

EGFR signaling is critical for maintaining the superficial layer of articular cartilage and preventing osteoarthritis initiation

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Osteoarthritis (OA) is the most common joint disease, characterized by progressive destruction of the articular cartilage. The surface of joint cartilage is the first defensive and affected site of OA, but our knowledge of genesis and homeostasis of this superficial zone is scarce. EGFR signaling is important for tissue homeostasis. Immunostaining revealed that its activity is mostly dominant in the superficial layer of healthy cartilage but greatly diminished when OA initiates. To evaluate the role of EGFR signaling in the articular cartilage, we studied a cartilage-specific *Egfr*-deficient (*CKO*) mouse model (*Col2-Cre EgfrWa5/flox*). These mice developed early cartilage degeneration at 6 mo of age. By 2 mo of age, although their gross cartilage morphology appears normal, *CKO* mice had a drastically reduced number of superficial chondrocytes and decreased lubricant secretion at the surface. Using superficial chondrocyte and cartilage explant cultures, we demonstrated that EGFR signaling is critical for maintaining the number and properties of superficial chondrocytes, promoting chondrogenic proteoglycan 4 (*Prg4*) expression, and stimulating the lubrication function of the cartilage surface. In addition, EGFR deficiency greatly disorganized collagen fibrils in articular cartilage and strikingly reduced cartilage surface modulus. After surgical induction of OA at 3 mo of age, *CKO* mice quickly developed the most severe OA phenotype, including a complete loss of cartilage, extremely high surface modulus, subchondral bone plate thickening, and elevated joint pain. Taken together, our studies establish EGFR signaling as an important regulator of the superficial layer during articular cartilage development and OA initiation.

EGFR | articular cartilage | chondrocyte | lubrication | osteoarthritis

Osteoarthritis (OA) is the most common chronic condition of the joints, affecting ~27 million adults in the United States alone. Mature articular cartilage consists of four zones: superficial, transitional, middle, and calcified zones (1). The combination of the first three zones are also termed uncalcified zone. Among them, the superficial layer is the first line of defense against OA initiation because of its surface location, unique composition, and functions. It has 2–4 layers of flat cells expressing unique molecules, such as proteoglycan 4 (*Prg4*, lubricin) and tenascin C, and contains a fine network of collagen fibrils that are oriented horizontally and parallel to the articular surface (2). Its multifaceted roles include, but are not limited to, producing lubricant proteins, harboring chondroprogenitors, resisting shear stresses, serving as a gliding surface, and isolating deeper layers from synovial fluid (2). In OA, degenerative changes initiate with cellular disorganization, gradual stiffening, and irregular surface of this layer (3–5). Despite the critical role of superficial chondrocytes in maintaining articular

cartilage and in blocking OA, its regulation by growth factors and hormones is still largely unknown.

Epidermal growth factor receptor (EGFR) signaling is important for tissue homeostasis. Multiple ligands bind to and activate the EGFR, including epidermal growth factor (EGF), transforming growth factor- α (TGF α), amphiregulin, heparin-binding EGF (HB-EGF), epiregulin, and betacellulin. Once activated, it stimulates an intracellular signal transduction cascade to markedly influence cell behavior (6). Our current knowledge of EGFR function in articular cartilage mostly came from previous studies of its ligands and its intracellular inhibitor, mitogen-inducible gene 6 (*Mig-6*). Large-scale gene profiling experiments have identified that the expression of TGF α (7) and HB-EGF (8) was increased in articular chondrocytes in both rodent surgical OA models and OA patients. *Mig-6* null mice develop a severe OA-like joint degeneration phenotype at an early adult stage (9). However, mice with limb- (*Prx1-Cre*) or cartilage- (*Col2-Cre*) specific deletion of *Mig-6* showed a much milder OA phenotype (10–12). Indeed, a

Significance

The uppermost superficial zone of articular cartilage plays multifaceted roles in maintaining cartilage structure, function, and mechanical properties and in preventing cartilage degeneration during osteoarthritis initiation. However, its regulation by growth factors and hormones is still largely unknown. Here we report that EGFR signaling is an important growth factor pathway that maintains superficial chondrocyte number, promotes boundary lubricant secretion and cartilage surface lubrication, and stimulates mechanical strength of articular cartilage. Reduction in EGFR activity leads to structurally, functionally, and mechanically compromised articular cartilage during development and drastically accelerates cartilage degeneration under normal and surgically induced osteoarthritis conditions. Thus, our studies strongly suggest that targeting cartilage surface EGFR signaling should be considered as a novel direction for osteoarthritis treatment.

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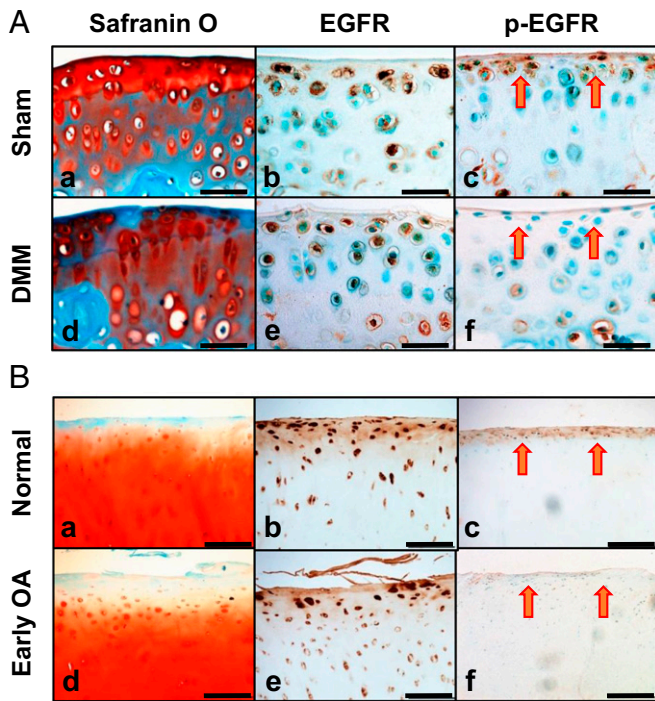


