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GLUCOCORTICOIDS INHIBIT NOTCH TARGET GENE EXPRESSION IN OSTEOBLASTS

Stefano Zanotti^{1,2,3}, Jungeun Yu^{1,3}, Suyash Adhikari⁴, Ernesto Canalis^{1,2,3}

¹Department of Orthopaedic Surgery, UConn Health, Farmington, CT 06030

²Department of Medicine, UConn Health, Farmington, CT 06030

³Department of UConn Musculoskeletal Institute, UConn Health, Farmington, CT 06030

⁴Department of University of Connecticut, Storrs, CT 06269

Abstract

Glucocorticoids in excess suppress osteoblast function and cause osteoporosis. We demonstrated that cortisol induces the expression of selected Notch receptors in osteoblasts, revealing a potential mechanism for the skeletal effects of glucocorticoids. However, it remains to be determined whether increased expression of Notch receptors results into enhanced signaling. Following activation of Notch, its intracellular domain (NICD) binds to the DNA-associated protein recombination signal binding protein for immunoglobulin kappa-J region (RBPJ) and induces the expression of target genes such as *Hey1*, *Hey2* and *HeyL*. To determine whether glucocorticoids modulate Notch signaling in the skeleton, 1 month old wild-type mice were administered prednisolone or placebo and sacrificed after 72 h, and gene expression was analyzed in femoral bone. Prednisolone induced *Tsc22d3*, a glucocorticoid target gene, and suppressed *Hey1* and *HeyL* expression, which is indicative of inhibited Notch receptor activity or direct *Hey* downregulation. To determine the mechanisms of *Hey* suppression, wild-type osteoblast-enriched cells were seeded on the Notch cognate ligand Delta-like (DLL)1 or transfected with constructs expressing the NOTCH1 NICD fragment and exposed to either cortisol or vehicle. Cortisol opposed the induction of mRNA and heterogeneous nuclear RNA for *Hey1*, *Hey2* and *HeyL* by DLL1, but had no effect on mRNA stability, indicating that glucocorticoids inhibit *Hey* expression by transcriptional mechanisms. Transactivation studies and electrophoretic mobility shift assays revealed that cortisol did not oppose RBPJ-mediated transcription or RBPJ/DNA interactions, respectively. In conclusion, glucocorticoids suppress expression of *Hey1*, *Hey2* and *HeyL* in osteoblasts by RBPJ-independent transcriptional mechanisms.

Keywords

Glucocorticoids; Hey; RBPJ; Osteoblast; Osteocyte

Address correspondence to: Stefano Zanotti, Ph.D., Department of Orthopaedic Surgery, UConn Health, Farmington, CT 06030-5456, Telephone: (860) 679-2355; Fax: (860) 679-1474; zanotti@uchc.edu.

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Osteoblasts derive from mesenchymal precursors and mature osteoblasts can differentiate further into bone lining cells or may become embedded in the newly mineralized bone matrix as osteocytes [Bianco and Gehron, 2000; Dallas et al., 2013]. Osteoblasts regulate bone formation, and in conjunction with osteocytes, govern osteoclast differentiation and bone resorption by secreting receptor activator of nuclear factor kappa-B ligand (RANKL) and its decoy receptor osteoprotegerin [Lacey et al., 1998]. Bone is continuously remodeled by the coordinated activities of osteoblasts and osteoclasts; this process ensures the renewal of bone tissue and allows the regulation of calcium metabolism [Canalis et al., 2007a]. The endocrine system is a central regulator of bone remodeling and multiple hormones influence osteoblast and osteoclast differentiation, function and lifespan.

