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14. ABSTRACT

Anterior cruciate ligament (ACL) is critical in providing stability of the knee joints. Injuries causing ruptures to the ACL are a common cause of knee instability and subsequent posttraumatic osteoarthritis.

Since Duffy antigen receptor for chemokines (DARC) binds inflammatory chemokines previously shown to be up-regulated in osteoarthritic knees, we proposed to analyze the role of DARC on the development of PTOA in the present study.

In addition to MCP-1 and RANTES, DARC binds to other chemokines previously shown to be present in OA knees such as CXCL1 and CXCL5, and which are known to be involved in neutrophil migration. Thus, to determine the role of these chemokines in PTOA development, we evaluated the effect of ACL injury on their mRNA levels. We predicted that expression of these chemokines would correlate with the level of inflammation and the magnitude of catabolic effects on cartilage and subchondral bone that occur in response to injury. At three days post ACL injury, both chemokines were found to be increased in response to knee injury, confirming the recruitment of neutrophils to the knee joints in response to ACL injury. No difference was observed in the expression of Cxcl1 or Cxcl5 between the two lines of mice suggesting that post-ACL injury inflammation was not affected by Darc deficiency.

To determine whether interfering with chemokine-DARC interaction will inhibit post-ACL injury inflammation, injured knees were treated with neutralizing antibodies against DARC or IgG control, at the knee joints. Then, the expression level of one of DARC ligands which is also one of the major inflammatory chemokines and the expression of *Mmp3* were measured. The mRNA levels of *Mcp-1* and Mmp3 were significantly greater in the injured knees compared to control un-injured knees. However, no difference in the expression of *Mcp-1* or *Mmp3* was found between DARC-Ab and IgG treated knees at three day-post ACL injury.

15. SUBJECT TERMS

ACL, DARC, Inflammation, PTOA

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A. INTRODUCTION

Joint injury is often associated with subsequent pathologic events, particularly when the joint is destabilized. These include inflammation, cell death, altered cartilage structure, and the release of blood into the joint space. Studies have shown that joint injuries are the main cause of osteoarthritis (OA) development. Every healthy knee is maintained by healthy tendons that connect the knee bones to the leg muscles and allow knee joint movements, and healthy ligaments that connect the femur to the tibia at the knee joint. Anterior cruciate ligament (ACL) is critical in providing stability of the knee. Injuries causing ruptures to the ACL are a common cause of knee instability and subsequent posttraumatic osteoarthritis (PTOA). US military personnel suffer from ACL injury at a rate 10 times that of the civilian population. In addition, even though ACL injuries can be surgically repaired, following reconstructive ACL surgery, 35% of patients will develop tibio-femoral osteoarthritis (OA) (Svoboda, 2014). Clearly, ligament damage, especially to the ACL is a significant factor in the initiation and progression of PTOA, and merits further study in order to develop effective therapeutic interventions.

Hypotheses. In this study, we proposed the following new hypotheses: (**1**) Anterior cruciate ligament (ACL) injury/rupture induces cytokine secretion in the synovial space, leading to increased chemokine expression; (**2**) then, through DARC-dependent chemokine transcytosis will increase chemokine secretion, in turn promoting the recruitment of inflammatory cells to the joint space, which will lead to cartilage degradation and PTOA. Thus, Darc-knockout mice should show less inflammation in response to knee injury and therefore will be protected from cartilage degradation; and (**3**) the local application of anti-DARC antibody to the joint space of the knee after ACL injury will inhibit chemokine binding to DARC, reducing the recruitment of inflammatory cells to the joint space, and reducing or preventing the onset and/or progression of PTOA.

Objectives. Developing treatments for PTOA requires an understanding of the underlying molecular mechanisms. For this purpose, we proposed to first identify those chemokines that bind to DARC, and whose expression is induced in response to ACL injury. Time course experiments will identify the temporal relationship between DARC-chemokine interactions and the development of PTOA pathology. Together, these studies will provide critical information about the molecular mechanisms that contribute to inflammation and cartilage damage, and will identify when chemokine-DARC interaction occurs. This will aid in the design of the second series of experiments to evaluate, in a mouse model of PTOA that mimics joint injury in humans, the effect of local administration of DARC neutralizing antibodies on PTOA development, as well as potentially identifying other targets that may be amenable for therapeutic intervention.

Therefore, we proposed two specific aims to confirm the above hypothesis:

Specific Aim 1. Identify the chemokines induced and the cells that migrated to the synovial fluid in response to knee injury during the development of PTOA

Specific Aim 2. Develop a strategy to locally reduce or inhibit post-ACL injury mediated inflammation and development of PTOA by direct administration of anti-DARC antibodies to the injured knee

B. BODY

Progress report during the 2nd year of the funding period

1. **Specific Aim 1.** Identify the chemokines induced and the cells that migrated to the synovial fluid in response to knee injury during the development of PTOA

a. **Gene expression profile in response to ACL injury caused by axial loading**

- **Animal Model.** Axial loading to induce ACL tear/rupture.

