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RESEARCH ARTICLE

Lin28a overexpression reveals the role of Erk signaling in articular cartilage development

Tatsuya Kobayashi* and Anastasia Kozlova

ABSTRACT

Adult articular cartilage shows limited tissue turnover, and therefore development of the proper structure of articular cartilage is crucial for life-long joint function. However, the mechanism by which the articular cartilage structure is developmentally regulated is poorly understood. In this study, we show evidence that activation of extracellular signal-regulated kinases (Erk1/2) in articular chondrocyte progenitors during developmental stages control articular cartilage thickness. We found that overexpression of Lin28a, an RNA-binding protein that regulates organismal growth and metabolism, in articular chondrocyte progenitor cells upregulated Erk signaling and increased articular cartilage thickness. Overexpression of a constitutively active Kras mimicked Lin28a overexpression, and inhibition of Erk signaling during embryonic stages normalized the cartilage phenotype of both Kras- and Lin28a-overexpressing mice. These results suggest that articular cartilage thickness is mainly determined during the process of embryonic synovial joint development, which is positively regulated by Erk signaling.

KEY WORDS: Articular cartilage, Kras, Erk, let-7, Lin28, MicroRNA, Osteoarthritis

INTRODUCTION

Osteoarthritis (OA) is a highly prevalent skeletal condition among the elderly (Dieppe and Lohmander, 2005). Although OA is considered a degenerative disease caused primarily by accumulated mechanical stresses, evidence suggests that other factors, such as genetic components, are also involved in OA pathogenesis. Recent genome-wide association studies (GWAS) have demonstrated associations between OA and genetic polymorphisms in several genes that regulate skeletal development, such as *GDF5* and *PTHLH* (Chapman et al., 2008; Day-Williams et al., 2011; Evangelou et al., 2009, 2011; Miyamoto et al., 2007; Valdes et al., 2011; Zeggini et al., 2012). These data suggest that subtle changes in joint morphogenesis during cartilage development can influence joint homeostasis and development of OA later in life. However, compared with the catabolic process of the articular cartilage of synovial joints in OA, understanding of articular cartilage development and growth is still limited. Particularly, mechanisms regulating the cartilage matrix volume and chondrocyte number in the articular cartilage are largely unknown.

Chondrocytes that form the articular cartilage arise from a distinct progenitor population from those of the growth plate (Hyde et al., 2007; Koyama et al., 2008); articular chondrocytes differentiate from interzone cells of the joint primordium that express *Gdf5* but not the matrilin gene (*Matn1*), whereas growth plate chondrocytes derive from *Gdf5*-negative, *Matn1*-positive cells. During articular cartilage development, cells expressing *Gdf5* and/or the lubricin gene (*Prg4*) at the articular surface serve as progenitors for chondrocytes in the mature articular cartilage. Using a lineage-tracing technique, a recent study demonstrated that articular cartilage increases its volume through appositional growth (Kozhemyakina et al., 2015). In this process, chondrocytes in the articular surface (superficial zone chondrocytes; SZCs) slowly divide and produce progeny in deeper layers of the articular cartilage to increase the cartilage thickness. This study suggests that the number and function of SZCs are major determinants of the cartilage volume during development and growth stages. However, the mechanism that regulates the function and/or number of SZCs during synovial joint development remains elusive.

Small regulatory microRNAs (miRNAs) play crucial roles in skeletal development and homeostasis by suppressing target gene expression (Mirzamohammadi et al., 2014; Papaioannou et al., 2014). We have shown that miRNAs play important roles in chondrocyte proliferation, differentiation and survival, both in the growth plate and in the articular cartilage (Kobayashi et al., 2008, 2015). We have also shown that overexpression of Lin28a, an RNA-binding protein that inhibits the biogenesis of let-7 (Mirlet7 – Mouse Genome Informatics) miRNAs (Viswanathan et al., 2008), reduces chondrocyte proliferation, whereas it causes limb overgrowth when expressed in early limb mesenchymal progenitor cells (Papaioannou et al., 2013). Thus, Lin28a overexpression differentially regulates the proliferation of chondrocytes and their mesenchymal progenitor cells in a cell type-dependent manner. Based on these findings, we hypothesized that Lin28a overexpression regulates articular cartilage development by controlling the cell number of articular chondroprogenitor cells through let-7 miRNA-dependent and -independent pathways.

