Sexual Dimorphism in Reactive Oxygen Species Production and a Role for Integrin alpha 1 beta 1 in Estrogen Receptor Expression in Articular Cartilage

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Abstract

Background

Integrin $\alpha 1\beta 1$ protects against osteoarthritis more robustly in females compared to males potentially through its influence on epidermal growth factor receptor (EGFR). The purpose of this study was to investigate the impact of *itga 1* on chondrocyte EGFR activity and downstream reactive oxygen species (ROS) production in male and female mice. Furthermore, chondrocyte expression of estrogen receptor (ER) α and ER β were measured to investigate the mechanism for sexual dimorphism in the EGFR/integrin $\alpha 1\beta 1$ signaling axis.

Methods

Femoral and tibial cartilage of male and female, wild type and *itga 1*-null mice were processed for *ex vivo* confocal imaging of ROS, immunohistochemical analysis of 3-nitrotyrosine, or immunofluorescence of pEGFR and ER α and ER β .

Results

We show that ROS producing chondrocytes are more abundant in female *itga 1*-null compared to wild type mice *ex vivo*, however *itga 1* had limited influence on the percent of chondrocytes stained positively for 3-nitrotyrosine or pEGFR *in situ*. In addition, we found that *itga 1* influenced ER α and β expression in femoral cartilage from female mice, and that ER α and β were co-expressed as well as co-localized in chondrocytes. Finally, we show sexual dimorphism in ROS and 3-nitrotyrosine production, but surprisingly not in pEGFR expression.

Conclusions

Together these data highlight sexual dimorphism in the EGFR/integrin $\alpha 1\beta 1$ signaling axis and underline the need for further investigation into the role of ERs in this biological paradigm. Understanding the molecular mechanisms underlying the development of osteoarthritis is essential for the development of individualized, sex-specific treatments in this age of personalized medicine.

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by articular cartilage degradation, meniscal damage, synovial inflammation, and osteophyte formation at the joint margins [1]. For the patient, this manifests as joint pain and stiffness, and reduced mobility and quality of life. As such, OA is a leading cause of disability in North America affecting 1 in 6 adults [2, 3]. Many risk factors have been associated with OA including obesity, age, sex, and joint trauma, though the exact etiology of OA remains

unknown [2]. Medication and therapy alleviate joint pain and inflammation and/or help to maintain or restore joint function, however treatment options to slow or stop the progression of the disease itself remain elusive [1, 2]. Understanding the responses of chondrocytes to the early stages of OA may reveal novel molecular targets for treatment strategies that might delay the remodelling of the extracellular matrix, fibrillation, fissuring and ultimate destruction of the articular cartilage in joints.

Integrins are heterodimeric transmembrane extracellular matrix receptors and mediators of cell-cell interactions involved in the activation of numerous intracellular signaling cascades including regulation of cell adhesion, differentiation, as well as matrix remodeling [4, 5]. Of particular interest to OA is integrin $\alpha 1\beta 1$ that is expressed by chondrocytes and binds collagens type II and VI and laminin in cartilage [6, 7]. Importantly, the $\alpha 1$ subunit pairs exclusively with the $\beta 1$ subunit and therefore deletion of the $\alpha 1$ subunit in mice (*itga 1*-null) renders them integrin $\alpha 1\beta 1$ deficient, while leaving the other partnerships of the $\beta 1$ subunit untouched [8]. Integrin $\alpha 1\beta 1$ is upregulated in both subclinical and end stage OA in cynomolgus macaque and mice [6, 9]. *Itga 1*-null mice develop spontaneous OA two to three months earlier than wild type mice [9] and post-traumatic OA one month earlier than wild type mice, with the latter result exclusive to females [10]. Together these data suggest that integrin $\alpha 1\beta 1$ protects against OA when it is upregulated in the early stages of disease, with this influence being more robust in females compared to males. The molecular mechanisms by which integrin $\alpha 1\beta 1$ affords this chondroprotection however, are not well understood.

