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Following the Trajectory of Osteoarthritis Development Through Serial Near Infrared Fluorescence Imaging of MMP Activities

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Abstract

Objectives—A major hurdle in osteoarthritis (OA) research is the lack of sensitive detection and monitoring methods. It is hypothesized that proteases, such as matrix metalloproteinases (MMPs), are upregulated at early stages of OA development. The aim of this study was to investigate if a near infrared fluorescence (NIRF) probe activated by MMPs could visualize *in vivo* OA progression starting from its early stages.

Methods—Using an MMP activatable NIRF probe (MMPsense680), we assessed the upregulation of MMP activity *in vitro* by incubating human chondrocytes with the pro-inflammatory cytokine IL-1 β . MMP activity was then evaluated *in vivo* serially in a chronic, injury-induced OA mouse model. For tracking MMP activity over time, mice were imaged 1 – 8 weeks post OA inducing surgery. Imaging results were correlated with histology.

Results—*In vitro* studies confirmed that NIRF imaging could identify enhanced MMP activity in IL-1 β -treated human chondrocytes. *In vivo* imaging showed significantly higher fluorescent intensity in OA knees compared to sham knees (control) of the same mice. Additionally, the total emitted fluorescence intensity steadily increased over the entire course of OA progression that was examined. NIRF imaging results correlated with histological analysis, which showed an increase in articular cartilage structural damage over time.

Conclusions—Imaging of MMP activity in an OA mouse model provided sensitive and consistent visualization of OA progression, beginning from the early stages of OA. In addition to

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facilitating the preclinical study of OA modulators, this approach has the potential for future human translation.

Osteoarthritis (OA) is a chronic disorder that causes long-term disability and is associated with joint injury, aging, and obesity (1). OA is characterized by joint cartilage destruction and is accompanied by chondrocyte cell death and osteophyte formation. Currently, OA research lacks optimal detection and monitoring methods for assessing *in vivo* OA progression, making it difficult to efficiently evaluate treatment option efficacy in preclinical models (2–4).

One key feature of OA joint destruction is the induction of matrix metalloproteinases (MMPs) that cleave cartilage matrix proteins (5, 6). However, the exact pattern of *in vivo* MMP induction is still unknown. For example, while MMP2 and MMP13 proteins were detected in synovial fluid from OA patients (7), MMP3 expression was shown to be downregulated in late stage OA human femoral cartilage compared to controls (8). Furthermore, a mild injury-induced OA mouse model caused by destabilization of the medial meniscus (DMM) (9), demonstrated increased MMP3 and MMP13 mRNA expression in OA knees compared to sham controls, but did not exhibit a consistent change over time (10, 11). It is also likely that MMP protein expression and activity differ from mRNA expression at different stages of OA development, as MMP inhibitors (TIMPs) are also induced in OA (11). As many of these MMPs have overlapping functions, the dynamics of the net MMP activity that shapes the landscape of matrix degradation throughout early and later OA stages is still not clear.

It is challenging to assess MMP activity over the course of disease development *in vivo* in OA research. While mouse strains are often used in preclinical studies to examine genetic determinants of OA, synovial fluid extraction from mice requires animal sacrifice (4). Furthermore, synovial fluid analysis does not directly reflect MMP activities within all joint tissues, which is important as OA is increasingly recognized as a whole joint disease (12, 13). However, this is an important area of investigation as a change in overall MMP activity may sensitively reflect metabolic changes in the OA joint prior to gross structural damage.

Environmentally-sensitive imaging probes combined with near infrared fluorescence (NIRF) technology can non-invasively reflect the real-time metabolic, enzymatic, and physiological tissue states. Using a synthetic enzyme-specific substrate conjugated with a fluorescence module that fluoresces upon enzymatic activation, NIRF imaging may be able to detect the net catabolic activity of enzymes activated under OA conditions. In this study, we used an MMP-activatable NIRF probe to examine the trajectory of MMP activity in the knee joint of the DMM mouse model (9). We found that joint NIRF signals steadily increased starting from early OA development to the end time point of our investigation, suggesting that NIRF can be used as a sensitive and minimally invasive measure for real-time joint degeneration in an *in vivo* OA mouse model.

MATERIALS AND METHODS

In Vitro Chondrocyte Cultures and RT-PCR analysis

Primary normal human knee articular chondrocytes (nHACs, Lonza) were redifferentiated in alginate beads according to Lonza's established protocol using chondrogenic differentiation media (Lonza) containing TGF β and IGF-1. For 2D cultures, redifferentiated nHACs were cultured for 4 days in chondrogenic differentiation media that contained 0, 1, or 4ng/mL IL-1 β (Peprotech). For 3D cultures, nHACs were seeded into the silk scaffolds (graciously provided by David Kaplan, pore size of 100–200 μ m generated with solvent evaporation using HFIP) and cultured for 4 days with chondrogenic differentiation media that contained 0, 5 or 10ng/mL IL-1 β . Total RNA from cultured nHACs was isolated with the Qiagen RNeasy Mini Kit (Qiagen) and cDNA was generated using M-MLV Reverse Transcriptase (Invitrogen). PCR was performed using the iQ5 Real-Time PCR Detection System (Bio-Rad). All RT-PCR analyses were normalized to TBP expression (14). Primer sequences are available upon request.

Experimental Animals

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committees at Tufts University and Massachusetts General Hospital. Wild-type CD1 male mice were purchased from Charles River Laboratories. All mice were caged in groups under standard conditions.

