The Regulatory Role of EGFR Signaling in Adult Cartilage Homeostasis and Osteoarthritis

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Introduction

Osteoarthritis (OA) is the most common chronic condition of the joints with debilitating clinical symptoms including continuous pain, stiffness and limited motion. The uppermost superficial zone of articular cartilage is the first line of defense against OA initiation. It plays multifaceted roles in maintaining cartilage structure, function, and mechanical properties. We previously demonstrated that epidermal growth factor receptor (EGFR), a tyrosine kinase receptor, plays an essential role in maintaining this superficial zone during articular cartilage development using a cartilage-specific *Egfr*-deficient mouse model (*Col2-Cre Egfr*$^{Wa5/flox}$, *Egfr CKO*^{Col2Cre})[1]. However, this system did not distinguish whether the severe OA phenotypes observed in the *Egfr CKO*^{Col2Cre} mice after DMM is due to the inferior nature of cartilage before the surgery or the requirement of EGFR signaling in response to acute insults. We hypothesized that EGFR signaling is necessary for protection of adult articular cartilage from OA, and examined EGFR activity in human OA samples at different stages and studied surgery-induced OA phenotypes in mice with EGFR inactivation at adult stage.

Methods

Human OA articular cartilage samples

They were prepared from de-identified specimens obtained at the total arthroplasty of the knee joints. Their paraffin sections were stained by Safranin O/Fast green to evaluate OA stage[2] and neighboring sections were used for p-EGFR staining. *Animals- Aggrecan-CreER* mice were first crossed with *Egfr*$^{Wa5/+}$ (*Wa5* is a dominant negative allele of *Egfr*) to obtain *aggrecan-CreER Egfr*$^{Wa5/+}$, which was then crossed with *Egfr*$^{flox/flox}$ to generate *Egfr CKO*^{AgcER} mice and their *WT* (*aggrecan-CreER Egfr*$^{flox/+}$ and *Egfr*$^{flox/+}$) and *Wa5* (*Egfr*$^{Wa5/flox}$) siblings. Male mice at 3 months of age received tamoxifen (tam) injections (75 mg/kg/day × 5) followed by surgical destabilization of the medial meniscus (DMM) in right knees and sham operation in left knees a week later. In DMM surgery, the joint capsule was opened and the medial meniscotibial ligament was cut to destabilize the meniscu. In sham surgery, the joint capsule was opened but no further damage. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

Histology and immunohistochemistry (IHC)

Knee joints were fixed in 4% PFA, decalcified in 10% EDTA, and processed for paraffin sections followed by Safranin-O/fast green staining or p-EGFR, Ki67, TUNEL staining.

AFM Nanoindentation

Freshly dissected femoral condyle cartilage at 1 month post surgery was indented at more than 10 locations by a borosilicate colloidal spherical tip (R = 5 μm, nominal spring constant k = 7.4 N/m) with maximum indentation depth of ~1 μm at a 10 μm/s indentation rate using a Dimension Icon AFM (BrukerNano). The effective indentation modulus, E_{ind} (MPa), was calculated by fitting the whole loading portion of each indentation force–depth curve using the Hertz model as previously described[3].

Statistics

Data are expressed as means±SEM and analyzed by paired, two-tailed Student's t-test.

Results

Healthy human articular cartilage exhibited strong staining of p-EGFR, the activated EGFR, in chondrocytes at the superficial layer and gradually decreased staining in chondrocytes deep into the middle zone (Figure 1A, B). This staining was remarkably reduced at early OA when superficial zone is still intact (Figure 1C, D). At middle OA stage when superficial layer is depleted, p-EGFR was detected in some chondrocytes in middle and deep zones (Figure 1E, F). At late stage OA, most cell clusters beneath damaged cartilage surface had strong p-EGFR staining (Figure 1G, H). To explore the role of EGFR signaling in adult cartilage and in OA progression, we injected tam into 3-month-old *Egfr CKO*^{AgcER} mice and their *WT* and *Wa5* controls and then performed sham/DMM surgeries. The sham legs in the tam-treated *CKO*^{AgcER} mice showed great decreases in p-EGFR and Ki-67 (proliferation marker) staining and an increase in TUNEL (apoptosis marker) staining in the superficial chondrocytes at 4 months of age (Figure 2). At 6 months of

