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The effect of PKG II deficiency on osteoblast-specific gene expression in mice

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by

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ABSTRACT OF THE THESIS

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C-*fos* and *fra-2,* both transcription factors under the control of PKGII, were seen in previous experiments to have significantly decreased mRNA transcript levels in PKGII -/- mice compared with wild type litter mates (Rangaswami 2010). Osteoblasts derived from PKGII -/- mice have impaired cGMP-dependent Src and Erk activation, steps along a PKGII signaling pathway (Rangaswami 2010). These findings, as well as the fact that PKGII -/- suffer from dwarfism with severe

endochondral ossification defects, all suggest that PKGII has an integral part in proper bone development and function (Pfeiffer 1996).

RNA was extracted from the tibial diaphysis of PKG -/- mice and wild type or heterozygous litter mates of three age groups: seven, eleven, and twenty-eight day-old mice, and used to analyze by reverse transcription/quantitative real-time polymerase chain reaction (RT-PCR) five genes of interest, all pertaining to osteoblast or osteoclast proliferation and function. Relative mRNA levels of the genes of interest were calculated via $2^{-\Delta Ct}$ method, where ΔCt represents the difference in threshold cycle values between the gene of interest and the housekeeping reference gene, Gapdh.

Out of the five genes of interest, and in all the age groups examined, only osteoprotegerin (OPG) in the seven and eleven day-old mice showed a significant difference in relative mRNA levels between wild-type and knock-out mice. OPG is produced by (pre)osteoblasts and negatively regulates osteoclast differentiation. The up-regulation of OPG in PKGII -/- mice suggests a novel role for PKGII in the regulation of osteoclast differentiation, and warrants further study.

INTRODUCTION

Dynamics of bone growth:

The skeleton begins in the early embryo with mesenchymal stem cells (MSCs), which develop into two of the three major cells that make up the skeletal system: chrondrocytes and osteoblasts (Lefebvre and Bhattaram 2010). From there chondrocytes form the cartilaginous early skeleton that eventually is replaced with bone tissue during pre and post natal development, a process known as endochondral ossification (Lefebvre and Bhattaram 2010). The bones which develop without the initial presence of cartilage, such as the skull and clavicles, undergo intramembranous ossification (Lefebvre and Bhattaram 2010).

During development chondrocytes form globular growth plates, epiphyses, at both ends of long bone shafts, diaphysis, where their staggered maturing layers allow for the growth of limbs (Lefebvre and Bhattaram 2010). During endochondral ossification, a highly complex network of communication exists between chondrocytes and bone remodeling cells, osteoblasts and osteoclasts. As chondrocytes proliferate and differentiate, osteoclasts enter the cartilage matrix to begin breaking it down, in order clear the way for osteoblasts to lay the mineral foundation of bone tissue (Lefebvre and Bhattaram 2010).

Osteoblasts also allow for intramembranous ossification, forming an organic matrix that later becomes calcified through radiating spicules from the center of the bone, called the ossification center (Lefebvre and Bhattaram 2010). The functioning of osetoblasts is dependent on their proper development

and proliferation, the regulation of which is incompletely understood (Lefebvre and Bhattaram 2010).

Also vital for proper skeletal formation are osteoclasts, which unlike chondrocytes and osteoblasts originate from monocyte/macrophage lineage (Boyle et. al. 2003). Osteoclastogenesis is thought to occur through direct contact between osteoclast precursor cells of hemopoietic lineage and pre-osteoblast cells (Quinn and Gillespie 2005). The promotion of osteoclasts is also regulated through complex intercellular signaling and depends especially on the functioning of osteoblasts. Both cells work together continually to remodel bone tissue, osteoblasts forming new tissue while osteoclasts remove older or damaged tissues.

The importance of the cGMP dependent protein kinase II pathway:

There are two c-GMP dependent protein kinases, PKG I and PKG II, found in various tissues throughout the body. The soluble cytosolic PKGI is found prevalently in smooth muscle, platelets and the brain, while the membrane bound PGKII is more limited to neuronal cells, as well as bone and kidney cells (Pfeifer et. al. 1998). Experiments with over-expression or deficiency in cGMP protein kinases has shown their integral importance in proper gene expression and development, in particular for cardiovascular, skeletal and nervous systems (Pilz and Broderick 2005). Mice deficient in PKGI have limited life spans due to dysfunction of smooth muscle, as well as neuronal abnormalities. PKGII deficient mice are viable, however, they express impairments of the circadian clock as

well as a dwarfism phenotype due to difficulties with endrochondral ossification (Pfeifer et. al. 1996).

The dwarfism phenotype expressed by PKGII deficient mice, as well as the phenotype of bone overgrowth seen in mice over-expressing natriuretic peptides suggests an important role for PKGII dependent signaling in skeletal development (Suda et. al. 1998). Natriuretic peptides stimulate guanylate cyclases (GCs) A and B, both found prominently in the epiphysis of the tibia; the stimulation of GCs produces cGMP, which activates PKGs, providing a correlation between the activity of protein kinases and bone growth (Suda et. al. 1998). While both PKGI and II are expressed in the growth plates of bones, only the deletion of PKGII shows an effect on bone elongation suggesting a novel and absolutely necessary role of PKGII in endochondral ossification (Hofmann 2005).

The role of PKGII in osteoblast proliferation and survival has been examined in various studies performed by our lab. It was found that PKGII is necessary for osteoblast proliferation upon either fluid shear stress stimulation (FSS) or the mimicking of fluid shear stress via a cGMP membrane permeable analogue (Rangaswami et. al. 2010). Osteoblast proliferation stimulated by FSS was found to be mediated through a Src dependent pathway, which in osteoblasts derived from PKGII deficient mice was defective (Rangaswami et. al. 2010). The examination of *fos* family genes, known to be under the control of PKGII, also showed defective amounts of *c-fos* and *fra-2* mRNAs in the tibial diaphyses of PKGII -/- mice vs. wild-type (Rangaswami et. al. 2010). It was

thought that other osteoblast and osteoclast specific genes may be under the control of PKGII signaling, therefore I chose to examine PKGII -/- mice for five genes of interest, all pertaining to proper osteoblast and osteoclast function and differentiation, to see if there was transcriptional down-regulation caused by the PKGII deficiency.

The genes in question:

One of the better known osteoblast-specific genes is the Runt-domain containing transcription factor, Runx2, known to act on the promoter region of the osteoblast specific matrix protein, osteocalcin (Ducy and Karsenty 1995). Runx2 is also known to affect osteoprogenitor cells very early on in development as well as regulating many osteoblast specific genes; non-osteoblast cells over-expressing Runx2 were seen to express osteoblast specific factors such as osteocalcin (Ducy et. al. 1997). The human disease CCD, cleidocranial dysplasia, is due to a mutation in the Runx2 locus, while Runx2 deficient mice are have defective osteoblast production (Lee et. al. 1997 and Komori et. al. 1997).

The second gene of interest was osteocalcin, a vitamin K dependent protein with three glutamic acid residues which are modified post-transcription by vitamin K dependent carboxylases (Maillard et. al. 1992). Such post-transcriptional modification allows for the ionic attraction of minerals such as calcium and their incorporation into the mineralized bone matrix (Maillard et. al. 1992). Osteocalcin is also vital for osteoblast differentiation and function, and has been shown to regulate glucose metabolism in osteoblasts (Ferron et. al. 2010). In osteocalcin -/-mice an abnormal amount of visceral fat deposition was

seen, suggesting a critical link between metabolism and bone growth (Ducy et. al. 1996).

Several osteoblast-produced factors are known to affect osteoclast differentiation and proliferation (Chambers 2000). RANKL, or receptor activator of nuclear factor kappa B (NF-κB) ligand, is thought to be a dominating signal produced by osteoblast/stromal cells to induce osteoclast differentiation (Boyle et. al. 2003). It is a membrane-bound protein produced by osteoblasts; when in contact with osteoclast precursors it binds to the osteoclast-precursor specific receptor, RANK, to ensure complete differentiation into osteoclasts (Boyle et. al. 2003). RANKL is also found in soluble form, however sRANKL is less effective in inducing osteoclast proliferation and survival (Hikita et. al. 2006). The lack of RANKL in mice causes osteopetrosis due to a lack of osteoclasts (Boyle et. al. 2003).

A decoy receptor for the RANKL ligand was also examined, OPG or osteoprotegerin, since its function is to down-regulate osteoclast differentiation (Simonet et. al. 1997). Over-expression of the osteoprotegerin receptor causes osteopetrosis, a thickening of the bone matrix due to a lack of osteoclasts; while OPG -/- mice suffer osteoporosis, a hollowing of the bone matrix. Both mouse models show that osteoprotegerin plays an important role in the formation and resorptive functions of osteoclasts (Bucay et. al. 1998).

The last osteoblast-specific gene examined was Fra1, a member of the *fos* family of transcription factors already examined by our lab for their role in osteoblast proliferation and function (Rangaswami et. al. 2010). The *fos* family

proteins are known to be one of two protein groups needed to form the dimeric transcription factor, activating protein-1, AP-1 (Eferl and Wagner 2003). The *fos* family is thought to be crucial for both osteoblast and osteoclast proliferation and differentiation, as seen by over-expression of *c-fos* causing osteosarcomas from hyper-proliferation of osteoblasts and deletion of c-fos causing osteopetrosis from under-production of osteoclasts (Eferl and Wagner 2003). Interestingly, the defective differentiation of osteoclasts in *c-fos* -/- mice can be rescued by the expression of fellow *fos* protein, Fra1, suggesting overlapping functionality of the two proteins (Eferl and Wagner 2003). Fra1 -/- mice are not viable, however the conditional knockout of Fra1 restricted to embryonic tissue results in viable mice with osteopenia, reduced bone mass (Fleischmann et. al. 2000). Fra1-deficient mice have normal osteoblast and osteoclast cell numbers, however the lack of bone formation was attributed to defective osteoblast functionality, in particular with secreted matrix forming proteins like osteocalcin (Fleischmann et. al. 2000).