Surgical Model of OA in Mice

Destabilization of the medial meniscus (DMM) surgery was performed on 7-week-old CD1 male mice according to the established protocol (9). Briefly, under isoflurane anesthesia, the right knee joint was opened along the medial border of the patellar ligament and the medial meniscotibial ligament (MMTL) was severed. Sham surgery was performed on the left knee of the same mice, which involves the visualization but not transection of the MMTL.

NIRF *in vitro* imaging

For *in vitro* MMPsense680 activation, 10ng of pro-enzymes (MMP-1, 2, 9, and 13; Anaspec) were added to 0.4 μ M MMPsense680 (Perkin Elmer) and 100 μ M APMA (Sigma) in a 96-well black bottom plate and incubated at 37°C. In the enzyme combination experiment, 2.5ng/enzyme was used. For inhibiting MMP activities, 100 μ M of the pan MMP inhibitor PD166793 (Anaspec) in 0.5% DMSO was used. As a control, 0.05ng of trypsin was incubated with 0.4 μ M of MMPsense680. For negative controls, pro-enzymes, APMA, MMPsense680 alone, or pro-enzymes with MMPsense680 but without APMA were used. Plates were read in the Xenogen IVIS 200 Biophotonic Imaging Unit, and signal intensities were analyzed using Living Image software. NIRF signals were calculated as the radiance from APMA activated MMPs subtracted by the radiance from the pro-MMP without APMA.

For *in vitro* NIRF imaging on cultured chondrocytes, 0.4 μ M MMPsense680 was added to the culture medium for 4 hours (2D cultures) or 16 hours (3D cultures). Live cultures were then examined using a Zeiss LSM 510 confocal microscopy system (Carle Zeiss) for NIR

fluorescence (633nm excitation, 700nm emission). Hoechst dye (Invitrogen) was added to localize the cells.

NIRF *in vivo* imaging and data analysis

For *in vivo* imaging, the hair of the mouse hind limb was removed to reduce autofluorescence. Under isoflurane anesthesia, MMPSense680 (4μL of 13.3μM) was directly injected through the patellar ligament into the knee joint cavity using a 30G needle. Afterward, the mice were brought out of anesthesia and allowed to roam freely for 2 hours until imaging. Unless otherwise noted, immediately before imaging an approximately 1.5 cm skin incision was created directly over the patellar ligament of each knee to allow imaging without skin overlying the joint. Images were acquired using the Multispectral Imaging System (In Vivo Pro; Carestream) in the following sequence 1) white light 2) X-ray 3) near infrared scanning with 530–680 nm excitation filters and 700 nm emission filter. The number of mice used for imaging at each time point are: n=7, 8, 7, 8, 4, 8, 4, 8, for weeks 1–8 respectively, with a total of 54 mice used.

Fluorescence signal intensity of the NIRF data was measured using the standard analysis software from Carestream. A region of interest was drawn over each knee joint to quantify the mean fluorescent signal intensity and the area of increased signal within the raw image. The fluorescence signal intensity of the mouse skin was measured as the background signal and subtracted from the raw data for each knee joint to internally calibrate each image. Total signal intensity ratio was calculated as (DMM knee mean fluorescence signal intensity × DMM knee area of increased signal) / (sham knee mean fluorescence intensity × sham knee area of signal). Using the 8-bit NIRF images in ImageJ, a straight line was placed over the regions of interest and the “plot profile” of the arbitrary signal intensity values was generated. Using the “surface plot” analysis, the arbitrary values of intensities over the whole raw images were shown in a three-dimensional plot.

Histological Analysis

Knee joints were isolated and fixed in 4% paraformaldehyde for 24 hours, decalcified in 10% EDTA for 1 week, and then embedded in paraffin. 5μm serial sections were cut sagittally across the femorotibial joint. Sections every 40μm were stained with 0.1% safranin O and counterstained with hematoxylin. Fast green counterstaining was also used when indicated. To quantify observed cartilage damage, 9 stained sections from each sample were pooled, randomized and blindly scored using established articular cartilage structure and safranin O scoring systems (15). The femur and tibia were scored separately and added together to calculate a total joint score for each section. The sections were blindly analyzed for two additional OA histological parameters, tibial chondrocyte cell death and osteophyte maturity, with established methods (10, 15, 16).

Light microscopy

Bright field images of chondrocytes were taken using the Olympus IX-71 microscopy and the Olympus DP70 digital camera. The optical parameters and camera exposure time were kept constant between samples.

Statistical Analysis

Data are shown as mean $\pm$ SEM. The semiquantitative histological scoring systems were evaluated using nonparametric statistical analyses. All other experiments were evaluated with a Student's t-test or analysis of variance (ANOVA) with post-hoc tests for pairwise comparisons. Normality of the data was tested using the Shapiro-Wilk normality test. Best-fit line equations and R^2 values were determined using a second order polynomial regression. Spearman correlation was used for correlation analyses. A p value of less than 0.05 was considered significant in all cases. All statistics were performed using GraphPad software (Version 6.0d).

RESULTS

MMP sensitive NIRF probe exhibits enhanced fluorescent signals in IL-1 β -treated chondrocytes *in vitro*

We first confirmed the ability of the NIRF probe MMPsense680 to detect MMP activity *in vitro*. Recombinant pro-MMPs were incubated with APMA, a known activator of MMPs (17), as well as with MMPsense680, and the resulting NIRF signal was measured. At 1hr, the signals were almost the same as the background or even slightly below, as was the case for MMP13 (Fig. 1A). However, in the following hours, the signals rose steadily. MMP13 caused the MMPsense680 strongest activation, followed by MMP9, MMP2, and then MMP1, respectively (Fig. 1A). An equal mixture of the recombinant MMPs (MMP-mix) resulted in a similar activation trend (Fig. 1B). A pan-MMP inhibitor, PD 166793, inhibited MMP-mix-induced NIRF signal, demonstrating that the observed fluorescence specifically resulted from active MMPs (Fig. 1B). Trypsin was used as a positive control to confirm that fluorescence activation of the probe was indeed caused by proteolytic cleavage (Fig. 1C).