10-week old C57/B6 wild type (WT) and Darc-KO mice were used during this funding period.

Mice were anesthetized by isoflurane inhalation. The right leg was subjected to a preload of 1-2N applied to the knee, followed by the single dynamic axial compressive load of 15 N (at a 40 N/sec loading rate), as previously described (**Christiansen et al., 2012**), and using Instron Hydraulic machine. This load was successful to induce knee injury at 12N, characterized by a discontinuity in the force-displacement curve at 12N (**Fig. 1**). Subcutaneous injection of buprenorphine analgesia (0.5 μg/g body weight) was administered to each animal after injury. The animals were allowed unrestricted movement following injury. The left knee served as contralateral control was not loaded.

Animals were sacrificed at different time points post-ACL injury. Tissues were collected by excising a region extending 1-2 mm above and below the middle of the knee joint, with samples snap frozen in liquid nitrogen, and stored at -80°C.

For RNA extraction, samples were pulverized in liquid nitrogen; total RNA was isolated using Trizol and RNeasy kit (Qiagen) and processed for real-time-PCR. Real-time quantitative PCR was performed using the Applied Biosystems ViiA7 RT-PCR systems instrument, and the SYBR Green PCR kit from Applied Biosystems Inc., as previously described (Edderkaoui et al., 2007).

In our first annual report, we have reported the response of inflammatory genes and Mmp genes to anterior cruciate ligament (ACL) injury at 1 and 3 days post-AL. In this 2nd annual report, we have evaluated the response of two other chemokines that bind to DARC and are known to control neutrophil migration in response to ACL injury at three days post knee injury. We have assessed the response of DARC ligands, chondrogenesis markers and Mmp genes to knee injury at 7 days and 3 weeks post-ACL injury from both WT and Darc-KO mice. In addition, we have tested the effect of blocking the function of DARC using neutralizing antibody against DARC on the gene expression after ACL injury (without affecting the genetic background of the animal). These data will be presented in this annual progress report.

- **Gene expression profile post-axial loading and ACL injury from both WT and Darc-KO mice**

  - <u>Gene expression profile of CXCL1 and CXCL5 that bind to DARC, at three days post ACL injury</u>.

In addition to MCP-1 and RANTES, DARC binds to other chemokines previously shown to be present in OA knees, such as CXCL1 and CXCL5 (Bay-Jensen et al., 2015, Smith et al., 2008), and which are known to be involved in neutrophil migration (Smith et al., 2008). Thus, to determine the role of these chemokines in PTOA development, we evaluated the change in their mRNA levels in response to ACL injury. We predicted

that expression of these chemokines would correlate with the level of inflammation in the knee, and the magnitude of catabolic effects on cartilage and subchondral bone that occur in response to joint injury. At three days post ACL injury, both chemokines were found to be increased in response to knee injury in both lines of mice (**Fig. 2**). This data confirmed the recruitment of neutrophils to the knee joint in response to ACL injury and suggest that post-ACL injury inflammation was not affected by *Darc* deficiency.

- Gene expression profile at seven-days and three weeks post-ACL injury

Each chemokine binds to its specific receptors, but they also bind to other receptors called multi-specific receptors, such as Duffy Antigen Receptor for Chemokines (DARC). Among these chemokines; MCP-1 and RANTES that are known to bind to their specific receptors as well as to DARC. In this study, we have evaluated the response of these two genes in response to ACL injury, in the presence and in the absence of *Darc* expression. *Mcp-1*, but not *Rantes*, was induced in response to ACL injury, and the expression level of *Mcp-1* was significantly greater in *Darc*-KO mice compared to WT mice at one day post-ACL injury, but no difference in the expression was observed between the two line of mice at three days post-ACL injury (previous annual report). Then, to determine the pattern of expression of the two chemokines during OA development, we have measured the mRNA levels of *Mcp1* and *Rantes* at seven days post-ACL injury by qPCR. The mRNA levels of *Mcp-1* was significantly increased at seven days post-injury in both WT and KO mice, but no change in the expression of *Rantes* was observed in response to ACL injury and no difference was observed in the expression level of *Mcp-1* between the two lines of mice at this time point (**Fig. 3**).

The mRNA levels of two matrix metalloproteinases known for their catabolic effects on the extracellular matrix; MMP3 and MMP13, were evaluated at seven day-post knee injury. The data about expression levels at one and three days post-ACL injury of these two genes have been already reported in our first annual report. *Mmp3* showed significant increase in mRNA levels in both lines of mice when compared to WT-unloaded knees at seven-days post knee injury (**Fig. 4**), and the magnitude of increase in the mRNA level was similar in both lines of mice (**Fig. 4**).

