Low Bone Turnover in Chronic Kidney Disease is associated with decreased VEGF-A expression and osteoblast differentiation

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Abstract:

Background: Low turnover bone (low bone formation rates) with decreased osteoblast number is common in CKD and attributed to "over- suppression" of parathyroid hormone (PTH) despite supra-physiologic levels. An alternative hypothesis is abnormal osteoblast differentiation resulting in low bone formation rates due to reduced VEGF-A.

Methods: We analyzed the expression of VEGF-A and mesenchymal stem cell (MSC) differentiation factors in freshly isolated BM cells, and in BM cell-derived MSC in rats with different levels of bone formation rates and PTH (modulated by calcium and zoledronic acid). The regulators of VEGF in MSC were also determined.

Results: VEGFA expression was reduced in the BM cells from CKD vs. normal animals ($p < 0.02$). In BM derived MSC from CKD there were decreased osteoblast transcription factors and mineralization. In CKD animals, the BM VEGF-A expression was positively correlated with BFR ($r = 0.80$, $p<0.001$). Reducing bone formation rates in CKD animals led to reductions in VEGF-A expression and osteoblast transcription factors regardless of PTH level. We therefore examined other regulators of VEGF-A and found decreased expression of hypoxia-inducible factor-1α and the master transcription factor of anti-oxidants Nuclear factor (erythroid-derived 2)-like 2 in CKD animals with low PTH.

Conclusion: Low bone formation rates in CKD are associated with a basal decrease in VEGFA expression in BM that may be driven by altered hypoxia and oxidative stress.

Introduction:

CKD is associated with increased fracture rates [1-4]. Abnormal bone remodeling is a major determinant of this increased fracture rate, and can be assessed by histomorphometry with dynamic measures using tetracycline labeling. In CKD, high turnover bone disease (high bone formation rates) has been associated with elevated PTH and low turnover disease (low bone formation rates) has been associated with "over suppression" of PTH [5], yet levels often remain greater than normal levels in the general population. Studies in the 1970s demonstrated skeletal resistance to PTH in patients with CKD[6] and, until recently, PTH was felt to be the primary bone hormone involved in this disease. However, there is now increased understanding of the complexity of bone differentiation, osteoblast-osteoclast cross talk, the importance of the osteocyte in facilitating cell-cell communication, and the role of the bone marrow microenvironment[7, 8]. These advances raise the possibility that low bone formation rates in patients with CKD may represent an inherent defect in bone cell differentiation or cell signaling rather than a direct result of lowering PTH levels. This hypothesis may also explain why normal or slightly elevated PTH levels may be associated with low bone formation rates in patients with CKD.

Osteoblasts, key cells in bone formation, differentiate from marrow derived mesenchymal stem cells (MSC) via regulation by multiple factors[8]. With aging[9], and in CKD[10], MSC preferentially differentiate to adipocytes rather than osteoblasts, leading to increased marrow fat and low bone formation rates. One regulator of normal osteoblast differentiation is vascular endothelial growth factor-A (VEGF-A)[11]. While initially identified as a key regulator of endochondral ossification[12], VEGF-A and its receptors (VEGFR-1 (Flt-1), VEGFR-2 (Kdr)) are highly expressed in osteoblasts and VEGF-A dose-dependently stimulates chemotactic migration and proliferation of primary human osteoblasts[13, 14]. In mouse models, aging is

associated with decreased VEGF-A expression in MSC[15]. Studies have demonstrated that PTH can regulate the expression of VEGF *in vitro* and *in vivo*[16, 17]. VEGF-A is also regulated by both hypoxia and oxidative stress, mediated by HIF-1α (hypoxia-inducible factor-1α) [18, 19] and Nrf-2 (Nuclear factor (erythroid-derived 2)-like 2)[20], respectively. Therefore, the goals of this study were to test the hypotheses that 1) altered VEGF-A expression in CKD is associated with decreased bone formation rates and 2) increased PTH would result in normalization of altered VEGF-A expression and increased bone formation rates.

Materials and Methods:

Animal model and experimental design:

This study examined bone marrow cells (BM) obtained at the time of sacrifice from male Cy/+ rats, Han:SPRD rats due to a missense mutation in the SamCystin (ANKS6) gene[21]. In the rat, this transmits polycystic kidney disease in an autosomal dominant manner rather than the autosomal recessive nephronophthisis in humans[22]. The Cy/+ rat, hereafter called CKD, spontaneously develops all three manifestations of CKD-Mineral Bone Disorder (MBD): biochemical abnormalities, extra skeletal calcification, and abnormal bone[23, 24]. The CKD rats, if untreated, develop secondary hyperparathyroidism and increased bone formation rates. However, the bone formation rates can be suppressed with either calcium (that also lowers PTH), or the bisphosphonate zoledronic acid[1, 25].

For the present study, we compared BM cells isolated and analyzed at the time of sacrifice to directly compare BM originating from CKD versus normal (NL) animals. We also isolated MSC from these BM cells to determine differentiation potential (via culture) in NL and CKD animals. The study design, biochemical, and bone histomorphometry methods and results were previously reported[1] and are summarized in Table 1. In brief, male heterozygote Cy/+ (CKD) animals (n=10 each group) were started treatment at 25 weeks for 10 weeks and received 1) no treatment (control CKD = high PTH and high bone formation rate), 2) received 3% calcium in the drinking water (CKD/Ca group= low PTH and low bone formation rate), or 3) received a single injection of zoledronic acid (CKD/Zol group = high PTH and low bone formation rate). These groups allowed us to analyze how differences in VEGF signaling and MSC differentiation relate to bone formation rate distinct from PTH levels. All procedures were

reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Isolation of bone marrow (BM) cells and culture of MSC:

At the time of euthanasia, bone marrow (BM) cells were collected using published protocols [26]. BM cells collected from tibia and femur were flushed with ice-cold α-MEM media (Gibco, Grand Island, NY) and centrifuged to pellet BM cells for RNA isolation to compare CKD versus NL animal VEGF-A and VEGF receptor expression. For MSC cultures, BM cells from tibia and femur from NL and CKD animals were cultured in α-MEM and 10% FBS. Non-adherent cells were removed by replacing the medium after 3 days. Adherent cells were further cultured in α-MEM with 10% FBS and 50 µg/ml ascorbic acid to obtain a homogenous population of MSC for use in the experiments. To induce osteogenesis, MSC were seeded at $1x10^4$ cells/cm^2 and cultured in α-MEM with 10% FBS containing 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone for 21 days[27]. For adipogenic differentiation, MSC were incubated in α-MEM with 10% FBS containing 1 µM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma, St. Louis, MO) for 21 days[27]. Media was changed every 3 days.

Real time (quantitative) RT-PCR analysis:

Total RNA was isolated from BM or MSC using miRNeasy Mini kit (Qiagen, Valencia, CA). The gene expression in cells was determined by real time PCR using 1 µg of total RNA in TaqMan Reverse Transcription reagent (Applied Biosystems, Foster City, CA). Target-specific PCR primers for VEGF-A, VEGF-R1, VEGF-R2, Runx2, peroxisome proliferator-activated receptor γ (PPARγ), and C/EBPα (CCAAT-enhancer-binding proteins) were obtained from Applied Biosystems. Real-time PCR amplification was performed using TaqMan Gene

Expression Assays (TaqMan MGP probes, FAM dye-labeled) using Applied Biosystems ViiA7 Real-Time PCR system (Applied Biosystems). The cycle number at which the amplification plot crosses the threshold was calculated (C_T), and the $\Delta\Delta C_T$ method was used to analyze the relative changes in gene expression using β-actin as a housekeeping gene[28].

Calcification in vitro:

Cultured MSC were decalcified with 0.6N HCl for 24 hours. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complex one method (Calcium kit; Pointe Scientific) and normalized to protein content as previously described[29].

Alkaline phosphatase activity:

Alkaline phosphatase activity was measured using p-nitrophenyl substrate supplied in an alkaline phosphatase assay kit (Pointe Scientific). ALP activity is normalized by protein content[30].

HIF-1α transcriptional activity measurement in MSC:

The HIF-1α activity in MSC was determined by HIF-1α Transcription Factor Assay Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instruction and normalized by nuclear protein concentration.

Western blot analysis:

Nuclear and cytosolic protein from cultured MSC was isolated using Cayman's Nuclear Extraction kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions. The expression of HIF-1α and Nrf2 were measured in the nuclear fraction, and the major regulator of Nrf2, Keap1(Kelch-like ECH-associated protein I) was measured in the cytosolic fraction by Western blot. Briefly, 30 µg of nuclear or cytosolic protein was loaded on 10% SDS-PAGE and the blots were incubated with antibody against HIF-1α (1:1000, Novus

Biologicals, Littleton, CO), Nrf2 or Keap 1(1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4^{o}C followed by incubating with peroxidase conjugated secondary antibody (1:5000 dilution), and immunodetection with the Enhanced Chemiluminescence Prime Western Blot Detection Reagent (Amersham, Piscataway, NJ). The blots were also stained for Ponceau S staining as loading control [31]. The band intensity was analyzed by ChemiDoc MP Imaging System (Imaging Lab 4.0, Bio-Rad, Richmond, CA) and normalized by Ponceau S staining.

Statistics:

Statistical analysis was conducted by ANOVA and within group comparisons by Fisher's post hoc analysis. Correlations were examined by the Pearson product-moment [32]. The results are expressed as means ± SD, with $p<0.05$ considered significant (StatView, SAS Institute, Cary, NC).

Results:

CKD compared to Normal Animals:

VEGF-A expression is lower in bone marrow cells (BM) from CKD compared to normal animals:

As shown in Figure 1, VEGF-A expression was significantly lower in BM from CKD rats compared to normal animals. However, there was no difference in the expression of the receptors, VEGF-R1 and VEGF-R2.

Mesenchymal stem cells (MSCs) from CKD rats show reduced mineralization and lower osteoblast differentiation along with increased adipocyte differentiation

MSC derived from CKD BM had decreased alkaline phosphatase activity (Figure 2A) and reduced calcification/mineralization (Figure 2B) compared to normal animals. In MSC from CKD animals compared to normal animals, there was increased expression of the adipogenic genes PPARγ and C/EBPα (Figure 3A & B) when cultured in adipogenic media, and decreased expression of the osteoblastic transcription factor Runx2 when cultured in osteogenic media (Figure 3C). This suggests that, compared to normal animals, BM derived MSC from CKD had reduced VEGF-A expression and impaired osteoblast differentiation and mineralization with enhanced potential for adipocyte differentiation.