We then tested whether chondrocytes treated with the pro-inflammatory cytokine IL-1 β could cause an increase in the MMPsense680 NIRF signal, as IL-1 β is known to induce MMP expression and activity (18–21). Indeed, IL-1 β treatment of normal human articular chondrocytes (nHACs) led to the induction of MMP1, MMP2, MMP9 and MMP13 expression (Fig. 1D). Furthermore, these chondrocytes exhibited a more elongated morphology (Fig. 1E) and enhanced MMP-activated NIRF signal by IL-1 β treatment in a dosage-dependent manner (Fig. 1F). Similarly, nHACs grown in 3D silk scaffolds also demonstrated IL-1 β dosage-dependency in the upregulation of MMP-activated NIRF signal (Fig. 1G).

MMP sensitive NIRF probe exhibits enhanced signals *in vivo* in DMM OA mouse

To determine whether MMP sensitive NIRF probes can be used *in vivo* to detect cartilage destruction in the OA knee, we performed destabilization of the medial meniscus (DMM) surgery on CD1 male mice. We chose the DMM surgery as an *in vivo* OA model because it has been shown to reliably cause mild injury-induced OA in mice (9). To minimize potential differences in intrinsic MMP activities between individuals, we performed DMM surgery on the right knee and sham surgery on the left knee of the same mouse as an internal control.

At 7 weeks post DMM we injected the NIRF probe MMPsense680 intraarticularly to measure current MMP activity level in the joint. Previous studies on MMP activities in the aorta and the brain have established that MMPsense680 is also activated by MMPs *in vivo*, as mice treated with an MMP13 inhibitor and MMP9^{-/-} mice demonstrated largely reduced MMPsense680 fluorescence (22, 23). To determine the optimal time for fluorescence imaging in DMM mice, mice were imaged at several time points after probe injection. At all measured time points, the DMM knee demonstrated a higher fluorescent signal than the sham knee of the same mouse (Fig. 2A). NIRF signals were quantified to calculate the mean fluorescence intensity for each knee and the DMM:sham total signal intensity ratio (i.e. mean fluorescence $\times$ area of signal) for each mouse (Fig. 2B, 2C). Results of both analyses indicated significantly higher MMP activity in the OA knee compared to the sham knee and that the most significant difference between the two knee joints was observed at 2 hours post injection (Fig. 2A–C). Therefore this time interval was used in all subsequent experiments. To delineate the location of the NIRF signal after probe injection, NIRF images were overlaid with X-ray images. When these images are limited to the brightest 50% or 25% of the collected NIRF signal, the X-ray overlay confirms that the imaged signal was localized to the knee joint (Fig. 2D).

As a control, mice that did not undergo DMM surgery were imaged before and after probe injection. Before probe injection, no NIRF signal was observed (Fig. 3A), suggesting that no endogenous fluorescence from the mice contributes to the detected NIRF signals. Following probe injection, mice demonstrated low NIRF signal intensities that were very similar between the right and left knees, indicating that the difference between DMM and sham knee NIRF signals was not due to intrinsic differences in MMP activity between the two knees (Fig. 3A). Mice 7-week post DMM were imaged before and after removal of the skin overlying the joint to confirm that the NIRF signal came specifically from the knee joint (Fig. 3B and 3C). We observed similar NIRF signal ratios between the DMM and sham knees with both transcutaneous and exposed joint imaging (Fig. 3B and 3C), which is suggestive of the potential for imaging the knees without skin excision in future translational applications. Since sharper images were obtained when the joint was directly exposed, we kept this practice in subsequent experiments.

MMP sensitive NIRF signal intensity steadily increases over OA progression in DMM mice

The observation that DMM surgery induced a significantly higher level of MMP sensitive NIRF signal compared to sham control led us to hypothesize that NIRF signal intensity would correlate with OA development as the joint degenerates. To test this hypothesis, we imaged individual mice serially every 2 weeks over 8 weeks post DMM. As the half-life of MMPsense680 is only 72 hours (PerkinElmer data sheet), we expected signals from the previous imaging session to have dissipated before the subsequent imaging session. To confirm this, we always pre-imaged all mice immediately before new probe injection at each imaging session. Strikingly, at every examined time point, the DMM knee exhibited a higher fluorescent signal than the sham knee, which received the same number of injections as the DMM knee (Fig. 4A). Signal quantification indicated that the mean fluorescent intensity of the DMM knee increased in a linear fashion from 2 to 8 weeks post surgery, while signals from the sham knee showed only a mild increase over time. When the DMM:sham total

signal intensity ratios were calculated, they indicated a significant increase over the course of OA development that was studied (Fig. 4C). This data indicates that MMP activities in the OA joint were induced shortly after surgery and continuously rose from the very early stages of OA to moderate OA.

Correlation of NIRF signal intensity with cartilage histological analysis over OA progression in DMM mice

We performed histological analysis of NIRF imaged mice over 8 weeks post DMM to correlate NIRF signals with histological parameters known to demonstrate progressive joint degeneration. As it is not possible to evaluate joint histology at each time point in serially imaged mice, additional surgeries were performed and mice were sacrificed at multiple time points immediately after NIRF imaging (Fig. 5A). Again, the average NIRF signal intensities of the DMM knee rose consistently over time, while those from the sham knee remained relatively flat (Fig. 5B). As a result, the ratio of DMM:sham total signal intensity increased significantly over time (Fig. 5B). When serial imaged animals were also included in the plot, it further confirmed the trend of increased MMP activity in the DMM knee compared to sham controls over the entire time-course studied and demonstrated the strong reliability and reproducibility of this trend (Fig. 5C).

