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Elucidating the Molecular Signatures Associated with Elevated Bone Formation Rate

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Abstract

Osteoporosis is a disease of decreased bone density that occurs when bone resorption exceeds bone formation, thereby placing individuals at greater risk of fracture and disability. We previously reported that deletion of the *Bmpr2* gene in embryonic skeletal progenitor cells causes substantially elevated bone density in young adulthood and reduced age-related decline in bone density, likely due to elevated bone formation rate. Thus, these mice may serve as a novel model in which to explore the mechanisms regulating bone formation in the aging skeleton. Here, we performed transcriptome profiling and identified a concise gene signature associated with elevated bone formation rate in *Bmpr2* mutant mice, with 120 transcripts up-regulated and 131 transcripts down-regulated. Candidate-driven qRT-PCR provided secondary confirmation of this dataset. Notably, only 8 of these differentially-expressed transcripts have been previously implicated in bone physiology (*Pak4, Rpl38, B2m, Fgf1, Nmu, Phospho1, Smpd3* and *Inhbe*), thus representing potentially novel regulators of osteoblast function in the aging skeleton. Additionally, we sought to examine the cell communication events that are associated with elevated bone formation rate. Using protein samples from control and mutant mice, we took advantage of recent advancements in high-throughput phospho-profiling antibody arrays, which allow simultaneous detection of >1,300 targets using very small quantities of protein. These results indicate that the phosphorylation status of at least 86 signaling effectors is differentially regulated in *Bmpr2* mutant mice as compared to control littermates, including numerous proteins known to regulate osteoblast differentiation and/or activity. Collectively, our work highlights novel factors associated with elevated bone formation rate and may identify new opportunities for treating low bone density in humans.

Bmpr2-cKO Model

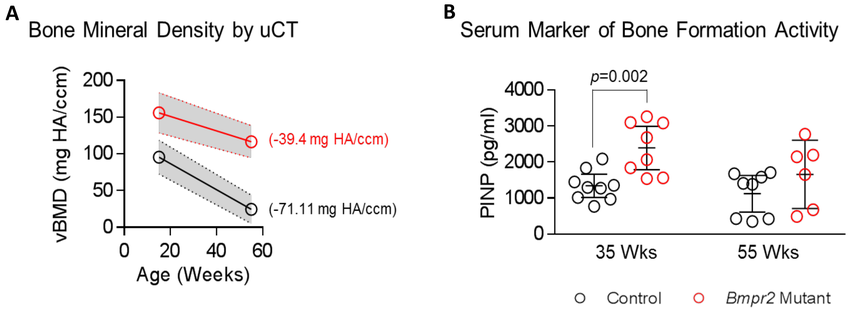

Figure 1: (A) *Bmpr2* mutant mice were generated by crossing *Bmpr2*$^{fl/fl}$*; Prx1*-Cre^{+} males with *Bmpr2*$^{fl/fl}$ females. Volumetric bone mineral density (vBMD) was quantified by micro-CT in females at 15 and 55 weeks of age. Mean decline in mg hydroxyapatite per cubic centimeter for each genotype between 15 and 55 week old cohorts is indicated (mg HA/ccm); gray bars denote 95% confidence intervals. **(B)** Quantification of the bone formation marker PINP in sera of control and *Bmpr2* mutant mice using ELISA. Individual samples are represented by circles and group mean by horizontal lines ± SEM; *p* values determined by unpaired t test.

Antibody Signaling Array Workflow

1. Femora obtained from n ≥ 4 each control and *Bmpr2* mutant mice at 35 weeks and 55 weeks of age
2. Marrow removed by gentle centrifugation
3. Bones homogenized, total protein collected, and concentration determined using BCA Assay
4. Each genotype pooled at equal protein amounts per mouse
5. Pooled samples were applied to Phospho Explorer Antibody Array slides
6. Protocol was carried out according to the manufacturer's directions with one modification of incubating at 4^{o}C in the protein labeling and coupling steps
7. Signal intensity was determined by Full Moon Biosystems on a GenePix4000B scanner Imager using GenePix Pro software and normalized against beta-actin, GAPDH or total protein isoform

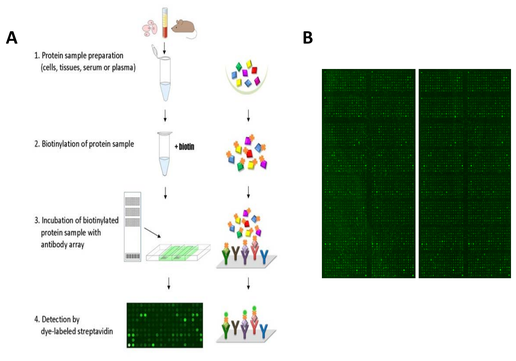

Figure 2: (A) Schematic of antibody array workflow provided by Full Moon Biosystems **(B)** Slides from our 55 week mutant and control samples, detection by Cy3-streptavidin.