In this study, we show that overexpression of Lin28a increases the articular cartilage volume via upregulation of extracellular receptor-regulated kinase (Erk) signaling. We also show that expression of a constitutively active form of Kras mimics the effect of Lin28 overexpression. Inhibition of Erk signaling during embryogenesis normalized the articular cartilage in these mice, thus suggesting that Erk signaling during articular cartilage development primarily determines the articular cartilage volume in postnatal stages.

RESULTS

Lin28a overexpression in chondroprogenitor cells increases cartilage thickness

We have previously demonstrated that let-7 miRNAs regulate skeletal growth using mouse models overexpressing Lin28a, an inhibitor of let-7 miRNA biogenesis. Lin28a overexpression in limb mesenchymal cells resulted in overgrowth of the limb skeleton, whereas Lin28a overexpression in chondrocytes decreased their

Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA.

*Author for correspondence (tkobayashi1@mgh.harvard.edu)

T.K., 0000-0003-4264-5117

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proliferation, causing mild growth impairment (Papaioannou et al., 2013). These results suggest that let-7 miRNAs differentially regulate tissue development and growth, dependent on cell types.

In order to test the effect of Lin28a overexpression, we used a Cre-mediated binary system (Papaioannou et al., 2013). To drive *Lin28a* expression, we first used *Col2* (*Col2a1*)-*Cre* transgenic mice, in which Cre is expressed in early chondro-osteo progenitor cells in the mesenchymal condensation, as well as in mature articular and growth plate chondrocytes. Using RNA isolated from embryonic day (E) 13.5 *Col2-Cre:Lin28a* mouse limbs, and also from primary limb bud cells of *Prx1* (*Prdx1*)-*Cre:Lin28a* mice, we confirmed Lin28a overexpression and concomitant suppression of let-7 miRNAs by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis (Fig. S1). The magnitude of *Lin28a* expression and let-7 miRNA reduction was not as robust as when *Lin28a* expression was driven by *Prx1-Cre* transgenic mice. This is likely because *Col2-Cre* expression starts later during limb morphogenesis and is more restricted to chondro-osteo progenitors compared with *Prx1-Cre* expression. Nevertheless, *Lin28a* overexpression in *Col2*-postive cells increased the thickness of the articular cartilage of the mid-tibial plateau when assessed at the age of 3 weeks (Fig. 1A,B, Fig. S2). This phenotype was replicated when *Lin28a* was overexpressed using *Gdf5-Cre* transgenic mice, in which Cre is expressed in progenitor cells of articular chondrocytes and synovial cells, but not those of growth plate chondrocytes (Rountree et al., 2004) (Fig. 1C,D, Fig. S2). Immunostaining revealed normal distribution of type II collagen in *Lin28a*-expressing cartilage, suggesting that the quality of the cartilage matrix is maintained (Fig. S3).

Lin28a overexpression in mesenchymal progenitor cells increases Erk signaling

It was previously shown that let-7 miRNAs suppress the Ras signaling pathway by direct binding to transcripts encoding Ras isoforms (Johnson et al., 2005). Ras activation mainly signals through the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K; PIK3R1) pathways (Castellano and Downward, 2011; Papaioannou et al., 2016). Therefore, we determined the activities of Erk1/2 (also known as Mapk3/1) and PI3K using primary mesenchymal progenitor cells (Fig. 2A). Serum stimulation induced greater responses in Lin28a-overexpressing cells than in control cells in Erk signaling, assessed by phosphorylation of Erk1/2, and modestly in PI3K signaling, assessed by the level of phosphorylated Akt (Akt1) (p-Akt). We did not find significant alteration in the expression of Ras, a predicted target of let-7 miRNAs, upon Lin28a overexpression (Fig. 2B). Immunohistochemical analysis also confirmed upregulation of Erk phosphorylation in articular chondrocytes in *Lin28a* transgenic mice (Fig. S4).