CKD animals with high versus low bone formation rates:

The basal decrease in VEGF-A expression in BM is associated with low bone formation in CKD

The expression of VEGF-A in BM cells was positively correlated with the bone formation rate by histomorphometry ($r = 0.81$, $p < 0.001$; Figure 4A) in CKD animals. VEGF-A expression in the BM cells was also positively correlated with the expression of Runx2 ($r= 0.92$, $p < 0.001$; Figure 4B) and PPARγ ($r = 0.68$, $p < 0.003$; Figure 4C). Similarly, the expression

of Runx2 and PPARγ in BM cells was positively correlated with bone formation rate ($r=0.71$, $p<0.001$; $r=0.53$ $p<0.02$, respectively). Thus, the magnitude of BM cell VEGF-A expression correlated with bone formation*in vivo*, and the magnitude of VEGF-A expression was associated with increased mesenchymal transcription factor expression (differentiation potential). Thus, VEGF-A may be a key factor for MSC differentiation from BM cells.

The abnormal bone formation and VEGF-A expression in BM is independent of PTH:

As shown in table 1 and previously published [1], when CKD rats were treated with calcium in the drinking water, the serum calcium is slightly increased, phosphorus is decreased and PTH is suppressed. The histomorphometry demonstrated that there is low bone formation rate (BFR). When CKD rats were treated with zoledronic acid, the PTH is not changed but the bone formation rate is also decreased. This model thus allows us to compare CKD animals with high bone formation rates and high PTH, to low bone formation rates with low or high PTH.

The analyses of BM cells allow us to directly relate an individual CKD animal BM to the histology. Treating CKD rats with calcium or zoledronic acid both significantly decreased VEGF-A expression in BM cells but had no effect on expression of VEGF receptors (Figure 5A). Furthermore, compared to untreated (high PTH) CKD rats, there was decreased expression of osteoblast transcription factor Runx2 and adipogenic transcription factors PPARγ and C/EBP in BM cells from CKD rats treated with calcium (low PTH) or zoledronic acid (high PTH)(Figure 5B). These results suggest that in CKD animals, low bone formation rates are associated with both decreased VEGF-A expression in the BM cells, and reduced expression of both adiopocyte and osteogenic transcription factors, regardless of the PTH level. Thus, low bone formation rates are associated with impaired potential of BM cells to differentiate down the MSC lineage, perhaps the result of reduced VEGF-A expression.

MSC from CKD rats with low PTH have altered transcriptional response to hypoxia and oxidative stress:

We isolated BM derived MSC (prior to differentiation), and demonstrated decreased nuclear HIF-1α protein expression (Figure 6A), and activity (6B) in CKD animals treated with calcium (low bone formation rates and low bone turnover) compared to that in CKD control. The nuclear expression of Nrf2 (Figure 6C) was similarly decreased, and the cytosolic repressor Keap1 (Figure 6D) was increased in these same cells. These results suggest that in the setting of CKD and low PTH, there is evidence of decreased hypoxia and oxidative stress.

Discussion:

In the current study, we have demonstrated that BM cells from CKD animals had decreased expression of VEGF-A compared to normal animals. When the BM derived MSC were incubated with osteoblast or adipocyte differentiating media for 21 days, CKD derived cells had decreased osteoblast differentiation and mineralization and increased adipocyte differentiation compared to normal derived cells. Thus, the reduced VEGF-A expression in BM cells and the preferential differentiation to adipocytes in culture in CKD may lead to impaired osteoblast function compared to normal animals. The bone marrow cells represent a static measure of the composition of the marrow at the time of the biopsy whereas the cultured cells represent the ability of the existing MSC to differentiate down a specific mesenchymal lineage. Thus, our results suggest that in CKD, compared to normal, there is both a defect in the overall mesenchymal lineage in the bone marrow, and that the differentiation is driven towards adipocytic rather than osteoblastic lineage.

Osteoblasts and adipocytes have a common origin in mesenchymal stem cells with transcription factor expression driving differentiation[33]. In bone marrow derived MSC, there is a progressive increase in adipocyte formation and a decrease in osteoblast number with aging[34]. We have also demonstrated increased bone marrow fat in patients with CKD compared to healthy age, gender and race matched individuals[10]. Older individuals have reduced bone formation rates and a high fat to bone ratio in their bone marrows compared with young and healthy controls [35] suggesting that control of the fates of osteoblast/adipocyte differentiation in bone marrow MSC is important in bone formation, similar to the findings in the CKD animals in the present study. In dialysis patients, over half of bone biopsies demonstrate low bone formation rates[36]. While reduction, or so called ‘over-suppression” of PTH may be

one factor, this alone cannot explain why young dialysis patients with normal or even mildly elevated PTH have reduced bone formation rates. Impaired osteoblast differentiation due to low VEGF-A may be an alternative, or secondary, mechanism.

In CKD, there is a broad range of bone turnover phenotypes, often categorized clinically by low and high PTH[37]. However, we found that low bone formation rates were associated with low expression of VEGF-A and osteoblast differentiating transcription factor expression in BM cells regardless of the animal's PTH level, suggesting an inherent defect in CKD. The level of VEGF-A correlated positively with bone formation rate, supporting the importance of VEGF-A in bone abnormalities. We further demonstrated that cultured MSC from CKD animals with low PTH had reduced expression and activity of HIF-1α and Nrf2, both of which could lead to suppressed VEGF-A among other potential factors. Unfortunately, we could not assess HIF-1a and Nrf2 in the BM cells, or in BM/MSC from zoledronic acid treated animals due to the limited volume of cells. Nonetheless, taken together, these results suggest that cultured MSC from CKD animals have reduced VEGF-A expression. This is associated with reduced osteoblast differentiation, perhaps due to decreased HIF-1α and Nrf2, and this may contributes to low-turnover bone disease. We hypothesize that elevated PTH directly stimulates VEGF-A[16], thereby bypassing (or overcoming) this inherent defect in CKD BM differentiation. Stated differently, PTH may protect against the basal low levels of VEGF-A in bone marrow in CKD. Additional studies where low turnover animals are given PTH would be required to confirm this hypothesis. The current single time point design of this study only allows us to identify associations, and does not allow us to definitively assess cause and effect of the various potential factors that may reduce VEGF-A expression in CKD.

Mice with conditional VEGF deficiency in pre-osteoblasts had reduced bone mass and increased bone marrow fat[11]. Isolated MSC from these animals revealed impaired osteoblast and increased adipocyte differentiation [11], similar to our observations in the BM cells from CKD animals. In a previous study in mice with CKD from partial renal ablation, Noh et al. showed decreased expression of VEGF-A in MSC with impaired migration and angiogenesis[38]. Our study takes this a step further to demonstrate that the decreased expression of VEGF-A in bone marrow cells, the precursors of MSC, is strongly correlated to reduced bone formation rates. Zoledronic acid is known to reduce cell VEGF-A expression *in vitro [32, 39]* and thus explains the reduced VEGF-A expression in zoledronic acid treated BM cells. However, the mechanism by which CKD rats treated with calcium have lower VEGF-A expression is unknown and calcium itself may or may not be related.

We examined other pathways known to regulate VEGF-A including hypoxia and oxidative stress. Tissue hypoxia is common in CKD[40] and bone marrow[41].Both the present study and the work of Noh demonstrated reduced HIF-1α in the marrow, suggesting an important role of marrow hypoxia. HIF-1α, induced by hypoxia, is a regulator of the expression of VEGF-A and VEGF receptors in multiple cell types [26]. Hypoxia can induce cis-acting hypoxia response elements in the VEGF gene promoter region, which contains a putative binding site for HIF-1α. In MSC, up-regulation or down-regulation of HIF-1α led to increased or decreased VEGF-A[26], confirming a direct regulatory effect in MSC. We further demonstrated reductions in Nrf2, a master transcription factor that responds to increased oxidative stress [42]. Nrf2 is held in the cytoplasm as an inactive complex bound to a repressor molecule Keap1, which facilitates its ubiquitination[43]. Oxidative stress leads to dissociation of Nrf2 from Keap1 and its translocation to the nucleus to activate gene expression of antioxidants. Nrf2

activation has been shown to induce VEGF expression in several cell types [44-46]. In the MSC of CKD animals treated with calcium to lower PTH, HIF-1α and Nrf2 expression was reduced and Keap1 expression was increased compared to CKD animals with high PTH. Several studies have suggested that Hypoxia is closely related to oxidative stress [47]. The activation of HIF-1α reduces, whereas its inhibition worsens reactive oxygen species (ROS) generation[48]. On the other hand, oxidative stress exacerbates the status of hypoxia [47]. The current studies support that in CKD, similar to aging, the impaired osteoblast differentiation is associated with reduced VEGF-A. Two possible mechanisms include altered hypoxia and oxidative stress in bone marrow. Hypothetically, PTH can overcome, or mask, this defect by direct stimulation of VEGF-A[16, 17] and explain the need for supra-physiologic levels of PTH to obtain normal bone formation rates in CKD.

In summary, compared to normal animals, BM cells and MSC from CKD animals have decreased expression of VEGF-A, decreased osteoblast differentiation and mineralization and increased adipocyte differentiation. In CKD animals, bone formation rates directly correlate with VEGF-A levels in the BM cells. These studies support the hypothesis that CKD is a state of VEGF-A deficiency and this, in turn, leads to reduced mesenchymal stem cell differentiation to osteoblasts resulting in reduced bone formation rates. Elevated PTH may bypass this defect by directly upregulating VEGF-A. In patients with very low PTH and low turnover or adynamic bone disease, we hypothesize that upregulation of VEGF-A (perhaps by altering oxidative stress) may improve bone formation rates. However, our results demonstrate associations and thus additional studies are warranted to confirm this hypothesis.

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Table 1: Serum biochemistry and bone parameters in CKD rats treated with calcium or zoledornic acid:

Figure legends:

Figure 1: *The expression of VEGF-A, but not* VEGF receptor 1(Flt-1) and VEGF receptor 2 (KDR)*, is decreased in bone marrow cells (BM) from CKD compared to Normal rats:*

RNA expression of VEGF-A is significantly decreased in BM from CDK rat compared to normal animals. However, there is no difference in the expression of VEGF-R1 and VEGF-R2. Data are shown as mean ± SD (n= 9 each animal group), *p<0.05, CKD compared to normal.

