

Supporting Information

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SI Materials and Methods

Zebrafish Experiments. Zebrafish experiments were performed as previously described (1) on approval by the Animal Care and Use Committee of Shinshu University School of Medicine. One nanogram of synthesized GFP or Lin28a RNA was coinjected with 50 μ M scrambled or microRNA-140 morpholino oligo (2) in one- or two-cell stage embryos. Fish were harvested at 6 d post-fertilization and subjected to whole-mount Alcian blue staining.

In Situ Hybridization. In situ hybridization for *Col10a1* and *Spp1* was performed as previously described (3).

Cell Cycle Analysis. Pregnant mice with embryo day (E) 11.5 embryos were injected with BrdU 1 h before euthanasia. Embryos

were harvested and genotyped, and limb buds were collected. Limb buds from both forelimbs and hind limbs were dispersed to single cells by trypsinization. Cells were subjected to cell cycle analysis using the APC BrdU Flow Kit (BD Pharmingen) according to the manufacturer's instructions.

LIN28A Immunohistochemistry. Frozen sections of E12.5 limb buds were cut, lightly fixed with 10% (vol/vol) formalin, and subjected to immunohistochemistry using the anti-LIN28A antibody (Cell Signaling) at a dilution of 1:50 and the ImmunoCruz Rabbit LSAB Staining System (Santa Cruz Biotechnology).

Whole-Mount X-Gal Staining. Whole-mount X-gal staining was performed as described previously (4).

1. Nakamura Y, et al. (2007) The CCN family member Wisp3, mutant in progressive pseudorheumatoid dysplasia, modulates BMP and Wnt signaling. *J Clin Invest* 117(10): 3075–3086.
2. Eberhart JK, et al. (2008) MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. *Nat Genet* 40(3):290–298.

3. Kobayashi T, et al. (2008) Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. *Proc Natl Acad Sci USA* 105(6):1949–1954.
4. Chung UI, Lanske B, Lee K, Li E, Kronenberg H (1998) The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. *Proc Natl Acad Sci USA* 95(22):13030–13035.

