Sex dimorphic regulation of osteoprogenitor progesterone in bone stromal cells

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Abstract
Increasing peak bone mass is a promising strategy to prevent osteoporosis. A mouse model of global progesterone receptor (PR) ablation showed increased bone mass through a sex-dependent mechanism. Cre-Lox recombination was used to generate a mouse model of osteoprogenitor-specific PR inactivation, which recapitulated the high bone mass phenotype seen in the PR global knockout mouse model. In this work, we employed RNA sequencing analysis to evaluate sex-independent and sex-dependent differences in gene transcription of osteoprogenitors of wild-type and PR conditional knockout mice. PR deletion caused marked sex hormone-dependent changes in gene transcription in male mice as compared to wild-type controls. These transcriptional differences revealed dysregulation in pathways involving immunomodulation, osteoclasts, bone anabolism, extracellular matrix interaction and matrix interaction. These results identified many potential mechanisms that may explain our observed high bone mass phenotype with sex differences when PR was selectively deleted in the MSCs.

Introduction
Osteoporosis is a disease that affects more than 53 million people in the United States (NIH 2016). Individuals with osteoporosis are usually treated with medications to either maintain or increase bone mass. These treatments include bisphosphonates, parathyroid hormone, RANK ligand inhibitors and selective estrogen receptor modulators. However, these therapeutic agents are often not prescribed until a patient is diagnosed with osteoporosis. Peak bone mass is associated with the risk of developing osteoporosis in an individual's lifetime. The greatest gain in bone mass occurs during a rapid bone growth period associated with puberty (Johnston & Slemenda 1993). Bone gain slows and bone mass is maintained through balanced remodeling starting around 25 years of age and lasting for nearly two decades, after which time bone loss begins (Johnston & Slemenda 1993). If a high peak bone mass is achieved, an individual’s risk of osteoporosis would be delayed as the risk of fracture is strongly associated with bone density (Johnston & Slemenda 1993). Attempts to improve peak bone mass through diet and weight-bearing exercise during or before puberty have had limited success, but there is a clear need for interventions with greater efficacy.
Progesterone receptor deletion in MSCs

Materials and methods

Mouse strains and animal procedures

PR-flox B6/129 mice were obtained from Dr John Lydon at the Baylor College of Medicine. A targeting vector designed to replace part of exon 2 of the PR gene with a selectable marker was used to create a strain of mice carrying a conditional null PR allele (Fernandez-Valdivia et al. 2010). These PR-flox C57BL/6J mice were then crossed with Prx1-Cre mice (Jackson Laboratory) to create PrKO mice. The Prx1; PrKO mouse line was backcrossed at least 10 generations. The efficiency of the Prx1-Cre PrKO system was validated through undetectable levels of PR in Western blot analysis of femoral protein (Zhong et al. 2017). One set of PRKO and WT (Cre-negative, PR-flox/flox) mice were either ovariectomized (OVX) or orchietomized (ORX) at two months of age and killed at three months of age. Gonadectomy was confirmed by the absence of ovaries or testes at necropsy, bone mass (microCT) and bone turnover. A second set of PRKO and WT mice were left intact and killed at 2 months of age. Mice were housed in an animal facility with controlled environmental conditions (12-h light/dark cycle, temperature 22°C) and had access to food/water ad libitum. PCR-based strategies were used to genotype the mice. At 2 months of age, the mice were exsanguinated by cardiac puncture and dissected. All animal experiments were performed per protocols approved by the University of California, Davis Institutional Animal Care and Use Committee.

Bone marrow-derived osteoprogenitor cells isolation and culture

The condyles of the proximal and distal femurs were removed and the bone marrow was flushed out with phosphate-buffered saline. The cell suspension was filtered through a 70 µm filter and plated into a dish. The first media change was done less than 72 h after plating the cells in the flask. These bone marrow stromal cells (BMSCs) were maintained in stem cell medium for 10 days and then were stimulated to differentiate into osteoblasts in osteogenic media containing 50 µg/mL ascorbic acid and 10 mM β-glycerol phosphate for 3 days (Life Technologies) before being used for RNA isolation and sequencing (n=3–4/genotype/sex).

RNA isolation and sequencing

Transcriptome profiling was performed using a directional, strand-specific mRNA-Seq approach. Briefly, total RNA was extracted using TRIzol (Invitrogen). RNA-Seq libraries were prepared from 0.1 µg total RNA using the KAPA Stranded mRNA-Seq Kit (Kapa Biosystems Inc., Wilmington, MA, USA) per the manufacturer's standard protocol. Poly-adenylated mRNA was purified from total