Possible Repressors of Osteoblast Activity

Antibody List		35 Weeks Old Fold Change Slide 2/Slide 1	55 Week Old Fold Change Slide 2/Slide 1
M-CSF Receptor (Phospho-Tyr561)	M-CSF Receptor (Ab-561)	0.46	1.49
Pyk2 (Phospho-Tyr881)	Pyk2 (Ab-881)	0.48	0.84
PKC zeta (Phospho-Thr410)	PKC zeta (Ab-410)	0.52	0.82
Cyclin B1 (Phospho-Ser147)	Cyclin B1 (Ab-147)	0.55	1.29
Tau (Phospho-Ser356)	Tau (Ab-356)	0.59	1.16
EGFR (Phospho-Tyr1069)	EGFR (Ab-1069)	0.59	1.00
ATP1A1/Na+K+ ATPase1 (Phospho-Ser23)	ATP1A1/Na+K+ ATPase1 (Ab-23)	0.60	1.12
CDK1/CDC2 (Phospho-Tyr15)	CDK1/CDC2 (Ab-15)	0.60	1.24
PAK1/2 (Phospho-Ser199)	PAK1/2 (Ab-199)	0.62	1.47
Ezrin (Phospho-Thr566)	Ezrin (Ab-566)	0.63	1.61
CDC25A (Phospho-Ser75)	CDC25A (Ab-75)	0.63	1.79
Myosin regulatory light chain 2 (Phospho-Ser18)	Myosin regulatory light chain 2 (Ab-18)	0.63	1.43
IRS-1 (Phospho-Ser636)	IRS-1 (Ab-636)	0.64	0.95
DAB1 (Phospho-Tyr220)	DAB1 (Ab-220)	0.64	0.92
ASK1 (Phospho-Ser966)	ASK1 (Ab-966)	0.65	0.80
FGFR1 (Phospho-Tyr766)	FGFR1 (Ab-766)	0.65	1.12
EGFR (Phospho-Tyr1197)	EGFR (Ab-1197)	0.66	0.80
Src (Phospho-Ser75)	Src (Ab-75)	0.66	1.41
CaMK4 (Phospho-Thr196/200)	CaMK4 (Ab-196/200)	0.67	0.91
Keratin 8 (Phospho-Ser431)	Keratin 8 (Ab-431)	0.67	1.10
Integrin beta-3 (Phospho-Tyr785)	Integrin beta-3 (Ab-785)	0.68	1.34
LCK (Phospho-Tyr504)	LCK (Ab-504)	0.68	1.32
Chk1 (Phospho-Ser345)	Chk1 (Ab-345)	0.68	1.23
Smad3 (Phospho-Ser213)	Smad3 (Ab-213)	0.68	1.49
B-RAF (Phospho-Ser446)	B-RAF (Ab-446)	0.69	1.00
Caspase 3 (Phospho-Ser150)	Caspase 3 (Ab-150)	0.69	0.81
PLD1 (Phospho-Ser561)	PLD1 (Ab-561)	0.69	1.28
Ezrin (Phospho-Tyr353)	Ezrin (Ab-353)	0.69	1.13
HDAC2 (Phospho-Ser394)	HDAC2 (Ab-394)	0.70	0.84
BLNK (Phospho-Tyr96)	BLNK (Ab-96)	0.71	1.23
PPAR-gamma (Phospho-Ser112)	PPAR-gamma (Ab-112)	0.71	1.24
Met (Phospho-Tyr1003)	Met (Ab-1003)	0.72	1.03
HER2 (Phospho-Tyr1221/Tyr1222)	HER2 (Ab-1221/1222)	0.72	0.97
TOP2A/DNA topoisomerase II (Phospho-Ser1106)	TOP2A/DNA topoisomerase II (Ab-1106)	0.72	0.81
ATF2 (Phospho-Thr69/51)	ATF2 (Ab-69/51)	0.73	1.09
ICAM-1 (Phospho-Tyr512)	ICAM-1 (Ab-512)	0.73	1.11
Connexin 43 (Phospho-Ser367)	Connexin 43 (Ab-367)	0.73	0.90
p53 (Phospho-Ser378)	p53 (Ab-378)	0.73	1.07
CaMK2-beta/gamma/delta (Phospho-Thr287)	CaMK2-beta/gamma/delta (Ab-287)	0.73	0.98
SYK (Phospho-Tyr525)	SYK (Ab-525)	0.74	0.88
EGFR (Phospho-Tyr1016)	EGFR (Ab-1016)	0.74	0.95

≤ 0.75
≥ 1.5

Figure 3: Antibody Array data indicates that phosphorylation of these proteins are reduced in *Bmpr2-cKO* mice at 35 weeks of age and are relatively normal at 55 weeks of age.