Safranin O staining was performed for histological analysis of OA joint damage at the time of NIRF imaging (Fig. 6A–C). For quantifying articular cartilage damage, histological images were randomized and blindly scored for changes in articular cartilage structure and safranin O staining based on published assessment methods (10, 15). Cartilage structural damage and loss of safranin O staining were significantly higher in the DMM knee as compared to the sham knee at all time points (Fig. 6A–B, 6D–E). Interestingly, fibrillation and clefts were consistently seen in the DMM samples 1-week post surgery, which resulted in a higher cartilage structure score (Fig. 6A–6C). However, such changes became less pronounced in the following weeks and only became visible again as OA progressed to 8 weeks (Fig. 6A–B, 6D). Safranin O scores did not significantly change over time (Fig. 6E), which is consistent with a previous report when C57 mice were examined between 2 and 8 weeks post DMM surgery (10).

Chondrocyte cell death and osteophyte maturation are also known indicators of progressive joint damage in the DMM OA model (10, 24). We observed an increase in chondrocyte cell death from 1 to 8 weeks post DMM surgery (Fig. 6F). Furthermore, all DMM knees at each time point demonstrated osteophyte formation in the medial tibial plateau, while none was seen in the sham knees (Fig. 6C, 6G). As osteophyte size has been shown to be stable over time in the DMM model, osteophyte size was not assessed in these samples (10). Rather, we assessed osteophyte maturity over time. As expected for the DMM model, these osteophytes matured from being entirely cartilaginous (week 1) to mostly mature bone (weeks 4–8) (Fig. 6C, 6G), indicating the progression of osteophyte pathology (10). Synovial changes were also examined; however no synovitis was seen at any time point examined, which is expected, as mild surgical OA models, such as DMM, do not appreciably demonstrate synovitis until very severe disease stages (9, 10, 25). Overall, the ratios of DMM to sham NIRF signals are positively correlated with examined histological parameters of OA

damage, except for the safranin O score (Fig. 6H). However, the only parameter with a statistically significant correlation was chondrocyte cell death, suggesting that higher NIRF signals generally reflect more joint destruction, but the NIRF signals cannot be used alone to indicate damage in histological analysis.

DISCUSSION

Real-time imaging of tissue metabolic state has been increasingly explored as a method for monitoring a diverse array of diseases including cancer and inflammatory conditions (26, 27). However, it has not been used to monitor OA progression, which typically occurs over a long time period and has been challenging to visualize due to the relative magnitude of changes that occur at any given time. Here, we utilized an MMP-sensitive probe, MMPsense680, which we have confirmed *in vitro* emits near infrared fluorescence upon MMP cleavage, with the most prominent activation caused by MMP13. MMP consensus cleavage sites clearly differ from several proteases known to be upregulated in OA (such as ADAMTS4 and cathepsin K) (28–30), suggesting it is unlikely that this probe also reflects the activities of these enzymes. However, we cannot exclude the possibility that this probe is also activated by other proteases. We demonstrate that MMPsense680's fluorescence intensity steadily increases as joint damage progresses in an *in vivo* OA mouse model from very early OA stages.

Interestingly, the signals from the *in vivo* experiment peaked at 2 hours, while those *in vitro* continued to rise over the course of 24 hours. This discrepancy is potentially due to clearance of the probe from the joint post injection, whereas it is confined locally within the culture wells in *in vitro* assays.

Given the lack understanding and monitoring methods for early OA, it is particularly exciting to observe a steady increase in total NIRF signal from week to week. This suggests that MMP activity may mark the stage of joint degeneration and that NIRF imaging may be used for *in vivo* tracking of OA joint destruction in DMM mice. This study differs from mRNA and protein analysis of MMPs in some prior reports which do not show a change in MMP levels over OA progression (10, 11), and suggests that the net MMP activity of the joint may serve as a more accurate measure of cartilage destruction during OA progression in DMM mice. Further investigations will be needed to determine the respective contributions of specific MMPs to the generated NIRF signals. This would allow examination of whether the MMPs most involved in OA joint damage, such as MMP13, are also the main producers of the NIRF signal, or if the two factors are unrelated.

NIRF probes have been used to detect increased MMP and cathepsin activities in the joints of rheumatoid arthritis animal models (31–37). For OA, a cathepsin B sensitive NIRF probe (38) and MMP activatable NIRF probes have also been used (19, 39–42). MMPsense680 has also been used to compare different regions of cartilage in late stage human OA specimens (43). Importantly, our work differs from these studies in that we continuously imaged mice starting from the beginning of OA to later stages to analyze the trajectory of MMP activity in OA *over time*. By pre-imaging, we assessed MMP activities at each given time point, rather than cumulative changes over OA development. The non-toxic nature and

short half-lives of the NIRF probes allow current MMP activity to be measured at multiple time points and eliminates the need to sacrifice the animals to assess MMP activities (44). Results from mice imaged at single time points followed by immediate sacrifice demonstrated the same trend as mice imaged at multiple times, arguing against the possibility of increased signal due to repeated incisions/injections alone. Furthermore, most of the previously published studies were performed using systemic administration (both intravenous and retroorbital) of the NIRF agent, as opposed to the intraarticular injections we used. This demonstrates that intraarticular and systemic administration of NIRF probes can both be used for metabolic imaging of the OA joint (19, 38–40).