Fig. 1. EGFR signaling in healthy and diseased articular cartilage. (A) Safranin O staining (a and d) and immunohistochemistry of EGFR (b and e) and p-EGFR (c and f) in tibial articular cartilage of WT mice at 1 mo postsham (a–c) or post-DMM (d–f) surgery. (Scale bars, 50 μ m.) (B) Safranin O staining (a and d) and immunohistochemistry of EGFR (b and e) and p-EGFR (c and f) in human joints with healthy (a–c) and early OA (d–f) cartilage. (Scale bars, 200 μ m.) Red arrows point to superficial cells.

common phenotype in these two conditional knockout models is an expansion of knee articular cartilage at a young age. In addition to EGFR, *Mig-6* also suppresses the signaling of several other

growth factor receptors, such as HGF/MET (13), implicating that the cartilage phenotypes observed in *Mig-6* null mice might not be solely due to EGFR overactivation.

In the past, we established a chondrocyte-specific *Egfr* knockout mouse model to demonstrate a pivotal role of EGFR signaling in growth plate development and secondary ossification center (SOC) formation (14, 15). In this study, we used this model to investigate the function of EGFR in articular cartilage development. A surgery model by destabilization of the medial meniscus (DMM) was applied to elucidate the action of EGFR in OA initiation. Together with human cartilage data, our results clearly demonstrated that chondrogenic EGFR signaling is an essential regulator of the superficial layer of articular cartilage by maintaining chondrocyte number, its mechanical properties, and lubrication function. Furthermore, it plays a critical role in protecting cartilage from OA initiation.

Results

EGFR Signaling in Healthy and Diseased Articular Cartilage. To study EGFR signaling in the articular cartilage, we first analyzed the gene expression of EGFR family members and its cognate ligands in mouse femoral head cartilage. Real-time RT-PCR revealed high levels of *Egfr*, its coreceptor *ErbB2*, and its ligands *Tgfa*, *Hb-egf*, *amphiregulin*, and *epiregulin* mRNA and relatively lower levels of other ligands, *Egf* and *betacellulin* (Fig. S1). Although all chondrocytes within articular cartilage were positive for EGFR staining (Figs. 1 A, b and 2 A, a), EGFR activity, indicated by phospho (p)-EGFR, was located mostly in surface chondrocytes and infrequently in calcified chondrocytes (Figs. 1 A, c and 2 A, b). DMM surgery induces OA-like phenotypes in mouse knees. At 1 mo after DMM, when articular cartilage had yet to show any morphological changes, EGFR staining remained throughout the cartilage, but p-EGFR staining had already been diminished at the surface (Fig. 1 A, d–f). In line with these data, healthy human articular cartilage exhibited strong EGFR staining throughout the cartilage (Fig. 1 B, b) and p-EGFR staining only at the superficial layer (Fig. 1 B, c). At an early OA stage, when the superficial zone (SZ) was still intact but proteoglycan staining was partially lost (Fig. 1 B, d), p-EGFR staining was remarkably reduced (Fig. 1 B, f). These expression and activity profiles strongly implicate a potential role of EGFR signaling in the SZ of healthy and diseased articular cartilage.

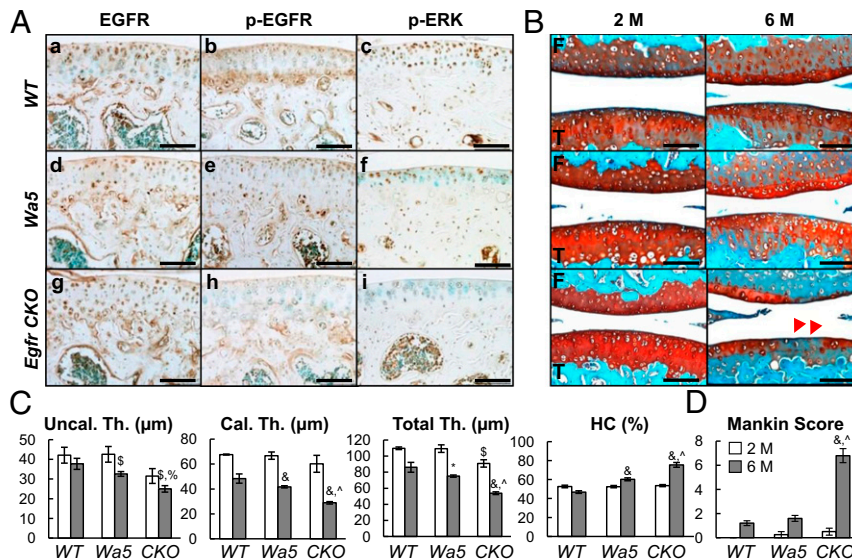


Fig. 2. EGFR signaling is important for articular cartilage structure. (A) Immunostaining of EGFR (a, d, and g), p-EGFR (b, e, and h), and p-ERK (c, f, and i) in tibial articular cartilage of 2-mo-old WT (a–c), *Wa5* (d–f), and *Egfr* CKO (g–i) mice. (Scale bars, 100 μ m.) (B) Safranin O staining of mouse joints at 2 and 6 mo of age. Red arrows point to reduced cartilage thickness and staining intensity in CKO mice. F, femur; T, tibia. (Scale bars, 100 μ m.) (C) Average thicknesses of uncalcified (Uncaled Th.), calcified (Cal. Th.), and total (Total Th.) articular cartilage in femoral condyle were quantified. The percentage of hypertrophic chondrocyte (HC) in both femoral condyle and tibial plateau was quantified together. (D) Mankin score confirms that CKO mice develop early OA symptoms at 6 mo of age. $n = 6$ per age per genotype. * $P < 0.05$, $^sP < 0.01$, $^{\&}P < 0.001$ vs. WT; $^{\%}P < 0.01$, $^{\wedge}P < 0.001$ vs. *Wa5*.