Figure 2: *Mesenchymal stem cells (MSCs) from CKD rats have decreased alkaline phosphatase activity and reduced ability to mineralize:*

BM cell derived MSC had decreased alkaline phosphatase activity (Figure 2A) and reduced mineralization (Figure 2B) compared to normal. Data are shown as mean ± SD. Data are shown as mean ± SD (n=4 normal or CKD rats, with three cell cultures from each animal for final n of 12). *$p<0.05$, normal vs. CKD.

Figure 3: *Mesenchymal stem cells (MSCs) isolated from CKD rats have increased adipocyte differentiation and decreased osteoblast differentiation:*

BM derived cultured MSC demonstrated increased expression of adipogenic genes PPARγ and C/EBPα (Figure 3A & B) in MSC from CKD rats compared to that from normal rats. In contrast, the expression of the osteoblastic transcription factor Runx2 was decreased in MSC from CKD rats compared to normal rats (Figure 3C). Data are shown as mean ± SD (n=4 normal or CKD rats, with three cell cultures from each animal for final n of 12). *$p<0.05$, normal vs. CKD.

Figure 4: *The basal decrease in VEGF-A expression in BM is associated with defective MSC differentiation and low bone formation rate in CKD rats treated with or without calcium or zoledronic acid**:***

tThe expression of VEGF-A in BM is positively correlated with the bone formation rate (Figure 4A). VEGF-A expression is also positively correlated with the expression of both Runx2 (Figure 4B) and PPARγ (Figure 4C) in BM from these CKD rats. The correlation of low VEGF-A with low expression of both Runx2 and PPARγ, suggests an overall suppression of multiple lineages that derive from MSC. Black circles = CKD animals with high PTH, open circles = CKD animals with low PTH treated with calcium, Black triangles = CKD animals treated with zoledronic acid. n= 9 each animal group.

Figure 5: *The reduced VEGF-A expression in BM from animals with low bone formation in CKD is independent of PTH:*

BM cells from CKD rats treated with calcium (low bone formation rates/low PTH) or zoledronic acid (low bone formation rates/high PTH) were compared to control animals (high bone formation rates/high PTH). The results demonstrated that both low bone formation rate groups had decreased BM cell VEGF-A RNA expression but no effect on expression of VEGF receptors (Figure 5A), whereas the control animals with high bone formation rate had increased VEGF-A expression. Furthermore, compared to CKD rats with high bone formation rate, there was decreased expression of the osteoblast transcription factor Runx2 and adipogenic transcription factors PPARγ and C/EBPα in BM cells from CKD rats with low bone formation rate regardless of PTH (Figure 5B). Data are shown as mean ± SD (n= 9 each animal group), *$p<0.05$, CKD vehicle vs. CKD-Zol or CKD-Ca.

Figure 6: Hypoxia and oxidative stress in *MSC from BM derived from CKD rats with low bone formation rates:*

MSC from CKD rats with low bone formation rates and low PTH had decreased HIF-1α protein expression (Figure 6A) and lower HIF-1α transcriptional activity (Figure 6B), compared to animals with high bone formation rates and high PTH. Furthermore, there is decreased Nrf2 translocation (Figure 6C) to the nucleus and increased expression of Nrf2 repressor Keap1in cytoplasm (Figure 6D) in MSC from CKD rats with low bone formation compared to animals with high bone formation rates. The Western blot images are from 6 samples (3 from CKD rats and 3 from CKD rats treated with Ca). Quantitative data are shown as mean ± SD (n= 5 rats each animal group), *p<0.05, CKD vs. CKD/Ca.

Low Bone Turnover in Chronic Kidney Disease is associated with decreased VEGF-A expression and osteoblast differentiation

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Abstract:

Background: Low turnover bone (low bone formation rates) with decreased osteoblast number is common in CKD and attributed to "over- suppression" of parathyroid hormone (PTH) despite supra-physiologic levels. An alternative hypothesis is abnormal osteoblast differentiation resulting in low bone formation rates due to reduced VEGF-A.

Methods: We analyzed the expression of VEGF-A and mesenchymal stem cell (MSC) differentiation factors in freshly isolated BM cells, and in BM cell-derived MSC in rats with different levels of bone formation rates and PTH (modulated by calcium and zoledronic acid). The regulators of VEGF in MSC were also determined.

Results: VEGFA expression was reduced in the BM cells from CKD vs. normal animals ($p < 0.02$). In BM derived MSC from CKD there were decreased osteoblast transcription factors and mineralization. In CKD animals, the BM VEGF-A expression was positively correlated with BFR ($r = 0.80$, $p<0.001$). Reducing bone formation rates in CKD animals led to reductions in VEGF-A expression and osteoblast transcription factors regardless of PTH level. We therefore examined other regulators of VEGF-A and found decreased expression of hypoxia-inducible factor-1α and the master transcription factor of anti-oxidants Nuclear factor (erythroid-derived 2)-like 2 in CKD animals with low PTH.

Conclusion: Low bone formation rates in CKD are associated with a basal decrease in VEGFA expression in BM that may be driven by altered hypoxia and oxidative stress.

Introduction:

CKD is associated with increased fracture rates [1-4]. Abnormal bone remodeling is a major determinant of this increased fracture rate, and can be assessed by histomorphometry with dynamic measures using tetracycline labeling. In CKD, high turnover bone disease (high bone formation rates) has been associated with elevated PTH and low turnover disease (low bone formation rates) has been associated with "over suppression" of PTH [5], yet levels often remain greater than normal levels in the general population. Studies in the 1970s demonstrated skeletal resistance to PTH in patients with CKD[6] and, until recently, PTH was felt to be the primary bone hormone involved in this disease. However, there is now increased understanding of the complexity of bone differentiation, osteoblast-osteoclast cross talk, the importance of the osteocyte in facilitating cell-cell communication, and the role of the bone marrow microenvironment[7, 8]. These advances raise the possibility that low bone formation rates in patients with CKD may represent an inherent defect in bone cell differentiation or cell signaling rather than a direct result of lowering PTH levels. This hypothesis may also explain why normal or slightly elevated PTH levels may be associated with low bone formation rates in patients with CKD.

Osteoblasts, key cells in bone formation, differentiate from marrow derived mesenchymal stem cells (MSC) via regulation by multiple factors[8]. With aging[9], and in CKD[10], MSC preferentially differentiate to adipocytes rather than osteoblasts, leading to increased marrow fat and low bone formation rates. One regulator of normal osteoblast differentiation is vascular endothelial growth factor-A (VEGF-A)[11]. While initially identified as a key regulator of endochondral ossification[12], VEGF-A and its receptors (VEGFR-1 (Flt-1), VEGFR-2 (Kdr)) are highly expressed in osteoblasts and VEGF-A dose-dependently stimulates chemotactic migration and proliferation of primary human osteoblasts[13, 14]. In mouse models, aging is

associated with decreased VEGF-A expression in MSC[15]. Studies have demonstrated that PTH can regulate the expression of VEGF *in vitro* and *in vivo*[16, 17]. VEGF-A is also regulated by both hypoxia and oxidative stress, mediated by HIF-1α (hypoxia-inducible factor-1α) [18, 19] and Nrf-2 (Nuclear factor (erythroid-derived 2)-like 2)[20], respectively. Therefore, the goals of this study were to test the hypotheses that 1) altered VEGF-A expression in CKD is associated with decreased bone formation rates and 2) increased PTH would result in normalization of altered VEGF-A expression and increased bone formation rates.

Materials and Methods:

Animal model and experimental design:

This study examined bone marrow cells (BM) obtained at the time of sacrifice from male Cy/+ rats, Han:SPRD rats due to a missense mutation in the SamCystin (ANKS6) gene[21]. In the rat, this transmits polycystic kidney disease in an autosomal dominant manner rather than the autosomal recessive nephronophthisis in humans[22]. The Cy/+ rat, hereafter called CKD, spontaneously develops all three manifestations of CKD-Mineral Bone Disorder (MBD): biochemical abnormalities, extra skeletal calcification, and abnormal bone[23, 24]. The CKD rats, if untreated, develop secondary hyperparathyroidism and increased bone formation rates. However, the bone formation rates can be suppressed with either calcium (that also lowers PTH), or the bisphosphonate zoledronic acid[1, 25].

For the present study, we compared BM cells isolated and analyzed at the time of sacrifice to directly compare BM originating from CKD versus normal (NL) animals. We also isolated MSC from these BM cells to determine differentiation potential (via culture) in NL and CKD animals. The study design, biochemical, and bone histomorphometry methods and results were previously reported[1] and are summarized in Table 1. In brief, male heterozygote Cy/+ (CKD) animals (n=10 each group) were started treatment at 25 weeks for 10 weeks and received 1) no treatment (control CKD = high PTH and high bone formation rate), 2) received 3% calcium in the drinking water (CKD/Ca group= low PTH and low bone formation rate), or 3) received a single injection of zoledronic acid (CKD/Zol group = high PTH and low bone formation rate). These groups allowed us to analyze how differences in VEGF signaling and MSC differentiation relate to bone formation rate distinct from PTH levels. All procedures were

reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Isolation of bone marrow (BM) cells and culture of MSC:

At the time of euthanasia, bone marrow (BM) cells were collected using published protocols [26]. BM cells collected from tibia and femur were flushed with ice-cold α-MEM media (Gibco, Grand Island, NY) and centrifuged to pellet BM cells for RNA isolation to compare CKD versus NL animal VEGF-A and VEGF receptor expression. For MSC cultures, BM cells from tibia and femur from NL and CKD animals were cultured in α-MEM and 10% FBS. Non-adherent cells were removed by replacing the medium after 3 days. Adherent cells were further cultured in α-MEM with 10% FBS and 50 µg/ml ascorbic acid to obtain a homogenous population of MSC for use in the experiments. To induce osteogenesis, MSC were seeded at $1x10^4$ cells/cm^2 and cultured in α-MEM with 10% FBS containing 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone for 21 days[27]. For adipogenic differentiation, MSC were incubated in α-MEM with 10% FBS containing 1 µM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma, St. Louis, MO) for 21 days[27]. Media was changed every 3 days.