It has been reported that abnormal mechanical stress due to knee destabilization caused by ACL injury induces osteophyte formation (Hsia et al., 2017; van der Kraan and van den Berg, 2007). Since, the formation of osteophytes starts with an abnormal chondrogenesis, we have analyzed the expression level of chondrocyte proliferation and differentiation markers at the knee joints, three weeks post ACL-injury in both lines of mice. Three genes were tested; type 2 collagen (*Col. 2*), type 10 collagen (*Col. 10*) and aggrecan (*Acan*). We found the three genes significantly greater in the injured knees compared to unloaded knees in both lines of mice (**Fig. 5**), suggesting that ACL injury causes an early osteophyte formation that was not affected by the lack of *Darc* expression.

b. Histologic assessment of knee joint after ACL injury

Injured and un-injured knee joints were collected, and muscle tissue was removed. The knees were fixed for one day in 10% buffered formalin, decalcified in Formical 4 for one day at room temperature and embedded in paraffin. Then, 5 µm sections were taken at 100-µm intervals from the posterior to anterior side of the knee joints. Histology sections were stained with either hematoxylin and Eosin (H & E) to assess the density of the cells at the synovial space, or Safranin-O and Fast green to visualize the chondro-osteophyte formation and fibrous tissue formation around the knee joints after knee injury.

Since gene expression profile showed increased expression of some chondrogenesis markers (**Fig. 5**) at three weeks post ACL injury, we predicted an earlier osteophyte formation due to ACL injury, so we analyzed the histology sections stained with Safranin-O that were collected from mice animals at two-weeks post ACL injury. Fibrous tissue (FT) was obvious around the injured knees from both lines of mice (**Fig. 6**). The injured knees showed synovial thickening and a swelling like phenotype (**Fig. 6**). Safranin-o staining appeared at the side of the femur condyle and tibia plate as well as at the connection of the menisci and the cruciate ligaments with the tibia bone (**Fig. 6**). The collateral un-loaded knee showed a smooth articular cartilage, smooth medial collateral and smooth lateral collateral ligament, no fibrous tissue was observed in un-loaded knees, no safranin-o staining was observed at the connection of the menisci and cruciate ligament with tibia bone. The apparition of safranin-o staining is a sign of osteophyte formation and explains the increased levels of type 2 collagen (Col. 2), Collagen X (Col. 10) and Aggrecan (Acan) detected at three weeks post-ACL injury with qPCR (**Fig. 5**).

2. **Specific Aim 2.** Develop a strategy to locally reduce or inhibit post-ACL injury mediated inflammation and development of PTOA by direct administration of anti-DARC antibodies to the injured knee

In this specific aim 2, we tested the effect of local blockade of DARC function on inflammation and Mmp gene expression post ACL injury. Intra-articular injection of the neutralizing antibody against DARC or IgG control was performed at one day-post ACL injury, we tested 2 doses; 0.4 µg and 4 µg DARC-antibody (Ab) that was diluted in PBS, we are only presenting the data from 4 µg DARC-Ab., in this annual report. Then, animals were sacrificed at three days post ACL injury.

To determine whether interfering with chemokine-DARC interaction will inhibit inflammation, we have evaluated the expression level of one of DARC ligands which is also one of the major inflammatory chemokines. In both DARC-Ab and IgG treated groups, the mRNA level of *Mcp-1* was significantly greater in the injured knees compared to control un-injured knees (**Fig. 7**). We have also compared the response to ACL injury of two major Mmp genes, in the presence and in the absence of DARC-Ab. The mRNA level of Mmp3 was greater in the injured knees compared to un-loaded knee, and no difference in the expression of Mmp3 was found between DARC-Ab treated knees and IgG treated knees.

C. KEY RESEARCH ACCOMPLISHMENTS DURING THE LAST 12 MONTHS OF FUNDING

We have made the following progress towards achieving the specific aims in this research project:

- We have evaluated the response to ACL injury of two other chemokines that bind to DARC; CXCL1 and CXCL5, at three days post ACL injury. The two chemokines are known to be involved in neutrophil migration (Smith et al., 2008) and were found in the knees of osteoarthritic patients (Bay-Jensen et al., 2015, Smith et al., 2008).
- The mRNA level of both *Cxcl1* and *Cxcl5* was increased in response to knee injury, in both lines of mice. This data confirmed the recruitment of neutrophils to the knee joint in response to ACL injury and suggests that post-ACL injury inflammation was not affected by *Darc* deficiency.
- We have evaluated the change in the expression levels of the two chemokines that bind to DARC as well as the two major effectors of the metabolism of articular cartilage, *Mmp3* and *Mmp13*at seven days post knee injury.
- The mRNA levels of *Mcp-1* and *Mmp3* remained significantly greater in the injured knees of both lines of mice as compared to the control un-injured knees.
- We have analyzed the expression levels of chondrocyte proliferation and differentiation markers at the knee joints, three weeks post ACL-injury in both lines of mice.
- The three genes tested were significantly greater in the injured knees compared to the control unloaded knees in both lines of mice, suggesting that osteophyte formation started early post ACL injury.
- Our examination of the histology sections prepared from the knees collected at two weeks post-knee injury, revealed synovial thickening and knee swelling as well as a starting of osteophyte formation.
- To block the function of DARC locally at the knee joints, we have tested 2 doses; 0.4 μg and 4 μg DARC-antibody (Ab). The antibody was diluted in 10 μl PBS,
- At one day-post ACL injury, DARC-Ab or IgG control were injected at the injured knees. Then, animals were sacrificed at three days post ACL injury.
- we have evaluated the expression level of *Mcp-1*, *Mmp3* and *Mmp13* in both control and DARC-Ab treated mice at three days post ACL injury.
- In both group of mice, the mRNA level of *Mcp-1* was significantly greater in the injured knees compared to control un-injured knees.
- The mRNA level of Mmp3 was greater in the injured knees compared to un-loaded knee, and no difference in the expression of Mmp3 was found between DARC-Ab treated knees and IgG treated knees.