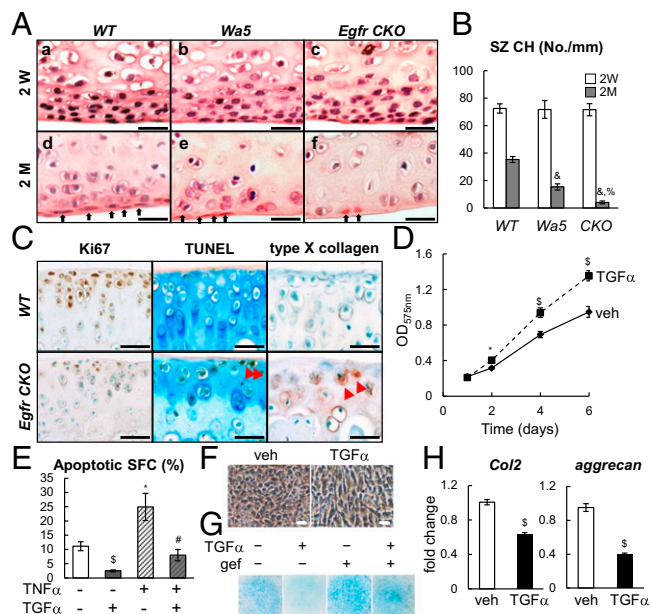


Fig. 3. EGFR signaling maintains the number of chondrocytes in the SZ. (A) H&E staining of femoral articular cartilage in WT (a and d), *Wa5* (b and e), and *CKO* (c and f) mice at 2 wk (a–c) and 2 mo (d–f) of age. Black arrows point to superficial chondrocytes. (Scale bars, 50 μ m.) (B) The number of superficial chondrocytes was quantified. $n = 5$ per age per genotype. $^*P < 0.001$ vs. WT; $^{\%}P < 0.01$ vs. *Wa5*. (C) Immunostaining of Ki67, TUNEL, and type X collagen in 2-mo-old mouse tibia. Red arrows point to positive cells. (Scale bars, 70 μ m.) (D) TGF α stimulates the proliferation of superficial chondrocytes. Superficial chondrocytes received either vehicle or TGF α (100 ng/mL) treatment on day 1. Cells were harvested at indicated time points for MTT assay. $^*P < 0.05$, $^{\$}P < 0.01$ vs. vehicle. (E) TGF α inhibits the apoptosis of superficial chondrocytes. Superficial chondrocytes were serum-starved overnight, pretreated with TNF α (25 ng/mL), and then treated with TGF α (100 ng/mL) for 2 d before apoptosis assay. $^*P < 0.05$, $^{\$}P < 0.01$ vs. serum starvation only; $^{\#}P < 0.05$ vs. serum starvation plus TNF α . (F) TGF α maintains the elongated cell shape in superficial cells after cells were cultured in chondrogenic differentiation medium for 10 d. (Scale bars, 20 μ m.) (G) Activating EGFR suppresses chondrogenic differentiation of superficial chondrocytes. Cells were cultured in chondrogenic differentiation medium with or without TGF α and gefitinib (gef, 10 μ M). Alcian blue staining was performed after 10 d. (H) Real-time RT-PCR analysis shows that 2 d of TGF α treatment suppressed the expression of cartilage matrix genes *Col2* and *aggrecan* in superficial chondrocytes. $^{\$}P < 0.01$ vs. vehicle.

Deficiency in Chondrogenic EGFR Signaling Leads to Abnormal Articular Cartilage Development.

Next we used a chondrogenic *Egfr* *CKO* (*Col2-Cre Egfr^{Wa5/flox}*) mouse model (Fig. S2) to investigate the action of EGFR in the articular cartilage. We previously demonstrated that EGFR activity in chondrocytes isolated from *CKO* mice and their sibling controls follows this sequence: WT > *Wa5* >> *CKO* (15). Although *CKO* mice expressed EGFR ubiquitously in the articular cartilage (Fig. 2A, g) due to the presence of a *Wa5* allele, the amounts of p-EGFR and p-ERK, both EGFR signaling targets, were indeed drastically reduced compared with WT (Fig. 2A, h and i). Consistent with cell culture results, the staining patterns of p-EGFR and p-ERK in *Wa5* cartilage were more similar to WT (Fig. 2A, e and f).

At 2 mo of age, compared with WT and *Wa5*, *Egfr* *CKO* mice had slightly but noticeably reduced total thickness of articular cartilage (Fig. 2B and C and Fig. S3). At 6 mo of age, this reduction became much more evident in *CKO* mice, and *Wa5* mice started to show similar changes. This drastic reduction of total thickness in *CKO* mice was accompanied by decreases in both uncalcified and calcified zones and an increase in the percentage of morphologically hypertrophied cells (Fig. 2C). In addition, *CKO* cartilage had less Safranin O staining and exhibited histological defects, such as minor fibrillation and cleaving at the

cartilage surface, resulting in an early OA-like phenotype with a Mankin score of 6.8 ± 0.6 (Fig. 2D).