Real time (quantitative) RT-PCR analysis:

Total RNA was isolated from BM or MSC using miRNeasy Mini kit (Qiagen, Valencia, CA). The gene expression in cells was determined by real time PCR using 1 µg of total RNA in TaqMan Reverse Transcription reagent (Applied Biosystems, Foster City, CA). Target-specific PCR primers for VEGF-A, VEGF-R1, VEGF-R2, Runx2, peroxisome proliferator-activated receptor γ (PPARγ), and C/EBPα (CCAAT-enhancer-binding proteins) were obtained from Applied Biosystems. Real-time PCR amplification was performed using TaqMan Gene

Expression Assays (TaqMan MGP probes, FAM dye-labeled) using Applied Biosystems ViiA7 Real-Time PCR system (Applied Biosystems). The cycle number at which the amplification plot crosses the threshold was calculated (C_T), and the $\Delta\Delta C_T$ method was used to analyze the relative changes in gene expression using β-actin as a housekeeping gene[28].

Calcification in vitro:

Cultured MSC were decalcified with 0.6N HCl for 24 hours. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complex one method (Calcium kit; Pointe Scientific) and normalized to protein content as previously described[29].

Alkaline phosphatase activity:

Alkaline phosphatase activity was measured using p-nitrophenyl substrate supplied in an alkaline phosphatase assay kit (Pointe Scientific). ALP activity is normalized by protein content[30].

HIF-1α transcriptional activity measurement in MSC:

The HIF-1α activity in MSC was determined by HIF-1α Transcription Factor Assay Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instruction and normalized by nuclear protein concentration.

Western blot analysis:

Nuclear and cytosolic protein from cultured MSC was isolated using Cayman's Nuclear Extraction kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions. The expression of HIF-1α and Nrf2 were measured in the nuclear fraction, and the major regulator of Nrf2, Keap1(Kelch-like ECH-associated protein I) was measured in the cytosolic fraction by Western blot. Briefly, 30 µg of nuclear or cytosolic protein was loaded on 10% SDS-PAGE and the blots were incubated with antibody against HIF-1α (1:1000, Novus

Biologicals, Littleton, CO), Nrf2 or Keap 1(1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4^{o}C followed by incubating with peroxidase conjugated secondary antibody (1:5000 dilution), and immunodetection with the Enhanced Chemiluminescence Prime Western Blot Detection Reagent (Amersham, Piscataway, NJ). The blots were also stained for Ponceau S staining as loading control [31]. The band intensity was analyzed by ChemiDoc MP Imaging System (Imaging Lab 4.0, Bio-Rad, Richmond, CA) and normalized by Ponceau S staining.

Statistics:

Statistical analysis was conducted by ANOVA and within group comparisons by Fisher's post hoc analysis. Correlations were examined by the Pearson product-moment [32]. The results are expressed as means ± SD, with $p<0.05$ considered significant (StatView, SAS Institute, Cary, NC).

Results:

CKD compared to Normal Animals:

VEGF-A expression is lower in bone marrow cells (BM) from CKD compared to normal animals:

As shown in Figure 1, VEGF-A expression was significantly lower in BM from CKD rats compared to normal animals. However, there was no difference in the expression of the receptors, VEGF-R1 and VEGF-R2.

Mesenchymal stem cells (MSCs) from CKD rats show reduced mineralization and lower osteoblast differentiation along with increased adipocyte differentiation

MSC derived from CKD BM had decreased alkaline phosphatase activity (Figure 2A) and reduced calcification/mineralization (Figure 2B) compared to normal animals. In MSC from CKD animals compared to normal animals, there was increased expression of the adipogenic genes PPARγ and C/EBPα (Figure 3A & B) when cultured in adipogenic media, and decreased expression of the osteoblastic transcription factor Runx2 when cultured in osteogenic media (Figure 3C). This suggests that, compared to normal animals, BM derived MSC from CKD had reduced VEGF-A expression and impaired osteoblast differentiation and mineralization with enhanced potential for adipocyte differentiation.

CKD animals with high versus low bone formation rates:

The basal decrease in VEGF-A expression in BM is associated with low bone formation in CKD

The expression of VEGF-A in BM cells was positively correlated with the bone formation rate by histomorphometry ($r = 0.81$, $p < 0.001$; Figure 4A) in CKD animals. VEGF-A expression in the BM cells was also positively correlated with the expression of Runx2 ($r= 0.92$, $p < 0.001$; Figure 4B) and PPARγ ($r = 0.68$, $p < 0.003$; Figure 4C). Similarly, the expression

of Runx2 and PPARγ in BM cells was positively correlated with bone formation rate (r=0.71, p<0.001; r=0.53p<0.02, respectively). Thus, the magnitude of BM cell VEGF-A expression correlated with bone formation*in vivo*, and the magnitude of VEGF-A expression was associated with increased mesenchymal transcription factor expression (differentiation potential). Thus, VEGF-A may be a key factor for MSC differentiation from BM cells.

The abnormal bone formation and VEGF-A expression in BM is independent of PTH:

As shown in table 1 and previously published [1], when CKD rats were treated with calcium in the drinking water, the serum calcium is slightly increased, phosphorus is decreased and PTH is suppressed. The histomorphometry demonstrated that there is low bone formation rate (BFR). When CKD rats were treated with zoledronic acid, the PTH is not changed but the bone formation rate is also decreased. This model thus allows us to compare CKD animals with high bone formation rates and high PTH, to low bone formation rates with low or high PTH.

The analyses of BM cells allow us to directly relate an individual CKD animal BM to the histology. Treating CKD rats with calcium or zoledronic acid both significantly decreased VEGF-A expression in BM cells but had no effect on expression of VEGF receptors (Figure 5A). Furthermore, compared to untreated (high PTH) CKD rats, there was decreased expression of osteoblast transcription factor Runx2 and adipogenic transcription factors PPARγ and C/EBP in BM cells from CKD rats treated with calcium (low PTH) or zoledronic acid (high PTH)(Figure 5B). These results suggest that in CKD animals, low bone formation rates are associated with both decreased VEGF-A expression in the BM cells, and reduced expression of both adiopocyte and osteogenic transcription factors, regardless of the PTH level. Thus, low bone formation rates are associated with impaired potential of BM cells to differentiate down the MSC lineage, perhaps the result of reduced VEGF-A expression.

MSC from CKD rats with low PTH have altered transcriptional response to hypoxia and oxidative stress:

We isolated BM derived MSC (prior to differentiation), and demonstrated decreased nuclear HIF-1α protein expression (Figure 6A), and activity (6B) in CKD animals treated with calcium (low bone formation rates and low bone turnover) compared to that in CKD control. The nuclear expression of Nrf2 (Figure 6C) was similarly decreased, and the cytosolic repressor Keap1 (Figure 6D) was increased in these same cells. These results suggest that in the setting of CKD and low PTH, there is evidence of decreased hypoxia and oxidative stress.

Discussion:

In the current study, we have demonstrated that BM cells from CKD animals had decreased expression of VEGF-A compared to normal animals. When the BM derived MSC were incubated with osteoblast or adipocyte differentiating media for 21 days, CKD derived cells had decreased osteoblast differentiation and mineralization and increased adipocyte differentiation compared to normal derived cells. Thus, the reduced VEGF-A expression in BM cells and the preferential differentiation to adipocytes in culture in CKD may lead to impaired osteoblast function compared to normal animals. The bone marrow cells represent a static measure of the composition of the marrow at the time of the biopsy whereas the cultured cells represent the ability of the existing MSC to differentiate down a specific mesenchymal lineage. Thus, our results suggest that in CKD, compared to normal, there is both a defect in the overall mesenchymal lineage in the bone marrow, and that the differentiation is driven towards adipocytic rather than osteoblastic lineage.

Osteoblasts and adipocytes have a common origin in mesenchymal stem cells with transcription factor expression driving differentiation[33]. In bone marrow derived MSC, there is a progressive increase in adipocyte formation and a decrease in osteoblast number with aging[34]. We have also demonstrated increased bone marrow fat in patients with CKD compared to healthy age, gender and race matched individuals[10]. Older individuals have reduced bone formation rates and a high fat to bone ratio in their bone marrows compared with young and healthy controls [35] suggesting that control of the fates of osteoblast/adipocyte differentiation in bone marrow MSC is important in bone formation, similar to the findings in the CKD animals in the present study. In dialysis patients, over half of bone biopsies demonstrate low bone formation rates[36]. While reduction, or so called ‘over-suppression” of PTH may be

one factor, this alone cannot explain why young dialysis patients with normal or even mildly elevated PTH have reduced bone formation rates. Impaired osteoblast differentiation due to low VEGF-A may be an alternative, or secondary, mechanism.

In CKD, there is a broad range of bone turnover phenotypes, often categorized clinically by low and high PTH[37]. However, we found that low bone formation rates were associated with low expression of VEGF-A and osteoblast differentiating transcription factor expression in BM cells regardless of the animal's PTH level, suggesting an inherent defect in CKD. The level of VEGF-A correlated positively with bone formation rate, supporting the importance of VEGF-A in bone abnormalities. We further demonstrated that cultured MSC from CKD animals with low PTH had reduced expression and activity of HIF-1α and Nrf2, both of which could lead to suppressed VEGF-A among other potential factors. Unfortunately, we could not assess HIF-1a and Nrf2 in the BM cells, or in BM/MSC from zoledronic acid treated animals due to the limited volume of cells. Nonetheless, taken together, these results suggest that cultured MSC from CKD animals have reduced VEGF-A expression. This is associated with reduced osteoblast differentiation, perhaps due to decreased HIF-1α and Nrf2, and this may contributes to low-turnover bone disease. We hypothesize that elevated PTH directly stimulates VEGF-A[16], thereby bypassing (or overcoming) this inherent defect in CKD BM differentiation. Stated differently, PTH may protect against the basal low levels of VEGF-A in bone marrow in CKD. Additional studies where low turnover animals are given PTH would be required to confirm this hypothesis. The current single time point design of this study only allows us to identify associations, and does not allow us to definitively assess cause and effect of the various potential factors that may reduce VEGF-A expression in CKD.