MATERIALS AND METHODS

PKGII Knock-out mice:

Homozygous PKGII deficient mice were generated via vector targeted disruption of the PKGII gene, where a 308 bp deletion of the second exon in the PKGII locus caused a disruption of one of the two necessary cGMP binding pockets (Pfeifer et. al. 1996). The genotypes of litter mates were determined via Nothern and Southern blots as well as reverse transcriptase-PCR and immunoblotting (Pfeifer et. al. 1996)). Immunoblotting also showed that the lack of PKGII had no effect on PKGI functionality, as well as not affecting cGMP production (Pfeifer et. al. 1996). Tibial diaphyses samples were collected from the PKGII knock out litters at the University of Bonn, Germany, in accordance to Animal Welfare Committee regulations (Rangaswami et. al. 2010). The tibial diaphyses samples were removed so that the epiphyseal growth plates were cut off and the perichondrium removed, as well as the bone marrow flushed out. The samples were flash frozen and shipped on dry ice to our lab and stored at -80°C until ready for RNA extraction.

RNA Extraction:

RNA was extracted from the tibiae and examined litter by litter, varying in age from one to four weeks. The samples were pulverized via a metal mortar and pestle and thawed on ice.. The crushed bone was then removed to 1.5mL centrifuge tubes with the addition of TRI reagent (Molecular Research Center), a combination of phenol and guanidine thiocyanate to inhibit RNAse activity. The

TRI reagent/bone mixture was vortexed for one minute to assist in homogenization and then allowed to rest at room temperature for five minutes for complete dissociation of nucleoprotein complexes. Cold BCP (1-bromo-3-chloropropane) was then added to help ensure the separation of aqueous and organic phases. After a ten minute rest at room temperature the mixture was centrifuged (4°C, 14K rpm) for fifteen minutes. After centrifugation, the RNA was isolated in the upper aqueous phase, while DNA remained in the interphase and proteins in the organic phase, and the upper phase was then removed to a fresh tube. The RNA was precipitated with the addition of isopropanol. The mixture was left at room temperature for eight minutes, followed by an eight minute centrifugation (4°C, 14K rpm). The supernatant was removed and the RNA pellet was washed with 75% ethanol and centrifuged for another five minutes (4°C, 12K rpm). Following removal of the supernatant the pellet was allowed to air dry for a few minutes and then re-suspended in 10μL of RNAse free H_2O. Heating of the samples for 10 minutes at 55-60°C allowed for complete dissolution of the RNA. The samples were allowed to chill on ice prior to being inspected via a NanoDrop (ND1000) Spectrophotometer, using 1.5μL of RNA.

The spectrophotometer allowed for quality and quantity examination of the RNA sample. Quality was determined by how well it fit within the 1.8-2.0 260/280 ratio, quantity was determined (by the machine) via a modified form of the Beer-Lambert equation. Concentration was determined by the equation: **c = A* e/b**, where **c** is the concentration in ng/μL, **A*** is the absorbance reading at 260, **e** is a wave dependent extinction coefficient, (in this case for RNA = 40

ng-cm/mL), and **b** is the path in cm. Based on the machine's determined concentration, cDNA was made from each RNA sample via reverse transcriptase using the volume equivalent of 1μg RNA, 4μL 5X iScript Reaction Mix (Bio-Rad), 1μL iScript Reverse Transcriptase (Bio-Rad), and RNAse free H_2O for the remaining of the 20μL reaction total. The RT-PCR was carried out using a Bio-Rad S1000 Thermal Cycler at the denoted iScript protocol cycles: 25°C 5 min, 42°C 30 min, 85°C 5 min, 25°C 5 min and then kept at 12°C. After cDNA synthesis the samples were stored at 4°C until needed for Real Time quantitative PCR analysis.

Optimization and use of Real Time Quantitative PCR:

Real Time quantitative PCR was performed using a Stratagene MX3005P qPCR machine, using the reagent mix 2X Brilliant II SYBR Green QPCR Master Mix (Stratagene) with the cycle settings: 95°C 30 sec, 60°C 45 sec, 72°C 1 sec and then the temperature was increased by 1°C/sec from 55°C to 95°C to produce a dissociation curve. Primers were optimized for each of the five aforementioned genes, as well as the reference housekeeping gene Gapdh, glyceraldehyde-3-phosphate dehydrogenase, using samples from eight week old normal, wild-type mice, (tibiae harvested in similar fashion as the PKGII knock out samples, and their RNA extraction as well as cDNA synthesis followed the above steps). Primers were also optimized for two additional genes, Osterix and c-fos, however readable results were only detected with RNA extracted from MC3T3 cells, therefore they were not examined further in the PKGII knock out samples.

Primers were optimized by producing standard curves for each primer, using variable amounts of cDNA input (dilution of cDNA sample). The thermal cycler determines the threshold cycle, Ct, the cycle at which the amount of DNA detected via fluorescence reaches a preset fluorescence intensity threshold. The amount of cDNA is shown in a logarithmic scale to account for the exponential amplification scale upon which the threshold cycle is based. The curve produced represented the linear range of each primer, corresponding to a perfect doubling of the cDNA amount at each cycle, (100% efficiency). Various concentrations of each primer were tested, from 50nM to 400nM, over a minimum of a three-fold dilution range of DNA concentrations, tested always in duplicate. To be acceptable, the standard curves derived had to have an efficiency between 95-105% with a correlation coefficient (how well the data points fit the curve produced) above 99%. Table I of the appendix shows the derived primer concentrations and sample dilution ranges needed for reproducible standard curves, as well as each primer's sequence.

After primer optimization, the actual PKGII knock out litter samples were analyzed. Each cDNA sample was examined at two-fold dilutions, each in duplicate, within the optimal dilution range for each primer, with no-template controls for each primer. Each sample was tested for all genes simultaneously, using Gapdh as the normalizing factor. The results were examined for raw Ct value reproducibility (between duplicates of a single dilution) as well as primer efficiency over the two-fold dilution scale. Inefficient results caused a rerun of the sample using a different two-fold dilution scale, but still within the optimized

range. Samples with extreme variation were re-run using a freshly made cDNA sample from the original RNA sample.

Since there were not always a single wild type control mouse of the same sex as the knockout mice in each litter, we decided not to use the $2^{-\Delta\Delta Ct}$ method traditionally used to express fold changes in gene expression based on a reference sample. Instead, then qPCR results were analyzed for differences in relative gene expression levels based on the calculation: $2^{-\Delta Ct}$, ΔCt being the difference in threshold cycle values between the gene of interest, and the endogenous control, the housekeeping gene Gapdh (Ct gene – Ct Gapdh). Since all the primers were optimized to be approximately 100% efficient, there was assumed to be a doubling of the DNA per cycle, therefore $2^{\text{cycle number}}$ corresponds to the increase in DNA. Representing the relative mRNA expression levels, as $2^{-\Delta Ct}$ allowed for exponential transformation of the threshold cycle value differences.

RESULTS

Primers for the five target genes as well as the endogenous control, Gapdh, were optimized to have relatively equal efficiencies, between 95-102%, where 100% efficiency corresponds to a doubling of DNA at each cycle. The standard curves produced for each primer are shown in Graphs I-VI, where the threshold cycle values are plotted against the input cDNA amount, shown both as copy number and the equivalent amount in μg cDNA. Linearity of the curves was also important for the optimization, since the trend should be a difference of 1 Ct for every doubling of DNA (doubling with each cycle), the Rsq value, or correlation coefficient, shows how well the data points fit this trend.

For the actual sample qPCR runs, two-fold dilutions within the optimized range were tested in duplicate for each primer, and the difference in Cts for the two-fold range was examined for its proximity to 1 Ct as an internal control. Tables II-IV show the averages of the raw Ct values for each dilution for each primer, as well as the quality and quantity (260/280 ratio and concentration, respectively) of the initial RNA sample from which the cDNA was made. Table II shows results for seven-day-old mice only, consisting of seven samples total, four wild-type and three knock-out. Table III shows results for eleven-day-old mice only, consisting of three different litters for a total of four wild-type and five knock-out mice. Lastly, table IV shows results for twenty-eight-day-old mice, three litters of four mice each, for a total of five wild-type, three heterozygote and four knock-out mice. Table IV also shows the sexes for each of the mice,

sexes were only recorded for the twenty-eight-day-old mice since it is too early to tell the sex at seven to eleven days.

From these averaged Ct values, relative changes in mRNA expression levels were calculated by taking the difference in Ct between the gene of interest and the endogenous control, Gapdh, (Ct gene – Ct Gapdh). Gapdh was chosen as the endogenous control because experiments in our lab have shown that its expression levels remain fairly constant between differing genotypes (PKGII -/- vs. wild-type) and that Gapdh mRNA levels in cultured cells are not affected by various stimuli in vitro. Gapdh is a housekeeping gene with high expression levels, therefore it was expected that the Ct values for Gapdh would be significantly lower than any of the genes of interest, (a lower Ct corresponds to a higher amount of cDNA in the sample). Since the Ct values for the genes of interest were always higher than those of Gapdh, the ΔCt (Ct gene – Ct Gapdh) was always a positive integer. A small ΔCt would therefore correspond to little difference between the Ct of the gene of interest and the Gapdh Ct, meaning the expression level of the target gene mRNA level was close to that of the housekeeping gene, Gapdh. Likewise a large ΔCt would show that the mRNA level of the gene of interest was lower relative to the mRNA level of Gapdh.