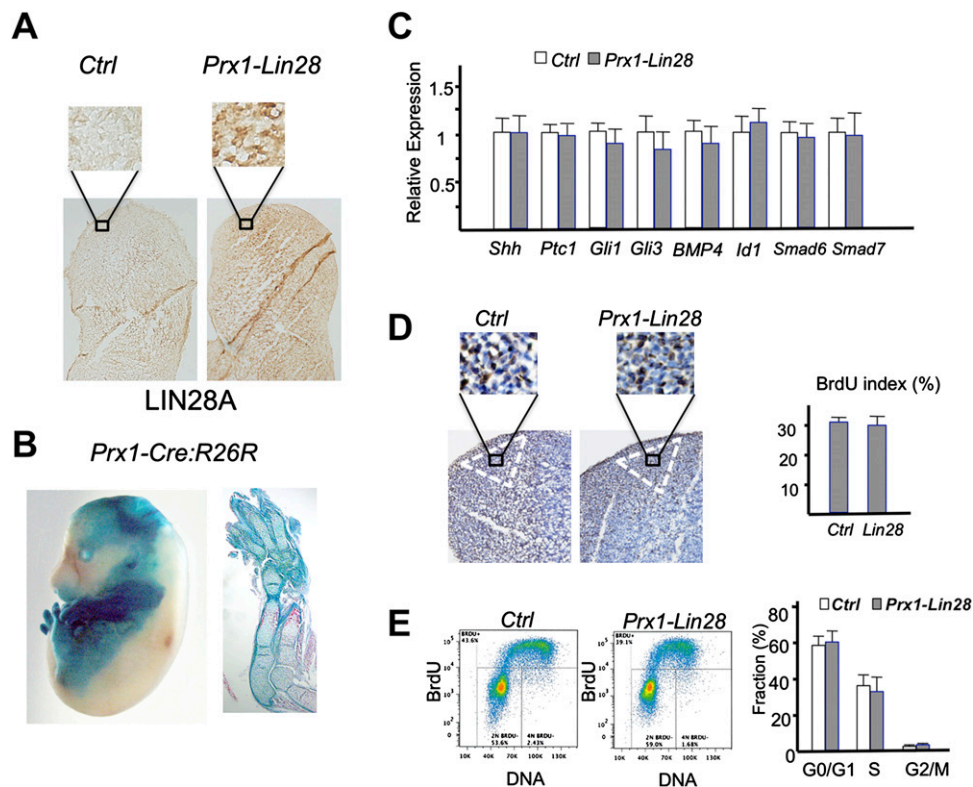


Fig. S1. Analysis of *Prx1-Cre:Lin28a^c* (*Prx1-Lin28*) limb buds. (A) LIN28A expression in *Prx1-Lin28* limb buds. Frozen sections of E12.5 forelimb buds were subjected to immunohistochemical analysis to detect LIN28A. In *Prx1-Lin28* limb buds, LIN28A signals are diffusely detected, although with variable intensities, whereas endogenous LIN28A is expressed at a low level in WT littermates (*Ctrl*). (Original magnification, $\times 40$.) (Insets) Magnified views are shown. (Magnification, $\times 200$.) (B, Left) Whole-mount X-gal staining of E14.5 mice carrying both the *Prx1-Cre* transgene and the *Rosa 26-R* (*R26R*) LacZ Cre reporter allele shows that *Prx1-Cre*-expressing cells give rise to cells in the skull and limbs. (Right) Sections of the forelimb show that chondrocytes are descendants of *Prx1-Cre*-expressing cells. (C) Quantitative RT-PCR analysis for Shh and BMP signaling. Expression of indicated genes was quantified in E11.5 limb bud cells. (D and E) BrdU labeling assay. (D) Frozen sections of BrdU-injected E12.5 forelimb buds were stained for BrdU. Interdigital ray regions (indicated by white dotted lines) were selected and counted. (Original magnification, $\times 100$.) (Insets) Magnified views are shown. (Magnification, $\times 200$.) The BrdU index in *Prx1-Cre:Lin28a^c* (*Lin28*) mice is not significantly increased compared with controls (*Ctrl*; *Prx1-Cre* or *Lin28a^c* single-transgenic or WT littermates; $n = 3$ in each group). (E) Flow cytometric analysis of E11.5 limb bud cells was performed to assess the cell cycle status. Representative flow pictures are shown. The cell cycle status of *Lin28* mice ($n = 3$) was similar to that of *Ctrl* ($n = 4$).

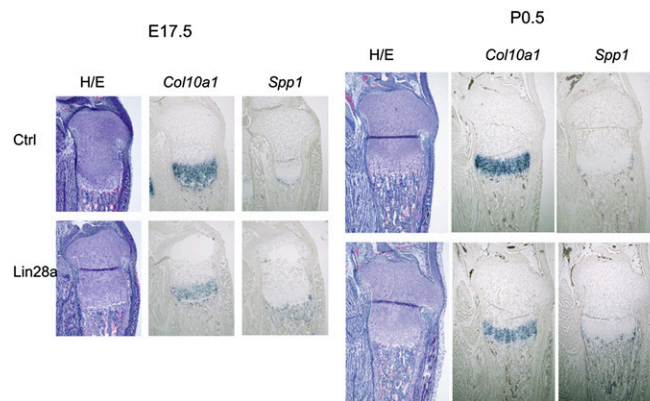


Fig. S2. Normal expression of type X collagen (*Col10a1*) and osteopontin (*Spp1*) mRNA expression in the tibial growth plate of *Col2-Cre:Lin28a^c* mice. In situ hybridization was performed on sections on E17.5 and postnatal day (P) 0.5 mice. (Original magnification, $\times 40$.)