Progesterone is a sex steroid known for its reproductive system effects. Clinical studies that evaluated the use of progesterone containing oral contraceptives found slight reductions in bone mineral density in the treated group compared to controls at both central and peripheral skeletal locations (Cundy et al. 1991, 1996, 1998, Cundy & Reid 1997, Bachrach et al. 2000, Petitti et al. 2000, Cromer et al. 2006). Progesterone's functions are largely mediated through the progesterone nuclear receptor (PR), a ligand-regulated transcription factor (Conneely & Lydon 2000). The PR is expressed by cultured osteoblasts and osteoclasts (Pensler et al. 1990, Wei et al. 1993, MacNamara et al. 1995, MacNamara & Loughrey 1998, Yao et al. 2010) and is present in vivo in mouse bone at 4 weeks of age (Zhong et al. 2015). However, PR's effect on bone metabolism remains unclear. Our research group reported that global PR-knockout (PRKO) mice had increased bone mass – female PRKO mice had increased bone formation and male PRKO mice had decreased bone resorption compared to 1- to 12-month-old wild-type (WT) mice (Yao et al. 2010). Conditional PR inactivation in mesenchymal stem cells (MSCs) driven by Prx1-Cre (PrKO) recapitulated the global PRKO phenotype with some differences: 2-month-old male and female mutants had a 85% and 51.5% greater peak bone mass compared to their WT controls, respectively, with increased trabecular surface-based bone formation observed in males and decreased osteoclast surface observed in females (Zhong et al. 2017). Notably, the male Prx1-Cre PrKO mice had increased MSC frequency in bone marrow stromal cells as well as increased osteogenic potential, as compared to control (Zhong et al. 2017). Conditional PR inactivation in more terminally differentiated cells using Bglap-Cre and Dmp1-Cre failed to completely reproduce the increased bone phenotype (Zhong et al. 2017). Therefore, we hypothesized that progesterone signaling through the PR in osteoprogenitors may be a key regulator of sexual dimorphism in the acquisition of peak bone mass.

In this work, we utilized Prx1-Cre to conditionally inactivate the PR gene in MSCs of male- and female-intact and gonadectomized mice and analyzed the differences in gene transcription.
RNA and ribosomal RNA was removed by binding to oligo(dT) beads, followed by RNA fragmentation at 94°C in the presence of magnesium. Double-stranded cDNA was generated by random-primed first-strand synthesis and subsequent second-strand synthesis in the presence of dUTP for strand marking. The double-stranded cDNA was 3'-A tailed and indexed, Illumina-compatible adapters were ligated. The libraries were then enriched by high-fidelity PCR amplification (13 cycles) with KAPA HiFi HotStart DNA Polymerase and adapter-specific primers. Subsequently, libraries were combined for multiplex sequencing on an Illumina HiSeqRNA HiSeq 3000 System (50-bp single read; 8 libraries/lane; ~37 million reads/sample) (Bentley et al. 2008).

QC and pre-processing

Image processing, base calling, quality scoring (Phred) and sample demultiplexing were executed by HiSeq Control Software with Real-Time Analysis (HCS v3.3.41/RTA 2.5.2) and bcl2fastq Conversion Software (Illumina; San Diego, CA, USA). RNA was size-selected, ribosomal RNA depleted and poly(A) enriched. Non-coding RNA was not our focus but some, like Xist (polyadenylated ncRNA), were present in our data. Sequence data were analyzed using a standard TopHat-Cufflinks workflow (Trapnell et al. 2012). RNA-seq sequence reads were aligned to the reference mouse genome assembly (Dec. 2011, GRCm38/mm10) using TopHat software (Trapnell et al. 2010), which performs splice junction mapping after read alignment with Bowtie2 (Langmead & Salzberg 2012). Using SubRead (Liao et al. 2014), aligned reads were summarized using featureCounts and counts-per-million (CPM) values were calculated for each gene. Genes with less than 1 CPM in fewer than 2 samples were removed. Trimmed mean of M-values normalization was applied to each filtered count dataset (Robinson & Oshlack 2010).

qPCR validation

Differential gene expression was validated in whole bone including bone marrow (n=4-6 biological replicates) using qPCR with primers for Rankl: 5′-CTGACATCGTCTCTTGCTCTGTA-3′ (forward) and 5′-CTGGTITTTCTATGAGTTCTCA-3′ (reverse), Opg: 5′-ACCCGAAAGACTGTCATCACGG-3′ (forward) and 5′-CTCAATACACACACCCTACCATCTC-3′ (reverse), Icam1: 5′-GTATGTCCTAGGTATCCATCCA-3′ (forward) and 5′-CACAGTTCTCAAGCAGCGG-3′ (reverse), Sdf1: 5′-CTGCACACGACTATAAA-3′ (forward) and 5′-CGGAAATCCCGCCAGTACT-3′ (reverse), Csf1: 5′-ATGACGAGAGTTGCAAGG-3′ (forward) and 5′-TCCATTCCAATCATGTGGCTA-3′ (reverse), Trap: 5′-CACCACCCCTCTGAGATTGT-3′ (forward) and 5′-CATCGTCTGACCGGGTTCTG-3′ (reverse), Ccdn1: 5′-GGTACCTTCGACACAAATCTC-3′ (forward) and 5′-CTCTTGCTGCTGCTGCTC-3′ (reverse), and Actb: 5′-GAATGGTGTCAGAAAGGACT-3′ (forward) and 5′-CATGTCGTCCCGATGTGAT-3′ (reverse). Whole bone RNA was harvested by snap freezing tissue in liquid nitrogen and homogenizing in TRizol (Bead Ruptor 12, Omni, Inc., Kennesaw, GA, USA; Invitrogen). Isolated RNA (2 µg/sample) was reverse transcribed to cDNA (RT2 First-Strand Kit; SABioscience, Inc., Frederick, MD, USA) and diluted to 10 µL. RT-PCR was carried out on an ABI Prism 7300 (Applied Bioscience) in 12.5 µL 2x SYBER Green Mix (SABioscience, Inc., Frederick, MD, USA), 1 µL cDNA, 1 µL primers and 10.5 µL H2O. PCRs were run in triplicate using 40 cycles of 95°C, 15 s and 60°C, 60 s. Threshold cycle (Ct) was determined for each gene and compared to the reference gene, Actb, using the delta–delta Ct method to find log2 (fold-change). RNA results were plotted against RNA-seq fold-change values. Linear regression was performed to assess correlation.