Currently, monitoring OA progression in animals largely relies on histological analysis. Our results demonstrated significantly more damage in articular cartilage structure in the DMM joint than the sham control. The severity of this damage increased over the course of 8 weeks post DMM surgery, following the same trend as chondrocyte cell death and osteophyte maturity. These data demonstrate the validity of this OA model and are consistent with published studies (10, 15, 24). We also found that the safranin O score did not increase over time, which is in accordance with prior published work (10), suggesting that singular analysis of GAG content with safranin O staining is not a reliable method for analyzing DMM damage over time, even though it reliably indicates the presence or absence of OA. Interestingly, we did consistently observe an increased level of articular fibrillation in the first week post DMM in all mice examined, which had decreased by two weeks post DMM, only to gradually increase again after that. This phenomenon has not been reported in the past and it is unclear how it took place. We suspect that the sudden change in mechanical loading of the joint could lead to articular surface damage, which was to some extent repaired in the subsequent weeks. Over the long term however, cartilage may be unable to compensate for the altered loading caused by DMM surgery, ultimately leading to increased structural damage. It will be interesting to determine whether similar changes occur at such an early stage in other injury-induced OA models. Despite the fact that histology is the gold standard for confirming OA damage, the observed differences were small and not significantly different from week to week (10, 45, 46). In contrast, NIRF imaging of MMP activity is able to detect larger differences between weekly time points than histology suggesting that this is a more sensitive approach for monitoring changes in OA.

Further investigations would be required to assess whether similar trends in MMP activity exist until the late stages of OA, or in other animal models or in human OA, including aging or obesity-related OA. It will also be important to correlate NIRF imaging with biochemical biomarkers, such as the cartilage degradation products CTX-II, C2C, C1, 2C, CPII, and Comp (4, 47), as well as with other imaging modalities such as MRI and μ CT, which provide higher resolution images than NIRF images (48–50). NIRF imaging could be used to provide complementary information to structural imaging modalities already currently available.

In summary, NIRF imaging allows us to directly examine the metabolic state of the joint and the microenvironment of the cells within the joint during the course of *in vivo* OA development in real time. As a minimally invasive and sensitive approach, it represents an important step towards incorporating metabolic imaging into various aspects of OA

research, such as facilitating high throughput *in vitro* screening and serial *in vivo* OA assessment for understanding OA pathogenesis or drug development in a time and cost-effective way. NIRF probes have been shown to be safe for patient use and are already being used in several clinical trials for detecting other diseases, such as cancer (26), thus this technique has the potential for translational OA applications.

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References

1. Felson, DT. Osteoarthritis. In: Longo, DL.; Fauci, AS.; Kasper, DL.; Hauser, SL.; Jameson, JL., editors. *Harrison's Principles of Internal Medicine*. 18. New York: McGraw-Hill; 2012.
2. Lafeber FP, van Spil EW. Osteoarthritis year 2013 in review: Biomarkers; reflecting before moving forward, one step at a time. *Osteoarthritis Cartilage*. 2013
3. Patra D, Sandell LJ. Evolving biomarkers in osteoarthritis. *The journal of knee surgery*. 2011; 24(4): 241–9. [PubMed: 22303753]
4. Kraus VB, Burnett B, Coindreau J, Cottrell S, Eyre D, Gendreau M, et al. Application of biomarkers in the development of drugs intended for the treatment of osteoarthritis. *Osteoarthritis Cartilage*. 2011; 19(5):515–42. [PubMed: 21396468]
5. Goldring MB, Berenbaum F. The regulation of chondrocyte function by proinflammatory mediators: prostaglandins and nitric oxide. *Clin Orthop Relat Res*. 2004; 427(Suppl):S37–46. [PubMed: 15480072]
6. Leong DJ, Hardin JA, Cobelli NJ, Sun HB. Mechanotransduction and cartilage integrity. *Ann N Y Acad Sci*. 2011; 1240:32–7. [PubMed: 22172037]
7. Ryu JH, Lee A, Huh MS, Chu J, Kim K, Kim BS, et al. Measurement of MMP Activity in Synovial Fluid in Cases of Osteoarthritis and Acute Inflammatory Conditions of the Knee Joints Using a Fluorogenic Peptide-Probe-Immobilized Diagnostic Kit. *Theranostics*. 2012; 2(2):198–206. [PubMed: 22375158]
8. Swingle TE, Waters JG, Davidson RK, Pennington CJ, Puente XS, Darrah C, et al. Degradome expression profiling in human articular cartilage. *Arthritis Res Ther*. 2009; 11(3):R96. [PubMed: 19549314]
9. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis Cartilage*. 2007; 15(9):1061–9. [PubMed: 17470400]
10. Loeser RF, Olex AL, McNulty MA, Carlson CS, Callahan M, Ferguson C, et al. Disease progression and phasic changes in gene expression in a mouse model of osteoarthritis. *PLoS One*. 2013; 8(1):e54633. [PubMed: 23382930]
11. Bateman JF, Rowley L, Belluoccio D, Chan B, Bell K, Fosang AJ, et al. Transcriptomics of wild-type mice and mice lacking ADAMTS-5 activity identifies genes involved in osteoarthritis initiation and cartilage destruction. *Arthritis Rheum*. 2013; 65(6):1547–60. [PubMed: 23436205]
12. Poole AR. Osteoarthritis as a whole joint disease. *HSS journal : the musculoskeletal journal of Hospital for Special Surgery*. 2012; 8(1):4–6. [PubMed: 23372516]
13. Zhen G, Wen C, Jia X, Li Y, Crane JL, Mears SC, et al. Inhibition of TGF-beta signaling in mesenchymal stem cells of subchondral bone attenuates osteoarthritis. *Nature medicine*. 2013; 19(6):704–12.