Kras overactivation increases articular cartilage thickness

Because *Lin28a* overexpression increases Erk signaling, we tested whether overactivation of Ras signaling could reproduce the cartilage phenotype of *Lin28a* transgenic mice. We overactivated Kras signaling in articular chondrocyte progenitor cells using Cre-inducible oncogenic mutant Kras ($Kras^{G12D}$) transgenic mice (Johnson et al., 2001). Kras overactivation increased the cartilage thickness to a similar extent to that induced by *Lin28a* overexpression (Fig. 3A,B). Erk phosphorylation was upregulated, whereas the p-Akt level was low, in both control and $Kras^{G12D}$ transgenic mice (Fig. 3C). We also tested whether PI3K activation via genetic deletion of *Pten*, a negative regulator of PI3K signaling, would mimic the cartilage phenotype of *Lin28a* transgenic mice. Although *Pten* deletion in growth plate chondrocytes causes overgrowth (Ford-Hutchinson et al., 2007; Yang et al., 2008), we did not find a significant increase in articular cartilage thickness in *Pten* conditional knockout mice (Fig. S5). These data suggest that Erk signaling, but not PI3K signaling, positively regulated the articular cartilage thickness.

Erk inhibition reverses the cartilage phenotype caused by Kras activation or *Lin28a* overexpression

Based on these results, we hypothesized that the upregulation of Erk signaling is primarily responsible for the increased cartilage thickness caused by Kras activation. To test this, we injected

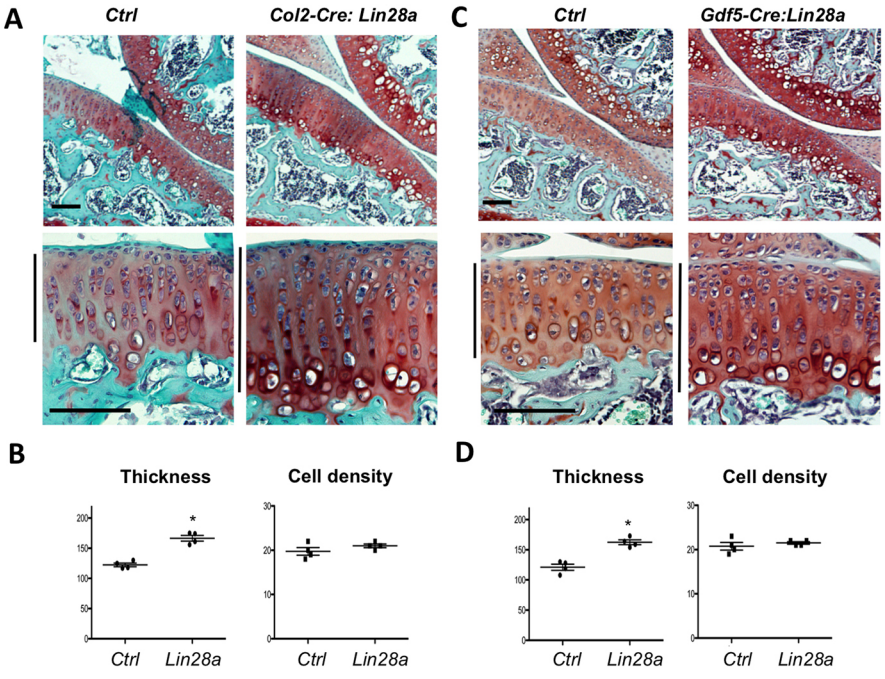

Fig. 1. *Lin28a* overexpression increases articular cartilage thickness. (A) Safranin O staining of knee joints of 3-week-old mice. Lin28a is overexpressed in *Col2*-positive cells and their descendants. Bottom images are magnified views of the tibial articular cartilage shown in corresponding images at the top. Sagittal sections at the level of the mid-medial tibial plateau are shown. (B) Measurements of cartilage thickness (in µm) and cell density of the *Col2-Cre:Lin28a* mice in A. Cell density was determined by counting cell numbers in a 5000 µm^2 square immediately below the articular surface. (C,D) Safranin O staining and cartilage thickness and cell density measurements of 3-week-old *Gdf5-Cre:Lin28* transgenic mice. Analyses were performed as in A and B. Data are mean±s.e.m. [n=4; *P<0.05 vs control (Ctrl)]. Scale bars: 100 µm.