One possible mechanism for the protection of integrin $\alpha 1\beta 1$ against OA is through its suppression of epidermal growth factor receptor (EGFR) signaling. Integrin $\alpha 1\beta 1$ can control EGFR through T-cell protein tyrosine phosphatase (TCPTP) that can either dephosphorylate EGFR or act on caveolin-1, a scaffolding protein involved in EGFR activation [11]. Non-cartilaginous tissues in the *itga 1*-null mouse have increased basal levels of phosphorylated EGFR and associated reactive oxygen species (ROS) production, increased levels of phosphorylated caveolin-1, and increased NADPH oxidase activity [11–15]. As such, one might expect EGFR activity to be upregulated in the chondrocytes of *itga 1*-null mice, leading to increased catabolic activity in cartilage contributing to an earlier onset of OA. In this regard, we have shown that treatment with the EGFR inhibitor erlotinib protected against cartilage degradation, with this effect being more robust in *itga 1*-null compared to wild type mice, and in female compared to male mice [10].

The research outlined above suggests that both integrin $\alpha 1\beta 1$ and EGFR signaling have sex dependent effects on cartilage. Interestingly, emerging evidence indicates crosstalk between EGFR and estrogen receptors (ERs), insinuating that ERs could be involved in the integrin-EGFR-mediated sex differences seen in chondrocyte signaling. The two isoforms, ER α and ER β , have been identified in the articular cartilage of humans and various animals [16–18], however the evidence regarding sex effects on ER expression is conflicting. Some studies report increased protein expression in females compared to males [19], while others report the opposite [20]. In articular cartilage, the ligand-independent ER activation pathway is connected to the development of OA where growth factors can activate ERs directly and indirectly through MAP or PI3/Akt kinase-mediated phosphorylation, in the absence of ER ligands [21].

Therefore, changes in EGFR activity, mediated by integrin $\alpha 1\beta 1$, could influence ER expression and contribute to the sexual dimorphism in knee OA in *itga 1*-null mice.

Therefore, the purpose of this study was to measure the impact of *itga 1* on EGFR activity and downstream ROS production in knee cartilage from male and female mice. In addition, chondrocyte expression of ER α and ER β were measured to investigate the mechanism for sexual dimorphism in the EGFR/integrin $\alpha 1\beta 1$ signaling axis. We hypothesized that there would be increased ROS production as well as increased expression of pEGFR and 3-nitrotyrosine in chondrocytes of *itga 1*-null mice compared to wild type controls, and that this effect would be greater in females compared to males. Finally, we also expected increased expression of ER α and β in females compared to males, with a greater effect seen in *itga 1*-null compared to wild type mice.

Materials And Methods

Animals

All methods were approved by the University of Guelph Animal Care Committee (AUP#3655). Skeletally mature (18 ± 2 weeks) wild type and *itga 1*-null [8], female and male BALB/c mice ($n = 3$ per group) were selected for experiments from breeding colonies at the University of Guelph. Genotype was determined through a multiplex polymerase chain reaction using DNA extracted from ear notches. Mice were weighed (mean $\pm$ standard deviation; wild type female (28.1 ± 1.2 g) and male (31.1 ± 2.1 g), *itga 1*-null female (27.4 ± 1.6 g) and male (33.0 ± 2.2 g)) then anesthetized with isoflurane and euthanized by cardiac puncture followed by cervical dislocation.

Ex vivo ROS measurement in intact femora

Left and right femora were dissected from male and female, wild type and *itga 1*-null BALB/c mice. Femora were kept hydrated with phosphate buffered saline during dissection, before being submerged in iso-osmotic (300 mOsm) media (Thermo Fisher Scientific, Mississauga, ON)). Femora were incubated (37°C and 5% CO_2) with the live cell stain calcein AM (4 μM , Thermo Fisher Scientific) followed by dihydroethidium (10 μM , Calbiochem, Etobicoke, ON) which reacts with a variety of ROS to form 2-hydroxyethidium [22,23].

For imaging, femora were placed condyles up in a 10 mm Falcon® polystyrene petri dish (Fisher Scientific, Mississauga, ON) and submerged in media, maintained at 37°C by a heat ring (Warner Instruments, Hamden, CT). Z-stack images of femoral condyles were generated using a 63x/0.9N.A. water immersion lens on a Leica DM 6000B confocal microscope connected to a Leica TCS SP5 scanner system (Leica Microsystems, Concord, ON). Both stains were excited using a 488 nm laser. Calcein fluorescence was captured at 505–525 nm and 2-hydroxyethidium fluorescence at 615–635 nm. These wavelength collection bands are typically used for live/dead assays and pilot tests staining with calcein or dihydroethidium alone confirmed that the emission bands were far enough apart to stop bleed through of fluorescence between the channels. Z-stacks (40 μm) had a resolution of 1024x1024 pixels and a step

size of 0.67 μ m with the pinhole set to 0.5 Airy units (74.25 μ m). To mitigate the effects of time post-staining on condylar differences in 2-hydroxyethidium fluorescence, the order of imaging was alternated between medial or lateral condyle being first per replicate.