14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25(4):402–8. [PubMed: 11846609]
15. McNulty MA, Loeser RF, Davey C, Callahan M, Ferguson C, Carlson CS. A Comprehensive Histological Assessment of Osteoarthritis Lesions in Mice. *Cartilage*. 2011; 2(4):354–63.
16. Little CB, Barai A, Burkhardt D, Smith SM, Fosang AJ, Werb Z, et al. Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development. *Arthritis Rheum*. 2009; 60(12):3723–33. [PubMed: 19950295]
17. Galazka G, Windsor LJ, Birkedal-Hansen H, Engler JA. APMA (4-aminophenylmercuric acetate) activation of stromelysin-1 involves protein interactions in addition to those with cysteine-75 in the propeptide. *Biochemistry*. 1996; 35(34):11221–7. [PubMed: 8780527]
18. Cortial D, Gouttenoire J, Rousseau CF, Ronziere MC, Piccardi N, Msika P, et al. Activation by IL-1 of bovine articular chondrocytes in culture within a 3D collagen-based scaffold. An in vitro model to address the effect of compounds with therapeutic potential in osteoarthritis. *Osteoarthritis Cartilage*. 2006; 14(7):631–40. [PubMed: 16527498]
19. Wang S, Wei X, Zhou J, Zhang J, Li K, Chen Q, et al. Identification of alpha2 -Macroglobulin as a Master Inhibitor of Cartilage-Degrading Factors That Attenuates the Progression of Posttraumatic Osteoarthritis. *Arthritis & rheumatology*. 2014; 66(7):1843–53. [PubMed: 24578232]
20. Goldring MB, Otero M, Tsuchimochi K, Ijiri K, Li Y. Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. *Ann Rheum Dis*. 2008; 67(Suppl 3):iii75–82. [PubMed: 19022820]
21. Freemont AJ, Byers RJ, Taiwo YO, Hoyland JA. In situ zymographic localisation of type II collagen degrading activity in osteoarthritic human articular cartilage. *Ann Rheum Dis*. 1999; 58(6):357–65. [PubMed: 10340960]
22. Quillard T, Tesmenitsky Y, Croce K, Travers R, Shvartz E, Koskinas KC, et al. Selective inhibition of matrix metalloproteinase-13 increases collagen content of established mouse atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2011; 31(11):2464–72. [PubMed: 21903941]
23. Klohs J, Baeva N, Steinbrink J, Bourayou R, Boettcher C, Royle G, et al. In vivo near-infrared fluorescence imaging of matrix metalloproteinase activity after cerebral ischemia. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2009; 29(7):1284–92.
24. McNulty MA, Loeser RF, Davey C, Callahan MF, Ferguson CM, Carlson CS. Histopathology of naturally occurring and surgically induced osteoarthritis in mice. *Osteoarthritis Cartilage*. 2012; 20(8):949–56. [PubMed: 22595226]
25. Glasson SS, Chambers MG, Van Den Berg WB, Little CB. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartilage*. 2010; 18(Suppl 3):S17–23. [PubMed: 20864019]
26. Sevcik-Muraca EM. Translation of near-infrared fluorescence imaging technologies: emerging clinical applications. *Annu Rev Med*. 2012; 63:217–31. [PubMed: 22034868]
27. Gounaris E, Martin J, Ishihara Y, Khan MW, Lee G, Sinh P, et al. Fluorescence endoscopy of cathepsin activity discriminates dysplasia from colitis. *Inflamm Bowel Dis*. 2013; 19(7):1339–45. [PubMed: 23591598]
28. Turk BE, Huang LL, Piro ET, Cantley LC. Determination of protease cleavage site motifs using mixture-based oriented peptide libraries. *Nat Biotechnol*. 2001; 19(7):661–7. [PubMed: 11433279]
29. Hills R, Mazzarella R, Fok K, Liu M, Nemirovskiy O, Leone J, et al. Identification of an ADAMTS-4 cleavage motif using phage display leads to the development of fluorogenic peptide substrates and reveals matrilin-3 as a novel substrate. *J Biol Chem*. 2007; 282(15):11101–9. [PubMed: 17311924]
30. Choe Y, Leonetti F, Greenbaum DC, Lecaille F, Bogoy M, Bromme D, et al. Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. *J Biol Chem*. 2006; 281(18):12824–32. [PubMed: 16520377]
31. Wunder A, Tung CH, Muller-Ladner U, Weissleder R, Mahmood U. In vivo imaging of protease activity in arthritis: a novel approach for monitoring treatment response. *Arthritis Rheum*. 2004; 50(8):2459–65. [PubMed: 15334458]