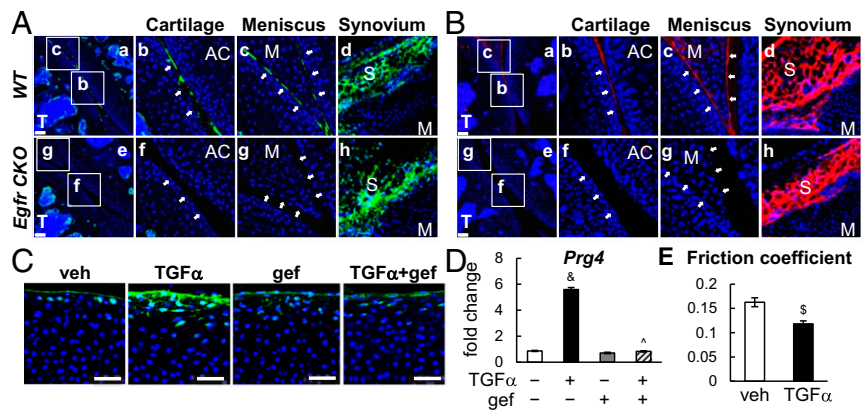
EGFR Is Required for Maintaining Superficial Chondrocytes. Because cartilage surface possesses a high EGFR activity, we next took a close look at the SZ in *CKO* mice. Interestingly, although the structure of articular cartilage appeared normal in 2-wk-old animals (Fig. 3A, a–c and B), the superficial layer in 2-mo-old *Wa5* and *CKO* mice had 56.6% and 88.5%, respectively, less cells than that in WT mice (Fig. 3A, d–f and B). Decreases in chondrocyte cellularity were also noticeable in the rest of uncalcified cartilage but not as prominent as in the SZ (Fig. 3A, f). Those superficial cells in *CKO* mice had less Ki67 staining but more TUNEL staining, and their deeper layer showed positive type X collagen (Fig. 3C and Fig. S4), suggesting that EGFR signaling is important for regulating the proliferative ability and cell survival of superficial chondrocytes and hypertrophy of chondrocytes. Consistently, activating EGFR by TGF α stimulated proliferation of cultured superficial chondrocytes (Fig. 3D) and increased resistance to apoptosis after serum depletion and TNF α treatment (Fig. 3E). Upon chondrogenic differentiation, TGF α -treated cells maintained a flat cell shape and a low level of proteoglycan accumulation, whereas vehicle-treated cells became polygonal in shape and accumulated alcian blue-stained proteoglycan (Fig. 3F and G). Cotreatment of gefitinib, an EGFR inhibitor, abolished this effect of TGF α (Fig. 3G). TGF α also attenuated the expression of two cartilage matrix genes, *Col2* and *aggrecan* (Fig. 3H). These data suggest a critical role of EGFR signaling in maintaining cell number and properties of superficial chondrocytes.

EGFR Signaling Promotes the Lubrication Function of Articular Cartilage. One key function of superficial chondrocytes is producing boundary lubricants Prg4 and hyaluronic acid (HA). In WT joints, most cartilage and meniscus surfaces were covered by Prg4 (Fig. 4A, a–c) and HA (Fig. 4B, a–c). Strikingly, Prg4 and HA signals were nearly absent at those surfaces in *CKO* joints (Fig. 4A, e–g and B, e–g). Note that their amounts in synovial membrane were not affected (Fig. 4A, d and h and B, d and h). These results are consistent with the p-EGFR staining pattern in our *CKO* model in which EGFR activity was also reduced at the meniscus surface but not in synovial membrane (Fig. S5).

The loss of lubricants in *CKO* articular cartilage could be a direct regulatory effect of EGFR signaling on *Prg4* expression or an indirect effect due to decreased superficial chondrocyte number. Indeed, primary culture experiments showed that TGF α stimulates *Prg4* expression in chondrocytes in a time-dependent fashion (Fig. S6A) and that this up-regulation can be abolished by gefitinib (Fig. S6B). Bovine articular cartilage explant represents a superior model to study the actions of growth factors under controlled and physiologically relevant conditions in a 3D environment. Using the uppermost 1 mm of bovine cartilage surface, we confirmed that TGF α increases *Prg4* expression at both mRNA and protein levels in an EGFR-dependent manner (Fig. 4C and D), proving that EGFR autonomously regulates *Prg4* in the articular cartilage. Most importantly, tribological testing revealed that 3 d of TGF α treatment significantly reduces the equilibrium friction coefficient of articular surface by 28% (Fig. 4E), demonstrating that EGFR signaling is an important regulator of cartilage lubrication function.

Deficiency of EGFR Signaling Weakens the Articular Cartilage. The principle function of articular cartilage is to provide load transmission and energy dissipative shock absorption during joint motion. Therefore, mechanical properties, mainly determined by cartilage extracellular matrix, are important parameters in characterizing cartilage function. Under the polarized light microscope, we observed that WT mice had mostly horizontally aligned collagen fibrils (in blue color, Fig. 5A, b) at the cartilage surface and perpendicularly aligned fibrils (in red color, Fig. 5A, b) with high collagen organization (high retardation values, Fig. 5A, c)

Fig. 4. EGFR signaling promotes lubricant secretion from cartilage and meniscus surfaces. (A) Immunofluorescence staining of Prg4 (green) in 1-mo-old WT and *CKO* joints. The articular cartilage surface (b and f) and meniscus surface (c and g), pointed by white arrows, are magnified images from a and e. Synovium is shown in d and h. AC, articular cartilage; M, meniscus; S, synovium; T, tibia. (Scale bars, 70 μ m.) (B) Detection of HA (red) in 1-mo-old WT and *CKO* joints. The articular cartilage surface (b and f) and meniscus surface (c and g) are magnified images from a and e. Synovium is shown in d and h. (Scale bars, 70 μ m.) (C) Immunofluorescence shows that TGF α increases the Prg4 amount (green) at the bovine cartilage explant surface in an EGFR-dependent manner. Blue, DAPI. (Scale bars, 50 μ m.) (D) TGF α increased *Prg4* mRNA in the top 1-mm region of cartilage explant in an EGFR-dependent manner as assayed by real-time RT-PCR. [&]*P* < 0.001 vs. vehicle; [^]*P* < 0.001 vs. TGF α . (E) TGF α treatment reduced the friction coefficient of the articular surface in cartilage explants. *n* = 5 per group. [&]*P* < 0.01 vs. vehicle.



in the deep layers of cartilage. In contrast, the collagen fibrillar structure changed remarkably in the middle and calcified zones of *CKO* mice, resulting in random orientation of collagen fibers (mixed blue and red colors, Fig. 5*A, e*) and reduced collagen organization (lower retardation values, Fig. 5*A, f*). Interestingly, these changes are very similar to the disruption of the collagen fibril network in OA. As shown in Fig. S7, WT articular cartilage developed disoriented collagen fibrils and reduced collagen organization in the middle zone of degenerated cartilage at 2 mo after DMM.