D. CONCLUSION

Knee ACL injury caused by axial loading induces the expression of multiple genes at different time points. The mRNA level of both Cxcl1 and Cxcl5 was increased in response to knee injury, in both lines of mice. This data confirmed the recruitment of neutrophils to the knee joint in response to ACL injury. Since no difference in the expression level of these two chemokines was found in the two lines of mice at three day-post ACL injury, we conclude that *Darc* deficiency does not affect post-ACL injury inflammation.

We have analyzed the expression levels of chondrocyte proliferation and differentiation markers at the knee joints, three weeks post ACL-injury in both lines of mice. The three genes tested were significantly greater in the injured knees compared to the control unloaded knees in both lines of mice, suggesting that osteophyte formation started early post ACL injury.

To determine whether interfering with chemokine-DARC interaction will inhibit inflammation, we have injected neutralizing antibodies against DARC at the knee joint after ACL injury, the expression level of one of DARC ligands which is also one of the major inflammatory chemokines was measured in both DARC-Ab and IgG treated groups, the mRNA level of *Mcp-1* was significantly greater in the injured knees compared to control un-injured knees. We have also compared the response to ACL injury of two major Mmp genes, in the presence and in the absence of DARC-Ab. The mRNA level of Mmp3 was greater in the injured knees compared to un-loaded knee. However, no difference in the expression of Mcp-1 or Mmp3 was found between DARC-Ab treated knees and IgG treated knees at three day-post ACL injury. Histologic assessments, of the knee joints collected from DARC-Ab and IgG treated mice after ACL injury, are in the process to determine the effect of blockade of DARC function on the inflammatory cell recruitment to the injured knees.

E. INVENTIONS, PATENTS AND LICENSES: Nothing to report

F. REPORTABLE OUTCOMES:

- ACL injury induced the expression of two other chemokines (CXCL1 and CXCL5), that binds DARC and are responsible for neutrophil migration, in the injured knees post ACL injury.
- ACL injury induced osteophyte formation as early as two weeks post ACL injury.
- Osteophyte formation starts with chondrogenesis around the injured knee joints.
- *Darc* deficiency did not affect the inflammation induced by ACL injury.

G. OTHER ACHIEVEMENTS: Nothing to report

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I. FIGURE LEGENDS

Figure 1. ACL injury during tibial compression loading identified by a release of compressive force during the loading cycle, with a continued increase in actuator displacement. X axis represents the time in milliseconds, the Y axis represents the loading force in Newton.

Figure 2. mRNA expression levels of *chemokines; Cxcl1 and Cxcl5* at the knee joints of Darc-KO and WT mice, at three days post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees), data are presented as fold change compared to WT unloaded knees, n=4 and **P*<0.05 vs WT left knees. Rk., for right knee, and Lk., for left knee.

Figure 3. mRNA expression levels of *Mcp-1* and *Rantes* at the knee joints of Darc-KO and WT mice, at seven days post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees). Then, RNA was isolated as described above, and qPCR was performed using pre-designed primers for the genes of interest. Data are presented as fold change compared to WT unloaded left knees, n=4-5 and **P*<0.05 vs WT left knees. Rk., for right knee, and Lk., for left knee.

Figure 4. mRNA expression levels of the two major matrix metalloproteinases, *Mmp-3*and *Mmp-13* at the knee joints, at seven days post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees), data are presented as fold change compared to WT unloaded knees (left knee), n=5 and **P*<0.05 vs WT left unloaded knees. #P<0.05 comparing the expression at the right knees between the two lines of mice. Rk., for right knee, and Lk., for left knee.

Figure 5. mRNA expression levels of three chondrogenesis markers, collagen type 2 alpha 1 (Col 2), Collagen, type x, alpha-1 (Col 10) and aggrecan at the knee joint of both WT and *Darc*-KO mice, three weeks post-ACL injury. RNA was isolated from knees of both sham unloaded knees (left knees) and loaded knees (right knees), data are presented as fold change compared to WT unloaded knees (left knee), n=5 and **P*<0.05 vs WT left unloaded knees. #P<0.05 comparing the expression at the right knees between the two lines of mice. Rk., for right knee, and Lk., for left knee.