Statistical analyses

Two-tailed Wilcoxon rank-sum tests were used on qPCR data to compare sex-matched WT and PRcKO groups. Statistical significance was determined at P<0.05. Data were analyzed using the GraphPad Prism 6 software package.

Differential expression analysis

Differential gene expression was determined using the edgeR (Robinson et al. 2010) R/Bioconductor package following the user’s guide examples (Chen et al. 2008). A negative binomial model was fitted to the filtered, normalized data and the trended dispersion was estimated using the Cox-Reid profile-adjusted likelihood. Batch effect was accounted for in generalized linear models, and likelihood ratio tests were performed over five comparisons: female WT OVX vs female PRcKO OVX; male WT ORX vs male PRcKO ORX; male WT vs female WT; male WT vs male PRcKO and female WT vs female PRcKO. Two biological replicates were run for the gonadectomy experiments and 3–4 biological replicates were run for the
intact experiments. Differentially expressed genes (DEGs) were identified as genes with $-1 > \log_2(\text{fold change}) > 1$ and false discovery rates (FDR) <0.05 by the Benjamini–Hochberg procedure. To reduce the number of identified DEGs and in the male WT vs male PRcKO comparison, the FDR cutoff was reduced to FDR <0.01. DEG lists were then analyzed for up and/or downregulation enrichment against Kyoto Encyclopedia of Genes and Genome (KEGG) pathways using hypergeometric tests and the Benjamini–Hochberg procedure with FDR <0.05 (Young et al. 2010).

Results

Hormone-dependent effect of PRcKO on MSC transcriptional profiles

No significant transcriptome differences were observed in the osteoprogenitor cells in our female WT OVX vs female PRcKO OVX or male WT ORX vs male PRcKO ORX contrasts. Hormonal deficiency induced similar changes in bone turnover in WT vs Prx1; PRcKO mice in both sexes (Fig. 1). These results suggested that bone turnover and transcriptome changes were hormone dependent. Further analyses were focused only on the intact WT and PRcKO groups.

DEG identification across comparisons

2796 DEGs were identified in the male WT vs male PRcKO comparison (851 upregulated in male PRcKO, 1945 downregulated in male PRcKO), 2 DEGs in the female WT vs female PRcKO comparison (2 downregulated in female PRcKO) and 14 DEGs in the male WT vs female WT comparison (9 upregulated in females, 5 downregulated in females). The DEGs identified in the female WT vs female PRcKO comparison, Tnxb ($\log_2$ FC −1.99, FDR 1.36E-02) and Gldc ($\log_2$ FC −2.23, FDR 1.36E-02), were not identified in the male WT vs male PRcKO comparison.

The male WT vs female WT sex difference comparison had 14 DEGs. 8 of the DEGs identified in the male WT vs female WT comparison were also identified as DEGs in the male WT vs male PRcKO comparison (Table 1).

The male WT vs male PRcKO comparison identified a substantial number of DEGs. We have shown 15 DEGs

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Figure 1

Prx1; PRcKO mice and their littermates had similar changes in bone turnover following gonadal deficiency in both sexes. Mice were ovariectomized (OVX) or orchiectomized (ORX) at two months of age and killed one month post surgery. Mice were given two fluorescents labeling at −7 and −1 day(s) before killing. (A). Dynamic bone histomorphometry was performed at the distal femurs from wildtype and Prx1; PRcKO mice of both sexes at 3 months of age. (B) Representative distal femurs from two-month-old female and male Prx1; PRcKO mice were OVX or ORX.

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with the smallest FDR values (Table 2). Because we had evidence supporting many differential gene expressions, we ran KEGG analysis on this comparison to identify biological pathways that may help to explain the increased bone mass phenotype present in the PRcKO animals including the following pathways (Fig. 2).

**Immunomodulatory pathways in male PRcKO** From KEGG analysis on the male WT vs male PRcKO comparison DEGs, some of the most significantly overrepresented pathways involved immunomodulation. These pathways included cytokine–cytokine receptor interaction, chemokine signaling pathway, complement and coagulation cascades, B cell receptor signaling pathway and T cell receptor signaling pathway (Table 3). Most of the identified DEGs involved in these pathways were downregulated.