Cartilage Preparation for Immunohistochemical Analysis

Femora and tibiae were isolated using micro-dissection, fixed overnight in 4% paraformaldehyde (Thermo Fisher Scientific, Mississauga, ON), decalcified for 32 hours (Cal-Ex II, Thermo Fisher Scientific, Mississauga, ON) and dehydrated (25% sucrose) overnight at 4°C. Femora were trimmed to two thirds their length and tibiae were cut midshaft before being embedded in cryomolds (15mm x 15mm x 5mm, VWR, Mississauga, ON) filled with optimal cutting temperature medium (Thermo Fisher Scientific, Mississauga, ON). In order to achieve physiological orientation of the femur, the shaft was balanced against the edge of the mold while the distal end was positioned in the center of the mold with the condyles facing up. Tibiae were oriented flat in the mold with the anterior side facing up. Cryosections (10 μ m) were cut in the coronal plane (CM 3050S, Leica Microsystems, Richmond Hill, ON) with four sections placed on each microscope slide (Superfrost Plus, Thermo Fisher Scientific, Mississauga, ON) and stored at -80°C until use. A single slide from each of the three animals in each group was selected for immunohistochemistry or immunofluorescence, with all slides representing the center of the joint, within the cartilage-cartilage contact region of the medial and lateral femoral condyles/tibial plateaus. Section quality (tissue flat to the slide, no folds or tears) was assessed using a brightfield microscope (Nikon Eclipse e400, Nikon, Mississauga, ON). Sections were then allocated to a treatment group (full antibody application, secondary only control, or blank control).

Immunohistochemistry for 3-nitrotyrosine

Endogenous peroxidases were quenched (3% hydrogen peroxide in methanol) followed by blocking (4% normal horse serum) for one hour in a humidified chamber. Sections were then incubated with mouse monoclonal anti-nitrotyrosine antibody (1:500, ab61392, Abcam, Cambridge, MA) overnight at 4°C. The Ultra-Sensitive ABC Peroxidase Mouse IgG Staining Kit (Thermo Fisher Scientific, Mississauga, ON) was used for the application of the secondary antibody as well as the ABC complex. Sections were developed with 3–3'-diaminobenzidine (Sigma-Aldrich, Oakville, ON) and counterstained with hematoxylin for imaging using light microscopy. Images were captured using a Nikon Eclipse e400 microscope outfitted with a Nikon Coolpix 990 digital camera (Nikon, Mississauga, ON). Using the 40x/0.65 N.A. air objective lens, sequential images of the tissue sections were taken to capture the entire length and depth of the articular cartilage across both the medial and lateral femoral condyles/tibial plateaus.

Immunofluorescence for ER α , ER β and pEGFR

Sections were blocked (5% normal goat serum) for one hour at room temperature. To determine the presence of ER α and ER β , anti-ER α mouse monoclonal (1:200, MA5-13191) and anti-ER β rabbit polyclonal (1:200, PA1-310B) primary antibodies (Thermo Fisher Scientific, Mississauga, ON) were diluted and applied simultaneously to sections to incubate overnight at 4°C. Goat anti-mouse IgG (H + L) F(ab')₂ fragment Alexa Fluor 647 (1:500, A21237) and goat anti-rabbit IgG (H + L) Alexa Fluor 555 (1:500,

A21428) secondary antibodies (Thermo Fisher Scientific, Mississauga, ON) were then diluted and applied simultaneously to sections to incubate at room temperature for an hour. To determine the presence of pEGFR, anti-EGFR (phosphor 1092) rabbit monoclonal primary antibody (1:100, ab40815, Abcam, Cambridge, MA) and goat anti-rabbit IgG (H + L) Alexa Fluor 555 secondary antibody (1:500, A21428, Thermo Fisher Scientific, Mississauga, ON) were applied as described above. All sections were counterstained with 0.1M Hoechst 33342 (Invitrogen, Burlington, ON), coverslips applied (ProLong Gold, Thermo Fisher Scientific, Mississauga, ON) and sealed with nail polish.