Possible Drivers of Osteoblast Activity

Antibody List		35 Week Old Fold Change Slide 2/Slide 1	55 Week Old Fold Change Slide 2/Slide 1
HDAC1 (Phospho-Ser421)	HDAC1 (Ab-421)	6.89	0.86
Abl1 (Phospho-Tyr204)	Abl1 (Ab-204)	3.54	1.20
Chk2 (Phospho-Thr383)	Chk2 (Ab-383)	3.22	0.81
E2F1 (Phospho-Thr433)	E2F1 (Ab-433)	2.34	0.96
NFAT4 (Phospho-Ser165)	NFAT4 (Ab-165)	2.08	1.39
p130Cas (Phospho-Tyr410)	p130Cas (Ab-410)	2.06	0.78
HDAC3 (Phospho-Ser424)	HDAC3 (Ab-424)	2.03	0.95
FAK (Phospho-Tyr397)	FAK (Ab-397)	2.00	1.18
FKHR/FOXO1A (Phospho-Ser329)	FKHR/FOXO1A (Ab-329)	1.97	0.74
Src (Phospho-Tyr418)	Src (Ab-418)	1.97	1.15
IRS-1 (Phospho-Ser323)	IRS-1 (Ab-323)	1.89	0.86
ACC1 (Phospho-Ser79)	ACC1 (Ab-79)	1.88	1.02
PAK1/2/3 (Phospho-Thr423/402/421)	PAK1/2/3 (Ab-423/402/421)	1.82	0.82
Myc (Phospho-Thr58)	Myc (Ab-58)	1.77	0.97
Caspase 9 (Phospho-Tyr153)	Caspase 9 (Ab-153)	1.76	0.77
CDK7 (Phospho-Thr170)	CDK7 (Ab-170)	1.74	0.89
BAD (Phospho-Ser91/128)	BAD (Ab-91/128)	1.72	0.68
14-3-3 beta/zeta (Phospho-Ser184/186)	14-3-3 beta/zeta (Ab-184/186)	1.72	1.22
LAT (Phospho-Tyr191)	LAT (Ab-191)	1.71	1.05
STAT6 (Phospho-Thr645)	STAT6 (Ab-645)	1.71	0.97
Caspase 9 (Phospho-Ser144)	Caspase 9 (Ab-144)	1.69	0.82
LCK (Phospho-Tyr192)	LCK (Ab-192)	1.67	1.25
S6 Ribosomal Protein (Phospho-Ser235)	S6 Ribosomal Protein (Ab-235)	1.67	0.77
BRCA1 (Phospho-Ser1457)	BRCA1 (Ab-1457)	1.65	1.47
Tuberin/TSC2 (Phospho-Ser939)	Tuberin/TSC2 (Ab-939)	1.65	1.16
DDX5/DEAD-box protein 5 (Phospho-Tyr593)	DDX5/DEAD-box protein 5 (Ab-593)	1.63	1.19
VEGFR2 (Phospho-Tyr951)	VEGFR2 (Ab-951)	1.63	1.39
IKK-gamma (Phospho-Ser31)	IKK-gamma (Ab-31)	1.63	0.82
P70S6K (Phospho-Thr229)	P70S6K (Ab-229)	1.61	0.97
Tau (Phospho-Ser214)	Tau (Ab-214)	1.60	0.96
PPAR-BP (Phospho-Thr1457)	PPAR-BP (Ab-1457)	1.60	1.22
PLK1 (Phospho-Thr210)	PLK1 (Ab-210)	1.59	0.97
Ezrin (Phospho-Tyr478)	Ezrin (Ab-478)	1.57	1.17
Rel (Phospho-Ser503)	Rel (Ab-503)	1.57	0.75
IGF2R (Phospho-Ser2409)	IGF2R (Ab-2409)	1.57	1.43
HSP90 co-chaperone Cdc37 (Phospho-Ser13)	HSP90 co-chaperone Cdc37 (Ab-13)	1.55	1.14
MEK1 (Phospho-Thr286)	MEK1 (Ab-286)	1.54	0.94
Tuberin/TSC2 (Phospho-Thr1462)	Tuberin/TSC2 (Ab-1462)	1.53	1.06
STAM2 (Phospho-Tyr192)	STAM2 (Ab-192)	1.53	1.23
Progesterone Receptor (Phospho-Ser190)	Progesterone Receptor (Ab-190)	1.53	0.99
ACC1 (Phospho-Ser80)	ACC1 (Ab-80)	1.53	0.98
VEGFR2 (Phospho-Tyr1214)	VEGFR2 (Ab-1214)	1.52	1.17
FGFR1 (Phospho-Tyr154)	FGFR1 (Ab-154)	1.52	0.88
c-PLA2 (Phospho-Ser505)	c-PLA2 (Ab-505)	1.51	0.87
VAV2 (Phospho-Tyr142)	VAV2 (Ab-142)	1.50	0.84
IL-10R-alpha (Phospho-Tyr496)	IL-10R-alpha (Ab-496)	1.50	1.47
AKT1 (Phospho-Ser124)	AKT1 (Ab-124)	1.50	0.97

≤ 0.75
≥ 1.5

Figure 4: Antibody Array data indicates that phosphorylation of these proteins are increased in *Bmpr2-cKO* mice at 35 weeks of age and are relatively normal at 55 weeks of age.