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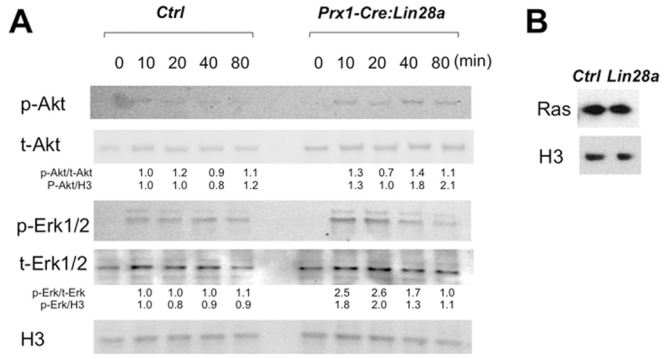

Fig. 2. *Lin28* overexpression upregulates Erk and PI3K signaling. (A) Limb bud mesenchymal cells were isolated from E11.5 embryos of Cre-inducible *Lin28* transgenic mice (*CAG-lsl-Lin28*). Erk and PI3K signaling pathways upon stimulation with 10% fetal calf serum (FCS) were evaluated at the indicated time points using phospho-specific antibodies. The band densities were measured, normalized to total Akt (t-Akt), total Erk1/2 (t-Erk1/2) or histone 3 (H3), and are indicated beneath each band. (B) Ras expression is unchanged upon *Lin28* overexpression. *Lin28a* was overexpressed in primary limb bud cells by adenoviral transduction of YFP (Ctrl) or Cre (*Lin28a*). Ras expression was assessed by immunoblot analysis using anti-pan Ras antibody.

U0126, a small chemical inhibitor of the Erk signaling pathway, into pregnant mothers during synovial joint development. Erk inhibition significantly reduced the articular cartilage thickness in mice with Kras overactivation (Fig. 4A,B). To determine whether Erk overactivation is also responsible for the cartilage phenotype of *Lin28a* transgenic mice, we treated pregnant mothers with U0126, using the same treatment protocol as that used on *Kras* transgenic mice. U0126 injection significantly reduced the cartilage thickness of *Lin28a* transgenic mice (Fig. 5A,B). These results suggest that overactivation of Erk signaling during cartilage morphogenesis increases cartilage thickness.

DISCUSSION

It has been shown that genetic components significantly contribute to the pathogenesis of OA. Recent GWAS indicate that OA is associated with genes involved in limb and cartilage morphogenesis, suggesting that proper development of joint tissues influences long-term cartilage health. However, understanding of the mechanisms that regulate development and growth of articular cartilage is still limited.

In this paper, we present evidence that Erk signaling during embryonic stages positively regulates the articular cartilage thickness. Overexpression of *Lin28a* in articular chondrocyte progenitors reduced let-7 miRNA expression, upregulated Erk signaling and increased the cartilage thickness, without affecting the cellular density. We show that this cartilage phenotype is mimicked by overexpression of a constitutively active oncogenic Kras, and that this cartilage phenotype is suppressed by inhibition of Erk signaling.

The cartilage phenotype caused by *Lin28a* or Kras activation is somewhat reminiscent of that of mice missing *Errfi1* (also called *Mig-6*) (Pest et al., 2014; Shepard et al., 2013; Staal et al., 2014; Zhang et al., 2005). *Errfi1* is expressed in the superficial zone of the articular cartilage and meniscus during cartilage development (Staal et al., 2014). *Errfi1* encodes a negative regulator of epidermal growth factor receptor (Egfr) signaling that is mediated mainly through the MAPK and PI3K signaling pathways (Avraham and Yarden, 2011). Therefore, loss of *Errfi1* increases Egfr and thus Erk activation in the articular cartilage (Staal et al., 2014). However, unlike *Errfi1*-deficient mice, mice with *Lin28a* overexpression or Kras overactivation do not exhibit an overt increase in cell density or cell proliferation in the 3-week-old articular cartilage or embryonic knee joint primordium (Fig. S6). It is possible that Ras-independent pathways of Egfr signaling are responsible for the continuous postnatal cell proliferation in *Errfi1*-deficient articular chondrocytes.

The finding that *Lin28a* overexpression increased Erk and PI3K signaling yet did not change Ras protein expression was unexpected, considering that RNAs encoding Ras proteins are conserved let-7 miRNA targets (Johnson et al., 2005), and that Lin28a overexpression significantly reduced let-7 expression. Let-7 miRNAs are also predicted to target other molecules associated with the Erk and PI3K signaling pathways, including Igf1 and its receptor Igf1r (Ghazal et al., 2015; Shen et al., 2014; Zhu et al., 2011). We found upregulation in Igf1r expression upon *Lin28a* overexpression in limb bud cells (Fig. S7A). However, genetic ablation of *Igf1r* was not able to mitigate the articular cartilage phenotype of *Lin28a* transgenic mice (Fig. S7B,C), suggesting that it is unlikely that Igf1r upregulation is responsible for the increased cartilage thickness. At the moment, the precise molecular mechanism by which *Lin28a* overexpression stimulates Erk and PI3K signaling is unclear. It is possible that modest upregulation of multiple let-7 miRNA targets cooperatively deregulates these signaling pathways. Because let-7 miRNAs are known to regulate oncogenic transcription factors, such as Mycn and Hmga2, they might indirectly regulate expression of genes that potentiate these signaling pathways (Balzeau et al., 2017; Nguyen and Zhu, 2015). It is also possible that let-7 miRNA-independent action of Lin28a contributes to the altered signaling (Jiang and Baltimore, 2016).