Figure 6. Representative images from histology sections of intact knee (A) and injured knees (B and C), at two week-post ACL injury. A. The medial side of the left (collateral un-touched) knee, collected from WT mice, shows smooth ligaments connecting femur bone to tibia bone (thick arrow) and smooth cruciate ligament (CT, thick arrow), no fibrous tissue was observed. **B.** The medial side of the right knee from WT mice, after ACL injury. The image shows fibrous tissue (FT), swelling like phenotype, and cartilage synthesis as evidenced by safranin-o staining (thin black arrows). **C.** The medial side of the right knee from *Darc*-KO mice, after ACL injury. The image shows fibrous tissue (FT), swelling like phenotype, and cartilage synthesis as evidenced by safranin-o staining (thin black arrows). F. for femur, T. for tibia. CT. for cruciate ligament. FT. for fibrous tissue.

Fig. 7 mRNA expression level of *Mcp-1* at the knee joints of WT mice injected with either DARC-antibody or IgG control, at three days post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees) from both DARC-Ab and IgG injected mice. Data are presented as fold change compared to WT injected with IgG unloaded left knees, n=6 and **P*<0.05 vs left knees from the animals injected with IgG control. Rk., for right knee, and Lk., for left knee.

Figure 8. mRNA expression levels of *Mmp-3* and *Mmp-13* at the knee joints of mice injected with either DARC-antibody or IgG control, at three- day-post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees) from both DARC-Ab and IgG injected mice. Data are presented as fold change compared to WT unloaded knees, n=4 and **P*<0.05 vs WT left unloaded knees. Rk., for right knee, and Lk., for left knee.