Mice with conditional VEGF deficiency in pre-osteoblasts had reduced bone mass and increased bone marrow fat[11]. Isolated MSC from these animals revealed impaired osteoblast and increased adipocyte differentiation [11], similar to our observations in the BM cells from CKD animals. In a previous study in mice with CKD from partial renal ablation, Noh et al. showed decreased expression of VEGF-A in MSC with impaired migration and angiogenesis[38]. Our study takes this a step further to demonstrate that the decreased expression of VEGF-A in bone marrow cells, the precursors of MSC, is strongly correlated to reduced bone formation rates. Zoledronic acid is known to reduce cell VEGF-A expression *in vitro [32, 39]* and thus explains the reduced VEGF-A expression in zoledronic acid treated BM cells. However, the mechanism by which CKD rats treated with calcium have lower VEGF-A expression is unknown and calcium itself may or may not be related.

We examined other pathways known to regulate VEGF-A including hypoxia and oxidative stress. Tissue hypoxia is common in CKD[40] and bone marrow[41].Both the present study and the work of Noh demonstrated reduced HIF-1α in the marrow, suggesting an important role of marrow hypoxia. HIF-1α, induced by hypoxia, is a regulator of the expression of VEGF-A and VEGF receptors in multiple cell types [26]. Hypoxia can induce cis-acting hypoxia response elements in the VEGF gene promoter region, which contains a putative binding site for HIF-1α. In MSC, up-regulation or down-regulation of HIF-1α led to increased or decreased VEGF-A[26], confirming a direct regulatory effect in MSC. We further demonstrated reductions in Nrf2, a master transcription factor that responds to increased oxidative stress [42]. Nrf2 is held in the cytoplasm as an inactive complex bound to a repressor molecule Keap1, which facilitates its ubiquitination[43]. Oxidative stress leads to dissociation of Nrf2 from Keap1 and its translocation to the nucleus to activate gene expression of antioxidants. Nrf2

activation has been shown to induce VEGF expression in several cell types [44-46]. In the MSC of CKD animals treated with calcium to lower PTH, HIF-1α and Nrf2 expression was reduced and Keap1 expression was increased compared to CKD animals with high PTH. Several studies have suggested that Hypoxia is closely related to oxidative stress [47]. The activation of HIF-1α reduces, whereas its inhibition worsens reactive oxygen species (ROS) generation[48]. On the other hand, oxidative stress exacerbates the status of hypoxia [47]. The current studies support that in CKD, similar to aging, the impaired osteoblast differentiation is associated with reduced VEGF-A. Two possible mechanisms include altered hypoxia and oxidative stress in bone marrow. Hypothetically, PTH can overcome, or mask, this defect by direct stimulation of VEGF-A[16, 17] and explain the need for supra-physiologic levels of PTH to obtain normal bone formation rates in CKD.

In summary, compared to normal animals, BM cells and MSC from CKD animals have decreased expression of VEGF-A, decreased osteoblast differentiation and mineralization and increased adipocyte differentiation. In CKD animals, bone formation rates directly correlate with VEGF-A levels in the BM cells. These studies support the hypothesis that CKD is a state of VEGF-A deficiency and this, in turn, leads to reduced mesenchymal stem cell differentiation to osteoblasts resulting in reduced bone formation rates. Elevated PTH may bypass this defect by directly upregulating VEGF-A. In patients with very low PTH and low turnover or adynamic bone disease, we hypothesize that upregulation of VEGF-A (perhaps by altering oxidative stress) may improve bone formation rates. However, our results demonstrate associations and thus additional studies are warranted to confirm this hypothesis.

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Table 1: Serum biochemistry and bone parameters in CKD rats treated with calcium or zoledornic acid:

Figure legends:

Figure 1: *The expression of VEGF-A, but not* VEGF receptor 1(Flt-1) and VEGF receptor 2 (KDR)*, is decreased in bone marrow cells (BM) from CKD compared to Normal rats:*

RNA expression of VEGF-A is significantly decreased in BM from CDK rat compared to normal animals. However, there is no difference in the expression of VEGF-R1 and VEGF-R2. Data are shown as mean ± SD (n= 9 each animal group), *p<0.05, CKD compared to normal.

Figure 2: *Mesenchymal stem cells (MSCs) from CKD rats have decreased alkaline phosphatase activity and reduced ability to mineralize:*

BM cell derived MSC had decreased alkaline phosphatase activity (Figure 2A) and reduced mineralization (Figure 2B) compared to normal. Data are shown as mean ± SD. Data are shown as mean ± SD (n=4 normal or CKD rats, with three cell cultures from each animal for final n of 12). *$p<0.05$, normal vs. CKD.

Figure 3: *Mesenchymal stem cells (MSCs) isolated from CKD rats have increased adipocyte differentiation and decreased osteoblast differentiation:*

BM derived cultured MSC demonstrated increased expression of adipogenic genes PPARγ and C/EBPα (Figure 3A & B) in MSC from CKD rats compared to that from normal rats. In contrast, the expression of the osteoblastic transcription factor Runx2 was decreased in MSC from CKD rats compared to normal rats (Figure 3C). Data are shown as mean ± SD (n=4 normal or CKD rats, with three cell cultures from each animal for final n of 12). *$p<0.05$, normal vs. CKD.

Figure 4: *The basal decrease in VEGF-A expression in BM is associated with defective MSC differentiation and low bone formation rate in CKD rats treated with or without calcium or zoledronic acid**:***

tThe expression of VEGF-A in BM is positively correlated with the bone formation rate (Figure 4A). VEGF-A expression is also positively correlated with the expression of both Runx2 (Figure 4B) and PPARγ (Figure 4C) in BM from these CKD rats. The correlation of low VEGF-A with low expression of both Runx2 and PPARγ, suggests an overall suppression of multiple lineages that derive from MSC. Black circles = CKD animals with high PTH, open circles = CKD animals with low PTH treated with calcium, Black triangles = CKD animals treated with zoledronic acid. n= 9 each animal group.

Figure 5: *The reduced VEGF-A expression in BM from animals with low bone formation in CKD is independent of PTH:*

BM cells from CKD rats treated with calcium (low bone formation rates/low PTH) or zoledronic acid (low bone formation rates/high PTH) were compared to control animals (high bone formation rates/high PTH). The results demonstrated that both low bone formation rate groups had decreased BM cell VEGF-A RNA expression but no effect on expression of VEGF receptors (Figure 5A), whereas the control animals with high bone formation rate had increased VEGF-A expression. Furthermore, compared to CKD rats with high bone formation rate, there was decreased expression of the osteoblast transcription factor Runx2 and adipogenic transcription factors PPARγ and C/EBPα in BM cells from CKD rats with low bone formation rate regardless of PTH (Figure 5B). Data are shown as mean ± SD (n= 9 each animal group), *$p<0.05$, CKD vehicle vs. CKD-Zol or CKD-Ca.

Figure 6: Hypoxia and oxidative stress in *MSC from BM derived from CKD rats with low bone formation rates:*

MSC from CKD rats with low bone formation rates and low PTH had decreased HIF-1α protein expression (Figure 6A) and lower HIF-1α transcriptional activity (Figure 6B), compared to animals with high bone formation rates and high PTH. Furthermore, there is decreased Nrf2 translocation (Figure 6C) to the nucleus and increased expression of Nrf2 repressor Keap1in cytoplasm (Figure 6D) in MSC from CKD rats with low bone formation compared to animals with high bone formation rates. The Western blot images are from 6 samples (3 from CKD rats and 3 from CKD rats treated with Ca). Quantitative data are shown as mean ± SD (n= 5 rats each animal group), *p<0.05, CKD vs. CKD/Ca.

Table 1:

Treatment	Serum Biochemistry			Histomorphometry
	Calcium	**Phosph**	**PTH**	**BFR/BS (Bone formation rate)**
CKD-Control (n=10)	↔	↑	↑↑	↑↑*
CKD- Low PTH/Ca treated (n=10)	↑	↓	↓↓	↓↓
CKD-Zol (n=10)	↔	↑	↑↑	↓↓

*↑↑, ↓↓, ↔ compare to normal animals; data adapted from[1]