In the cytokine–cytokine receptor interaction pathway, we found that PRcKO down-regulated CXC chemokines, CXC chemokine receptors, CC chemokines and CC chemokine receptors. Downstream of the chemokine receptors, PRcKO osteoprogenitors had differential expression in chemokine signaling. Notably, regulatory genes, Jak-STAT signaling genes and actin cytoskeleton regulation genes were all downregulated.

In the complement cascade, there was a downregulation of genes in the PRcKO males included the classical and lectin pathways, alternative pathway and their downstream receptors. Three complement cascade genes, Cfd, F2r and Masp1, were upregulated in these PRcKO animals. C2 and C4b, central genes in the classical and lectin pathways, were more than 8-fold downregulated in the PRcKO males. Anaphylatoxin chemotactic receptors C3ar1, C5ar1 and C5ar2 were downregulated in the PRcKO males. Similarly, we observed downregulation in many central genes involved in the coagulation cascade.

The B cell and T cell receptor signaling pathways had many downregulated genes in the PRcKO males, which included those that encode receptors, key signaling molecules and downstream transcription factors.

### Table 1 Wild-type sex difference DEGs in common with male wild-type vs male knockout.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>log₂ (FC) between sexes</th>
<th>FDR between sexes</th>
<th>log₂ (FC) in males</th>
<th>FDR in males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xist</td>
<td>X-inactive specific transcript</td>
<td>−9.71</td>
<td>6.39E-12</td>
<td>3.54</td>
<td>3.81E-03</td>
</tr>
<tr>
<td>Mtus2</td>
<td>Microtubule associated tumor suppressor candidate 2</td>
<td>−1.36</td>
<td>1.59E-03</td>
<td>−1.77</td>
<td>2.18E-07</td>
</tr>
<tr>
<td>Aldh1a7</td>
<td>Aldehyde dehydrogenase family 1, subfamily A7</td>
<td>−2.57</td>
<td>7.33E-03</td>
<td>3.96</td>
<td>1.07E-06</td>
</tr>
<tr>
<td>Tusc5</td>
<td>Tumor suppressor candidate 5</td>
<td>−1.46</td>
<td>8.74E-03</td>
<td>1.31</td>
<td>2.04E-03</td>
</tr>
<tr>
<td>Cd300c</td>
<td>CMRF35-like molecule 6</td>
<td>5.15</td>
<td>2.66E-02</td>
<td>−7.90</td>
<td>9.92E-10</td>
</tr>
<tr>
<td>Aldh1a1</td>
<td>Aldehyde dehydrogenase family 1, subfamily A1</td>
<td>−1.39</td>
<td>2.66E-02</td>
<td>2.45</td>
<td>4.29E-09</td>
</tr>
<tr>
<td>Pde3a</td>
<td>Phosphodiesterase 3A</td>
<td>−1.51</td>
<td>2.66E-02</td>
<td>1.65</td>
<td>3.63E-04</td>
</tr>
<tr>
<td>Emx2</td>
<td>Empty spiracles homebox 2</td>
<td>1.14</td>
<td>3.12E-02</td>
<td>−1.95</td>
<td>3.95E-08</td>
</tr>
</tbody>
</table>

Negative fold-change values indicate downregulation in males and downregulation in the PRcKO group, respectively.

### Table 2 Top 15 DEGs in male WT vs PRcKO by FDR.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>log₂ (FC)</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Msr1</td>
<td>Macrophage scavenger receptor 1</td>
<td>−6.72</td>
<td>8.86E-105</td>
</tr>
<tr>
<td>Myo1g</td>
<td>Myosin IG</td>
<td>−6.94</td>
<td>3.56E-100</td>
</tr>
<tr>
<td>Hcls1</td>
<td>Hematopoietic lineage cell-specific Lyn substrate 1</td>
<td>−5.46</td>
<td>1.32E-87</td>
</tr>
<tr>
<td>Arhgap30</td>
<td>Rho GTPase activating protein 30</td>
<td>−6.91</td>
<td>1.54E-81</td>
</tr>
<tr>
<td>Dok2</td>
<td>Docking protein 2</td>
<td>−5.71</td>
<td>1.85E-81</td>
</tr>
<tr>
<td>Hk3</td>
<td>Hexokinase 3</td>
<td>−7.08</td>
<td>4.82E-81</td>
</tr>
<tr>
<td>Serping1</td>
<td>Serpin family G member 1</td>
<td>−5.76</td>
<td>9.91E-80</td>
</tr>
<tr>
<td>Fmn1</td>
<td>Formin like 1</td>
<td>−5.21</td>
<td>4.35E-76</td>
</tr>
<tr>
<td>Vav1</td>
<td>Vav guanine nucleotide exchange factor 1</td>
<td>−6.68</td>
<td>6.34E-75</td>
</tr>
<tr>
<td>Llrb4a</td>
<td>Leukocyte immunoglobulin-like receptor, subfamily B, member 4A</td>
<td>−6.28</td>
<td>6.16E-70</td>
</tr>
<tr>
<td>Ccr1</td>
<td>C-C chemokine receptor type 1</td>
<td>−5.43</td>
<td>1.26E-68</td>
</tr>
<tr>
<td>Innp5d</td>
<td>Inositol polyphosphate-5-phosphatase D</td>
<td>−6.45</td>
<td>1.23E-67</td>
</tr>
<tr>
<td>Alox5ap</td>
<td>Arachidonate 5-lipoxygenase activating protein</td>
<td>−7.08</td>
<td>5.30E-66</td>
</tr>
<tr>
<td>Fermt3</td>
<td>Fermitin family member 3</td>
<td>−5.66</td>
<td>3.59E-65</td>
</tr>
<tr>
<td>Ncf2</td>
<td>Neutrophil cytosolic factor 2</td>
<td>−6.23</td>
<td>8.88E-65</td>
</tr>
</tbody>
</table>