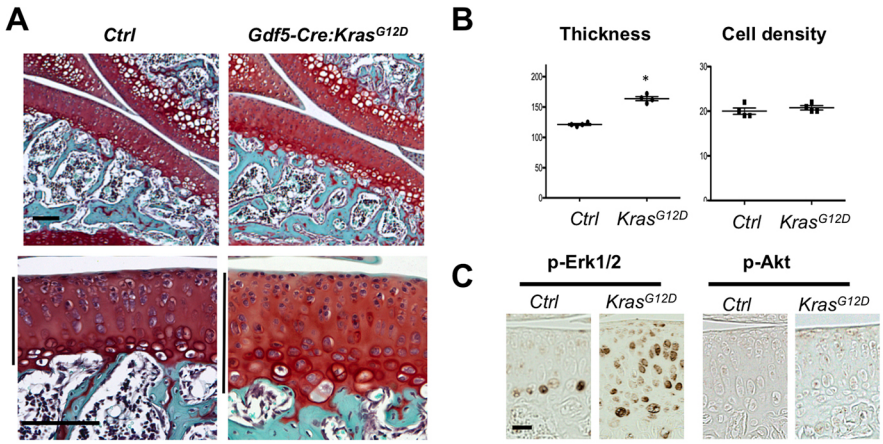

Fig. 3. Overexpression of *Kras^{G12D}* causes a phenotype similar to that induced by *Lin28a* overexpression. (A) Sagittal sections of the knee joint of control (Ctrl) and *Kras^{G12D}* transgenic (*Gdf5-Cre:Kras^{G12D}*) mice at the age of 3 weeks. (B) Quantification of cartilage thickness (μm) and cell density (cells/5000 μm^2) was performed as in Fig. 1. Data are mean±s.e.m. (n=4; *P<0.05 vs Ctrl). (C) Immunohistochemical detection of p-Erk and p-Akt in the tibial articular cartilage of *Kras^{G12D}* transgenic mice at 3 weeks of age. Scale bars: 100 μm.

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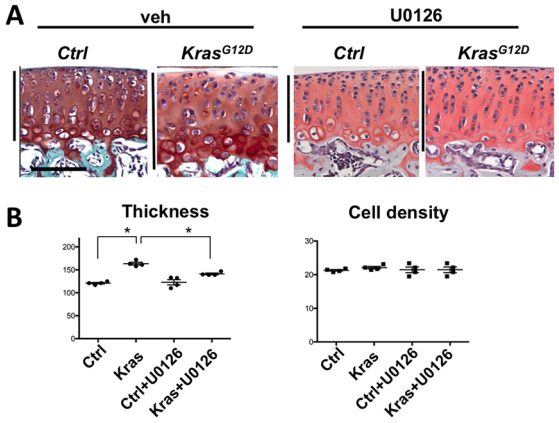

Fig. 4. Erk inhibition reverses the cartilage phenotype of *Kras^{G12D}* transgenic mice. (A) Sagittal sections of the medial tibial plateau of 3-week-old control (Ctrl) and *Gdf5-Cre:Kras^{G12D}* transgenic (*Kras^{G12D}*) mice treated with U0126 from E11.5 to E18.5, by daily intraperitoneal (i.p.) injection. Scale bar: 100 µm. (B) Quantification of cartilage thickness (µm) and cell density (cells/5000 µm^2) of the articular cartilage was performed as in Fig. 1. Data are mean±s.e.m. (*n*=4 each; **P*<0.05 vs Ctrl).