Figure 1

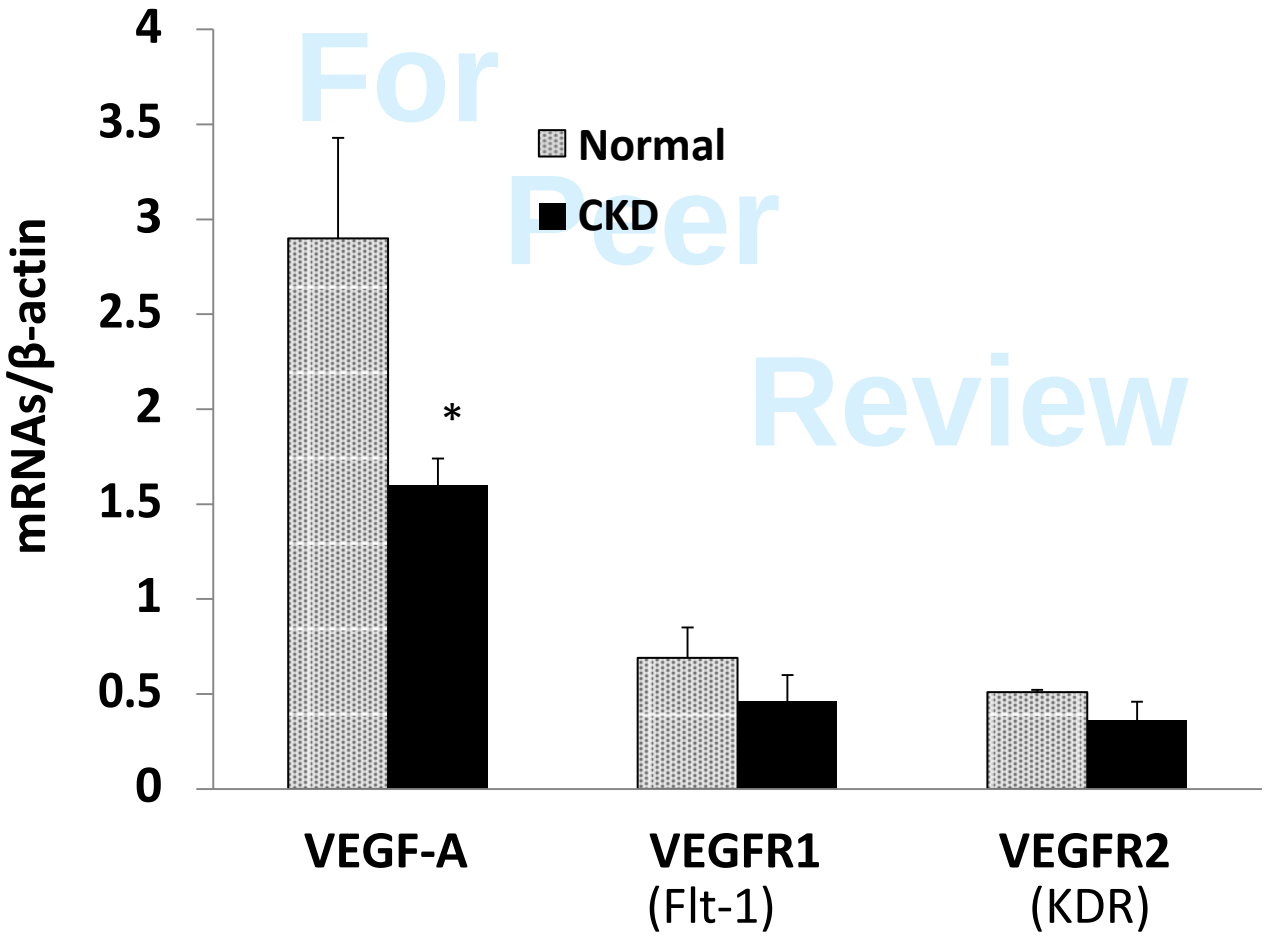


Figure 2

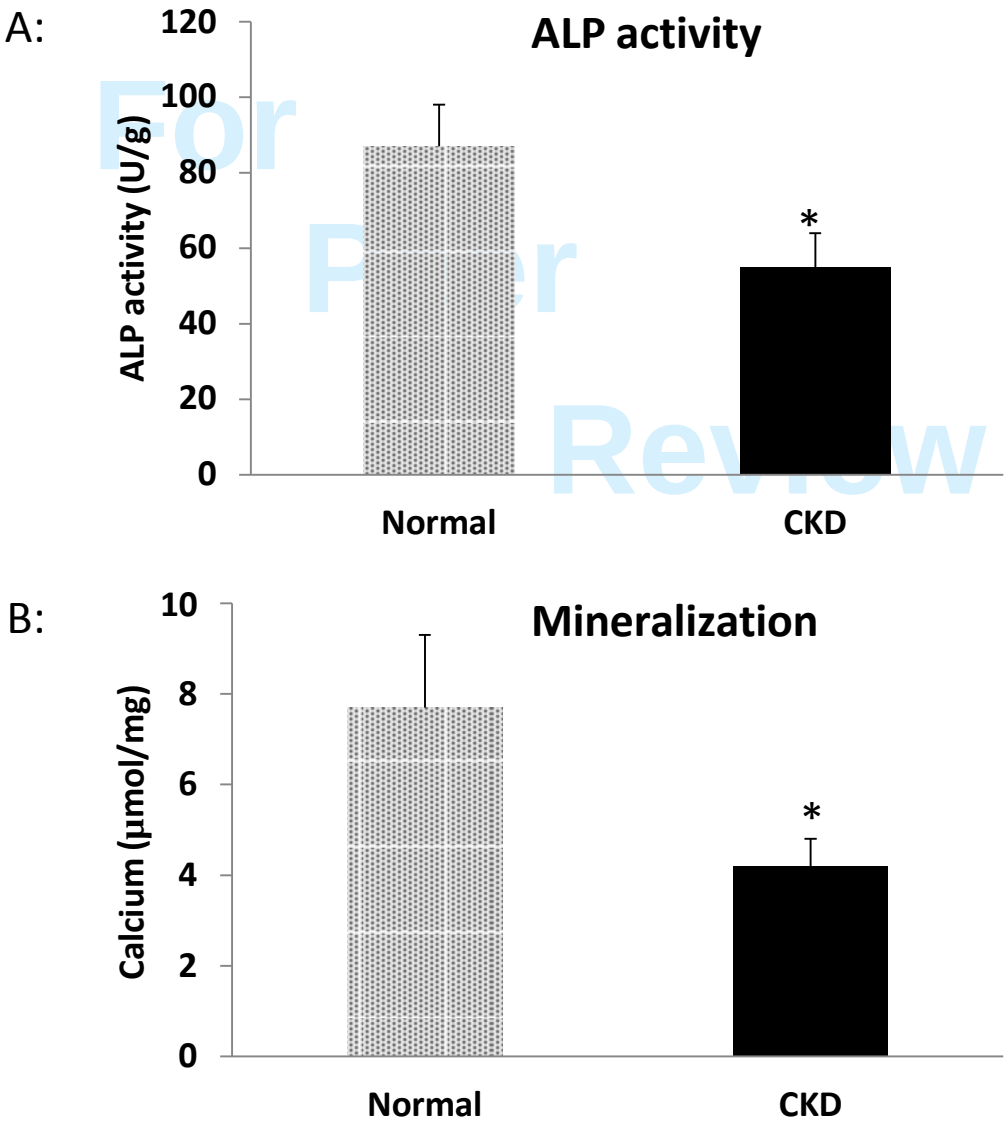


Figure 3

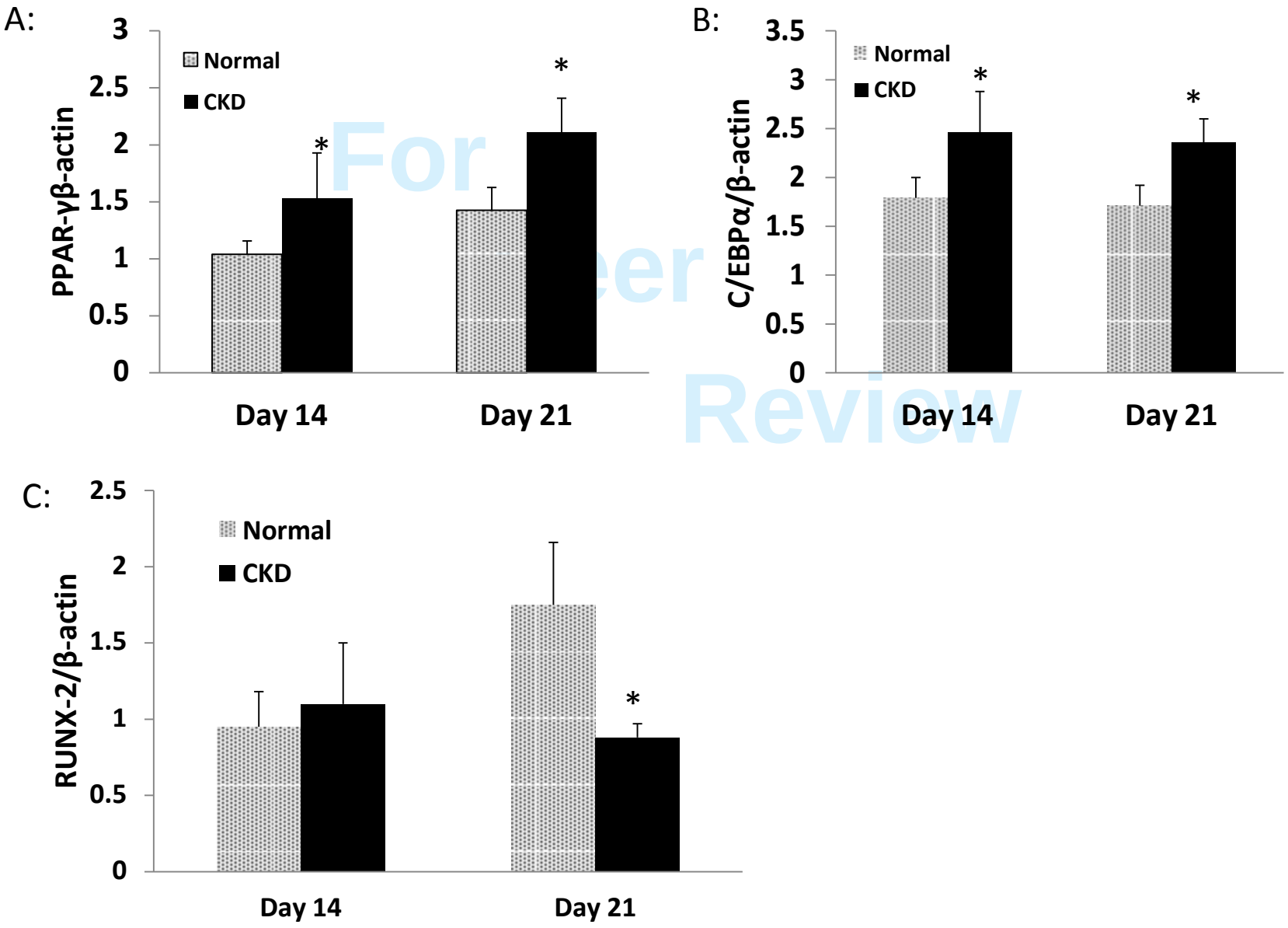


Figure 4A

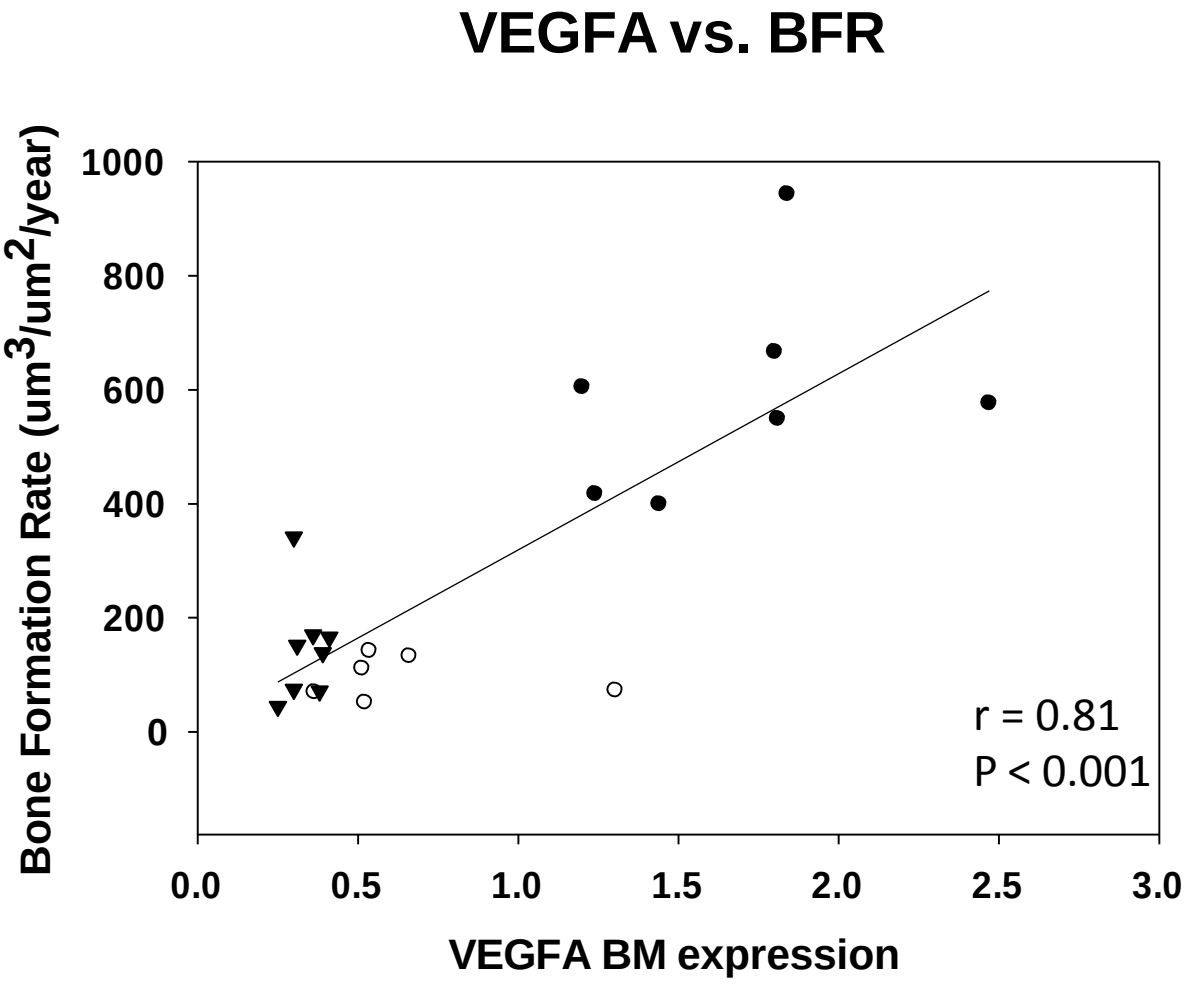

VEGFA vs. BFR
Bone Formation Rate (um3/um2/year)
1000
800
600
400
200
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
VEGFA BM expression
r = 0.81
P < 0.001

Figure 4B