The relative difference in mRNA levels was then expressed by calculating $2^{-\Delta Ct}$ to account for the exponential derivation of Ct values, since DNA amplification is exponential with a doubling of DNA with every cycle within the linear range of each primer (determined from the standard curve). By

calculating $2^{-\Delta Ct}$, a relative mRNA expression level of 1 would mean no difference in mRNA levels between the gene of interest and the housekeeping gene, Gapdh ($2^{-\Delta Ct}$ =1, $\Delta Ct = 0$). However, I chose to increase the $2^{-\Delta Ct}$ results by a scale of 100 since the large ΔCt differences in my results accounted for very small decimal ranges when calculated as $2^{-\Delta Ct}$. The results for the seven-day-old mice are shown in Graphs VII-VIII. Graph VII shows a cluster plot of the relative mRNA expression values ($2^{-\Delta Ct}$ x 100) pertaining to each gene for both the wild-type (blue diamonds, n=4), and knock-out (red circles, n=3). The cluster plot helps illustrate how the relative mRNA levels overlap for both genotypes examined. Significant differences between the wild-type vs. knock-out groups are not seen except in the levels of osteoprotegerin. For the seven-day-old mice, the mean Gapdh Ct was 23.98 +/- 1.57 (S.D.), with one knock-out lying more than one standard deviation above the mean, and one knock-out lying more than one standard deviation below the mean.

For Runx2, all the wild-type mice had a ΔCt between 6.01-7.05, meaning the Ct value for Runx2 was 6-7 cycles above that of Gapdh, corresponding to normalized relative mRNA levels of 0.75-1.55, (where the larger ΔCt corresponds to the smaller relative mRNA level). The knock-outs meanwhile had slightly smaller ΔCts, between 5.5-6, signifying slightly increased Runx2 levels relative to wild-type, and their normalized mRNA levels are shown between 1-2.2. For osteocalcin the wild-type ΔCts were between 6.09-6.49, with one outlier at 4.59, corresponding to relative mRNA levels of 1.11-4.15 (outlier). The knock-outs had ΔCts between 4.04 and 6.04, corresponding to relative mRNA levels between

1.52-6.08. Rankl had a tight clustering of the ΔCts for both wild-type and knock-out, all between 8.01-8.78, with one wild-type outlier at 9.69 ΔCt. The corresponding relative mRNA levels were all between 0.12 (outlier) to 0.38, meaning that the mRNA levels were about ten-fold lower compared to the relative mRNA levels of osteocalcin. The levels for osteoprotegerin seemed to suggest a difference between the wild-type and knock-out, where wild-types had ΔCts between 5.51-6.08 corresponding to relative mRNA levels of 1.48-2.19, and knock-outs between 4.63 to 5.23, corresponding to 2.66 to 4.04 relative mRNA levels. Fra-1 mRNA levels were not examined because their raw Ct values generated were well off the linear scale and unreliable.

This same data for the seven-day-old mice is shown again as a bar graph in Graph VIII, where the average of the relative mRNA levels was taken for both the wild-type (n=4) and knock-out (n=3) groups. The error bars for each average show +/- the standard error, calculated as standard deviation/square root of sample pool. The values of relative mRNA levels for both the wild-type and knock-outs were compared using a two-tailed T Test in Excel, where a p value less than 0.05 indicated a significant difference. Only osteoprotegerin showed a trend toward a difference between wild-type vs. knock-out relative mRNA levels, with p=0.054.

The results for the eleven-day-old mice are shown in Graphs IX-X. Graph IX is a cluster plot of the relative mRNA levels calculated ($2^{-\Delta Ct} \times 100$) to show the overlapping results between wild-type (blue diamonds, n=4) and knock-out (red circles, n=5). For all the eleven-day-old mice, the mean Gapdh Ct was 23.45 +/-

0.63 (S.D.), with one wild-type lying at one standard deviation above the mean, and one knock-out lying more than one standard deviation below the mean. For Runx2 the wild-types had ΔCts between 5.39-5.75, corresponding to relative mRNA levels between 1.86-2.38, ΔCts for the knock-outs were very similar, between 4.97 to 5.44, corresponding to relative mRNA levels between 2.3-3.19. Osteocalcin had ΔCts for the wild-types between 4.94-6.14, with corresponding relative mRNA levels between 1.42 to 3.26, while the knock-outs had slightly decreased ΔCts, between 4.93-5.69, corresponding to relative mRNA levels of 1.94 – 3.28. Rankl, similar to the seven-day-old mice results, had large ΔCts, between 7.44 to 8.34 for both wild-type and knock-out, corresponding to relative mRNA levels of 0.31-0.58, (therefore relative mRNA levels were three-fold lower compared to the other genes with relative mRNA levels above 1). The last gene analyzed, osteoprotegerin, had wild-type ΔCts between 5.25 to 5.75, corresponding to relative mRNA levels of 1.86 to 2.38, while the knock-outs had ΔCts between 4.97-5.44, corresponding to relative mRNA levels of 2.3-3.19, suggesting higher levels than those in wild type mice. Fra-1 mRNA levels were not examined because their raw Ct values generated were well off the linear scale and unreliable.

Graph X shows the same data as Graph IX in the form of a bar graph, where averages of the relative mRNA expression levels have been calculated between the wild-types (n=4) and knock-outs (n=5). Error bars are shown correlating to +/- the standard error for each average. A two tailed T test was carried out for each gene's results to see if a significant difference could be

detected between the wild-type vs. knock-out. Only osteoprotegerin indicated a significant difference, with a p=0.028.

The twenty-eight-day-old mice consisted of three different litters of four mice each, they were not analyzed collectively however due to inconsistencies between the litters in diet and exact age upon sacrifice. Therefore, their data was normalized only within each litter, and since the sample numbers for each genotype were very small (either one or two total per litter), neither averages nor statistical analysis was performed. Instead, the results of relative mRNA expression levels are shown as cluster plots for each litter (Graphs XI-XIII) which help to show the overlapping results between genotypes.

Littler one consisted of four mice, one male wild-type, one female wild-type, one male heterozygote and one male knock-out mouse. The litter had a mean Gapdh Ct of 20.98 +/- 2.63 (S.D.), with the heterozygote lying more than one standard deviation below the mean, and the knock-out lying more than one standard deviation above the mean. Graph XI is a cluster plot of the relative mRNA levels for litter 1. For Runx2 the wild-types and heterozygote had ΔCts between 6.13-6.32, corresponding to relative mRNA levels between 1.25-1.43. The knock-out had a ΔCt of 3.89, corresponding to 6.75 relative mRNA level. For osteocalcin the wild-types and heterozygote had ΔCts between 5.77 and 6.37, corresponding to relative mRNA levels of 1.21-1.83, while the knock-out had a ΔCt of 5.1, corresponding to a relative mRNA level of 2.92. Rankl showed ΔCts between 7.92 and 8.23 for the wild types and heterozygote, corresponding to relative mRNA levels of 0.33-0.41, the knock-out had a ΔCt of 5.76, with a

relative mRNA level of 1.85. For osteoprotegerin, the ΔCts were between 4.97-5.21 for the wild-types and heterozygote, corresponding to relative mRNA levels of 2.11-3.19, while the knock-out had a ΔCt of 3.18 corresponding to a relative mRNA level of 11.03. Fra-1 mRNA levels were not examined because their raw Ct values generated were off the linear scale and unreliable.

Litter two of the twenty-eight-day-old mice had four mice total, two male wild-types, one female heterozygote and one female knock-out. The litter had a mean Gapdh Ct of 27.19 +/- 1.37 (S.D.), with one of the male knock-outs lying more than one standard deviation above the mean. Graph XII shows a cluster plot of the relative mRNA levels calculated for the litter. For Runx2 the wild-types had ΔCts of 5.68-6.41, corresponding to relative mRNA levels of 1.18-1.95, while the heterozygote had a ΔCt of 4.86 corresponding to a 3.44 relative mRNA level, and the knock-out had a ΔCt of 5.54 corresponding to a relative mRNA level of 2.15. For osteocalcin, the wild-types had ΔCts of 4.15 and 2.27 (outlier) corresponding to a relative mRNA level of 5.63 and 20.73 (outlier), while the heterozygote had a ΔCt of 3.03 corresponding to a 12.24 relative mRNA level and the knock-out had a ΔCt of 1.46, corresponding to a relative mRNA level of 36.35. Rankl again showed larger ΔCts, between 6.75 and 7.22 for the wild-types and heterozygote, corresponding to relative mRNA levels between 0.67-0.93, while the knock-out had a ΔCt of 6.56 with a relative mRNA level of 1.06. Osteoprotegerin had ΔCts for the wild-types and heterozygote clustered between 3.9-4.14, corresponding to relative mRNA levels of 5.67-6.6, while the knock-out had a ΔCt of 3.21, corresponding to a relative mRNA level of 10.81.