Using a 40x/0.75–1.25 N.A. oil objective (Leica DM 6000B confocal microscope with a TCS SP5 scanner system, Leica Microsystems, Richmond Hill, ON), a single confocal image was taken in the central cartilage-cartilage contact region of each compartment (medial/lateral) and site (femoral condyle/tibial plateau). Image resolution was set to 2048x2048 pixels with a two-line average and an optical slice thickness of 7.419 μm . A brightfield image and sequential fluorescent scans were captured to avoid interference between the fluorescent dyes. The following excitation and emission settings were used for each dye; ER α (ex 633nm, em 670-720nm), ER β and pEGFR (ex 543nm, em 570-600nm or 625–640 respectively) and Hoechst (ex 405nm, em 440-460nm).

Image Analysis

For the *ex vivo* ROS experiment, 2-D Z-projections of each condylar Z-stack were made using FIJI software (Fig. 1A). Cells were manually categorized as either live (calcein present throughout), or dead (calcein not present or speckled, indicating cell lysis) and the viability of the chondrocytes on each femoral condyle was calculated. Only condyles that had > 84% viability were included. To measure 2-hydroxyethidium fluorescence, 2-D Z-projections of 30 individual live cells per condyle were generated from the original Z-stacks of the condyle. For each 2-D Z-projection of an individual chondrocyte, background fluorescence was calculated as the average of the extracellular fluorescence and subtracted from the entire 2-D Z-projection prior to calculating the mean intensity of 2-hydroxyethidium fluorescence.

For 3-nitrotyrosine immunohistochemistry, images were merged to create a collage of each femoral condyle/tibial plateau and the tidemark was marked using the 'draw' tool (PowerPoint). Sample collages were randomized and the total number of chondrocytes in the articular cartilage, and those stained positively for 3-nitrotyrosine were manually quantified by two blinded graders. Zonal analysis across the depth of the cartilage was not conducted as the zones could not be distinguished in the thin femoral cartilage (20–30 μm , 3–4 cell layers). For pEGFR, ER α and ER β immunofluorescence, the tide mark and articular surface of the cartilage seen on the differential interference contrast images were marked (ImageJ, National Institutes of Health, Maryland, United States), saved and randomized as .tiff files ready for counting. The total number of chondrocytes and those stained positively for pEGFR, ER α alone, ER β alone or both ER α and β were manually counted by two blinded graders and tallied electronically (ImageJ, National Institutes of Health, Maryland, United States).

Statistical Analysis

For the immunohistochemical and immunofluorescence analyses, Pearson correlations were run between the data sets from the two graders. Pearson correlation coefficients of 0.88 for 3-nitrotyrosine counts, 0.97 for pEGFR counts, and 0.97 for ER α and β counts were obtained and therefore the cell counts of the two graders were averaged and percent positive cells was calculated [24]. The mean 2-hydroxyethidium fluorescence measurements as well as the percent of chondrocytes positively stained for 3-nitrotyrosine or pEGFR were subject to an ANOVA, while the ER data was subject to a repeated measures ANOVA, all with sex (male/female), genotype (wild type/*itga 1*-null), site (femoral condyle/tibial plateau) and condyle (medial/lateral) as factors (Statistica™, Tibco, Palo Alto, CA). Fisher LSD post hoc tests were conducted where appropriate and significance was set at $p < 0.05$.

Results

Ex vivo ROS measurement on intact femora

2-hydroxyethidium is an *ex vivo* fluorescent product of a direct reaction between a variety of ROS and the small molecule dihydroethidium which can be analyzed as a measure of ROS activity in live cells [22, 23]. On average, basal levels of 2-hydroxyethidium were larger in chondrocytes from male compared to female mice with this effect being more pronounced on the medial compared to the lateral condyle ($p < 0.04$) (Fig. 1B). Furthermore, 2-hydroxyethidium was unaffected by genotype in male mice but was greater in female *itga 1*-null compared to female wild type mice ($p < 0.001$) (Fig. 1B).