Glucocorticoids are steroid hormones that bind to the glucocorticoid receptor and are necessary for osteoblastic function and bone homeostasis [Henneicke et al., 2014]. Glucocorticoids in excess, however, induce a transient increase in bone resorption caused by enhanced RANKL expression and by the suppression of osteoprotegerin. The bone resorptive response is followed by a decrease in bone formation due in part to a suppression of osteoblast differentiation and function, and the induction of osteoblast apoptosis. The effects of glucocorticoids on osteoblast differentiation and function and on the RANKL/osteoprotegerin axis are considered prominent mechanisms driving glucocorticoid-induced osteoporosis [Mazziotti et al., 2006]. Interactions of glucocorticoid signaling with the transcription factors peroxisome proliferator-activated receptor (PPAR)- γ and CCAAT-enhancer binding protein (CEBP)- β are believed to stimulate the differentiation of bone marrow precursor cells toward the adipocytic lineage [Rosen and Bouxsein, 2006]. As a consequence, osteoblastogenesis might be suppressed. The downregulation of insulin-like growth factor and Wnt/ β -catenin signaling by glucocorticoids further contributes to the inhibitory effects of glucocorticoids on bone formation [Canalis et al., 2007b].

The Notch receptors and cognate ligands of the Delta-like (Dll) and Jagged (Jag) families are single-pass transmembrane proteins that relay signals between adjoining cells [Kopan and Ilagan, 2009]. Receptor-ligand interactions lead to the cleavage of Notch and the release of its intracellular domain (NICD). The latter translocates to the nucleus and associates with a DNA-associated protein termed recombination signal binding protein for immunoglobulin kappa J region (RBPJ), leading to the formation of an active transcriptional complex [Kovall, 2007]. Classical target genes induced by these events are *Hey1*, *Hey2* and *HeyL*, which encode for closely related transcriptional repressors [Iso et al., 2003]. NOTCH1 and NOTCH2 regulate bone remodeling and their actions, like those of glucocorticoids, are determined by the degree of osteoblast maturity [Zanotti and Canalis, 2016]. Activation of NOTCH1 or NOTCH2 in osteoblasts suppresses cancellous bone formation, whereas activation of NOTCH1 in osteocytes causes osteopetrosis due to the induction of osteoprotegerin and the inhibition of osteoclastogenesis [Canalis et al., 2013a; Canalis et al., 2015; Canalis et al., 2013b; Canalis et al., 2016; Hilton et al., 2008; Yorgan et al., 2016; Zanotti et al., 2011; Zanotti et al., 2008; Zanotti et al., 2017].

Previous work demonstrated that cortisol induces *Notch1* and *Notch2* transcripts in the MC3T3 osteoblastic cell line, indicating that Notch signaling may contribute to the inhibitory effects of glucocorticoids on bone formation [Pereira et al., 2002]. In support of

this possibility, overexpression of the NOTCH1 NICD in MC3T3 cells synergizes with cortisol to favor the acquisition of an adipocytic phenotype at the expense of osteoblastic differentiation by enhancing the expression of PPAR- γ and CEBP- β [Sciaudone et al., 2003]. However, it was not established whether the reported effects of cortisol translate into enhanced Notch receptor activation. In the present study, we sought to determine whether glucocorticoids regulate Notch signaling in osteocytes as well as in osteoblasts and explored the mechanisms involved.

MATERIALS AND METHODS

Prednisolone Administration

To test the effects of glucocorticoids on Notch signaling in bone cells, pellets containing 5 mg of prednisolone (Innovative Research of America, Sarasota, FL), released over a 21-day period, were implanted subcutaneously in 1 month old wild-type C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME). Pellets containing 5 mg of placebo were implanted in littermates of the same sex and served as controls. Mice were sacrificed 72 h after implantation, which was equivalent to the administration of $\sim 700 \mu\text{g}$ of prednisolone or placebo per mouse. Femurs were dissected, the epiphyses excised, the bone marrow removed by centrifugation and the remaining bone fragments frozen in liquid nitrogen and stored at -80°C .