32. Hansch A, Frey O, Hilger I, Sauner D, Haas M, Schmidt D, et al. Diagnosis of arthritis using near-infrared fluorochrome Cy5.5. *Invest Radiol*. 2004; 39(10):626–32. [PubMed: 15377942]
33. Hansch A, Frey O, Sauner D, Hilger I, Haas M, Malich A, et al. In vivo imaging of experimental arthritis with near-infrared fluorescence. *Arthritis Rheum*. 2004; 50(3):961–7. [PubMed: 15022340]
34. Ryu JH, Lee A, Chu JU, Koo H, Ko CY, Kim HS, et al. Early diagnosis of arthritis in mice with collagen-induced arthritis, using a fluorogenic matrix metalloproteinase 3-specific polymeric probe. *Arthritis Rheum*. 2011; 63(12):3824–32. [PubMed: 22127700]
35. Chen WT, Mahmood U, Weissleder R, Tung CH. Arthritis imaging using a near-infrared fluorescence folate-targeted probe. *Arthritis Res Ther*. 2005; 7(2):R310–7. [PubMed: 15743478]
36. Ibarra JM, Jimenez F, Martinez HG, Clark K, Ahuja SS. MMP-Activated Fluorescence Imaging Detects Early Joint Inflammation in Collagen-Antibody-Induced Arthritis in CC-Chemokine Receptor-2-Null Mice, In-Vivo. *International journal of inflammation*. 2011; 2011:691587. [PubMed: 21755029]
37. Galligan CL, Fish EN. Circulating fibrocytes contribute to the pathogenesis of collagen antibody-induced arthritis. *Arthritis Rheum*. 2012; 64(11):3583–93. [PubMed: 22729466]
38. Lai WF, Chang CH, Tang Y, Bronson R, Tung CH. Early diagnosis of osteoarthritis using cathepsin B sensitive near-infrared fluorescent probes. *Osteoarthritis Cartilage*. 2004; 12(3):239–44. [PubMed: 14972341]
39. Zhou J, Chen Q, Lanske B, Fleming BC, Terek R, Wei X, et al. Disrupting the Indian hedgehog signaling pathway in vivo attenuates surgically induced osteoarthritis progression in Col2a1-CreERT2; Ihhf1/fl mice. *Arthritis Res Ther*. 2014; 16(1):R11. [PubMed: 24428864]
40. Vermeij EA, Koenders MI, Blom AB, Arntz OJ, Bennink MB, van den Berg WB, et al. In vivo molecular imaging of cathepsin and matrix metalloproteinase activity discriminates between arthritic and osteoarthritic processes in mice. *Mol Imaging*. 2014; 13(2):1–10. [PubMed: 24881106]
41. Ryu JH, Lee A, Na JH, Lee S, Ahn HJ, Park JW, et al. Optimization of matrix metalloproteinase fluorogenic probes for osteoarthritis imaging. *Amino Acids*. 2011; 41(5):1113–22. [PubMed: 20953646]
42. Lee S, Park K, Lee SY, Ryu JH, Park JW, Ahn HJ, et al. Dark quenched matrix metalloproteinase fluorogenic probe for imaging osteoarthritis development in vivo. *Bioconjug Chem*. 2008; 19(9):1743–7. [PubMed: 18729392]
43. Jones EF, Schooler J, Miller DC, Drake CR, Wahnische H, Siddiqui S, et al. Characterization of human osteoarthritic cartilage using optical and magnetic resonance imaging. *Mol Imaging Biol*. 2012; 14(1):32–9. [PubMed: 21384207]
44. Frangioni JV. In vivo near-infrared fluorescence imaging. *Curr Opin Chem Biol*. 2003; 7(5):626–34. [PubMed: 14580568]
45. Oh H, Chun CH, Chun JS. Dkk-1 expression in chondrocytes inhibits experimental osteoarthritic cartilage destruction in mice. *Arthritis Rheum*. 2012; 64(8):2568–78. [PubMed: 22488261]
46. Ma HL, Blanchet TJ, Peluso D, Hopkins B, Morris EA, Glasson SS. Osteoarthritis severity is sex dependent in a surgical mouse model. *Osteoarthritis Cartilage*. 2007; 15(6):695–700. [PubMed: 17207643]
47. Cibere J, Zhang H, Garnero P, Poole AR, Lobanok T, Saxne T, et al. Association of biomarkers with pre-radiographically defined and radiographically defined knee osteoarthritis in a population-based study. *Arthritis Rheum*. 2009; 60(5):1372–80. [PubMed: 19404937]
48. Buck RJ, Wyman BT, Le Graverand MP, Hudelmaier M, Wirth W, Eckstein F. Does the use of ordered values of subregional change in cartilage thickness improve the detection of disease progression in longitudinal studies of osteoarthritis? *Arthritis Rheum*. 2009; 61(7):917–24. [PubMed: 19565557]
49. Hayashi D, Guermazi A, Hunter DJ. Osteoarthritis year 2010 in review: imaging. *Osteoarthritis Cartilage*. 2011; 19(4):354–60. [PubMed: 21320616]
50. Lakin BA, Grasso DJ, Stewart RC, Freedman JD, Snyder BD, Grinstaff MW. Contrast enhanced CT attenuation correlates with the GAG content of bovine meniscus. *J Orthop Res*. 2013

Figure 1. MMP sensitive near infrared fluorescence (NIRF) probe detects enhanced MMP activities upon pro-inflammatory cytokine IL-1 β treatment in 2D and 3D cultures of human normal primary articular chondrocytes (nHACs)

A. Activation of MMP-Sense680 by active recombinant MMPs minus background fluorescence over 24 hours. **B.** MMP-Sense680 activation by recombinant MMPs plus the pan-MMP inhibitor PD 166793, demonstrating that PD166793 inhibits recombinant MMP activation of MMP-Sense680. **C.** MMP-Sense and trypsin activation of MMP-Sense680 after 24-hour incubation. **D.** qRT-PCR analysis of MMP expression in nHACs (Lonza) treated with or without 1 ng/ μ L of IL-1 β for 3 days, confirming MMP induction with IL-1 β treatment. **E.** Bright field images of IL-1 β -treated nHACs in 2D cultures. **F.** Confocal images after 4 hours of MMP-Sense680 (0.4 μ M, PerkinElmer) addition, indicating IL-1 β induced MMP activity in nHACs in a dosage-dependent way (arrows). **G.** Confocal images of HACs grown in 3D silk porous scaffolds (100–200 μ m pore size) after 16 hours of MMP-Sense680 (0.4 μ M) addition. MMP activity was induced by IL-1 β in a dosage-dependent way (arrows). Hoechst dye labeling indicates the locations of live cells. Black * = p < 0.05 and white * indicates the presence of the silk scaffold.