Lastly for Fra-1 one wild-type and the heterozygote had ΔCts of 4.35-4.62, corresponding to relative mRNA levels of 4.07-4.9, while the other wild-type (outlier) had a ΔCt of 3.68, close to the ΔCt of the knock-out at 3.5, corresponding to relative mRNA levels of 7.8 and 8.83, respectively.

The last litter examined of the twenty-eight-day-old mice was litter three, consisting of one female wild-type, one male heterozygote and two male knock-outs. The mean Gapdh Ct for the litter was 24.75 +/- 2.6 (S.D.) with the one female wild-type lying more than one standard deviation above the mean. Graph XIII is a cluster plot of the relative mRNA levels for this litter. For Runx2 the wild-type and knock-outs had overlapping results, with ΔCt for the wild-type at 7.2, heterozygote 6.54, and knock-outs 6.04 and 7.31, together corresponding to relative mRNA levels between 0.63-1.52. Osteocalcin had ΔCts for the wild-type and heterozygote between 3.1-3.68, with relative mRNA levels between 7.8-11.66, while the knock-outs had ΔCts between 4.26-5.49, corresponding to relative mRNA levels between 2.22 to 5.22. Rankl again had larger ΔCts, of 8.62-9.62 for the heterozygote and wild-type respectively, and 7.73-9.61 for the knock-outs, together corresponding to relative mRNA levels of 0.13-0.47 for all genotypes. For osteoprotegerin all the samples were clustered between a ΔCt of 4.35-5.06, with relative mRNA levels of 3.4-4.9 for the wild-type and heterozygote respectively, and 3-3.4 for the knock-outs. Lastly for Fra-1, the wild-type and heterozygote had ΔCts of 4.66-5.42, corresponding to relative mRNA levels of 2.34-3.96, while the knock-outs had ΔCts of 6.1-6.23, corresponding to relative mRNA levels between 1.33-1.46.

DISCUSSION

Tibiae taken from mice deficient in cGMP-dependent Protein Kinase II were analyzed, along with wild-type and heterozygote litter-mates, via quantitative RT-PCR to see if there was a difference in gene expression levels between the genotypes. The genes of interest were all pertaining to bone growth and maintenance, in particular genes related to the proper differentiation and function of bone-forming cells, osteoblasts, as well as genes related to the regulation of bone-resorbing cells by osteoblasts. Three age groups of mice were analyzed overall, seven-day-old, eleven-day-old, and twenty-eight-day-old. Out of all three age groups, and all five genes in question, only osteoprotegerin in the seven-day and eleven-day-old mice showed a (near) significant difference in relative mRNA levels between the wild-type and knock-out.

The mice of differing genotypes were examined for five genes of interest, Runx2 being a master osteoblast transcription factor, osteocalcin a osteoblast secreted matrix protein, Rankl a ligand needed for proper osteoclast differentiation, osteoprotegerin its decoy receptor allowing for down-regulation of osteoclasts, and Fra-1 a member of the fos family of transcription factors, known to be important for bone development. Having seen significantly reduced levels of c-fos and fra-2 mRNA transcription levels in tibiae of newborn PKGII -/- compared to wild type mice, and knowing that PKGII -/- mice have impaired osteoblast function, it was expected that the osteoblast specific genes, Runx2 and osteocalcin as well as the fos family gene, Fra-1, would have down-

regulated mRNA levels compared to the wild-type (Rangaswami 2010). The osteoclast specific genes, Rankl and osteoprotegerin, were examined without an expectation of being either up or down-regulated, however a difference either way was expected between the wild-type and knock-out mice.

Out of the seven-day-old mice analyzed, with a sample pool of four wild-type and three knock-out, there was no significant difference in relative mRNA levels between the wild-type vs. knock-out mice, except for the gene, osteoprotegerin. The two tailed T test done for the relative mRNA expression levels of OPG had a $p=0.054$, where $p<0.05$ indicates a significant change. While the p value lies just outside the significant range, I believe it to be a matter of having such a small sample pool that prevents the change from falling within the significant range.

The nine total eleven-day-old mice analyzed also had no significant differences between the mRNA transcription levels of the genes of interest between the wild-type and knock-out mice, except for osteoprotegerin. When a two tailed T test was performed for the relative mRNA levels of OPG, there was a corresponding p score of 0.028, where $p<0.05$ indicates a significant difference in values between the wild-type and knock-out samples. Therefore, both the seven-day and eleven-day-old mice suggest a statistically important difference in mRNA transcription levels of osteoprotegerin between wild-type and PKGII knock-out mice.

However, such a correlation is not seen in the twenty-eight-day-old mice. The twenty-eight-day-old mice consisted of three litter of four mice each, for a

total of five wild-type, three heterozygote, and four knock-out mice. However, there were inconstancies between the litters that prevented the collective analysis of all the twenty-eight-day-old results together. Firstly, the actual age of the mice (date of sacrifice) is not exact between all litters, with some being sacrificed at closer to thirty-five days instead of twenty-eight. Secondly, the weights of the twenty-eight-day-old old mice varied greatly between wild-type and knock-outs, Table V shows the weights in grams for all the twenty-eight-day-old mice.

As can be seen, in litter two both the wild-type males were significantly heavier than the heterozygote and knock-out female littermates (22.7 and 20.1g vs. 15.42 and 13.87g). While in litter three it can be seen that both the male knock-outs were of equivalent weight with the male heterozygote and female knock-out (13.3 and 14.6g vs. 13.7 and 12.7g). Such fluctuations in weight, along with being told that the diets of the mice were not strictly regulated (some were mistakenly fed the diet of pregnant females), made comparisons between the litters unreliable, and analysis was therefore only within each litter. However, being only able to compare within each litter meant samples pools of one or two for each genotype, which prevented any real statistical analysis of the differences due to insufficient numbers.

For litter one, the results seem to show an up-regulation of all the genes of interest in the knock-out sample. This particular litter's samples were run twice (with a fresh cDNA sample made for the second run), and still the results showed a comparatively low Gapdh Ct for the knock-out (24.45 compared to a mean of

20.45 +/- 2.63 S.D.). The Ct values for the target genes remained closer to the Gapdh Ct, showing a slightly increased relative mRNA expression level of those genes compared to the relative mRNA levels of the wild-types and heterozygote. The RNA sample itself could have been contaminated or have impurities that affected the Gapdh primers, since both cDNA samples made from the RNA showed low Gapdh values.

The results for litter two show much overlap for the knock-out and wild-type results. For Runx2 and Rankl all relative mRNA levels are clustered together, while for OC the results are spread out but the range for wild-type overlaps with knock-out, not presenting any significant difference in the two groups. The mRNA levels for osteoprotegerin in litter two do show the possibility of a significant difference, with the knock-out having a relative mRNA level of 10.8 while the wild-types and heterozygote remain between 5.67-6.69, however having such a small sample pool made it difficult to analyze the data any further. It is also difficult to assess how much this difference is attributed to the fact that the knock-out was a female and the wild-types were male, since males and females have different age-dependent skeletal growth patterns.

Litter three had high variation between the values of Gapdh, with the knock-outs being significantly lower (~22 Gapdh Ct vs ~27 for the wild-type and heterozygote). The samples were rerun using a freshly made cDNA sample and such wide variation in Gapdh values was seen again. Therefore, there could be something in the RNA samples of the wild-type and heterozygote affecting the efficiency of the Gapdh primers. For litter three, Runx2, Rankl and OPG are all

clustered within the same overlapping range between wild-type and knock-out. However, both OC and Fra-1 show down-regulation of the relative mRNA levels in the knock-outs. This could be a skewed representation of the data since low Gapdh Cts would cause higher ΔCts, therefore corresponding to lower relative mRNA levels than the wild-type and heterozygote.

Over all the three age groups, my results show the only possible gene affected by the PKGII -/- genotype is osteoprotegerin. Both the seven-day and eleven-day-old mice showed a significant difference in values between wild-type and knock-out (calculated via a two tailed T test). Out of the twenty-eight-day-old mice, litter two also indicated the possibility of having up-regulated relative mRNA levels for OPG in the knock-out compared to wild-type. I believe that the up-regulation seen in the knock-out mice for these age groups could indicate a novel role of PKGII in OPG transcription, and is possibly worth being pursued by further research.

My results overall indicate that while there are fluctuations in relative mRNA levels for the genes of interest between the wild-type and knock-out mice, their wide variation between litters indicates the need for a much wider sample pool for accurate statistical analysis. The twenty-eight-day-old litters showed great variation between them, indicating the need for strict regulation of the diet and environment of the mice, as well making sure the bone samples are harvested at the same time between all litters. It is also possible that PKGII has a more active role in osteoblast signaling and proliferation due to mechanical loading and stress, since its affects on osteoblast proliferation and

survival were seen after fluid shear stress stimulation. Therefore, the lack of alteration in relative mRNA levels of the genes examined could be attributed to the fact that these mice did not have the active lives necessary to show altered transcriptional regulation between wild-type and knock-out.

APPENDIX

TABLE I: Optimized primer sequences, concentrations, and dilution fold scale. Genes referenced all pertain to mice.