Positive fold-change values indicate upregulation in the PRcKO group.
Osteoclast-related pathways in male PRcKO KEGG analysis revealed downregulation of genes in pathways related to osteoclasts including: osteoclast differentiation, NF-kappa B signaling and TNF signaling (Table 4). In the osteoclast differentiation pathway, three genes were upregulated in male PRcKO osteoprogenitors: Csf1, Pparg and Tnfrsf11b. Many receptor genes and their ligands were downregulated, along with several important signaling genes.

Many genes in the NF-kappa B signaling pathway were downregulated in PRcKO males. Notably, genes in the antigen receptor signaling and genes involved in regulating inflammation downstream of NFkB were downregulated. Three of the four upregulated genes, Bcl2, Bcl2l1 and Pidd1, act on cell survival downstream of NFkB.

In the TNF signaling pathway, most of the DEGs were found in genes controlled by TNF signaling including downregulation in the genes associated with leukocyte recruitment (chemokines), surface receptors (Fas), inflammatory cytokines (Il1b and Il6), intracellular signaling (Bcl3, Ifi47, Socs3, Tnafip3 and Traf1), other transcription factors (Fos), remodeling extracellular matrix (Mmp3 and Mmp9) and cell adhesion (Icam1 and Vcam1). Notably, the Tnf gene was 26-fold downregulated and the Tnfrsf11b gene was 2.5-fold downregulated in the PRcKO males.

Bone anabolic pathways in male PRcKO Next, we selected those pathways which were related to bone anabolism including Wnt signaling and TGF-beta (Table 5). In our male PRcKO osteoprogenitors, we observed multiple genes upregulated within the canonical Wnt signaling pathway. Genes of inhibitory proteins, which were downregulated were mostly inhibitors for Wnt signaling. Similarly, in the TGF-beta signaling pathway, many central genes were
Progesterone receptor deletion in MSCs

Extracellular signaling pathways in male PRcKO
Some important pathways involving extracellular signaling were dysregulated in the male PRcKO including PI3K-Akt signaling, MAPK signaling, cGMP-PKG signaling and cAMP signaling (Table 6). In the PI3K-Akt signaling pathway, genes of many receptors upstream to PI3K and their ligands were dysregulated and many of the pathway’s central genes were downregulated in the PRcKO males. Notably, some genes encoding proteins phosphorylated by PDK1 were upregulated in the PRcKO males.

Table 3 KEGG pathway analysis results – immunomodulation.