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- Dr. Vicki Rosen (HSDM)
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- Indiana Academy of Science Senior Research Grant

For a video presentation of this poster and to join the conversation:

http://bit.ly/2nPBTHS

RNA-Sequencing Data

A

	35 Weeks of Age	55 Weeks of Age
Unchanged	18835 genes	16957 genes
Up-regulated	120 genes	2381 genes
Down-regulated	131 genes	172 genes
Not Detected in Control	105 genes	395 genes
Not Detected in Mutant	129 genes	65 genes

B

Zones	35 Weeks of Age	55 Weeks of Age
Zone 1: 9 genes	Up-regulated	Not Detected
Zone 2: 4 genes	Up-regulated	Down-regulated
Zone 3: 59 genes	Up-regulated	Unchanged
Zone 4: 32 genes	Up-regulated	Up-regulated
Zone 5: 618 genes	Unchanged	Not Detected
Zone 6: 89 genes	Unchanged	Down-regulated
Zone 7: 15799 genes	Unchanged	Unchanged
Zone 8: 2118 genes	Unchanged	Up-regulated
Zone 9: 12 genes	Down-regulated	Not Detected
Zone 10: 8 genes	Down-regulated	Down-regulated
Zone 11: 68 genes	Down-regulated	Unchanged
Zone 12: 26 genes	Down-regulated	Up-regulated
Zone 13: 44 genes	Not Detected	Down-regulated
Zone 14: 196 genes	Not Detected	Up-regulated
Zone 15: 951 genes	Not Detected	Unchanged

Figure 5: (A) Results of RNA-Seq analyses at 35 and 55 weeks of age; expressed relative to control. **(B)** Comparison of *Bmpr2* mutant results relative to control at 35 and 55 weeks of age represented in tabular forms.

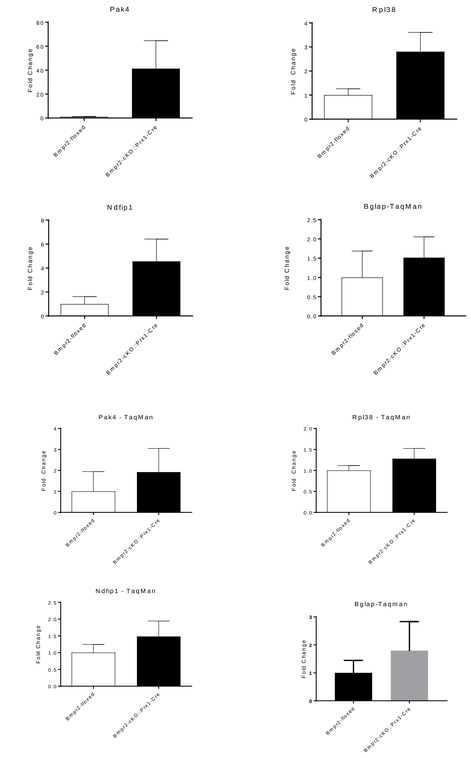

Figure 6: (A) qRT-PCR validates Pak4, Rpl38, Ndfip1 are up in Bmpr2-cKO mice at 35 weeks of age and Bglap (osteocalcin) is relatively normal. **(B)** qRT-PCR validates that Pak4, Rpl38, Ndfip1 and Bglap are all relatively normal in Bmpr2-cKO mice at 55 weeks of age.

Conclusion, Significance & Future Directions:

- *Bmpr2* mutant mice display high bone mass in young adulthood and reduced age-related bone loss.
- High throughput antibody signaling arrays of *Bmpr2* mutant bones identified 86 possible proteins that can act as either a repressor or driver of gene expression.
- Genome-wide transcriptome profiling of *Bmpr2* mutant bones identified 179 differentially expressed genes associated with increased osteoblast activity.
- Several genes corresponding with osteoblast differentiation and activity are up-regulated in *Bmpr2* mutant mice.
- Collectively, our findings provide insight into the mechanisms regulating age-related bone loss and highlight potential targets for therapeutic modulation of bone mass.
- Future studies will involve functional studies to narrow the gene signature to those that regulate osteoblast function.