3-nitrotyrosine Immunohistochemistry

3-nitrotyrosine is an *in situ* indirect measure of ROS (the byproduct of superoxide reacting with nitric oxide and then with proteins [25]). Chondrocytes stained positively for 3-nitrotyrosine throughout cartilage depth across the tibial plateaus and femoral condyles relative to secondary only control sections (Fig. 2A). On average, males had 10% more chondrocytes stained positively for 3-nitrotyrosine compared to females ($p = 0.009$) (Fig. 2B). Genotype, site, and compartment did not affect 3-nitrotyrosine staining ($(p > 0.05)$ Fig. 2C or data not shown).

pEGFR Immunofluorescence

Chondrocytes stained positively for pEGFR throughout cartilage depth across the tibial plateaus and femoral condyles relative to secondary only control sections (Fig. 3A). Chondrocyte expression of pEGFR was affected by a genotype and site interaction ($p = 0.014$) (Fig. 3B). Specifically, 15% more *itga 1*-null chondrocytes stained positively for pEGFR on the tibial plateaus compared to the femoral condyles ($p < 0.001$), but there was no site effect in wild type mice. Furthermore, 8% more femoral chondrocytes stained positively for pEGFR in wild type compared to *itga 1*-null mice ($p = 0.01426$), however there was no genotype effect in the tibia. Sex and compartment had no influence upon chondrocyte expression of pEGFR ($(p > 0.05)$ Fig. 3C or data not shown).

Estrogen Receptor Immunofluorescence

Chondrocytes stained positively for ER α and β throughout cartilage depth across the tibial plateaus and femoral condyles relative to secondary only control sections (Fig. 4A). ER α and β were present in the nucleus as well as the cytoplasm of chondrocytes and the receptors were sometimes colocalized in the cytoplasm (Fig. 5). The percent of chondrocytes stained positively for ERs was influenced by sex ($p = 0.028$) as well as a genotype and site interaction ($p = 0.004$). In the tibia (Fig. 4B), the percent of chondrocytes stained positively for both ER α and β was at least 40% greater than either receptor alone or no receptor, independent of genotype and sex ($p < 0.001$). This expression pattern was also observed in femoral chondrocytes (Fig. 4C) from male and female *itga1*-null mice ($p < 0.001$) and male wild type mice ($p < 0.0055$). In femoral chondrocytes from female wild type mice however, similar numbers (40%) of chondrocytes were stained positively for either ER β alone or both ER α and β .

Discussion

The purpose of this study was to measure the impact of *itga1* on EGFR activity and downstream ROS production in knee cartilage from male and female mice. In addition, chondrocyte expression of ER α and ER β was measured as a first step in understanding the mechanism for sexual dimorphism in the EGFR/integrin $\alpha1\beta1$ signaling axis. We show that ROS producing chondrocytes are more abundant in female *itga1*-null compared to female wild type mice *ex vivo* however *itga1* had limited influence on the percent of chondrocytes stained positively for 3-nitrotyrosine or pEGFR *in situ*. In addition, we found that *itga1* influenced ER α and β expression in femoral cartilage from female mice, and that ER α and β were co-expressed as well as co-localized in chondrocytes. Finally, we show sexual dimorphism in ROS and 3-nitrotyrosine production, but surprisingly not in pEGFR expression.

Firstly, in agreement with our hypothesis we show that *itga1*-null chondrocytes have more ROS production compared to their wild type counterparts when measured *ex vivo* in murine femora, but only in female mice. This is in agreement with previous work from our lab that saw earlier cartilage damage in female *itga1*-null compared to wild type mice following surgery to destabilize the medial meniscus, which was ameliorated with the EGFR antagonist erlotinib [10]. Together, these studies provide evidence to support the protective role of integrin $\alpha1\beta1$ in cartilage through suppression of EGFR activity.

In contrast to our hypothesis however we show that *itga1* had limited influence on the percent of chondrocytes stained positively for 3-nitrotyrosine and pEGFR *in situ*. The divergence in these results may be reflective of our use of a subclinical as opposed to a surgical intervention model of OA in mice, and the contrasting measures of ROS production used. The expression of integrin $\alpha1\beta1$ in cartilage increases significantly during matrix remodeling associated with early OA [9]. Such remodeling was not apparent in the 4-month-old mice used in our experiments. In terms of our measures of ROS production, 3-nitrotyrosine is an *in situ* indirect measure of ROS (the byproduct of superoxide reacting with nitric oxide and then with proteins [25]) whereas 2-hydroxyethidium is an *ex vivo* fluorescent product of a direct reaction between a variety of ROS and the small molecule dihydroethidium [22, 23]. Thus, the dampened

expression of integrin $\alpha 1\beta 1$, and the indirect measure of ROS used, may have hampered our ability to demonstrate a genotypic effect in pEGFR and ROS *in situ*.