We recently developed an atomic force microscopy (AFM)-based nanoindentation approach to accurately measure mouse cartilage surface mechanical properties. In line with the above polarized light microscopy data, we detected remarkable decreases in the effective nanoindentation modulus, E_{ind} , in both *Wa5* (58.7%) and *CKO* (74.7%) cartilage compared with WT (Fig. 5*B*). These data also match our current observation that DMM surgery rapidly reduced surface E_{ind} of WT mouse cartilage (16). Taken together, our results clearly demonstrate that EGFR signaling regulates mechanical properties of articular cartilage.

Loss of EGFR in Chondrocytes Remarkably Accelerates Cartilage Damage After DMM Surgery. Because the articular cartilage of *Egfr CKO* mice shows structural, functional, and mechanical defects, they should be prone to develop OA after joint instability. One month after DMM, while WT joints remained intact, *CKO* joints showed prominent cartilage damage, including surface fibrillation, clefts, and erosion (Fig. 6*A, c*), as well as remarkable reductions in uncalcified and calcified cartilage thicknesses (Fig. 6*B*). At 2 mo postsurgery, *CKO* joints developed a more severe

phenotype with a large part of articular cartilage eroded at the medial site (Fig. 6*A, f*), resulting in a maximum Mankin score (Fig. 6*C*). At 3 mo postsurgery, *CKO* joints lost almost the entire articular cartilage, with no measurements in both uncalcified and calcified cartilage thicknesses (Fig. 6*A, i* and *B*). Consistent with our previous report (17), *Wa5* DMM joints (Fig. 6*A, b, e*, and *h*) had modestly accelerated OA development compared with WT, but their symptoms were much milder compared with *CKO*.

Consistent with these cartilage phenotypes, *Egfr CKO* mice developed several late OA phenotypes. First, although the lateral site of WT joints remained healthy, OA symptoms in *CKO* mice had propagated from the medial to lateral site (Fig. 7*A* and *B*). Second, we observed a substantial increase in the thickness of the subchondral bone plate under the damaged cartilage area at the medial site in *CKO* mice (Fig. 7*C*), suggesting a subchondral sclerosis phenotype. Third, E_{ind} of the *CKO* DMM site increased remarkably to 4.3-fold of WT DMM joints, even more than WT sham joints (Fig. 7*D*), suggesting that the indented surface on *CKO* DMM joints is most likely calcified cartilage or even subchondral bone. Note that at the same time, DMM resulted in a 67.2% reduction in E_{ind} of articular cartilage surface in WT mice (Fig. 7*D*). As OA further develops, the DMM site of WT mice also exhibited the same or elevated E_{ind} compared with the sham site (16). Fourth, pain is the major symptom in OA. To detect mechanical allodynia, we performed von Frey assay (18) and found that *CKO* DMM legs were extremely sensitive to the stimulus, with a paw withdrawal threshold (PWT) only 29.7% of that observed in WT DMM legs (Fig. 7*E*), implicating that they experience much more pain than others after DMM. Lastly, although we did not observe any osteophyte in sham and DMM joints of 16 WT mice and 9 *Wa5* mice, 6 out of 18 sham and 8 out of 18 DMM joints of *Egfr CKO* had osteophytes originating from either the tibial or femoral growth plate (Fig. 7*F*). Combining all evidence together, we conclude that *Egfr CKO* mice reached the late stage of OA at 2 mo postsurgery through a pace much faster than WT mice. Additionally, we found that there are no changes in the trabecular bone structure in the metaphyseal area among WT, *Wa5*, and *CKO* mice regardless of surgery (Fig. S8*A* and *B*). Serum levels of bone formation (osteocalcin) and resorption (CTX) markers also remained the same among these mice (Fig. S8*C*). These data clearly demonstrate that our *CKO* model has no effect on overall bone metabolism.

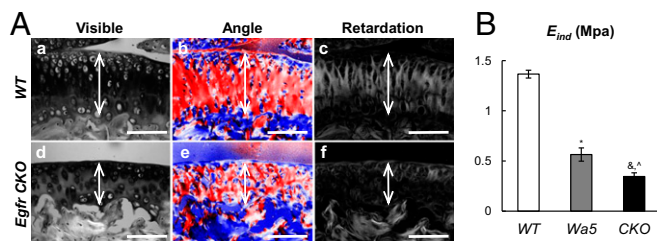


Fig. 5. EGFR signaling regulates the mechanical properties of articular cartilage. (A) Representative polarized light microscopy images of WT (*a–c*) and *Egfr CKO* (*d–f*) tibial articular cartilage at 5 mo of age. The blue and red colors in angle images are 90° apart in orientation. Double arrows depict the articular cartilage. (Scale bars, 100 μ m.) (B) AFM-based nanoindentation shows that EGFR deficiency significantly reduces the indentation modulus (E_{ind}) of mouse femoral cartilage surface as early as 2 mo of age. *n* = 6 per genotype. ^{*P} < 0.05, [&]*P* < 0.001 vs. WT; [^]*P* < 0.001 vs. *Wa5*.

Discussion

The SZ is an indispensable part of articular cartilage and plays critical roles in maintaining the integrity and function of articular cartilage and preventing their degeneration. However, little is known about how growth factors and hormones, as well as their downstream signaling pathways, regulate superficial chondrocytes and their biological and mechanical functions. Our current studies

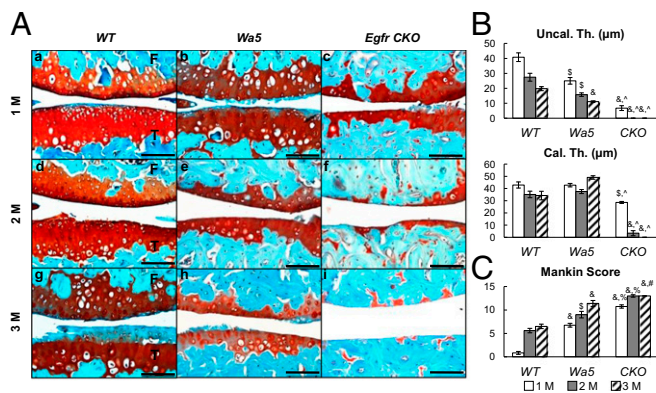


Fig. 6. EGFR chondrogenic deficiency causes severe OA after DMM surgery. (A) Safranin O staining of WT (a, d, and g), *Wa5* (b, e, and h), and *Egfr* CKO (c, f, and i) mouse joints at the medial site at 1 (a–c), 2 (d–f), and 3 (g–i) mo postsurgery. (Scale bars, 100 μm.) (B) Average thicknesses of uncalcified (Uncal. Th.) and calcified (Cal. Th.) cartilage were quantified. $n = 6$ per age per genotype. $^{\$}P < 0.01$, $^{\&}P < 0.001$ vs. WT; $^{\wedge}P < 0.001$ vs. *Wa5*. (C) The OA severity was measured by Mankin score. $^{\$}P < 0.01$, $^{\&}P < 0.001$ vs. WT; $^{\#}P < 0.05$, $^{\%}P < 0.01$ vs. *Wa5*. $n = 6$ per age per group.