<table>
<thead>
<tr>
<th>KEGG pathway</th>
<th>Upregulated genes in PRcKO</th>
<th>Downregulated genes in PRcKO</th>
<th>FDR upregulated</th>
<th>FDR downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine–cytokine interaction</td>
<td>Bmp7, Clcf1, Csfr1, Gdf5, Met, Pdgfc, Tnfrsf10b, Tnfrsf11b</td>
<td>Ackr3, Acvr1b, Ccl2, Ccl3, Ccl4, Ccl5, Ccl6, Ccl7, Ccl8, Ccl9, Ccl11, Ccl12, Ccr2, Ccr3, Ccr5, Cntfr, Csfr1, Csfr2a, Csfr2b, Csfr2b2, Csfr3, Csfr3r, Cxc3r1, Cxc1, Cxc2, Cxc5, Cxc10, Cxc12, Cxc14, Cxc16, Cxc3, Cxc4, Egfr, Fas, Flt1, Ifnar2, I1a, I1b, I6, I1r1, I1r2, I1rap, I1rg, I1raa, I1r4a, I6ra, I17r, I17ora, I17rora, I17rora, I17ra1, I17ra1a, I17ra5, I17ora, I20ora, I20rora, I21r, I7, Lif, Osm, Pdgfb, P44, Ppba, Tnf, Tnfrsf1b, Tnfrsf9, Tnfrsf11a, Tnfrsf13b, Tnfrsf14, Tnfrsf19, Tnfrsf21, Tnfrsf28, Tnfrsf9, Tnfrsf11, Tnfrsf13b, Tnfrsf14, Vegfa, Vegfc</td>
<td>1.19E-02</td>
<td>4.33E-67</td>
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<tr>
<td>Chemokine signaling</td>
<td>Adcy7, Gnb4, Prkcx</td>
<td>Adcy5, Arrb2, Ccl2, Ccl3, Ccl4, Ccl5, Ccl6, Ccl7, Ccl8, Ccl9, Ccl11, Ccl12, Ccr2, Ccr3, Ccr5, Cxc3r1, Cxc1, Cxc2, Cxc5, Cxc10, Cxc12, Cxc16, Cxc4, Dock2, Fgr, Gnb5, Gng2, Gng4, Gng7, Gngt2, Hck, Jak2, Lyn, Ncf1, Nfkb1a, Nfkb1b, Pik3cd, Pibcb2, Ppbb, Prex1, Prkcb, Ptk2b, Rac2, Rasgpl2, Sdc2, Sdc2p, Sdc73, Sdf1, Sdc73p, Tnf, Vav1, Vav3, Was</td>
<td>6.18E-01</td>
<td>3.37E-33</td>
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<tr>
<td>Complement and coagulation cascades</td>
<td>Cfd, F2r, Masp1</td>
<td>Badrbr1, Clqqa, Clqqa, Clqqa, C1ra, C1s1, C2, C3, C3ar1, C4b, Csar1, Csar2, Cd55, Cbf, Chf, F10, F3, F7, Igtarm, Itgam, Itgam, Itgb2, Itgb2b, Kng1, Kng2, Knt1, Plat, Prost, Serpin1, Serpinc1, Tfpi, Wwf</td>
<td>2.41E-02</td>
<td>8.92E-29</td>
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<tr>
<td>B cell receptor signaling</td>
<td>N/A</td>
<td>Blnk, Btk, Cdk2, Cdk7, Fger2b, Fos, Ifitm1, Inpp5d, Lyn, Nftak2, Nfkb1a, Nfkb1b, Pik3ap1, Pik3cd, Pirb, Plcg2, Prkcb, Ptpn6, Rac2, Rac3, Syk, Vav1, Vav3</td>
<td>1.00E+00</td>
<td>5.34E-15</td>
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<tr>
<td>T cell receptor signaling</td>
<td>N/A</td>
<td>Cd4, Fos, Lcp2, Map3k8, Nfat2, Nfkb1a, Nfkb1b, Pik3cd, Prkcq, Ptpn6, Ptprc, Tec, Tnf, Vav1, Vav3</td>
<td>1.00E+00</td>
<td>7.24E-08</td>
</tr>
</tbody>
</table>

Table 4 KEGG pathway analysis results – osteoclast related.

<table>
<thead>
<tr>
<th>KEGG pathway</th>
<th>Upregulated genes in PRcKO</th>
<th>Downregulated genes in PRcKO</th>
<th>FDR upregulated</th>
<th>FDR downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoclast differentiation</td>
<td>Csfr1, Pparg, Tnfrsf11b</td>
<td>Acp5, Blnk, Btk, Calcr, Csf1r, Csk, Cyba, Fcgr1, Fcgr2b, Fcgr3, Fcgry, Fog, Gab2, Gm14548, Ifnar2, I1a, I1b, I1l1, Irf9, Itgb3, Junb, Lcp2, Lira5, Lira6, Lirrb4a, LOC100038947, Ncf1, Ncf2, Ncf4, Nfct2, Nfkb1a, Oscar, Pik3cd, Pir1a, Pir2, Sirpa, Sirpb1a, Socs3, Spi1, Syp, Tec, Tnf, Tnfrsf11a, Tnfrsf11, Trem2, Tyrobp</td>
<td>4.84E-01</td>
<td>1.72E-33</td>
</tr>
<tr>
<td>NF-kappa B signaling</td>
<td>Bcl2, Bcl21, Card10, Pidd1</td>
<td>Bcl2a1b, Birc3, Blnk, Btk, Ccl4, Ccl4, Ccxd2, Ccxd12, Icam1, I1b1, I1r1, Lbp, Lyn, Nfkb1a, Plcg2, Prkcb, Prkcg, Syp, Ticam2, Tnf, Tnfaip3, Tnfrsf11a, Tnfrsf11, Tnfrsf13b, Tnfrsf14, Traf1, Vcam1</td>
<td>1.04E-01</td>
<td>5.13E-18</td>
</tr>
<tr>
<td>TNF signaling</td>
<td>Csfr1, Rps6ka4</td>
<td>Bcl3, Birc3, Ccl2, Ccl5, Cebbp, Creb31, Creb31c, Ccxi1, Ccxl2, Ccxl3, Ccxl10, Fas, Fos, Icam1, Ifi47, I1b1, I6b, Junb, Lif, Map3k8, Mmp3, Mmp9, Nfkb1a, Nov2, Pik3cd, Socs3, Tnf, Tnfaip3, Tnfrsf11b, Traf1, Vcam1</td>
<td>7.02E-01</td>
<td>1.36E-19</td>
</tr>
</tbody>
</table>
interaction, focal adhesion and regulation of actin cytoskeleton (Table 7). We observed dysregulation in ECM components. Many of these ECM–receptor interactions are also involved in the focal adhesion pathway, which was also enriched in both upregulation and downregulation. Additionally, some genes of components that associate/bind with integrins were also upregulated and downregulated. Some genes involved in regulating focal adhesion were also dysregulated and have been mentioned above in cytokines–cytokine receptor interaction, Wnt signaling, PI3K-Akt signaling and MAPK signaling.