GENE	SEQUENCE	PRIMER CONC. [NM]	DILUTION RANGE
Gapdh	**F:** 5' GCC AAA AGG GTC ATC ATC TC 3'	300	1:2 to 1:64
	R: 5' GGC CAT CCA CAG TCT TCT G 3'	300	1:2 to 1:64
Rankl	**F:** 5' CCA AGA TCT CTA ACA TGA CG 3'	100	1:2 to 1:16
	R: 5' CAC CAT CAG CTG AAG ATA GT 3'	100	1:2 to 1:16
OC	**F:** 5' AGC CCT TAG CCT TCC ATA GTG 3'	200	1:1 to 1:16
	R: 5' AGC CCT CTG CAG GTC ATA GA 3'	200	1:1 to 1:16
Runx2	**F:** 5' GAC AGA AGC TTG ATG ACT CTA AAC 3'	400	1:1 to 1:16
	R: 5' TCT GTA ATC TGA CTC TGT CCT TGT 3'	400	1:1 to 1:16
OP	**F:** 5' ACC CAG AAA CTG GTC ATC AGC 3'	300	1:4 to 1:16
	R: 5' CTG CAA TAC ACA CAC TCA TCA CT 3'	300	1:4 to 1:16
Fra1	**F:** 5' CAA AAT CCC AGA AGG AGA CAA G 3'	200	1:4 to 1:32
	R: 5' AAA AGG AGT CAG AGA GGG TGT G 3'	200	1:4 to 1:32

TABLE II: Averaged raw Ct values for one week (seven-day) old tibiae samples. Dilution ranges shown for Gapdh, normalizing gene, all other genes same dilution range shown.

Samples	Set	Sac	RNA	RNA Conc.	cDNA made	Dilutions	Gapdh Cts	Dilutions	Runx2	OC	Rankl	OP	Fra1
+/+ 5	1	2-Jun	1.99	924.4	1.0 ug	1 to 16	22.78	1 to 4	29.72	29.27	30.79	28.62	No Ct
						1 to 32	23.55	1 to 8	31.08	29.82	31.56	29.17	No Ct
+/+ 6	1	2-Jun	2.01	897.7	1.0 ug	1 to 16	22.92	1 to 4	29.97	29.01	32.61	29.00	No Ct
						1 to 32	23.70	1 to 8	30.78	30.28	32.40	29.63	No Ct
+/+ 7	1	2-Jun	1.99	450.6	1.0 ug	1 to 16	24.53	1 to 4	30.54	29.12	32.54	30.04	No Ct
						1 to 32	25.21	1 to 8	31.57	29.91	33.69	30.95	No Ct
+/+ 9	1	2-Jun	2.02	1492.1	1.0 ug	1 to 16	25.05	1 to 4	31.71	31.27	33.83	30.56	No Ct
						1 to 32	26.16	1 to 8	32.73	32.17	34.95	31.33	No Ct
-/- 3	1	2-Jun	2	322.4	1.0 ug	1 to 16	26.55	1 to 4	32.05	30.59	35.04	31.50	No Ct
						1 to 32	27.00	1 to 8	32.41	31.75	36.04	32.54	No Ct
-/- 4	1	2-Jun	1.98	548.3	1.0 ug	1 to 16	24.08	1 to 4	30.64	29.80	32.63	28.71	No Ct
						1 to 32	24.88	1 to 8	31.71	30.88	33.49	29.78	No Ct
-/- 10	1	2-Jun	1.98	887.7	1.0 ug	1 to 16	21.96	1 to 4	28.16	28.00	29.97	27.19	No Ct
						1 to 32	22.96	1 to 8	28.96	28.07	31.23	28.12	No Ct

TABLE III: Averaged raw Ct values for eleven-day-old tibiae samples. Dilution ranges shown for Gapdh, normalizing gene, all other genes same dilution range, shown below.

Samples	Set	Sac	RNA	RNA Conc.	cDNA made	Dilutions	Gapdh Cts	Dilutions	Runx2	OC	Rankl	OP	Fra1
+/+ 1 lit.1	7	9-May	1.97	295.3	1.0 ug	1 to 16	23.25	1 to 4	28.70	28.19	30.91	29.00	No Ct
						1 to 32	24.17	1 to 8	29.93	29.17	31.73	29.94	No Ct
-/- 1 lit. 1	7	9-May	1.89	305.9	1.0 ug	1 to 16	23.69	1 to 4	29.21	28.67	31.42	28.98	No Ct
						1 to 32	24.48	1 to 8	30.03	29.36	32.35	29.65	No Ct
-/- 2 lit. 1	7	9-May	1.86	255.3	1.0 ug	1 to 16	21.96	1 to 4	27.79	27.65	29.34	27.40	No Ct
						1 to 32	22.76	1 to 8	28.90	28.41	30.24	28.35	No Ct
+/+ 1 lit.2	7	9-May	1.98	1101.5	1.0 ug	1 to 16	24.08	1 to 4	29.85	30.22	32.42	29.47	No Ct
						1 to 32	24.93	1 to 8	30.59	31.10	33.38	30.21	No Ct
+/+ 2 lit.2	7	9-May	2.04	680.2	1.0 ug	1 to 16	23.16	1 to 4	28.66	28.55	30.67	28.80	No Ct
						1 to 32	24.07	1 to 8	29.65	29.53	31.69	29.76	No Ct
-/- 1 lit.2	7	9-May	2	433.7	1.0 ug	1 to 16	23.60	1 to 4	29.04	29.07	31.04	28.89	No Ct
						1 to 32	24.34	1 to 8	29.97	29.77	32.58	29.91	No Ct
-/- 2 lit.2	7	9-May	1.99	328.6	1.0 ug	1 to 16	23.90	1 to 4	28.86	28.83	31.50	29.15	No Ct
						1 to 32	24.72	1 to 8	29.78	29.71	32.56	30.04	No Ct
+/+ 2 lit.3	7	9-May	2.06	391.8	1.0 ug	1 to 16	23.64	1 to 4	29.31	28.84	32.13	29.11	No Ct
						1 to 32	24.50	1 to 8	30.16	29.78	33.16	29.56	No Ct
-/- 1 lit.3	7	9-May	2.02	292.4	1.0 ug	1 to 16	23.80	1 to 4	29.10	28.96	31.91	28.77	No Ct
						1 to 32	24.51	1 to 8	29.89	29.81	32.88	30.08	No Ct

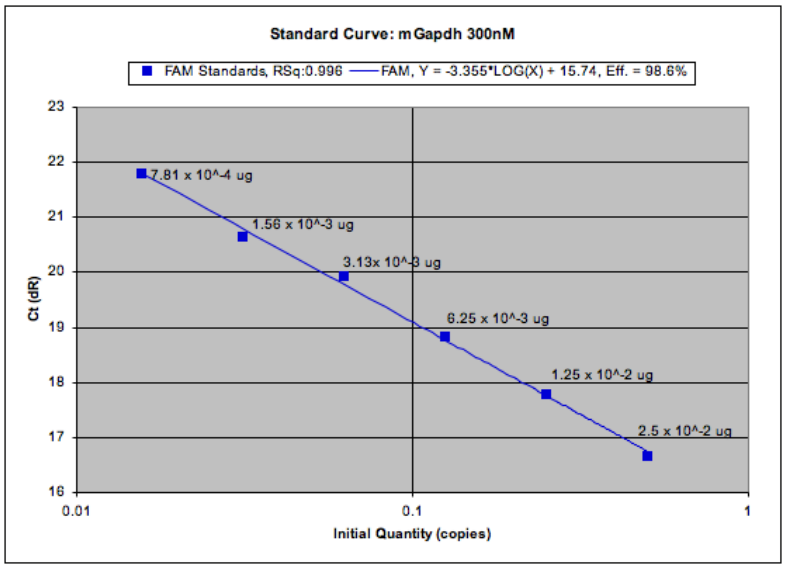
TABLE IV: Averaged raw Ct values for four-week (twenty-eight-day) old tibiae samples. Dilution ranges shown for Gapdh, normalizing gene, all other genes same dilution range, shown below.

Samples	Set	Sac	RNA	RNA Conc.	Sex	Dilutions	Gapdh Raw Cts	Dilutions	Runx2	OC	Rankl	OP	Fra1
+/+ 2	4	2-Jun	1.84	867.3	F	1 to 16	19.57	1 to 2	25.97	25.85	27.37	24.89	32.68
						1 to 32	20.58	1 to 4	26.71	26.51	28.50	25.79	33.66
+/+ 3	4	2-Jun	1.81	1067.5	M	1 to 16	19.52	1 to 2	26.03	25.82	27.49	24.91	32.68
						1 to 32	20.83	1 to 4	27.12	26.60	28.75	25.80	33.59
+/- 4	4	2-Jun	1.94	1122.4	M	1 to 16	17.57	1 to 2	23.81	23.88	25.72	22.74	30.70
						1 to 32	18.06	1 to 4	24.38	24.43	26.29	23.63	31.16
-/- 1	4	2-Jun	1.89	223.1	M	1 to 16	23.30	1 to 2	27.99	28.52	30.71	27.21	No Ct
						1 to 32	24.45	1 to 4	28.34	29.55	30.21	27.63	
+/+ 5	6	17-Mar	2.02	1056	F	1 to 32	27.49	1 to 4	34.69	30.59	37.11	32.37	32.15
						1 to 64	28.48	1 to 8	36.21	31.60	39.85	32.78	33.30
+/- 2	6	17-Mar	1.95	1378	M	1 to 32	26.42	1 to 4	32.96	30.10	35.04	30.77	31.84
						1 to 64	27.26	1 to 8	34.54	31.17	36.52	31.14	32.72
-/- 1	6	17-Mar	2.03	358.9	M	1 to 32	22.82	1 to 4	28.86	27.08	30.55	27.70	28.92
						1 to 64	23.49	1 to 8	29.89	28.23	31.44	28.52	29.93
-/- 7	6	17-Mar	2.09	1125.1	M	1 to 32	22.26	1 to 4	29.57	27.75	31.87	27.32	28.49
						1 to 64	23.10	1 to 8	30.62	28.88	32.93	28.42	29.39
+/+ 1 L	5	12-May	2.12	1270.8	M	1 to 16	25.93	1 to 2	31.89	30.28	32.88	30.58	30.66
						1 to 32	26.97	1 to 4	32.65	31.12	33.80	31.11	31.59
+/+ 2 L	5	12-May	2.07	1196.7	M	1 to 16	28.68	1 to 2	33.57	30.31	35.24	32.38	32.52
						1 to 32	29.18	1 to 4	35.59	31.45	36.40	33.08	32.86
+/- 3 L	5	12-May	2.11	875.1	F	1 to 16	25.00	1 to 2	30.28	28.09	31.64	29.28	29.45
						1 to 32	26.10	1 to 4	30.96	29.13	32.85	30.02	30.45
-/- 4 L	5	12-May	2.04	201.8	F	1 to 16	25.54	1 to 2	30.68	27.22	32.03	28.91	29.59
						1 to 32	26.51	1 to 4	32.05	27.97	33.07	29.72	30.01