The observation that Erk inhibition was able to rescue the articular cartilage phenotype caused by Kras overactivation or *Lin28a* overexpression strongly suggests that the overactivation of Erk, but not PI3K, signaling increases the articular cartilage thickness. This is consistent with the finding that *Pten* deletion, a negative regulator of PI3K signaling, did not increase the cartilage thickness. It is noteworthy that Erk inhibition only during embryonic stages is enough to prevent the effect of Kras overactivation or *Lin28a* overexpression. Considering that *Lin28a* overexpression in chondrocytes in the growth plate reduces cell proliferation, whereas *Lin28a* overexpression in mesenchymal progenitor cells causes limb overgrowth (Papaioannou et al., 2013), the major cell population responsible for the articular cartilage phenotype of *Lin28a* transgenic mice is likely to be chondroprogenitor cells of articular cartilage, but not differentiated articular chondrocytes. Consistent with this notion, when we overexpressed *Lin28a* in postnatal stages using tamoxifen-inducible Cre lines, we did not find increases in the articular cartilage thickness (Fig. S8A,B).

It is also an interesting question as to whether endogenous Lin28a contributes to articular cartilage morphogenesis, because germ-line Lin28a deficiency causes growth and skeletal defects in embryos (Shinoda et al., 2013). Endogenous Lin28a is strongly expressed in developing limb buds, although its expression wanes around the time when chondroprogenitor cells first start to appear in the limb bud (Yokoyama et al., 2008). In this regard, experiments to conditionally delete Lin28a will provide insight into the role of the endogenous Lin28/let-7 axis in articular cartilage morphogenesis.

Together, these results suggest that Erk activation in chondroprogenitor cells during cartilage morphogenesis increases their number, and subsequently increases the articular cartilage thickness (Fig. 5C).

MATERIALS AND METHODS

Mice

Cre-inducible *Lin28* transgenic mice (*CAG-lsl-Lin28*) (Papaioannou et al., 2013), Cre-inducible oncogenic *Kras* knock-in mice (Johnson et al., 2001), *Col2-Cre* transgenic mice (Ovchinnikov et al., 2000), *Gdf5-Cre* transgenic mice (Rountree et al., 2004) and *Prx1-Cre* transgenic mice (Logan et al., 2002) were previously described. Floxed *Igf1r* mice, floxed *Pten* mice (Lesche et al., 2002), floxed *Col2-CreER* mice (Nakamura et al., 2006) and *Prg4-CreER* mice (Kozhemyakina et al., 2015) were previously described. Mice were kept in a C57/B6-dominant mixed background. At the age of 3 weeks, no significant morphological differences were noted in the articular cartilage between males and females; therefore, sexes were not discriminated.

To suppress Erk signaling *in vivo*, the Mek inhibitor, U0126 [Cell Signaling Technology; 5 mg/kg body weight diluted in 100 µl phosphate buffered saline (PBS)/mouse], was injected daily intraperitoneally from E11.5 to E18.5.

The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital.

Histological analyses

Formalin-fixed mouse hindlimbs were paraffin-processed, cut, de-waxed and rehydrated, and subjected to Hematoxylin-Eosin and Safranin-O staining, and other histological analyses according to standard procedures. Midsagittal sections of the medial tibial plateau were selected for measurements.

Immunohistochemistry was performed using anti-phosphoErk1/2 antibody (4370, Cell Signaling Technology; diluted 1:400), anti-phospho-Akt antibody (4060, Cell Signaling Technology; diluted 1:100), anti-type II collagen alpha 1 antibody (15943-1-AP, Proteintech; diluted 1:50) and anti-Ki67 (Mki67) antibody (bs-2130R, Bioss; diluted 1:250) using an ImmunoCruz rabbit ABC Staining System (sc-2018, Santa Cruz Biotechnology) according to the manufacturer's instructions.

Bromodeoxyuridine staining

Pregnant mice were injected with 50 µg/g 5-bromo-2'-deoxyuridine (BrdU) 2 h before sacrifice. E13.5 embryonic hind limbs were dissected, fixed in 10% formalin/PBS overnight, paraffin processed and sectioned at 5 µm thickness. BrdU detection was performed using a BrdU Staining Kit (Invitrogen) according to the manufacturer's instructions.