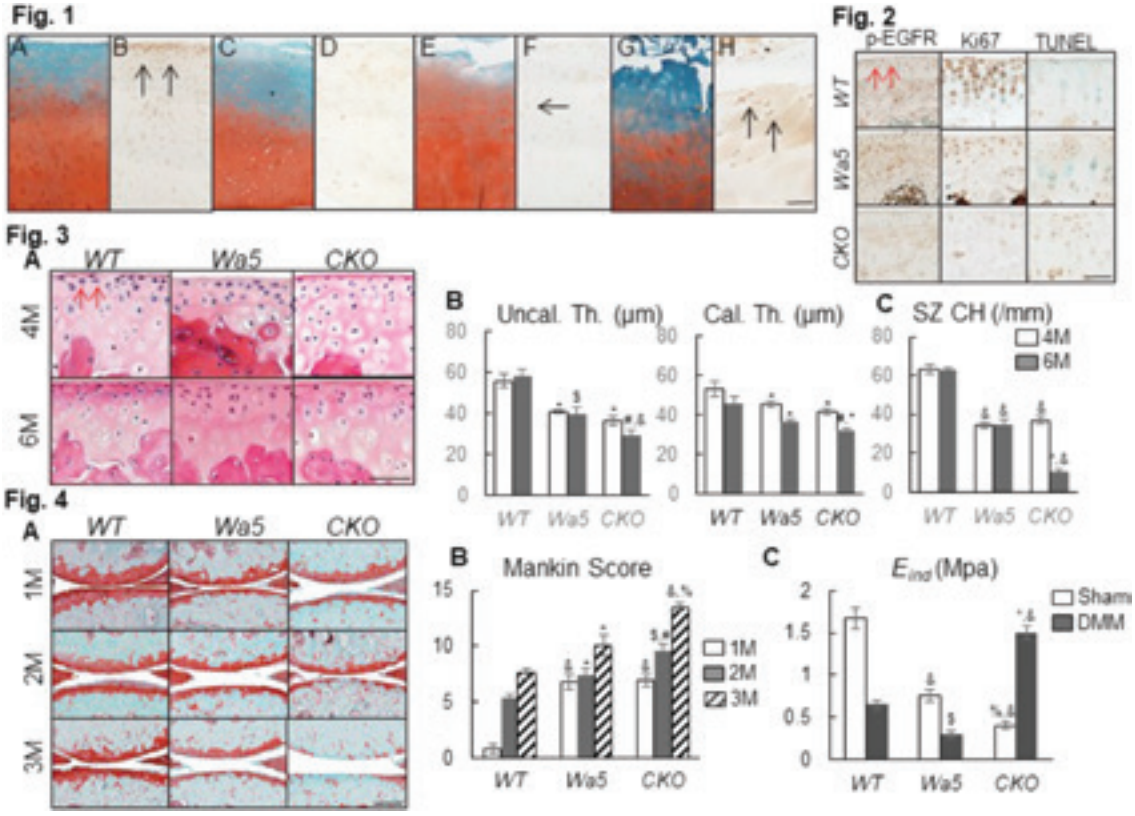

Figure 1. EGFR signaling in human healthy and diseased articular cartilage samples. **(A, B)** Healthy cartilage. **(C, D**) OA cartilage at early stage (OARSI Score 1-2). **(E, F)** OA cartilage at middle stage (OARSI Score 3-4). **(G, H)** OA cartilage at late stage (OARSI Score 5-6). **(A, C, E, G):** Safranin O/fast green staining; **(B,D,F,H):** IHC of p-EGFR. Arrows point to positive cells. n= 3/stage. Bar: 200 μm. **Figure 2.** IHC of p-EGFR, Ki67, and TUNEL in 4-mo-old sham tibia of *WT*, *Wa5*, and *Egfr CKO*^{AgcER} (Tam: 3-mo-old). Arrows point to superficial layer. Bar, 100 μm. **Figure 3.** EGFR signaling maintains adult cartilage structure. **(A)** H&E staining of femoral articular cartilage in 4 and 6-mo-old mice (Tam: 3-mo-old). Bar, 50 μm. **(B)** The thicknesses of uncalcified and calcified cartilage were quantified. **(C)** The number of superficial chondrocytes was quantified. n = 5/group. **Figure 4.** Chondrogenic EGFR deficiency in adult mice causes severe OA after DMM. **(A)** Safranin O staining of *WT*, *Wa5*, and *Egfr CKO*^{AgcER} joints at 1, 2, and 3 mo post DMM (Tam: 3-mo-old). Bar, 200 μm. **(B)** OA severity was measured by Mankin score. n = 6/age/genotype. **(C)** Femoral cartilage surface E_{ind} was measured at 1 mo post DMM. n = 6/genotype. *: $P < 0.05$, $: $p < 0.01$, &: $p < 0.001$ vs. *WT*; #: $p < 0.05$, %: $p < 0.01$, ^: $P < 0.001$ vs. *Wa5*.

age, these mice displayed reduced cartilage thickness at both uncalcified and calcified zones and diminished superficial chondrocytes (Figure 3), demonstrating that EGFR signaling is critical for adult cartilage homeostasis. One month after DMM, while WT joints remained intact, both *CKO*^{AgcER} and *Wa5* joints showed moderate cartilage damage (Figure 4A, B). Nanoindentation of femoral cartilage surface revealed that DMM causes a similar decrease of E_{ind} in *WT* and *Wa5* cartilage but an increase of E_{ind} in *CKO*^{AgcER} surface (Figure 4C), suggesting that *CKO*^{AgcER} has more advanced OA progression than *WT* and *Wa5*. At 2 months postsurgery, *CKO*^{AgcER} joints developed severer phenotype than controls with erosion of a large part of articular cartilage (Figure 4A, B). At 3 months postsurgery, *CKO*^{AgcER} joints developed late OA phenotypes characterized by loss of most articular cartilage (Figure 4A, B) and subchondral bone plate thickening at the medial site (data not shown). The OA phenotypes were also seen at the lateral sites in the *CKO*^{AgcER} (data not shown), which were not observed in control mice.

Discussion

Analyzing EGFR activity profile in human articular cartilage samples with different OA stages strongly implicates a potential role for EGFR signaling in the superficial layer of healthy and diseased articular cartilage. By using an inducible system, we were able to demonstrate that EGFR is an essential growth factor pathway in adult mouse cartilage that regulates the superficial layer by preserving their proliferation ability and promoting their survival. Reduction in EGFR activity in adult articular cartilage leads to accelerated cartilage degeneration under surgically induced OA conditions, indicating protective function of EGFR signaling on articular cartilage against OA degeneration.

Significance

Our studies uncover critical EGFR actions in adult knee articular cartilage and provide proof-of-principle evidence for targeting this novel pathway as OA therapies.

References

1. Jia H, et al. Proc Natl Acad Sci U S A 2016;113:14360–5
2. Pritzker KP, et al. OsteoArthritis and Cartilage, 2006;14:13-29.
3. Doyran B, et al. Osteoarthritis Cartilage 2017; 25:108-117.