qPCR expression of select genes The RNA-seq results were validated using qPCR on the same RNA samples. Linear regression on the qPCR vs RNA log2 (fold-change) values indicated good correlation (y = 0.9203x − 0.6699; R² = 0.8916; P < 0.0001; Fig. 3A). In the osteoprogenitor RNA, the Rankl/Opg ratio was decreased in the PRcKOs (9.270 in male WT vs −37.037 in male PRcKO; 6.131 in male WT vs 37.037 in male PRcKO).
female WT vs 3.006 in female PRcKO). qPCR was also used to examine transcription of select genes in the entire bone. Whole bone responded similarly to PRcKO as osteoprogenitors – most genes were downregulated and males experience a greater effect (Fig. 3B). Rankl, Opg, Trap and Ccnd1 were all significantly downregulated in the male PRcKOs. None of the genes examined were significantly dysregulated in the female PRcKOs.

Discussion

Compared to other sex steroid receptors such as the estrogen and androgen receptors, less is known about the PR effect on bone cell metabolism. The PR has been shown to be important for sex differences in bone mass acquisition, as genetic and chemical strategies to inhibit PR signaling have been shown to increase peak bone mass accrual, with sex differences (Yao et al. 2010). Previous studies with the Prx1-Cre-driven PRcKO system have demonstrated efficient PR inactivation by Western blot analysis, a sex-dependent increase in MSC frequency in BMSCs, and greater osteogenic potential of those MSCs (Zhong et al. 2017). This report revealed a critical role of PR in MSCs and that the lack of PR signaling in the MSCs significantly affected pathways involving immunomodulation, bone remodeling, extracellular signaling and matrix interactions. Most importantly, these effects were only seen in the males and were hormone (ligand) dependent. These finding suggest that the PR signaling in MSCs intrinsically regulates sex differences in health and disease conditions.

In cultured osteoprogenitors, the male WT vs male PRcKO comparison had many more DEGs compared to the other comparisons (male WT vs female WT and female WT vs female PRcKO). Further analysis identified many KEGG pathways as enriched for DEGs in this male WT vs male PRcKO comparison. In line with our group's

| Table 7 KEGG Pathway analysis results – extracellular matrix interaction. |
|---------------------------|---------------------------|---------------------------|
| KEGG Pathway              | Upregulated genes in PRcKO | Downregulated genes in PRcKO |
|                           |                          | FDR upregulated | FDR downregulated |
| Focal adhesion             | Actn4, Bcl2, Ccnd1, Chad, Col2a1, Col4a5, Col4a6, Col6a3, Col9a1, Col9a2, Col9a3, Comp, Flnb, Itga2, Itga3, Itga6, Itga10, Itgb7, Lamb3, Met, Mylk2, Pdgfc, Prkca, Prkgp, Thbs1, Tnn | Birc3, Cav3, Col11a1, Col16a6, Egfr, Flt1, Igf1, Itga4, Itga7, Itga9, Itgb3, Itgb6, Itgb8, Lama1, Lama4, Lama5, Lamb1, Myl9, Mylk, Mylk4, Mlyplf, Parvg, Pdgfb, Pik3cd, Prkcbl, Rac2, Rac3, Shc2, Thbs3, Vav1, Vav3, Vegfa, Vegfc, Vwff | 4.88E-19 | 6.60E-14 |
| Regulation of actin cytoskeleton | Actn4, Diap3, F2r, Fgd1, Fgf2, Fgfr2, Fgfr3, Igapap3, Itga2, Itga3, Itga6, Itga10, Itgb7, Myh10, Mylk2, Pdgfc, Pfn2, Ras2 | Bdkrb1, Cd14, Cypip2, Egfr, Fgf10, Fgf11, Fgf13, Fgf21, Itga4, Itga7, Itga9, Itgam, Itgax, Itgb2, Itgb21, Itgb3, Itgb6, Itgb8, Myl9, Mylk, Mylk4, Mlyplf, Nckap1, Pdgfb, Pik3cd, Pip5k1b, Rac2, Rac3, Ras, Scn, Vav1, Vav3, Was | 4.87E-09 | 1.01E-13 |
| ECM-receptor interaction | Chad, Col2a1, Col4a5, Col4a6, Col6a3, Col9a1, Col9a2, Col9a3, Comp, Hmmr, Itga2, Itga3, Itga6, Itga10, Itgb7, Lamb3, Thbs1, Tnn | Cd36, Col11a1, Col16a6, Itga4, Itga7, Itga9, Itgb3, Itgb6, Itgb8, Lama1, Lama4, Lama5, Lamb1, Npnt, Sdc1, Thbs3, Vwff | 5.04E-20 | 2.92E-09 |

Figure 3
Validation of select gene expressions by qRT-PCR. (A) qPCR vs RNA-seq for selected genes. log2 (fold-change) values plotted and used to assess the correlation. (B) Whole bone RT-PCR for selected genes (n=4–6/group). Mean ± s.e.m. (*P<0.05; Mann–Whitney test vs WT).
reported bone mass findings (Zhong et al. 2017), PRcKO had a greater effect on male gene transcription compared to females.