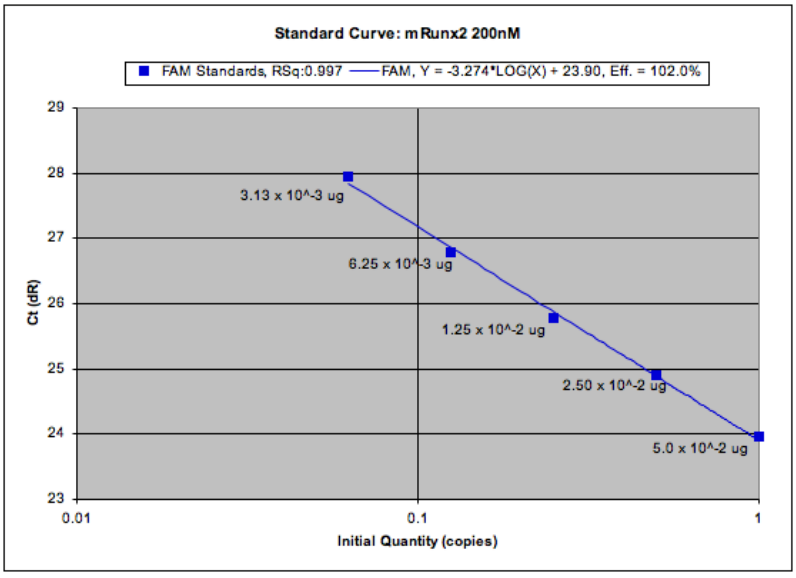
TABLE V: Table showing the genotype, sex and weight of all the four-week (twenty-eight-day) old specimens, three litters of four each, twelve total. Litter 1 corresponds to Set 4, Litter 2 corresponds to Set 5, and Litter 3 corresponds to Set 6 in Table IV listed above.

Litter	Genotype	Sex	Weight (g)
1	WT -2	F	15.9
1	WT -3	M	18.0
1	Het -4	M	15.5
1	KO -1	M	15.7
2	WT -1	M	22.7
2	WT -2	M	20.1
2	Het -3	F	15.4
2	KO -4	F	13.9
3	WT -5	F	12.7
3	Het -2	M	13.7
3	KO -1	M	13.3
3	KO -7	M	14.6

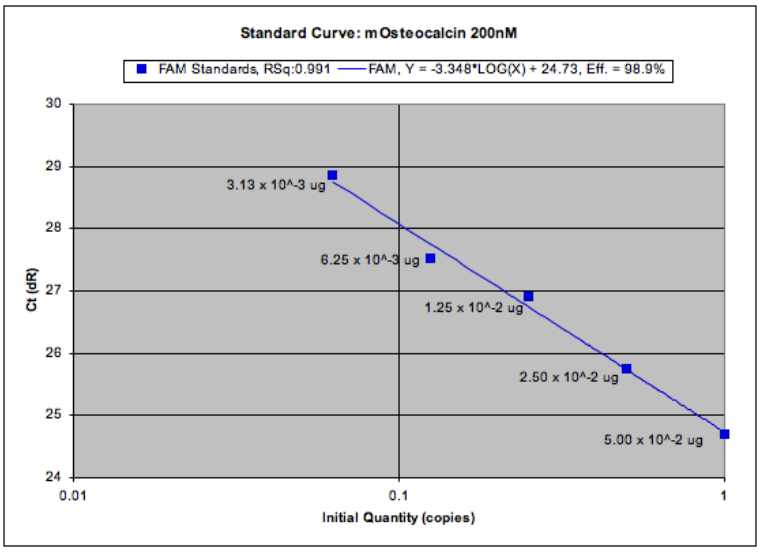
GRAPH I: Standard curve optimized for mouse Gapdh primers at 300 nM concentration. Threshold cycle on Y-axis, plotted against the initial quantity of DNA input, x-axis shows quantity as copy numbers, the correlation to concentration in μg cDNA is shown by each data point, beginning with a 1:64 cDNA dilution corresponding to 7.81×10^{-4} μg and ending with a 1:2 cDNA dilution corresponding to 2.5×10^{-2} μg. Primer efficiency quantified as 98.6%, where 100% equals a doubling of DNA at each cycle. Data points fit the curve with a Rsq value of 0.996.



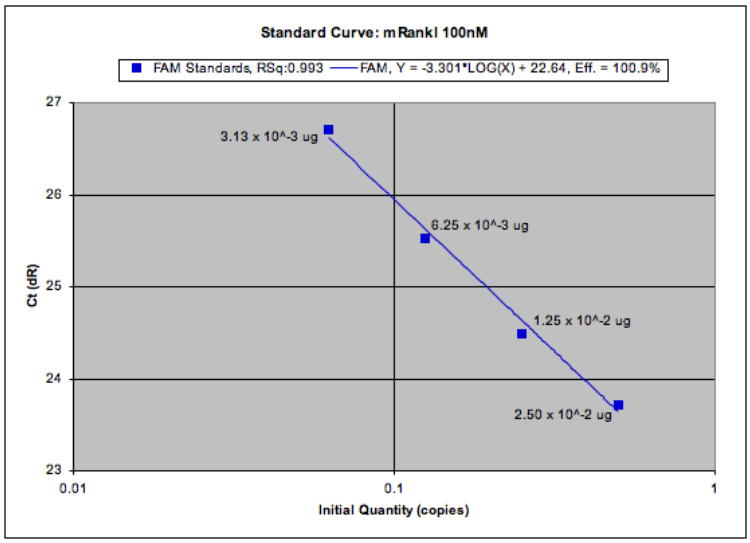
GRAPH II: Standard curve optimized for mouse Runx2 primers at 400 nM concentration. Threshold cycle on Y-axis, plotted against the initial quantity of DNA input, x-axis shows quantity as copy numbers, the correlation to concentration in μg cDNA is shown by each data point, beginning with a 1:16 cDNA dilution corresponding to 3.13×10^{-3} μg and ending with a 1:1 cDNA dilution corresponding to 5.0×10^{-2} μg. Primer efficiency quantified as 102%, where 100% equals a doubling of DNA at each cycle. Data points fit the curve with a Rsq value of 0.997.



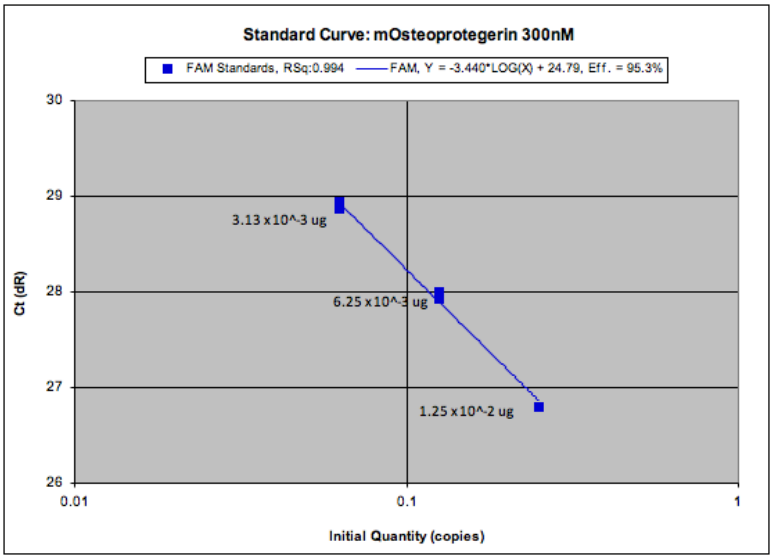
GRAPH III: Standard curve optimized for mouse osteocalcin primers at 200 nM concentration. Threshold cycle on Y-axis, plotted against the initial quantity of DNA input, x-axis shows quantity as copy numbers, the correlation to concentration in μg cDNA is shown by each data point, beginning with a 1:16 cDNA dilution corresponding to 3.13×10^{-3} μg and ending with a 1:1 cDNA dilution corresponding to 5.0×10^{-2} μg. Primer efficiency quantified as 98.9%, where 100% equals a doubling of DNA at each cycle. Data points fit the curve with a Rsq value of 0.991.