identify EGFR and its ligands as an essential growth factor pathway that regulates chondrocyte number, boundary lubrication, and mechanical properties of this superficial layer. We found several EGFR ligands, such as *Tgfa*, *Hb-egf*, *amphiregulin*, and *epiregulin*, are expressed at abundant levels in mouse articular cartilage. Previous studies revealed that both *TGFα* (7) and *HB-EGF* (8) mRNA levels are increased in OA articular chondrocytes. Because activating EGFR by those ligands has both anabolic and catabolic activities on primary chondrocytes, whether an increase of EGFR ligand amount plays a destructive or a compensatively repairing role in OA is unclear. Nevertheless, our data demonstrate that reduction in EGFR activity leads to structurally, functionally, and mechanically compromised articular cartilage during development and drastically accelerates cartilage degeneration under normal and surgically induced OA conditions.

Our data clearly showed that EGFR signaling is required for obtaining a sufficient number of superficial chondrocytes by preserving their proliferation ability, promoting their survival, and maintaining their superficial properties. Previous studies with chondrocyte-specific knockout of *Mig-6* (10–12) also support our conclusion, as young mice exhibit hyperproliferation and hypercellularity in the top part of articular cartilage. A recent study identified *Prg4*-expressing cells located at the cartilage surface as chondrogenitors for deeper layers of the mature articular cartilage (19). We also observed a decrease in chondrocyte cellularity in an uncalcified area below the superficial layer, accompanied with an increase in the percentage of hypertrophic chondrocytes. These data indicate that EGFR might regulate chondrogenitors in the superficial layer of articular cartilage.

Prg4, an O-linked glycosylated protein, and HA, a polymer of disaccharides, coat the surface of articular cartilage, meniscus, and synovium and serve as two major boundary lubricants in the knee joints (20). Our studies clearly identify EGFR signaling as an important pathway that promotes the production of *Prg4* at the cartilage surface. Many studies suggest a synergistic interaction between *Prg4* and HA to promote more effective boundary lubrication (21–23). Interestingly, our data reveal that both lubricants are remarkably decreased in EGFR-deficient joints. How EGFR signaling regulates HA on the cartilage surface is not known. Because *Prg4* is required to concentrate HA to the tissue surface (21), an intriguing possibility is that EGFR indirectly regulates surface HA attachment by maintaining *Prg4*. Most importantly, our cartilage explant study explicitly demonstrated that EGFR signaling stimulates cartilage lubrication function. The reduced friction coefficient on the articular surface after *TGFα*

treatment could result from the increased secretion of either PRG4 or both PRG4 and HA. In summary, future studies on the interactions among the superficial chondroprogenitor pool, lubricant secretion, and mechanical strength of cartilage surface using our *Egfr* CKO mouse model will likely provide more mechanistic insights into articular cartilage homeostasis.

Defective cartilage surface in CKO mice inevitably results in cartilage degeneration. At 2–3 mo after DMM, although WT and *Wa5* mice only developed mild to moderate OA, CKO mice had already exhibited many late OA symptoms, such as a complete depletion of articular cartilage at the medial site, subchondral bone plate thickening underneath the damaged cartilage, moderate OA development at the lateral site, osteophyte formation, and a high level of joint pain. One potential concern of using our *Egfr* CKO mice to study OA progression, a disease in which subchondral bone also plays an important role, is that this model might not be chondrocyte-specific. A recent study implicated that *Col2-Cre* is capable of inducing fluorescence reporter expression in bone marrow mesenchymal progenitors (24). Because our previous studies have demonstrated an important regulatory role of EGFR signaling on bone marrow mesenchymal progenitors (25–28), it is possible that OA symptoms observed in *Egfr* CKO mice might partially result from changes in bone remodeling. However, we found that trabecular bone in the secondary spongiosa and serum bone markers are not altered in CKO mice, suggesting that *Col2* promoter-driven Cre does not efficiently delete the EGFR gene in bone marrow mesenchymal progenitors and thus does not affect bone formation directly. Our data indicate that *Rosa-Tomato* used in lineage tracing experiments seems to be a much more sensitive target for Cre than floxed *Egfr* allele in this *Col2-Cre* model.

Previous studies of EGFR signaling in articular cartilage development and degeneration focused on an indirect approach analyzing mice lacking *Mig-6*, an EGFR inhibitor (9–12). Because *Mig-6* is not specific to EGFR, those results are too complicated to clearly interpret EGFR function. To our knowledge, this is the first study unambiguously demonstrating the important roles of