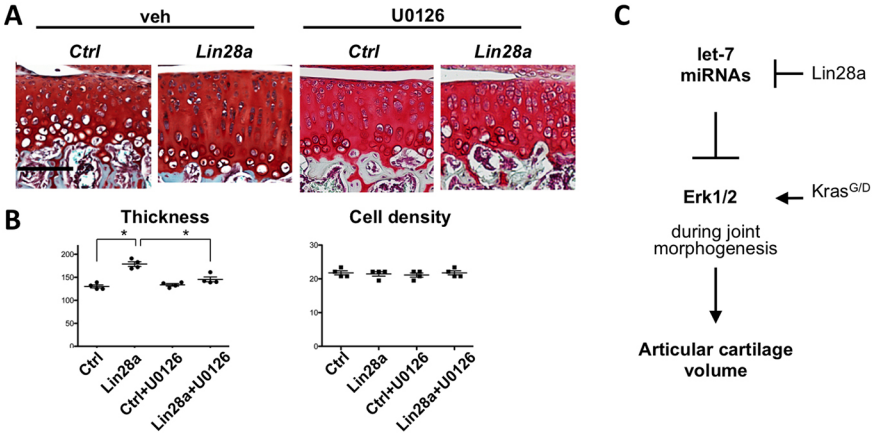

Fig. 5. Erk inhibition reverses the cartilage phenotype of *Lin28a* transgenic mice. (A) Sagittal sections of the medial tibial plateau of 3-week-old control (Ctrl) and *Gdf5-Cre:Lin28a* transgenic (*Lin28a*) mice treated with U0126 (5 mg/kg body weight) from E11.5 to E18.5, by daily i.p. injection. Scale bar: 100 µm. (B) Quantification of cartilage thickness (µm) and cell density (cells/5000 µm^2) of the articular cartilage was performed as in Fig. 1. Data are mean± s.e.m. (*n*=4 each; **P*<0.05 vs Ctrl). (C) Summary. Upregulation of Erk signaling via *Lin28a* overexpression, through an undefined mechanism mediated by let-7 miRNA-dependent and/or -independent pathways, or *Kras^{G12D}* overexpression, increases articular cartilage thickness, suggesting that let-7 miRNAs negatively regulate the process of articular cartilage development.

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qRT-PCR

qRT-PCR analysis was performed using a Sybr-green based method with a StepOne Realtime PCR System (Applied Biosystems). Quantification of miRNAs was performed using either a mirVana qRT-PCR miRNA detection kit (Invitrogen) or a qScript microRNA cDNA synthesis kit (Quantabio). Values were internally normalized by the level of Actb or U6.

Western blot analysis

Mesenchymal progenitor cells were isolated from E11.5 limb buds or postnatal day (P) 0.5 skull. Cells were trypsinized to disperse, and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS and antibiotics. Cells were serum starved for 2 h before FCS stimulation.

Experiments were performed according to the standard western blot procedure. Anti-phospho-Erk1/2 antibody (4370; diluted 1:2000), anti-phospho-Akt antibody (4060; diluted 1:2000), anti-histone3 antibody (9717; diluted 1:2000) and anti-pan Akt antibody (4691; diluted 1:1000) were purchased from Cell Signaling Technology. Anti-total Erk1/2 antibody was purchased from eBioscience (14-9108-80; diluted 1:1000). Anti-pan Ras antibody (61001; diluted 1:1000) was purchased from BD Biosciences. Anti-Igf1r antibody (bs-4985R) and anti-Actb antibody (845) were purchased from Bioss and Cell Signaling Technology, respectively. Fluorescent and horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Azure Biosystems (goat anti-mouse IgG IR700, and goat anti-rabbit IgG IR800) and Cell Signaling Technology (goat anti-rabbit IgG HRP, 7074), respectively.

Whole-mount Cre reporter assay

Whole-mount X-gal staining using *R26R* reporter mice (Soriano, 1999) was performed as previously described (Kobayashi et al., 2002).

Statistical analysis

Nonparametric, unpaired, two-tailed Student's *t*-test was used for analysis of statistical significance between two groups. $P<0.05$ was considered statistically significant. The sample size was determined based on the effect size and variability obtained during preliminary experiments.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.K.; Methodology: T.K., A.K.; Validation: T.K., A.K.; Formal analysis: T.K., A.K.; Investigation: T.K.; Resources: T.K., A.K.; Data curation: T.K.; Writing - original draft: T.K., A.K.; Writing - review & editing: T.K.; Visualization: T.K., A.K.; Supervision: T.K.; Project administration: T.K.; Funding acquisition: T.K.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.162594.supplemental

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