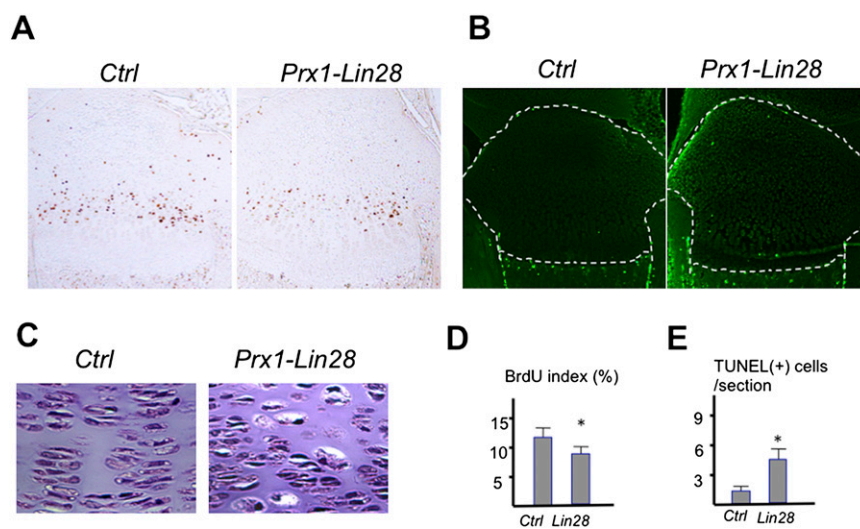


Fig. S3. Growth plate phenotypes of *Prx1-Cre:Lin28a^c* mice. (A) BrdU labeling assay on P9 tibial growth plates shows a reduction in cell proliferation in *Prx1-Cre:Lin28a^c* (*Prx1-Lin28*) chondrocytes compared with *Ctrl*. (Original magnification, $\times 40$.) (B) TUNEL-positive cells are increased in *Prx1-Lin28* mice. (Magnification, $\times 40$.) (C) Morphological changes similar to those in *Col2-Cre:Lin28a^c* mice are also present in the *Prx1-Lin28* growth plate. (Magnification, $\times 200$.) (D and E) Quantification of A and B ($n = 3$; * $P < 0.05$ vs. *Ctrl*).