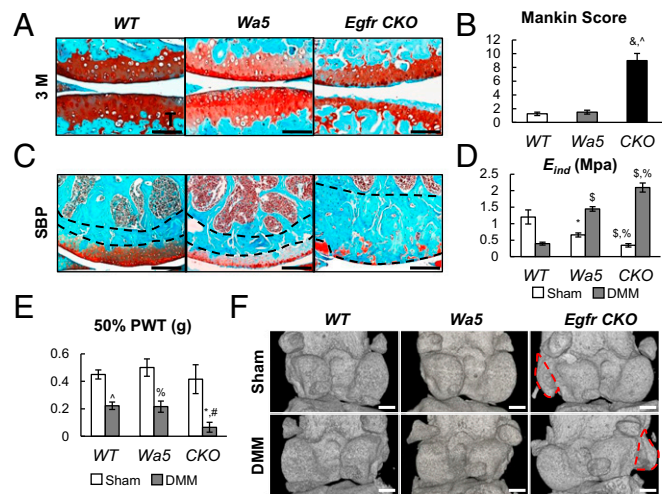


Fig. 7. *Egfr* CKO mice develop late OA symptoms after DMM surgery. (A) Safranin O staining of WT, *Wa5*, and *Egfr* CKO mouse joints at the lateral site at 3 mo postsurgery. (Scale bars, 100 μm.) (B) OA severity of the lateral site was measured by Mankin score. $n = 5$ per genotype. $^{\&}P < 0.001$ vs. WT; $^{\wedge}P < 0.001$ vs. *Wa5*. (C) *Egfr* CKO mice had thickened subchondral bone plates at the medial site at 2 mo after DMM surgery. Lines depict the area of the subchondral bone plate. (Scale bars, 100 μm.) (D) Both sham and DMM femurs were harvested at 2 mo postsurgery for nanoindentation to measure cartilage surface modulus. $n = 6$ per genotype. $^{\$}P < 0.05$, $^{\&}P < 0.01$ vs. WT; $^{\%}P < 0.01$ vs. *Wa5*. (E) von Frey assay was performed at 1 mo postsurgery. $n = 8$ per genotype. $^{\$}P < 0.05$ vs. WT; $^{\#}P < 0.05$, $^{\%}P < 0.01$, $^{\wedge}P < 0.001$ vs. sham. (F) 3D microCT images show osteophytes were only found in *Egfr* CKO joints. Red circles indicate osteophytes. (Scale bars, 500 μm.)

EGFR signaling in maintaining articular cartilage homeostasis. Our results illustrate that EGFR mainly promotes anabolic activity in articular cartilage, which is opposite from its catabolic activity during growth plate development and SOC formation discovered in our previous reports (14, 15). Because articular chondrocytes arise from a distinct progenitor population from growth plate and epiphyseal chondrocytes (29), the EGFR function we identified in endochondral ossification may not be the same in articular cartilage. Furthermore, the severe OA progression observed in *Egfr* *CKO* mice after DMM clearly indicates a protective function of EGFR signaling on articular cartilage against OA degeneration, which is consistent with our previous observation that EGFR inhibitor (gefitinib) treatment via oral gavage in mice enhances cartilage destruction after DMM (17). However, continuous infusion of another EGFR inhibitor, AG1478, in rats was shown to have opposite effects because it modestly delays OA progression after anterior cruciate ligament transection and partial medial meniscectomy (30). Because *Egfr* *CKO* mice already have defects in the superficial layer of articular cartilage before injury, it is difficult to dissociate the level of importance of EGFR signaling in this region from its function in other regions during OA development. Interestingly, we observed a loss of EGFR activity in superficial chondrocytes in both human and mouse samples at the OA initiation stage. The underlying mechanism is not known, but it provides substantial evidence that EGFR signaling must play an important role in OA disease. Currently we believe that EGFR

signaling has context-, stage-, and dose-dependent roles in OA progression. Future studies using inducible *Egfr* *CKO* mice with *aggrecan-CreER* (31) and superficial layer-specific knockout *Prg4-CreER* (19) are required to further elucidate spatiotemporal target cells and actions of this pathway and its effects on articular cartilage degeneration. Nevertheless, according to our studies, therapeutic agents specifically targeting cartilage surface EGFR should be considered as a novel direction for OA treatment.

Materials and Methods

All animal work performed in this report was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Details on animal studies, surgery, histology, human articular cartilage sample, AFM-nanoindentation analysis, chondrocyte culture, bovine cartilage explant culture and friction test, real-time RT-PCR analysis, polarized light microscopy analysis, OA pain analysis, microcomputed tomography (microCT) analysis, and statistical analysis are presented in *SI Materials and Methods* and real-time PCR primer sequences are given in *Table S1*.