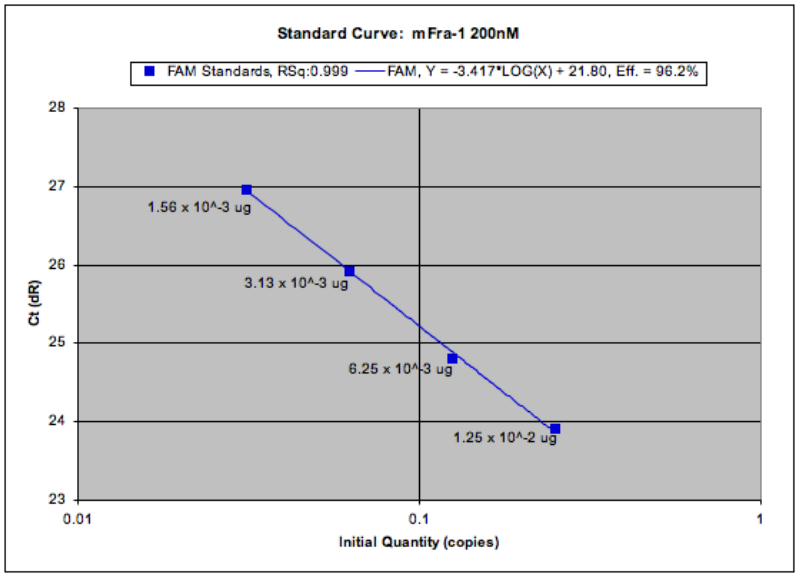
GRAPH IV: Standard curve optimized for mouse Rankl primers at 100 nM concentration. Threshold cycle on Y-axis, plotted against the initial quantity of DNA input, x-axis shows quantity as copy numbers, the correlation to concentration in μg cDNA is shown by each data point, beginning with a 1:16 cDNA dilution corresponding to 3.13×10^{-3} μg and ending with a 1:2 cDNA dilution corresponding to 2.5×10^{-2} μg. Primer efficiency quantified as 100.9%, where 100% equals a doubling of DNA at each cycle. Data points fit the curve with a Rsq value of 0.993.



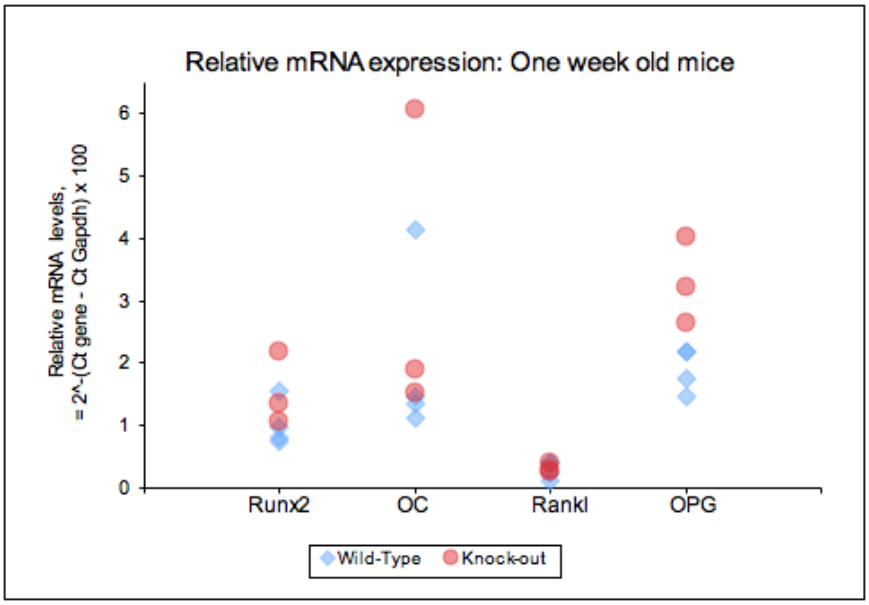
GRAPH V: Standard curve optimized for mouse osteoprotegerin primers at 300 nM concentration. Threshold cycle on Y-axis, plotted against the initial quantity of DNA input, x-axis shows quantity as copy numbers, the correlation to concentration in μg cDNA is shown by each data point, beginning with a 1:16 cDNA dilution corresponding to $3.13x10^{-3}$ μg and ending with a 1:4 cDNA dilution corresponding to $1.25\ x10^{-2}$ μg. Primer efficiency quantified as 95.3%, where 100% equals a doubling of DNA at each cycle. Data points fit the curve with a Rsq value of 0.994.



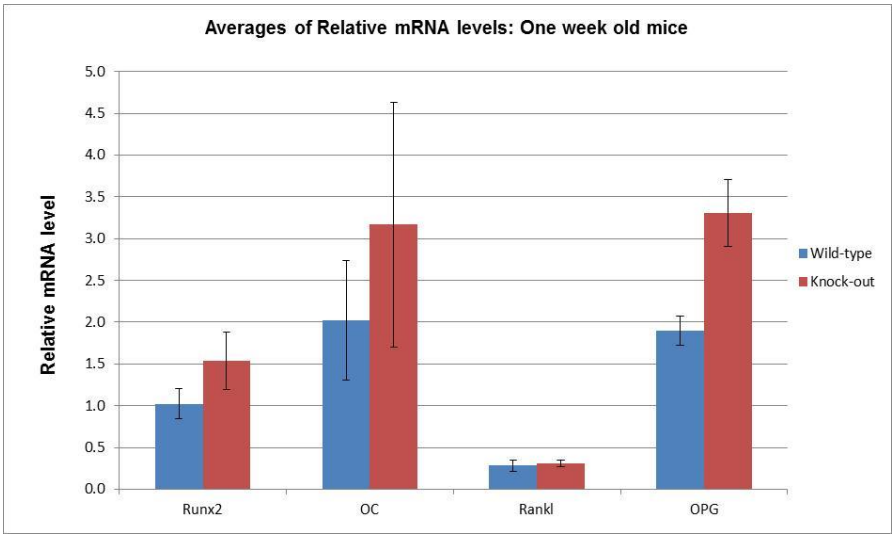
GRAPH VI: Standard curve optimized for mouse Fra-1 primers at 200 nM concentration. Threshold cycle on Y-axis, plotted against the initial quantity of DNA input, x-axis shows quantity as copy numbers, the correlation to concentration in μg cDNA is shown by each data point, beginning with a 1:32 cDNA dilution corresponding to $1.56x10^{-3}$ μg and ending with a 1:4 cDNA dilution corresponding to $1.25\ x10^{-2}$ μg. Primer efficiency quantified as 96.2%, where 100% equals a doubling of DNA at each cycle. Data points fit the curve with a Rsq value of 0.999.



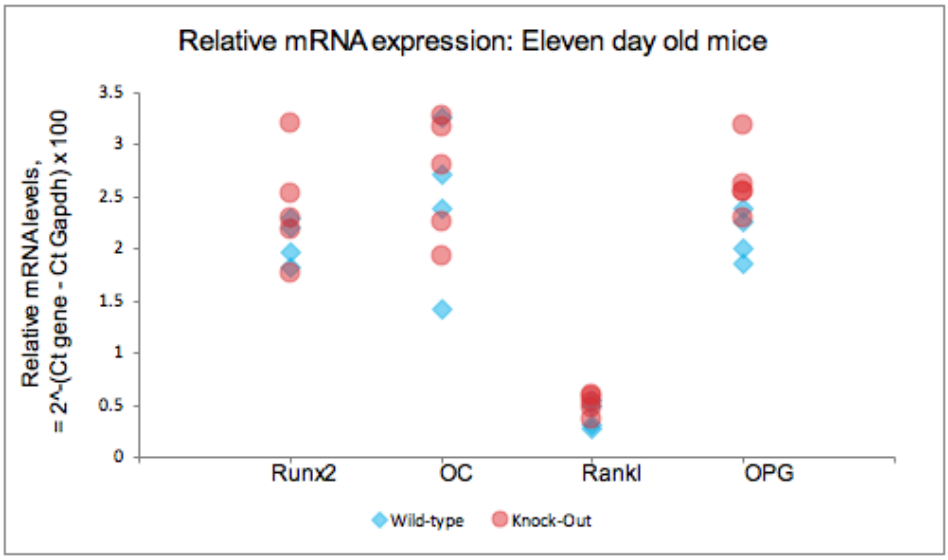
GRAPH VII : Cluster plot showing the relative mRNA expression levels for one-week (seven-day) old samples. Relative mRNA levels calculated as $2^{-(Ct\ target\ gene-Ct\ Gapdh)}$ and increased in scale by 100. Relative mRNA levels based on Ct values for endogenous control, Gapdh, with a mean of 23.98 +/- 1.57 (S.D.). Sample pool: four wild-type, three knock-out.