Figure 2. MMP sensitive NIRF probe exhibits enhanced signals *in vivo* in DMM OA mouse

A. *In vivo* NIRF images overlain on X-ray images from DMM mice after 4 μ L intraarticular injection of 13.3 μ M MMPsense680 in each knee. Mice were imaged at multiple time points post probe injection, demonstrating enhanced fluorescent signal in the DMM knee as compared to sham knee of the same mouse, with two hours post probe injection showing the largest difference. **B.** Mean fluorescent intensity emitted from each knee was quantified from collected NIRF images. **C.** Total signal intensity was quantified for each knee from the collected NIRF images and the signal intensity ratio of DMM:sham was calculated for each mouse. **D.** NIRF signal overlain on X-ray from 7-week DMM mouse with plotting thresholds set so only the brightest 50% (left) or brightest 25% (right) of NIRF signal is shown, demonstrating that the NIRF signal comes specifically from the knee joint. * = $p < 0.05$ for groups at the same time point, unless otherwise indicated by brackets.

Figure 3. NIRF signals are derived from the joint instead of the overlaying skin

A. Raw images of a control mouse prior to and after MMPsense680 intraarticular injection and processed NIRF image overlain on X-ray image of the control mouse after probe injection. **B.** Transcutaneous NIRF image overlain on X-ray image from a DMM mouse taken 2 hours after 4 μ L intraarticular 13.3 μ M MMPsense680 injection. ImageJ was used to quantify emitted fluorescence across the width of the image and to create a surface plot analysis to generate a three dimensional plot of the intensities of the whole raw transcutaneous NIRF image. **C.** NIRF image taken with the skin pulled open to expose the knee overlain on X-ray image from a DMM mouse taken 2 hours after 4 μ L intraarticular 13.3 μ M MMPsense680 injection. ImageJ was used to quantify emitted fluorescence across the width of the image and to create a surface plot analysis to generate a three dimensional plot of the intensities of the whole raw exposed knee NIRF image.

Figure 4. MMP-activatable NIRF signal intensity steadily increases over the course of OA progression in repeatedly imaged DMM mice

A. *In vivo* NIRF images overlain on X-ray images from DMM mice taken two hours after 4 μ L intraarticular injection of 13.3 μ M MMPsense680 in each knee. The same mice were imaged repeatedly every 2 weeks for 8 weeks total. Representative images show the combined X-ray and NIRF image of one individual mouse at 2, 4, 6, and 8 weeks post DMM surgery. Increasing levels of MMP activities were observed over OA development. **B.** Mean fluorescent intensity emitted from each knee was quantified from collected NIRF images. * = $p < 0.05$ for groups at the same time point. **C.** Total signal intensity was quantified for each knee from the collected NIRF images and the signal intensity ratio of DMM:sham was calculated for each mouse. Data was fit with a best-fit line using a second order polynomial regression, equation: $y = 1.6x^2 - 1.42x + 2.5$, R^2 value: 0.9463. # = $p < 0.05$ compared to all groups.

Figure 5. MMP-activatable NIRF signal intensity steadily increases over OA progression in DMM mice imaged at single time points

A. *In vivo* NIRF images overlain on X-ray images from DMM mice taken two hours after 4 μ L intraarticular injection of 13.3 μ M MMPsense680 in each knee. Mice were imaged at a single time point (1, 2, 3, 4, or 7 weeks post DMM surgery). **B.** Mean fluorescent intensity and total signal intensity emitted from each knee were quantified from collected NIRF images. The total signal intensity ratio of DMM:sham was calculated for each mouse. Data was fit with a best-fit line using a second order polynomial regression, equation: $y = 2.5x^2 - 4.6x + 4.5$, R^2 value: 0.9595. **C.** All quantified imaging data, including serially imaged mice and mice imaged at only a single time point, were pooled. Mean fluorescence intensity and total signal intensity ratio from pooled data was plotted over OA development. Data was fit with a best-fit line using a second order polynomial regression, equation: $y = 0.31x^2 + 2.8x + 5.6$, R^2 value: 0.9401. * = $p < 0.05$ for groups at the same time point. # = $p < 0.05$ compared to all groups.

Figure 6. Histological analysis of OA severity over the course of OA progression in DMM mice
A. Representative histologic appearance of safranin O/hematoxylin stained sagittally sectioned mouse knees 1–8 weeks post DMM surgery. Scale: 250 μ m. Arrows indicate cartilage damage. **B.** Representative images of tibial articular damage in fast green/safranin O stained sagittally sectioned mouse knees 1–8 weeks post DMM surgery. Scale: 25 μ m. Arrows indicate cartilage damage. **C.** Representative images of osteophyte formation in safranin O/hematoxylin stained sagittally sectioned mouse knees 1–8 weeks post DMM surgery. Scale: 100 μ m. Arrows indicate osteophytes. **D.** Cartilage structure scoring of OA

severity in mice 1–8 weeks post DMM. **E.** Safranin O scoring of OA severity in mice 1–8 weeks post DMM. **F.** Tibial chondrocyte cell death scoring of OA severity in mice 1–8 weeks post DMM. **G.** Scoring of osteophyte maturity in mice 1–8 weeks post DMM. **H.** Correlation analyses between histological parameters and NIRF DMM:sham total signal intensity ratio results. * = $p < 0.05$ for indicated groups. # = $p < 0.05$ for indicated sample compared to all other samples. At each time point, comparison of DMM and sham score has $p < 0.05$.