We also found that *itga1* influenced ER α and β expression in cartilage from female mice. Specifically, 20% more chondrocytes stained positively for both ER α and β in cartilage from female *itga1*-null femora and this was compensated for by 20% fewer chondrocytes stained positively for ER β alone, when compared to wild type mice. In parallel to our result, it has been shown that cells induced to express ER β increased integrin $\alpha 1\beta 1$ mRNA and protein content as well as increased adhesion to extracellular matrix components such as laminin in breast cancer cell culture [26]. Interestingly however, the *in vitro* treatment of endometrial cells with estrogen had no effect on the expression of various integrins, including integrin $\alpha 1\beta 1$ [27]. These studies together with our findings suggest that integrin $\alpha 1\beta 1$ and ERs can influence the expression and activation of each other independent of estrogen, and thus may contribute to the sexual dimorphism seen in the effect of integrin $\alpha 1\beta 1$ on the development of post-traumatic OA in mice [10].

Independent of the effects of *itga1*, we observed that the vast majority (70%) of chondrocytes express both ER α and β which are often colocalized and are present at the cell surface, in the cytoplasm, and nucleus. Our data are consistent with other reports of ER isoform distribution in various models including rats and cultured human chondrocytes [28–30], however we show their colocalization in chondrocytes for the first time. It is known that both ligand-dependent and -independent ER activity can influence chondrocyte biological activity, and that ER can translocate to the nucleus to influence gene expression in chondrocytes [21]. ER α and β form homodimers and/or heterodimers upon activation [31] and bind to the same DNA sequences (estrogen response elements) *in vitro*, thus jointly regulating DNA transcription in response to activation [32, 33]. Together with our work, this implies that through co-localization of the two dominant isoforms, ER α and β , the receptors may act cooperatively to elicit their cellular actions in chondrocytes.

Independent of *itga1*, we measured sexual dimorphism in chondrocyte ROS production, with male mice expressing more ROS than their female counterparts. Specifically, ROS levels *ex vivo* were at least 30% greater in chondrocytes from male compared to female mice and 10% more chondrocytes *in situ* stained positively for 3-nitrotyrosine in males compared to females. While additional research in chondrocytes is lacking, our findings agree with other studies in non-cartilaginous tissues that saw increased antioxidant enzyme activity in various organs in female compared to male mice [34], and increased ROS production in males compared to females in the analysis of human blood samples [35], and in microvessels of hypertensive rats [36]. In contrast to our ROS results and contrary to our hypothesis, sexual dimorphism was absent in our measurements of pEGFR. This is in contrast to our previous work that saw increased pEGFR expression in male compared to female mice 12 weeks following surgery to destabilize the medial meniscus [10]. As the same antibody was used in both experiments, this disparity is likely due to surgery exacerbating the sexual dimorphism making it measurable in the post-traumatic OA model but not in our subclinical model. Taken together, this evidence suggests that activation of EGFR is stimulated by an injurious or traumatic event, potentially affecting chondrocyte ROS production and thus cartilage degradation in a sex-dependent manner.

Conclusions

We show that ROS producing chondrocytes are more abundant in female *itga1*-null compared to wild type mice *ex vivo* however the influence of *itga1* was not apparent in our *in situ* measurements of 3-nitrotyrosine or pEGFR in this subclinical mouse model. In addition, we found that *itga1* influenced ER α and β expression in femoral cartilage from female mice, and that ER α and β were co-expressed as well as co-localized in chondrocytes. Finally, we show sexual dimorphism in 3-nitrotyrosine production, but surprisingly not in pEGFR expression. Taken together these data provide further support for sexual dimorphism in the EGFR/integrin $\alpha1\beta1$ signaling axis and underline the need for further investigation into the role of ER in this biological paradigm.

