ECM proteins (43–45). Because our data show that TGF-β signaling components are stimulated by miR-30c during osteoblast differentiation, the resulting sensitization of TGF-β/SMAD3 signaling could synergize with miR-30c to repress RUNX2 and the expression of osteoblast-specific ECM proteins. In contrast, miR-30c inhibits TGF-β signaling components during TRPS1-dependent chondrocyte differentiation. Because the chondrogenic master regulator SOX9 is activated by TGF-β signaling (44), miR-30c-dependent inhibition of the TGF-β/SMAD3/SOX9 axis may synergize with its direct inhibition of TRPS1.

Thus, miR-30c exemplifies a critical regulatory node in a complex posttranscriptional regulatory network that targets master regulators of skeletal development and that indirectly control signaling components and ECM proteins in osteoblastic and chondrocytic cells. This interpretation is consistent with the increasing appreciation that cofunctioning miRNAs generate biological effects by the combinatorial actions that affect multiple gene targets (11). Our observations suggest that TRPS1/RUNX2-targeting miRNAs may control a key developmental node embodied by precommitted bipotential progenitor cells that preferentially differentiate into osteoblasts or chondrocytes. In addition, these miRNAs appear to have important biological roles in controlling phenotypic maturation and the ECM-producing functions of osteoblasts or chondrocytes. The latter proposal is consistent with prior observations in mice with an osteoblast-specific deletion of Dicer that exhibit increased cortical bone mass associated with increased ECM production (5).

In C3H10T1/2 mesenchymal stem cells, TRPS1/RUNX2-targeting miRNAs that repress osteochondrogenic lineage selection promote adipogenesis, suggesting that the panel of TRPS1/RUNX2-targeting miRNAs is capable of altering lineage direction of multipotent mesenchymal stem cells independent of external induction of differentiation by altering the osteochondrogenic cell fate to adipogenic cell fate.

In conclusion, we have defined a set of miRNAs that regulate differentiation of chondrocytes and osteoblasts to support for-
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4. Li, Z., Hassan, M. Q., Volinia, S., van Wijnen, A. J., Stein, J. L., Croce, C. M., Lian, J. B., and Stein, G. S. (2008) A microRNA signature for a BMP2-induced osteoblast lineage commitment program. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13906–13911

5. Gaur, T., Hussain, S., Mudhasani, R., Parulkar, I., Colby, I. J., Frederick, D., Kream, B. E., van Wijnen, A. J., Stein, J. L., Stein, G. S., Jones, S. N., and Lian, J. B. (2010) Dicer inactivation in osteoprogenitor cells compromises fetal survival and bone formation, while excision in differentiated osteoblasts increases bone mass in the adult mouse. *Dev. Biol.* 340, 10–21

6. Inose, H., Ochi, H., Kimura, A., Fujita, K., Xu, R., Sato, S., Iwasaki, M., Sunamurata, S., Takeuchi, Y., Fukumoto, S., Saito, K., Nakamura, T., Siomi, H., Ito, H., Arai, Y., Shinomiya, K., and Takeda, S. (2009) A microRNA regulatory mechanism of osteoblast differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20794–20799

7. Li, X., Xie, H., Liu, W., Hu, R., Huang, B., Tan, Y. F., Xu, K., Sheng, Z. F., Zhou, H. D., Wu, X. P., and Luo, X. H. (2009) A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. *J. Clin. Invest.* 119, 3666–3677

8. Zhang, Y., Xie, R. L., Croce, C. M., van Wijnen, A. J., and Stein, G. S. (2011) A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9863–9868

9. Hassan, M. Q., Gordon, J. A., Beloti, M. M., Croce, C. M., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. (2010) A network connecting Runx2, SATB2, and the miR-23a−27a−24-2 cluster regulates the osteoblast differentiation program. *Proc. Natl. Acad. Sci. U.S.A.* 107, 19879–19884

10. Oskowizt, A. Z., Lu, J., Penfornis, P., Yllostalo, J., McBride, J., Flemington, E. K., Prockop, D. J., and Pochampally, R. (2008) Human multipotent stromal cells from bone marrow and microRNA: Regulation of differentiation and leukemia inhibitory factor expression. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18372–18377