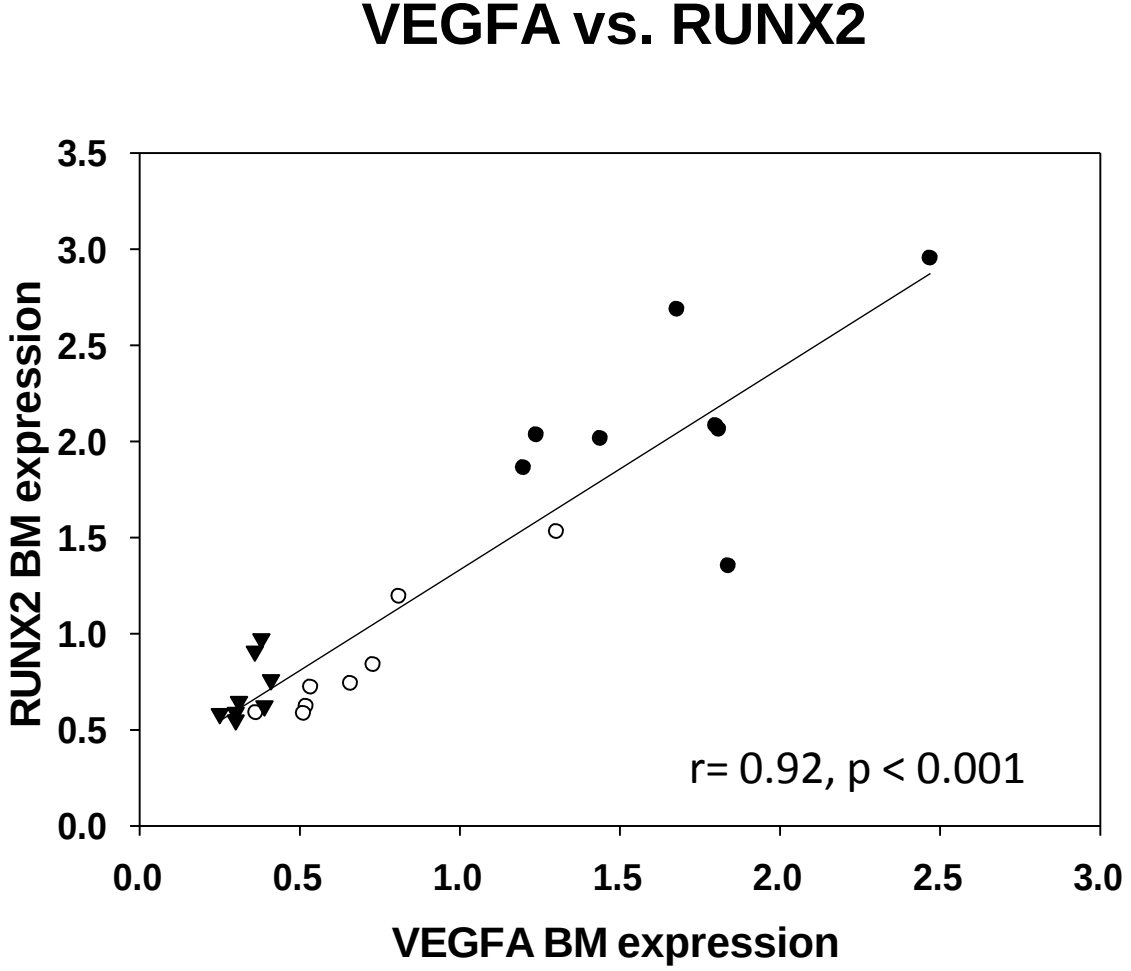

VEGFA vs. RUNX2
RUNX2 BM expression
VEGFA BM expression
r= 0.92, p < 0.001
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

Figure 4C

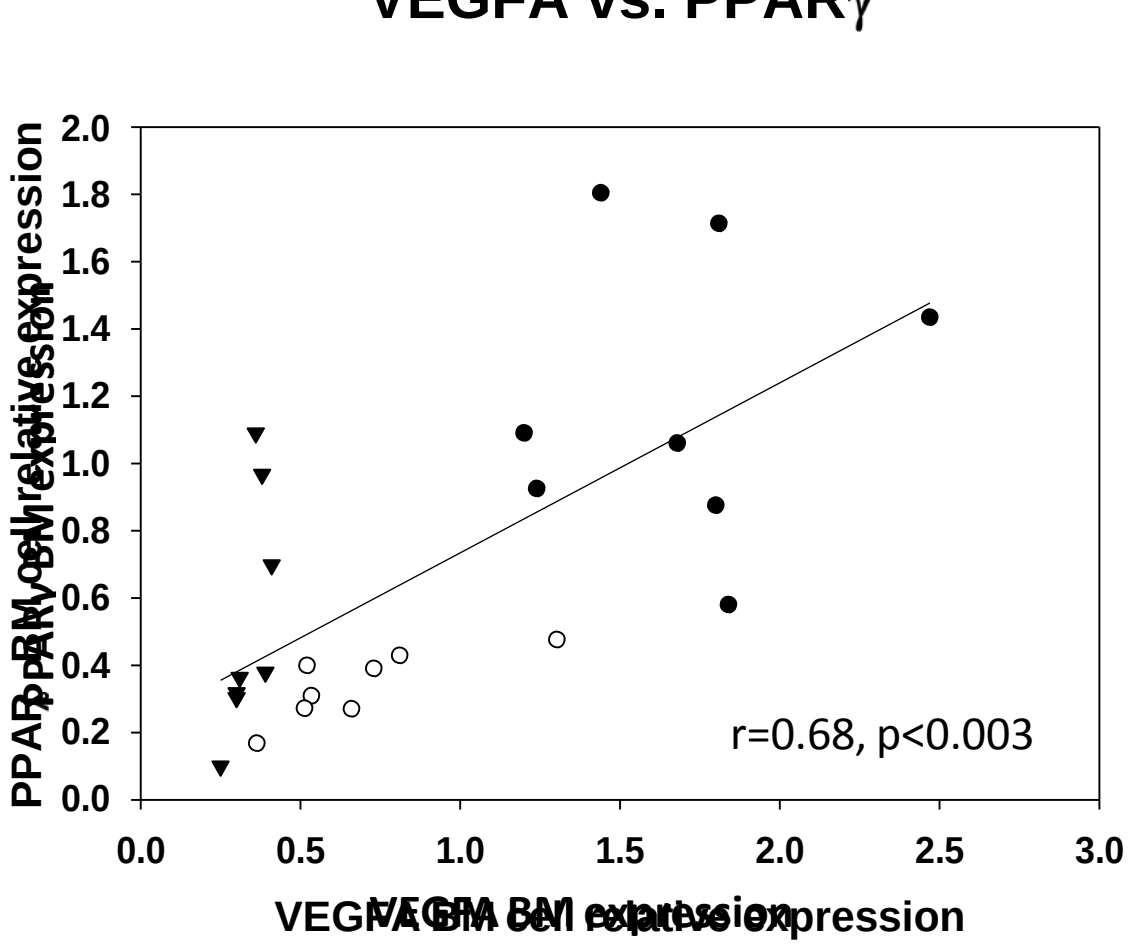

VEGFA vs. PPARγ
PPARγ BM cell relative expression
VEGFA BM cell relative expression
r=0.68, p<0.003
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
CKD
CKD + Calcium
CKD-ZOL