Abbreviations

EGFR – Epidermal growth factor receptor

pEGFR – Phosphorylated epidermal growth factor receptor

ER – estrogen receptor

OA - Osteoarthritis

ROS – Reactive oxygen species

TCPTP - T-cell protein tyrosine phosphatase

Declarations

Ethics Approval and Consent to Participate

All methods were approved by the University of Guelph Animal Care Committee (AUP#3655).

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that there are no conflicts of interest.

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Declaration of Interests

The authors declare that they have no competing interests.

Author Contributions

All authors have read and approved the final submitted manuscript. All authors contributed to the research design, or the acquisition, analysis, or interpretation of data; AB and AC were primary contributors to drafting the paper and revising the paper critically.

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Figures

Figure 1

Representative 2-D projections of summed fluorescence throughout a Z-stack of *ex vivo* chondrocytes stained with calcein (live cell dye) and dihydroethidium (that reacts with a variety of ROS to form 2-hydroxyethidium) and the two images merged (A). Images taken from a medial femoral condyle of a male wild type mouse. Mean intensity (arbitrary units) of 2-hydroxyethidium in femoral chondrocytes from the medial and lateral condyles of male and female wild type and *itga1*-null mice (B). Data points

are means (N = 3 femora, n = 90 cells) $\pm$ 95% CI. **a** Different from male equivalent ($p < 0.001$). **b** Different from *itga1*-null equivalent ($p < 0.001$). **c** Different from lateral equivalent ($p \leq 0.011$).

Figure 2

Light microscopy images of the full thickness articular cartilage of the medial tibial plateau or medial femoral condyle from female (i-iv) or male (v-xii) wild type mice (A). Sections are peroxidase stained for 3-nitrotyrosine (i-viii) or secondary only control (ix-xii). Scale bar 20 μ m (i,iv,v,viii,ix,xii) or 50 μ m (ii,iii,vi,vii,x,xi). Note the increased staining in tissues from male compared to female mice. Percent of chondrocytes stained positively for 3-nitrotyrosine as a function of sex (B) or genotype (C). Data points are means (N = 6 femora, n > 1000 cells) $\pm$ 95% CI. **a** Significantly different from female ($p = 0.009$).

Figure 3

Confocal microscopy images of femoral (i,iv,vii) and tibial (ii,iii,v,vi,viii,ix) cryosections showing the articular cartilage from the lateral compartment of *itga1*-null (i-iii) and wild type (iv-ix) male mice immunostained green for pEGFR (i,ii,iv,v) (A). Nuclei were counterstained blue with Hoechst as seen in the differential interference contrast images (iii,vi,ix). Secondary antibody only control sections (vii,viii) show negligible autofluorescence from the murine cartilage. Scale bar 50 μ m. Percent of chondrocytes stained positively for pEGFR as a function of genotype and site (B) or sex (C). Data points are means (N = 6 femora, n > 800 cells) $\pm$ 95% CI. **a** Different from all other data points ($p \leq 0.014$).

Figure 4

Confocal microscopy images of full thickness articular cartilage from the lateral tibial plateau (i-iv) and the medial femoral condyle (v-xii) of female and male *itga1*-null and wild type mice (A). Chondrocytes stained red for ER α (i-viii), green for ER β (i-viii) or secondary only controls (ix-xii). All sections counterstained blue with Hoechst (i-xii). Scale bar 50 μ m. Percent of tibial (B) and femoral (C) chondrocytes stained positively for ER α , ER β , ER α + β , or no receptor as a function of genotype and sex. Note the expression of ER α + β is significantly greater than either receptor alone or no receptor, except in femoral chondrocytes from wild type females. Data points are means (N = 3 femora, n > 400 cells) $\pm$ 95% CI. **a** Significantly different from all other receptor combinations of equivalent sex, genotype and bone ($p < 0.001$). **b** Significantly different from sex counterpart ($p = 0.002$).

Figure 5

Digitally zoomed confocal microscopy images of chondrocytes from the lateral tibial plateaus of female wild type (A-D), female *itga 1*-null (E-H), and male *itga 1*-null (I-L) mice. Images show differential interference contrast (A,E,I), ER α stained red (C,G,K), ER β stained green (D,H,L) or overlay of ER α and β (B,F,J). All nuclei counterstained blue with Hoechst. Note the presence of ER α and ER β in the cytoplasm (C,G,K;D,H,L) and nuclei (G,K;H,L) and colocalization of ER α and β in the cytoplasm (B,F,J). Scale bar 2 μ m.