Table S1. Up-regulated genes in *Lin28a*-overexpressing chondrocytes

Probe set	Gene	Accession no.	Lin28 Ctrl1	Lin28 Tg1	Lin28 Ctrl2	Lin28 Tg2	Tg/Ctrl	TargetScan
10517159	Lin28: Lin-28 homolog (<i>Caenorhabditis elegans</i>)	NM_145833	600.26	8,663.37	418.02	10,872.87	19.18552854	let-7 target
10360145	B930036N10Rik: (Refbp2?)RIKEN cDNA B930036N10 gene	AK047216	360.64	775.48	452.78	1,053.99	2.249108702	
10515755	Mpl: Myeloproliferative leukemia virus oncogene	NM_001122949	597.29	1,055.97	745.31	1,955.61	2.243095486	
10400510	Clec14a: C-type lectin domain family 14, member a	NM_025809	406.45	634.52	348.52	1,013.24	2.1825503	
10372796	Hmga2: High-mobility group AT-hook 2	NM_010441	5,677.33	13,099.42	5,595.73	10,355.23	2.080593024	let7 target
10351224	F5: Coagulation factor V	NM_007976	700.94	1,244.65	653.44	1,534.83	2.052215774	
10483150	Fign: Fidgetin	NM_021716	594.14	1,064.44	707.98	1,598.71	2.045241606	let7 target
10455967	2610318N02Rik: RIKEN cDNA 2610318N02 gene	ENSMUST0000093336	753.29	1,466.25	1,207.62	2,455.85	2.000142791	
10537026	Cpa4: Carboxypeptidase A4	NM_027926	429.68	823.51	294.43	605.26	1.97313944	let7 target
10569399	Trpm5: Transient receptor potential cation channel, subfamily M, member 5	NM_020277	645.2	1,154.25	837.78	1,735.35	1.948509083	
10444236	H2-DMb2: Histocompatibility 2, class II, locus Mb2	NM_010388	743.71	1,316.8	699.16	1,449.64	1.917317568	
10530772	Nmu: Neuromedin U	NM_019515	435.34	733.93	448.72	896.94	1.844750356	
10444229	H2-DMA: Histocompatibility 2, class II, locus DMA	NM_010386	2,716.6	4,806.13	3,168.74	6,025.54	1.84044932	
10523701	Ibsp: Integrin binding sialoprotein	NM_008318	2,244.29	3,666.17	1,561.14	3,275.24	1.824080327	
10390211	Igf2bp1: Insulin-like growth factor 2 mRNA binding protein 1	NM_009951	1,434.45	2,345.93	1,992.75	3,738.67	1.775385154	let7 target
10387821	Alox12: Arachidonate 12-lipoxygenase	NM_007440	3,306.09	5,359.84	960.93	1,983.51	1.72095514	
10569429	Cdkn1c: Cyclin-dependent kinase inhibitor 1C (P57)	NM_009876	4,727.38	7,776.59	5,655.81	10,021.44	1.714119649	
10582658	Agt: Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	NM_007428	1,860.8	2,916.9	1,857.63	3,451.06	1.71253997	
10485948	Grem1: Gremlin 1	NM_011824	5,854.1	10,113.31	3,120.97	5,181.05	1.704093673	
10583044	Mmp13: Matrix metalloproteinase 13	NM_008607	2,261.39	3,626.99	2,432.45	4,363.51	1.702337532	
10552494	Klk9: Kallikrein related-peptidase 9	NM_028660	738.92	1,387.28	337.76	442.72	1.699669354	
10567355	Gprc5b: G protein-coupled receptor, family C, group 5, member B	NM_022420	758.67	1,131.56	907.87	1,639.55	1.662792372	
10478048	Lbp: LPS binding protein	NM_008489	3,014.08	4,714.69	1,820.57	3,319.92	1.661880384	
10436590	2810055G20Rik: RIKEN cDNA 2810055G20 gene	AK148800	688.23	1,031.61	584.31	1,076.87	1.656906659	
10356240	Slc16a14: Solute carrier family 16 (monocarboxylic acid transporters), member 14	NM_027921	1,245.99	1,873.27	1,432.07	2,546.61	1.65040365	
10359689	Atp1b1: ATPase, Na ⁺ /K ⁺ transporting, beta-1 polypeptide	NM_009721	2,926.58	5,280.96	2,865.56	4,269.75	1.648908694	
10511333	Plag1: Pleiomorphic adenoma gene 1	NM_019969	817.47	1,391.17	1,546.15	2,396.03	1.602288016	
10488626	Zcchc3: Zinc finger, CCHC domain containing 3	NM_175126	2,604.47	3,999.39	2,072.64	3,371.06	1.575855603	let7 target
10471814	Gpr21: G protein-coupled receptor 21	NM_177383	467.25	770.29	573.65	864.54	1.570592756	
10564726	Rlbp1: Retinaldehyde binding protein 1	NM_020599	1,182.4	1,747.6	897.64	1,518.41	1.57016692	
10571840	Hpgd: Hydroxyprostaglandin dehydrogenase 15 (NAD)	NM_008278	5,256.55	7,676.49	4,059.4	6,939.88	1.568961834	
10516735	Tinagl1: Tubulointerstitial nephritis antigen-like 1	NM_023476	29,24.23	5,454.64	2,937.33	3,715.01	1.564370236	
10585186	1600029D21Rik: RIKEN cDNA 1600029D21 gene (placenta-expressed transcript 1)	NM_029639	3,985.17	6,332.57	1,318.63	1,939.92	1.559728874	
10360053	Pcp4l1: Purkinje cell protein 4-like 1	NM_025557	1,055.93	1,940.56	1,072.02	1,372.32	1.556841091	
10488697	Plagl2: Pleiomorphic adenoma gene-like 2	NM_018807	2,995.36	4,385.7	2,932.34	4,841.16	1.556566628	let7 target
10490894	E2f5: E2F transcription factor 5	NM_007892	3,379.48	5,010.32	2,929.66	4,755.58	1.547897178	let7 target
10505911	Dmrta1: Doublesex and mAb-3-related transcription factor-like family A1	NM_175647	1,262.36	2,127.76	1,375.2	1,953.48	1.547354373	
10597258	Tmie: Transmembrane inner ear	NM_146260	502.95	794.82	569.14	863.03	1.54637204	
10489985	Atp9a: ATPase, class II, type 9A	NM_015731	790.86	1,152.25	704.76	1,159.23	1.545499525	
10586118	Calml4: Calmodulin-like 4	NM_138304	737.19	1,212.5	1,016.97	1,468.66	1.528458065	

Table S1. Cont.