Figure 5A

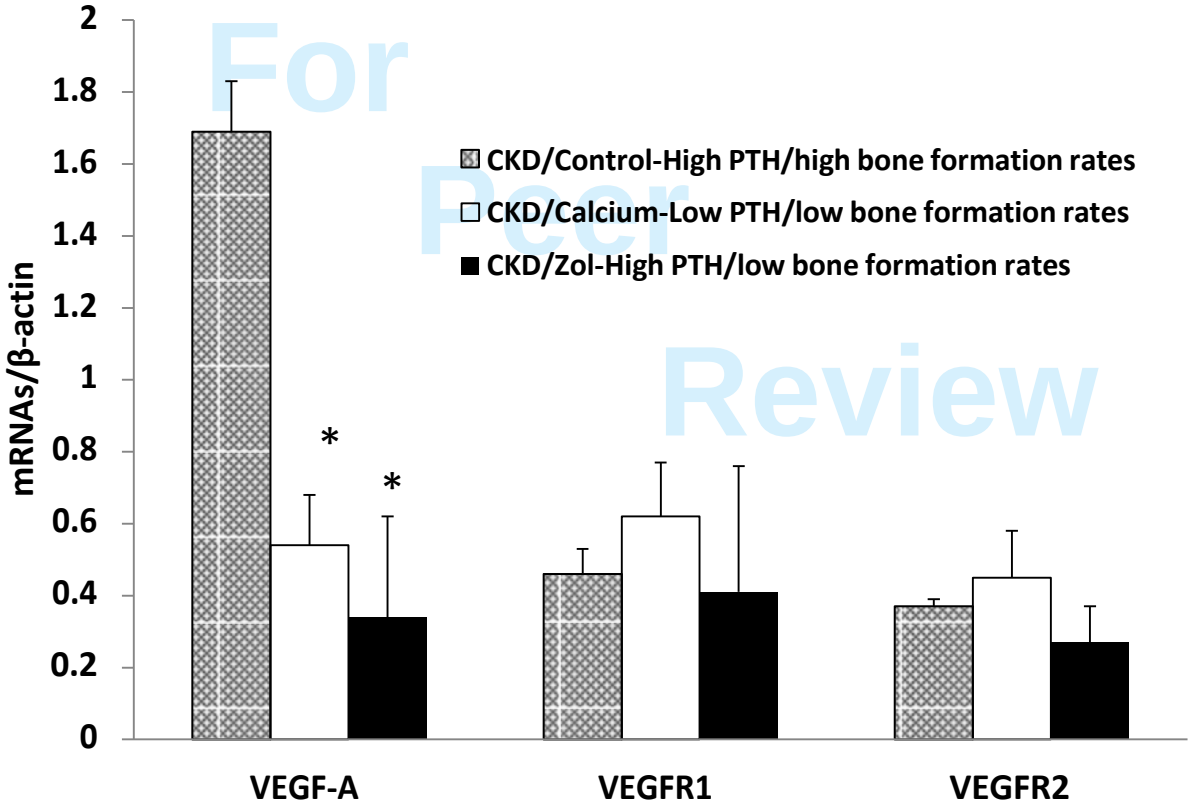


Figure 5B

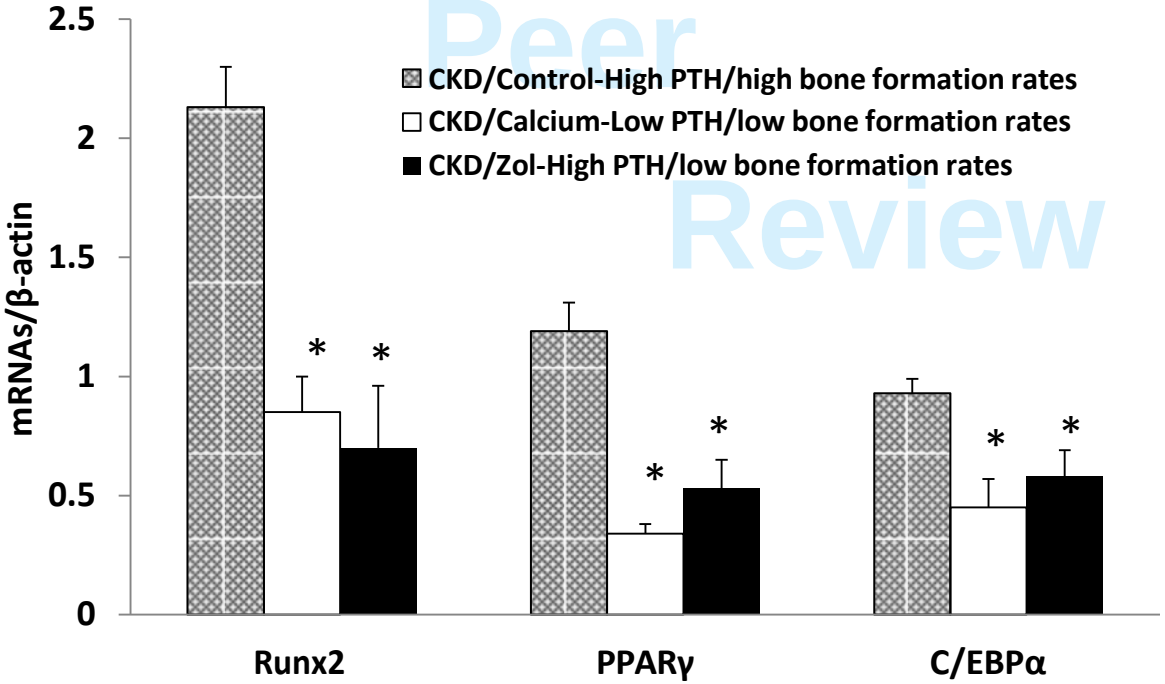
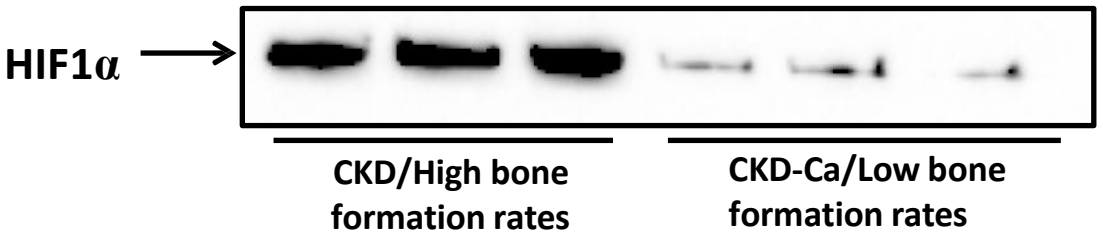


Figure 6

A:



B:

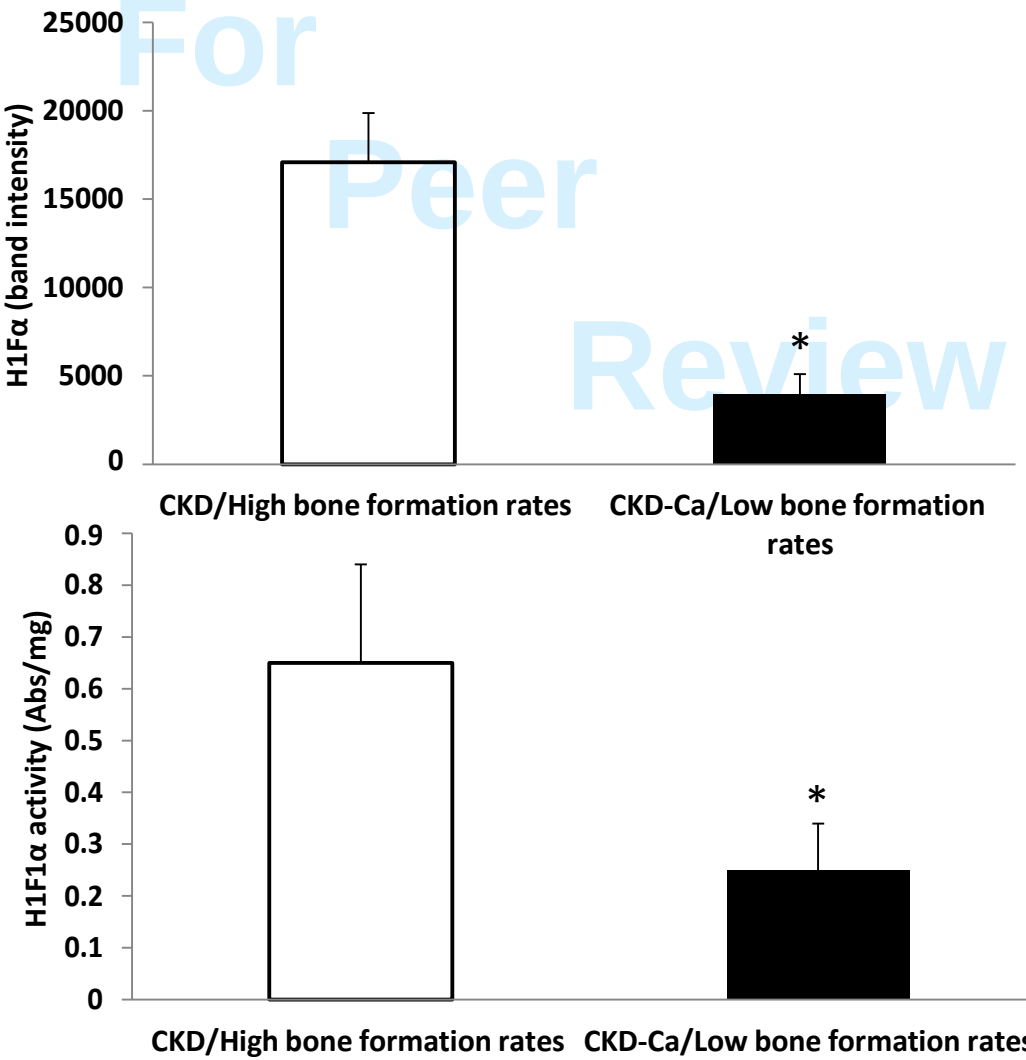
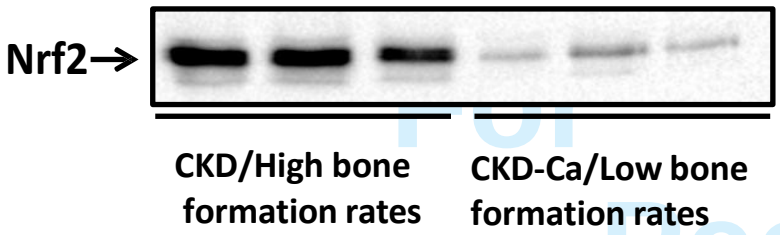


Figure 6

C: Nrf2 expression in nuclear fraction



D: Keap1 expression in cytosolic fraction