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- Buckwalter JA, Mankin HJ, Grodzinsky AJ (2005) Articular cartilage and osteoarthritis. *Instr Course Lect* 54:465–480.
- Becerra J, et al. (2010) Articular cartilage: Structure and regeneration. *Tissue Eng Part B Rev* 16(6):617–627.
- Glasson SS, Chambers MG, Van Den Berg WB, Little CB (2010) The OARSI histopathology initiative—Recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartilage* 18(Suppl 3):S17–S23.
- Pritzker KP, et al. (2006) Osteoarthritis cartilage histopathology: Grading and staging. *Osteoarthritis Cartilage* 14(1):13–29.
- Rolauffs B, et al. (2011) Onset of preclinical osteoarthritis: The angular spatial organization permits early diagnosis. *Arthritis Rheum* 63(6):1637–1647.
- Citri A, Yarden Y (2006) EGF-ERBB signalling: Towards the systems level. *Nat Rev Mol Cell Biol* 7(7):505–516.
- Appleton CT, Usmani SE, Bernier SM, Aigner T, Beier F (2007) Transforming growth factor alpha suppression of articular chondrocyte phenotype and Sox9 expression in a rat model of osteoarthritis. *Arthritis Rheum* 56(11):3693–3705.
- Long DL, Ulici V, Chubinskaya S, Loeser RF (2015) Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is increased in osteoarthritis and regulates chondrocyte catabolic and anabolic activities. *Osteoarthritis Cartilage* 23(9):1523–1531.
- Zhang YW, et al. (2005) Targeted disruption of Mig-6 in the mouse genome leads to early onset degenerative joint disease. *Proc Natl Acad Sci USA* 102(33):11740–11745.
- Pest MA, Russell BA, Zhang YW, Jeong JW, Beier F (2014) Disturbed cartilage and joint homeostasis resulting from a loss of mitogen-inducible gene 6 in a mouse model of joint dysfunction. *Arthritis Rheumatol* 66(10):2816–2827.
- Shepard JB, Jeong JW, Mailhe NJ, O'Brien S, Dealy CN (2013) Transient anabolic effects accompany epidermal growth factor receptor signal activation in articular cartilage in vivo. *Arthritis Res Ther* 15(3):R60.
- Staal B, Williams BO, Beier F, Vande Woude GF, Zhang YW (2014) Cartilage-specific deletion of Mig-6 results in osteoarthritis-like disorder with excessive articular chondrocyte proliferation. *Proc Natl Acad Sci USA* 111(7):2590–2595.
- Pante G, et al. (2005) Mitogen-inducible gene 6 is an endogenous inhibitor of HGF/Met-induced cell migration and neurite growth. *J Cell Biol* 171(2):337–348.
- Zhang X, et al. (2011) The critical role of the epidermal growth factor receptor in endochondral ossification. *J Bone Miner Res* 26(11):2622–2633.
- Zhang X, et al. (2013) Epidermal growth factor receptor (EGFR) signaling regulates epiphyseal cartilage development through β -catenin-dependent and -independent pathways. *J Biol Chem* 288(45):32229–32240.
- Dooran B, et al. (2016) Nanoindentation modulus of murine cartilage: A sensitive indicator of the initiation and progression of post-traumatic osteoarthritis. *Osteoarthritis Cartilage* 25(16):30243–30246.
- Zhang X, et al. (2014) Reduced EGFR signaling enhances cartilage destruction in a mouse osteoarthritis model. *Bone Res* 2:14015.
- Piel MJ, Kroin JS, Im HJ (2015) Assessment of knee joint pain in experimental rodent models of osteoarthritis. *Methods Mol Biol* 1226:175–181.
- Kozhemyakina E, et al. (2015) Identification of a Prg4-expressing articular cartilage progenitor cell population in mice. *Arthritis Rheumatol* 67(5):1261–1273.
- Daniel M (2014) Boundary cartilage lubrication: Review of current concepts. *Wien Med Wochenschr* 164(5–6):88–94.
- Bonnevie ED, Galesso D, Secchieri C, Cohen I, Bonassar LJ (2015) Elastoviscous transitions of articular cartilage reveal a mechanism of synergy between lubricin and hyaluronic acid. *PLoS One* 10(11):e0143415.
- Das S, et al. (2013) Synergistic interactions between grafted hyaluronic acid and lubricin provide enhanced wear protection and lubrication. *Biomacromolecules* 14(5):1669–1677.
- Kwiecinski JJ, et al. (2011) The effect of molecular weight on hyaluronan's cartilage boundary lubricating ability—Alone and in combination with proteoglycan 4. *Osteoarthritis Cartilage* 19(11):1356–1362.
- Ono N, Ono W, Nagasawa T, Kronenberg HM (2014) A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. *Nat Cell Biol* 16(12):1157–1167.
- Chandra A, Lan S, Zhu J, Siclari V, Qin L (2013) Epidermal Growth Factor Receptor (EGFR) signaling promotes proliferation and survival in osteoprogenitors by increasing Early Growth Response Protein (Egr2) expression. *J Biol Chem* 288(28):20488–98.
- Qin L, et al. (2005) Amphiregulin is a novel growth factor involved in normal bone development and in the cellular response to parathyroid hormone stimulation. *J Biol Chem* 280(5):3974–3981.
- Zhu J, Shimizu E, Zhang X, Partridge NC, Qin L (2011) EGFR signaling suppresses osteoblast differentiation and inhibits expression of master osteoblastic transcription factors Runx2 and Osterix. *J Cell Biochem* 112(7):1749–1760.
- Zhu J, et al. (2012) Amphiregulin-EGFR signaling mediates the migration of bone marrow mesenchymal progenitors toward PTH-stimulated osteoblasts and osteocytes. *PLoS One* 7(12):e50099.
- Koyama E, et al. (2008) A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. *Dev Biol* 316(1):62–73.
- Appleton CT, et al. (2015) Reduction in disease progression by inhibition of transforming growth factor α -CCL2 signaling in experimental posttraumatic osteoarthritis. *Arthritis Rheumatol* 67(10):2691–2701.
- Henry SP, et al. (2009) Generation of aggrecan-CreERT2 knockin mice for inducible Cre activity in adult cartilage. *Genesis* 47(12):805–814.
- Zhang X, et al. (2011) Epidermal growth factor receptor plays an anabolic role in bone metabolism in vivo. *J Bone Miner Res* 26(5):1022–1034.
- Miettinen PJ, et al. (1999) Epidermal growth factor receptor function is necessary for normal craniofacial development and palate closure. *Nat Genet* 22(1):69–73.
- Wang K, Yamamoto H, Chin JR, Werb Z, Vu TH (2004) Epidermal growth factor receptor-deficient mice have delayed primary endochondral ossification because of defective osteoclast recruitment. *J Biol Chem* 279(51):53848–53856.
- Schneider MR, et al. (2012) Normal epidermal growth factor receptor signaling is dispensable for bone anabolic effects of parathyroid hormone. *Bone* 50(1):237–244.
- Aigner T, et al. (2010) Histopathology atlas of animal model systems—Overview of guiding principles. *Osteoarthritis Cartilage* 18(Suppl 3):S2–S6.
- Batista MA, et al. (2014) Nanomechanical phenotype of chondroadherin-null murine articular cartilage. *Matrix Biol* 38:84–90.
- Yasuhara R, et al. (2011) Roles of β -catenin signaling in phenotypic expression and proliferation of articular cartilage superficial zone cells. *Lab Invest* 91(12):1739–1752.
- Zhou Y, Park M, Cheung E, Wang L, Lu XL (2015) The effect of chemically defined medium on spontaneous calcium signaling of in situ chondrocytes during long-term culture. *J Biomech* 48(6):990–996.
- Zimmerman BK, et al. (2015) Role of interstitial fluid pressurization in TMJ lubrication. *J Dent Res* 94(1):85–92.
- Xia Y, Moody JB, Burton-Wurster N, Lust G (2001) Quantitative in situ correlation between microscopic MRI and polarized light microscopy studies of articular cartilage. *Osteoarthritis Cartilage* 9(5):393–406.