Probe set	Gene	Accession no.	Lin28 Ctrl1	Lin28 Tg1	Lin28 Ctrl2	Lin28 Tg2	Tg/Ctrl	TargetScan
10438603	Igf2bp2: Insulin-like growth factor 2 mRNA binding protein 2	NM_183029	6,001.02	9,205.94	7,163.64	10,905.27	1.527666495	let7 target
10526487	Alkbh4: alkB, Alkylation repair homolog 4 (<i>Escherichia coli</i>)	NM_028070	3,222.58	4,573.01	2,937.59	4,823.22	1.525319918	
10454235	Asxl3: Additional sex combs like 3 (<i>Drosophila</i>)	ENSMUST00000062608	676.77	1,046.54	766.31	1,152.32	1.523727028	
10583669	AB124611: cDNA sequence AB124611	AB124611	1,308.19	1,938.68	1,101.87	1,678.63	1.500921139	
10438091	2610318N02Rik: RIKEN cDNA 2610318N02 gene	BC039993	240.91	349.68	424.07	640.72	1.489368101	
10403834	Sfrp4: Secreted frizzled-related protein 4	NM_016687	573.37	886.7	356.79	491.43	1.481605315	
10380415	Cdc34: Cell division cycle 34 homolog (<i>Saccharomyces cerevisiae</i>)	BC094502	2,977.95	4,903.79	2,914.46	3,780.67	1.473838379	let7 target
10492355	Mme: Membrane metalloendopeptidase	NM_008604	757.47	1,157.36	1,030.71	1,477.95	1.473738662	
10408928	Hspb1: Heat shock protein 1	NM_013560	7,089.37	10,757.31	4,143.68	5,630.32	1.458876262	
10526410	Hspb1: Heat shock protein 1	NM_013560	6,830.69	10,322.43	3,979.15	5,439.28	1.458089111	
10353102	Cpa6: Carboxypeptidase A6	NM_177834	474.82	669.54	341.33	520.18	1.457722232	
10523190	9130213B05Rik: RIKEN cDNA 9130213B05 gene	BC006604	2,652.16	3,860.99	3,107.85	4,508.52	1.453037408	
10552037	Sbsn: Suprabasin	NM_172205	1,404.61	2,103.41	1,251.56	1,742.27	1.447829017	
10415413	BC030046: cDNA sequence BC030046	BC057379	3,360.22	4,738.76	3,081.82	4,467.97	1.429163743	
10364455	Cdc34: Cell division cycle 34 homolog (<i>S. cerevisiae</i>)	NM_177613	7,230.34	10,766.08	6,680.04	9,110.3	1.428888355	let7 target
10488954	Gdf5: Growth differentiation factor 5	NM_008109	541.54	759.06	539.94	772.11	1.415809816	
10438778	Tmem207: Transmembrane protein 207	AK142524	280.35	400.52	245.76	336.13	1.400182471	
10595840	Acpl2: Acid phosphatase-like 2	NM_153420	2,226.9	3,170.25	1,120.72	1,404.5	1.36656789	
10544523	Rny1: RNA, Y1 small cytoplasmic, Ro-associated	NR_004419	750.45	1,106.44	2,663.13	3,267.37	1.281297055	

Alterations in the gene expression profile of primary rib chondrocytes after *Lin28a* overexpression was investigated. Among those whose signal intensity was greater than 200 (30,725 probes of a total 35,512 probes, with an average intensity of 2,838), we found that expression of 56 genes was up-regulated by more than 40% by *Lin28a* overexpression. Among these 56 genes, 10 (17.8%) were predicted to be let-7 target genes.