11. Lian, J. B., Stein, G. S., van Wijnen, A. J., Stein, J. L., Hassan, M. Q., Gaur, T., and Zhang, Y. (2012) MicroRNA control of bone formation and homeostasis. *Nat. Rev. Endocrinol.* 8, 212–227

12. Kobayashi, T., Lu, J., Cobb, B. S., Rodda, S. J., McMahon, A. P., Schipani, E., Merkenschlager, M., and Kronenberg, H. M. (2008) Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1949–1954

13. Huang, J., Zhao, L., Xing, L., and Chen, D. (2010) MicroRNA-204 regulates Runx2 protein expression and mesenchymal progenitor cell differentiation. *Stem Cells* 28, 357–364

14. Hornstein, E., Mansfield, J. H., Yekta, S., Hu, J. K., Harfe, B. D., McManus, M. T., Baskerville, S., Bartel, D. P., and Tabin, C. J. (2005) The microRNA miR-196 acts upstream of Hoxb8 and Shh in limb development. *Nature* 438, 671–674

15. Yekta, S., Tabin, C. J., and Bartel, D. P. (2008) MicroRNAs in the Hox enhancer. *J. Biol. Chem.* 283, 25221–25231

16. Liu, J., Shi, Y., Xie, R. L., Croce, C. M., van Wijnen, A. J., and Stein, G. S. (2008) A microRNA signature for a BMP2-dependent osteoblastic lineage. *J. Cell Physiol.* 212–227

17. Merkenschlager, M., and Kronenberg, H. M. (2008) Dicer-dependent miRNA-dependent transcriptional repression. *Nat. Genet.* 40, 53–60

18. Lengner, C. J., Lepper, C., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. (2004) Primary mouse embryonic fibroblasts. A model of mesenchymal cartilage formation. *J. Cell Physiol.* 200, 327–333

19. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, RESEARCH0034

20. John, B., Enright, A. J., Aravin, A., Tuschl, T., Sander, C., and Marks, D. S. (2004) Human microRNA targets. *PLoS Biol.* 2, e363

21. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002) Accurate normalization of real-time 19. Jones, S. W., Watkins, G., Le Good, N., Roberts, S., Iwasaki, M., Sunamurata, S., Takeuchi, Y., Fukumoto, S., Saito, K., Nakamura, T., Siomi, H., Ito, H., Arai, Y., Shinomiya, K., and Takeda, S. (2009) A microRNA regulatory mechanism of osteoblast differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20794–20799
39. Wuelling, M., and Vortkamp, A. (2010) Transcriptional networks controlling chondrocyte proliferation and differentiation during endochondral ossification. *Pediatr. Nephrol.* **25**, 625–631

40. Itoh, S., Kanno, S., Gai, Z., Suemoto, H., Kawakatsu, M., Tanishima, H., Morimoto, Y., Nishioka, K., Hatamura, I., Yoshida, M., and Muragaki, Y. (2008) Trps1 plays a pivotal role downstream of Gdf5 signaling in promoting chondrogenesis and apoptosis of ATDC5 cells. *Genes Cells* **13**, 355–363

41. Shi, S., Mercer, S., Eckert, G. J., and Trippel, S. B. (2009) Growth factor regulation of growth factors in articular chondrocytes. *J. Biol. Chem.* **284**, 6697–6704

42. Miraoui, H., and Marie, P. J. (2010) Fibroblast growth factor receptor signaling cross-talk in skeletogenesis. *Sci. Signal.* **3**, re9

43. Alliston, T., Choy, L., Ducy, P., Karsenty, G., and Derynck, R. (2001) TGF-β-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. *EMBO J.* **20**, 2254–2272

44. Furumatsu, T., Tsuda, M., Yoshida, K., Taniguchi, N., Ito, T., Hashimoto, M., Ito, T., and Asahara, H. (2005) Sox9 and p300 cooperatively regulate chromatin-mediated transcription. *J. Biol. Chem.* **280**, 35203–35208

45. Qiao, B., Padilla, S. R., and Benya, P. D. (2005) Transforming growth factor (TGF)-β-activated kinase 1 mimics and mediates TGF-β-induced stimulation of type II collagen synthesis in chondrocytes independent of Col2a1 transcription and Smad3 signaling. *J. Biol. Chem.* **280**, 17562–17571