Fig. 1

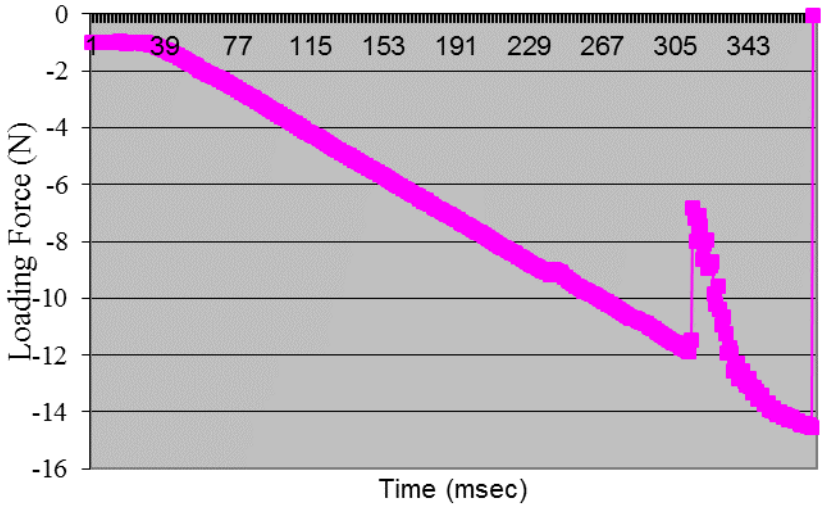
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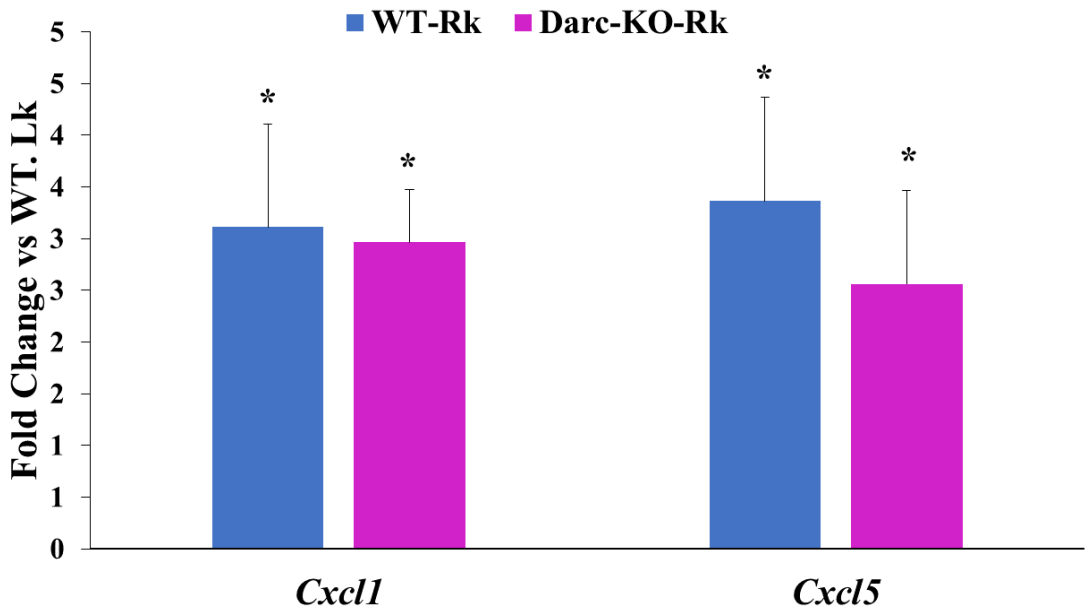
Fig. 2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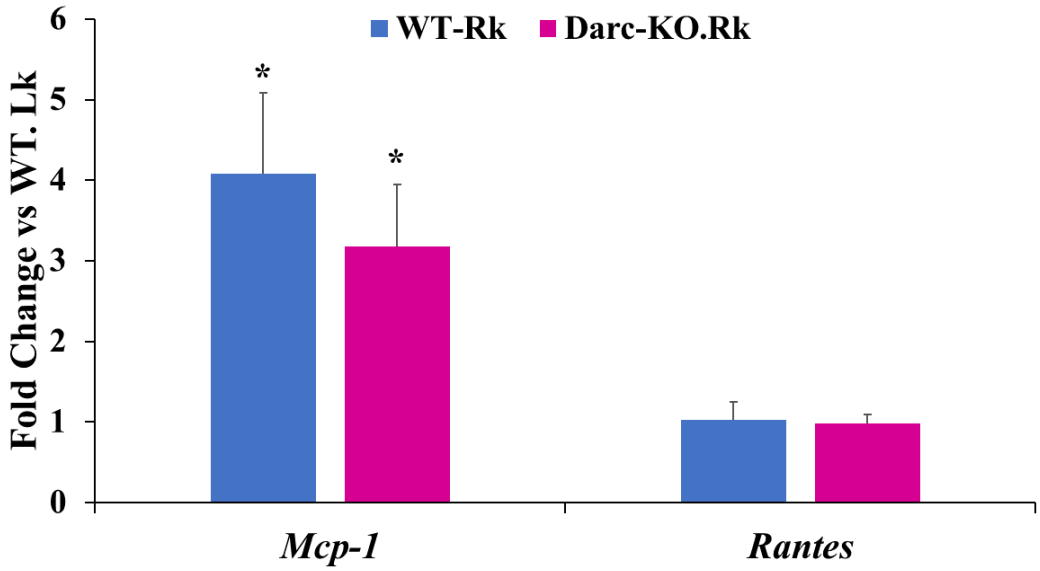