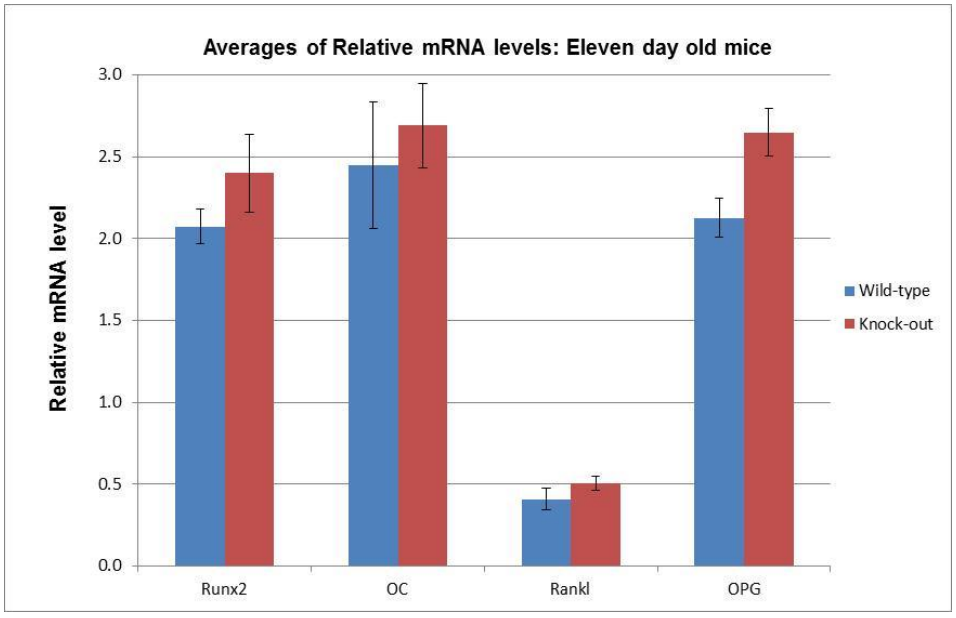
GRAPH VIII: Bar graph showing the averages for the relative mRNA expression levels of one-week (seven-day) old mice, same data as Graph I. Relative mRNA expression level based on $2^{-(Ct\ target\ gene-Ct\ Gapdh)}$ averages then taken for wild-type (n=4) and knock-out (n=3). Error bars showing +/- standard error (calculated as standard deviation/square root of sample pool).



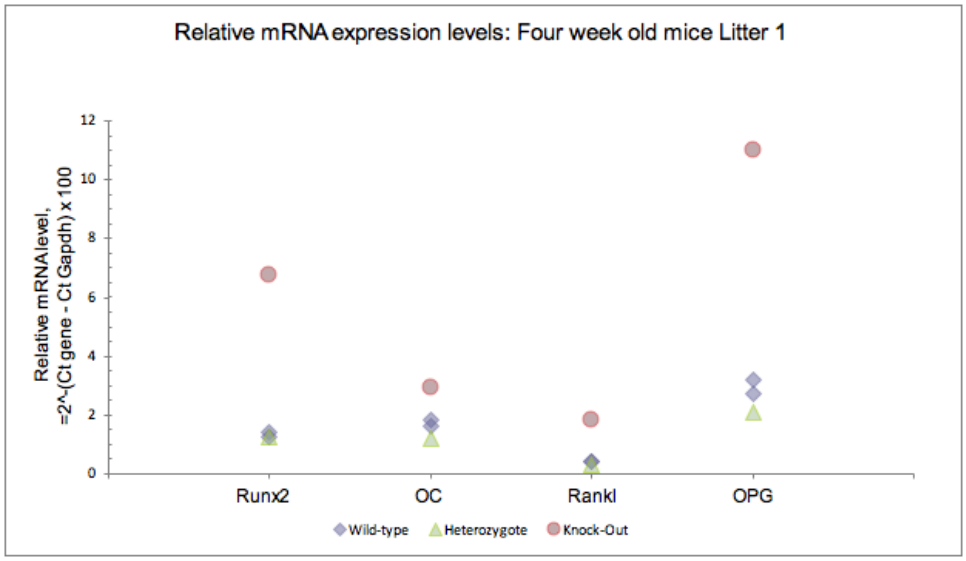
GRAPH IX : Cluster plot showing the relative mRNA expression levels for eleven-day-old mice. Relative mRNA levels calculated as $2^{-(Ct\ target\ gene-Ct\ Gapdh)}$ and increased in scale by 100. Relative mRNA levels based on Ct values for endogenous control, Gapdh, with a mean of 24.28 +/- 0.63 (S.D.). Sample pool: four wild-type and five knock-out.



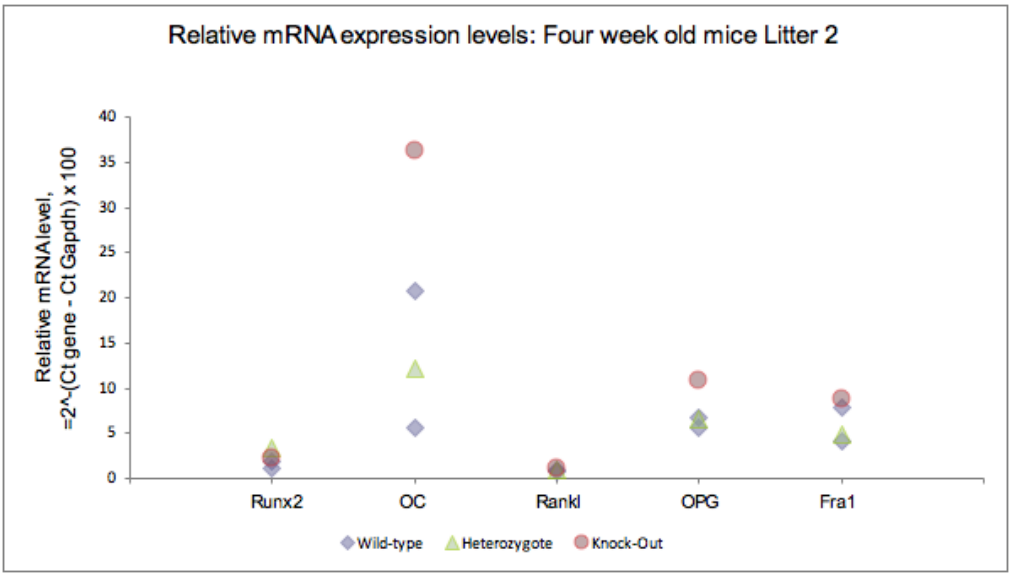
GRAPH X: Bar graph showing the averages for the relative mRNA expression levels of eleven-day-old mice, same data as Graph III. Relative mRNA expression level based on $2^{-(Ct\ target\ gene-Ct\ Gapdh)}$ averages then taken for wild-type (n=4) and knock-out (n=5). Error bars showing +/- standard error (calculated as standard deviation/square root of sample pool).



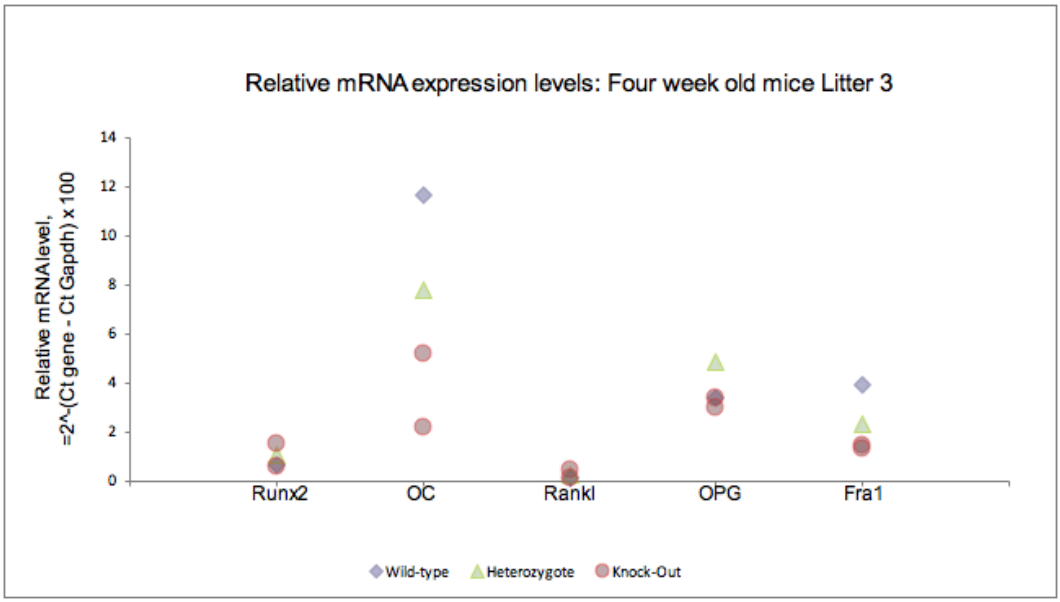
GRAPH XI: Cluster plot showing the relative mRNA expression levels for litter one of four-week (twenty-eight-day) old mice. Relative mRNA levels calculated as $2^{-(Ct\ target\ gene-Ct\ Gapdh)}$ and increased in scale by 100. Relative mRNA levels based on Ct values for endogenous control, Gapdh, with a mean of 20.98 +/- 2.63 (S.D.). Sample pool: two wild-type, one heterozgote, one knock-out.



GRAPH XII: Cluster plot showing the relative mRNA expression levels for litter two of four-week (twenty-eight-day) old mice. Relative mRNA levels calculated as $2^{-(Ct\ target\ gene-Ct\ Gapdh)}$ and increased in scale by 100. Relative mRNA levels based on Ct values for endogenous control, Gapdh, with a mean of 27.19 +/- 1.37 (S.D.). Sample pool: two wild-type, one heterozygote, one knock-out.



GRAPH XIII: Cluster plot showing the relative mRNA expression levels for litter three of four-week (twenty-eight-day) old mice. Relative mRNA levels calculated as $2^{-(Ct\ target\ gene - Ct\ Gapdh)}$ and increased in scale by 100. Relative mRNA levels based on Ct values for endogenous control, Gapdh, with a mean of 24.75 +/- 2.60 (S.D.). Sample pool: one wild-type, one heterozygote, two knock-out.



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