The effects of progesterone and PR on immunomodulation are well described for pregnancy and regulation of menstrual cycles. Progesterone, mediated through PR, decreased bone marrow-derived dendritic cells and the effects were more pronounced in the females than the males (Butts et al. 2008). The presence of progesterone and its interaction with PRs at the decidua level appears to play a major role in the modulation of maternal immune response and suppression of inflammation (Di Renzo et al. 2016). Blocking PR was associated with high NK activity as well as induction of pro-inflammatory cytokines TNFα, IFN-γ and NF-κB that increased resorption rate (Szekeres-Bartho et al. 2005, Tiwari et al. 2016). In the nervous system, after a spinal cord injury, PR modulates the astrocyte response and commitment of oligodendrocyte progenitor cells to enhance remyelination (Labombarda et al. 2015) and progesterone treatment decreases neuropathic pain associated with spinal cord injury (Coronel et al. 2016). The absence of PR signaling in the MSCs exerted an immunosuppressive response that was more pronounced in the males, suggesting that PR expression levels may elucidate disparities in gender-specific immune disorders and the potential role for targeting PR as gender-specific medicine.

In the female reproductive tract and brain, the PR is reported to regulate complement components, particularly C3 (Hasty et al. 1994, Lundeen et al. 2001, Brinton et al. 2008). Anaphylatoxins, C3a and C5a, have been reported to be chemotactic factors for MSCs and osteoblasts, with stronger effects in osteoblasts, which may be due to increased C5aR expression (Ignatius et al. 2011, Schraufstatter et al. 2009, Schoengraf et al. 2013). C3 expression is increased by osteoclastogenesis and C3arR signaling has been shown to stimulate osteoblast differentiation (Matsuoka et al. 2014). Inactivating the PR in male MSCs decreased the mRNA for both C3arR and C5arR, as well as many other complement-related genes, which may signal resistance to complement.

Apart from the classical progesterone nuclear receptor, membrane-associated nongenomic-binding protein for progesterone has been cloned. These membrane-binding sites for progesterone have been described as the membrane-associated binding protein and the mPRs (Kazeto et al. 2005). The membrane-associated progesterone-binding proteins are termed 25-Dx, PR membrane component 1 (PGRMC 1) and Hpr 6 (Meyer et al. 1996, Krebs et al. 2000, Selmin et al. 2005, Peluso et al. 2006). These proteins are associated with progesterone’s nongenomic actions including the activation of signal transduction pathways that stimulate the intracellular MAPK signaling (Maller 2001, Duckworth et al. 2002, Hammes 2003, Krietsch et al. 2006). Activation of the MAPK pathway is the key extranuclear signaling action of the steroid’s regulation of cell proliferation and survival in various cell types including osteoblasts and osteocytes (Plotkin et al. 1999, Marzia et al. 2000, Martin 2001). The MAPK pathway promotes osteoblast survival and has been shown to contribute to the anabolic effects of parathyroid hormone (Kwok et al. 2005, Qin et al. 2005, Aghaloo et al. 2006), basic fibroblast growth factor (Jackson et al. 2007) and lactoferrin (Naot et al. 2005, Cornish et al. 2006, Grey et al. 2006). PGRMC1 may serve as an adaptor protein to regulate immunoreceptor tyrosine-based activation motif/ITAM signaling, which is known for its roles in vesicle targeting and osteoclast signaling (Humphrey et al. 2005, Takayanagi 2005, Fodor et al. 2006, Shinohara & Takayanagi 2007). However, the net effect of MAPK signaling in osteoprogenitors is currently unclear (Schindeler & Little 2006). The effects of PR inactivation may have been amplified in males and/or offset in females. The biosynthesis of the sex hormones is very closely related, and it is challenging to study effects of individual hormones (Heffner et al. 2010). It is possible that other hormones played a role in creating the sexual dimorphism in bone mass and gene transcription phenotypes. Additionally, the PR shares the same DNA response element with the receptors for androgens, glucocorticoids and mineralcorticoids (Beato et al. 1995). Inactivating PR signaling may have allowed other nuclear receptors to access the shared response elements.

This study was initiated after our previous findings that global PR KO mice had increased bone mass acquisition compared to their wild-type littermates, with sex differences and identified those genes that may control this observation. However, there were some limitations to this study. We used a single Cre recombinase to inactivate PR signaling and analyzed RNA from a single cell-type, which limited our findings. Because of our osteoprogenitor isolation and culture technique, we expected some levels of bone marrow contamination, which may have confounded some of our results. Due to the relatively small sample sizes, we were not powered to find lower differential gene expressions, particularly in our female experiment. Also, we were
not able to differentiate between PR’s direct targets and indirect changes. These limitations are largely inherent to the techniques that we used and can be mitigated through additional investigations by using Cre recombinases that target different cell populations.

Overall, there are many potential mechanisms that may explain our observed high bone mass phenotype with sex differences when PR was selectively deleted in the MSCs. Additional functional studies on progesterone receptor signaling in both osteoprogenitors or in the osteoblast lineage are necessary to better understand the context of PR signaling in the regulation of sexual dimorphism in health and disease conditions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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