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Mcp-1
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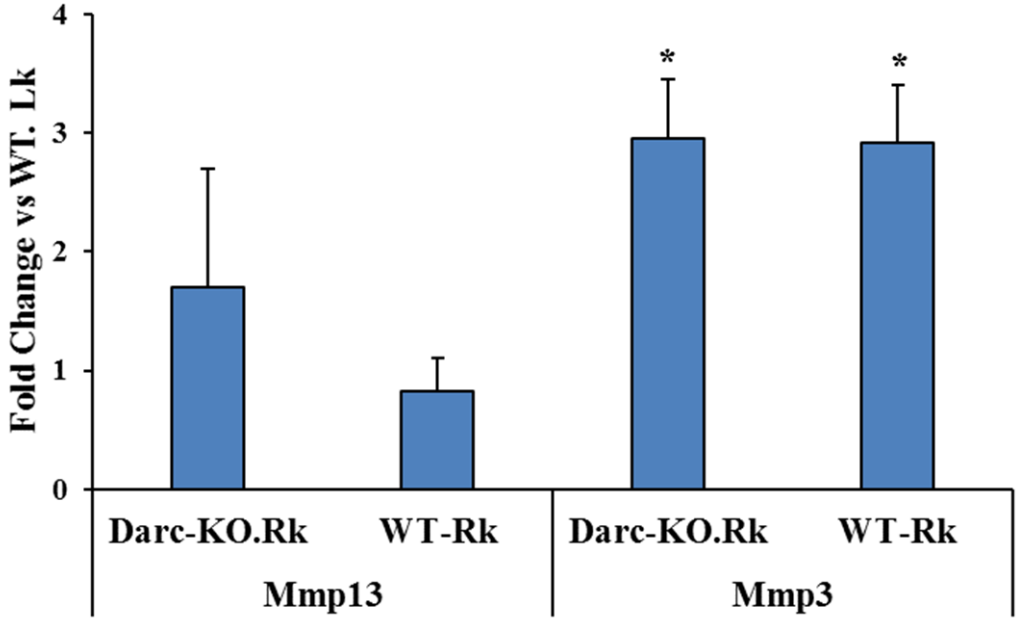
Fig. 4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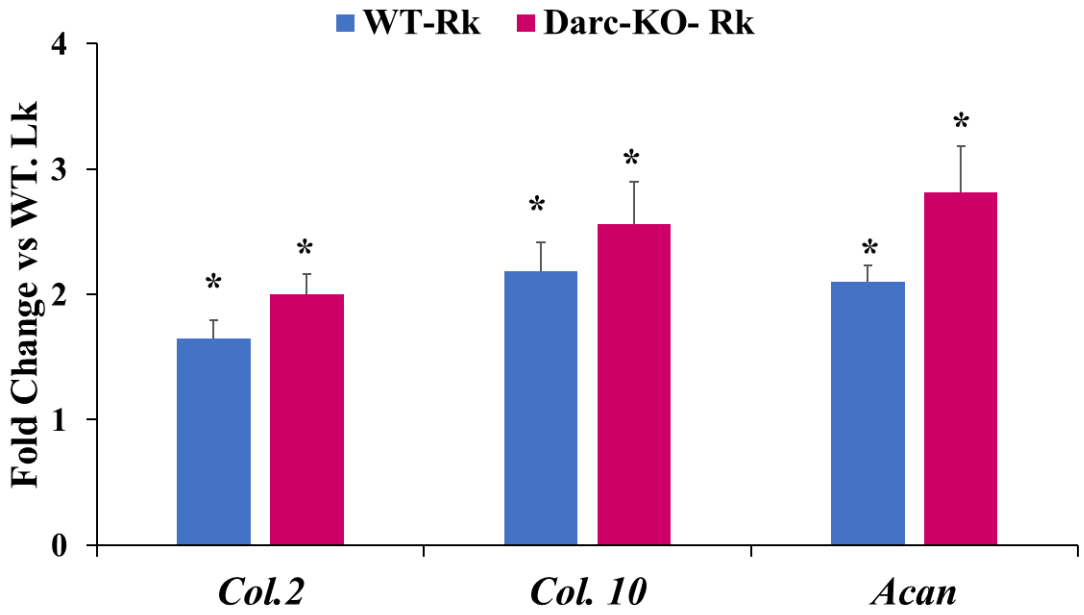
Mmp13
Mmp3

Fig. 5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Fold Change vs WT. Lk
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Fig. 6

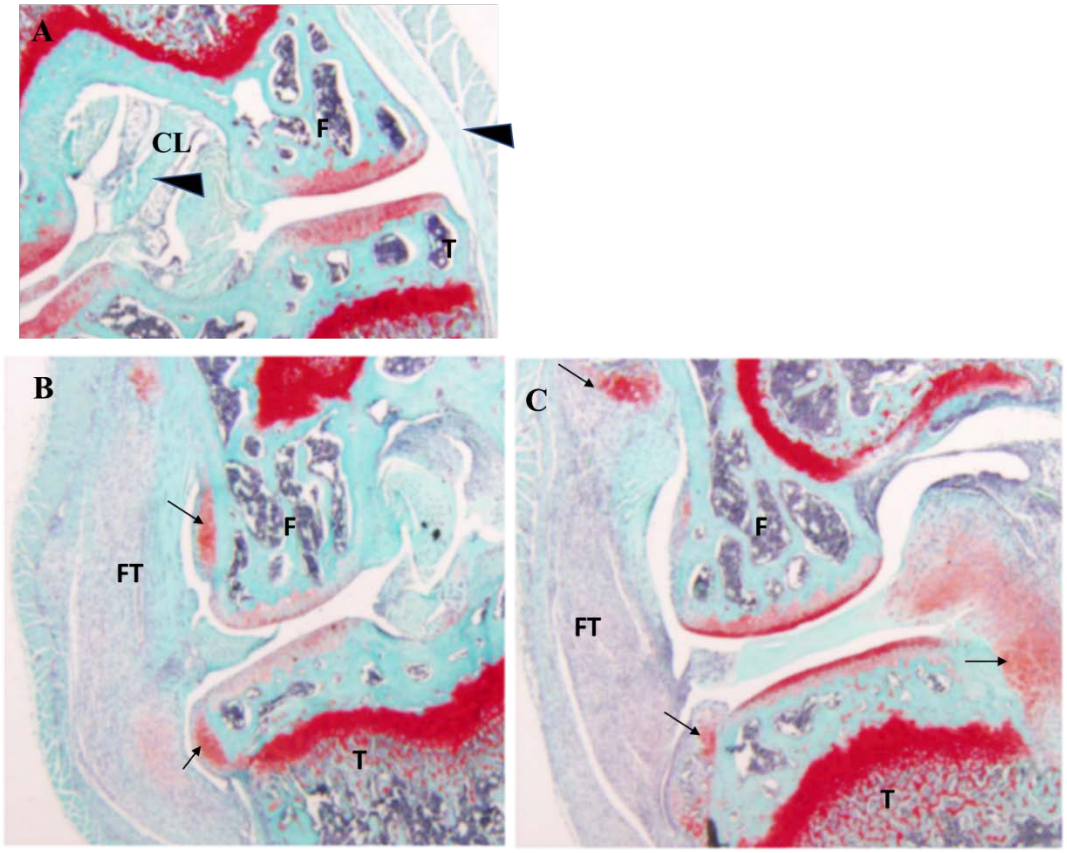
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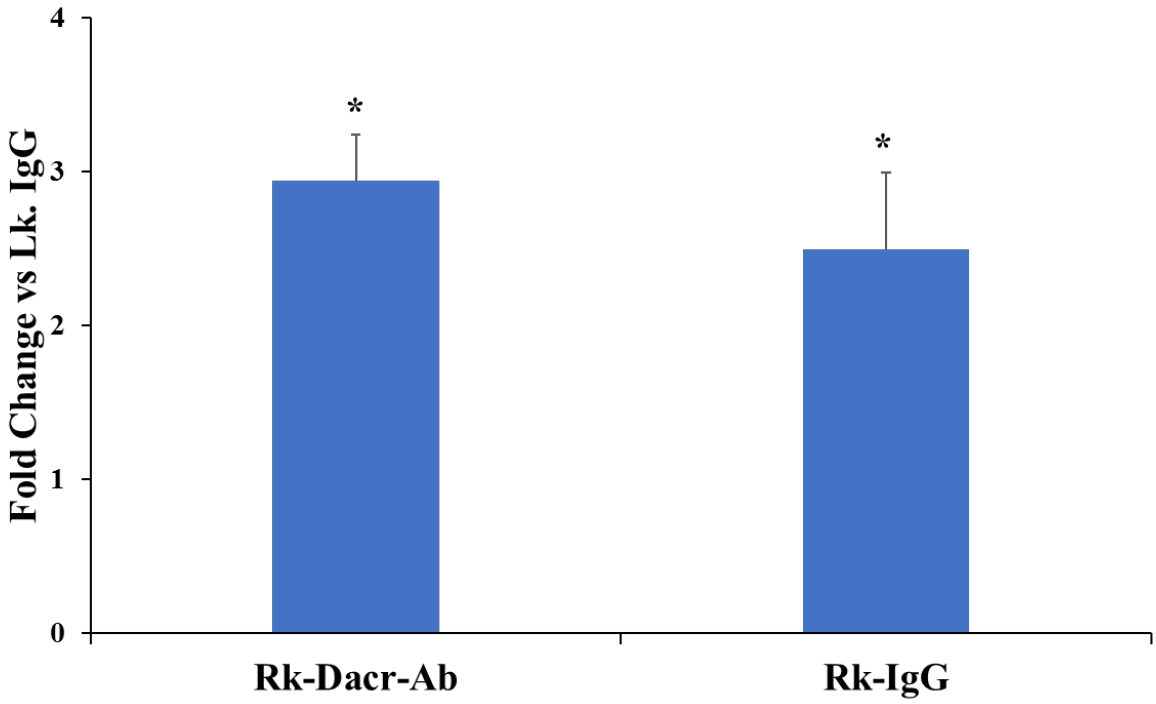
Fig. 7


Fold Change vs Lk. IgG
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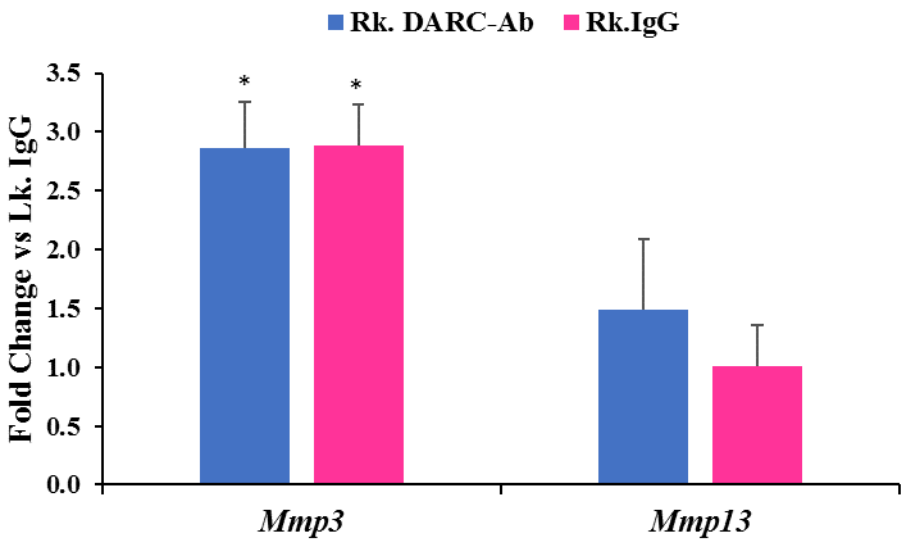
Rk-Dacr-Ab
Rk-IgG

Fig. 8


Rk. DARC-Ab
Rk.IgG
Fold Change vs Lk. IgG
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Mmp3
Mmp13