Osteoblast-enriched Cultures

Parietal bones of 3 to 5 day old C57BL/6J wild-type mice were digested at 37°C with either Liberase TL 1.2 U/ml (Sigma-Aldrich, St. Louis, MO) or type-II collagenase (Worthington Biochemical, Lake View, NJ) pretreated with N- α -tosyl-L-lysyl-chloromethylketone hydrochloride (Calbiochem, La Jolla, CA), for 20 min at 37°C and cells extracted in 5 sequential digestions [Yesil et al., 2009]. Cells extracted from the last 3 digestions were pooled and cultured in Dulbecco's modified Eagle's medium supplemented with non-essential amino acids (both from Thermo Fisher Scientific, Waltham, MA), 20 mM HEPES, 100 $\mu\text{g}/\text{ml}$ ascorbic acid (both from Sigma-Aldrich) and heat-inactivated 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) in a humidified 5% CO_2 incubator at 37°C . To activate Notch receptors, osteoblasts were trypsinized and seeded at a density of 22,000 cells/ cm^2 on a substrate coated with a DLL1-IgG $_2\Delta$ fragment crystallizable recombinant fusion protein (referred to as DLL1, R&D Systems, Minneapolis, MN) at a concentration of 125 ng/ cm^2 , as described [Nobta et al., 2005]. Cells seeded on an equimolar amount of bovine serum albumin (BSA, Sigma-Aldrich) served as controls [Hsu et al., 1999]. Cultures were allowed to recover for 24 h, serum-deprived for 24 h and subsequently exposed to cortisol 1 μM or ethanol, as control vehicle (both from Sigma-Aldrich), for 6 to 24 h. The effects of cortisol on mRNA decay were determined in selected cultures of osteoblasts seeded on DLL1 and exposed to either cortisol or control vehicle, and collected after different times of exposure to the transcriptional inhibitor actinomycin D (Sigma-Aldrich) at a concentration of 5 $\mu\text{g}/\text{ml}$ [Chen et al., 2008; Rydziel et al., 2004]. To establish slopes of mRNA decay, percent of corrected gene expression before exposure to actinomycin D was transformed by a base 10 logarithmic function and fitted against time by

linear regression. Experimental protocols were approved by the Institutional Animal Care and Use Committees of UConn Health.

RNA Integrity and Quantitative Reverse Transcription-polymerase Chain Reaction (qRT-PCR)

Femoral bone and osteocyte-enriched bone fragments were homogenized prior to chloroform/phenol extraction of total RNA with the micro RNeasy kit, whereas total RNA from osteoblast-enriched cells was extracted with the RNeasy kit (both from Qiagen, Valencia, CA). Integrity of the RNA was assessed by microfluidic electrophoresis on an Experion system (Bio-Rad, Hercules, CA), and gene expression measured by qRT-PCR in RNA of high quality, as defined by an RNA quality indicator number of 7.0 or higher [Nazarenko et al., 2002a; Nazarenko et al., 2002b]. Equal amounts of RNA were reverse-transcribed using the iScript RT-PCR kit (Bio-Rad) according to manufacturer's instructions, and amplified in the presence of specific primers (all from Integrated DNA Technologies (IDT), Coralville, IA; Table 1) and iQ or Sso Advanced Universal SYBR Green Supermix (Bio-Rad) at 60°C for 35 cycles. Transcript copy number was estimated by comparison to a serial dilution of cDNA for *Hey1* and *Hey2* (T. Iso, Los Angeles, CA), *HeyL* (D. Srivastava, Dallas, TX), *Rpl38* (American Type Tissue Culture Collection, Manassas, VA) and *Tsc22d3* (GE Healthcare Dharmacon, Lafayette, CO) [Iso et al., 2001a; Nakagawa et al., 1999].

To determine the effects of cortisol on *Hey1*, *Hey2* and *HeyL* transcription, expression of the respective heterogeneous nuclear (hn)RNA was measured in reverse-transcribed RNA with specific primers (IDT; Table 1) and Sso Advanced Universal SYBR Green Supermix (Bio-Rad) at 60°C for 40 cycles. Synthetic DNA fragments (IDT) homologous to the ~100 bp spanning the exon2-intron2 junction of *Hey1*, or the exon1-intron1 junctions of *Hey2* or *HeyL* were cloned into pcDNA3.1(–) (Thermo Fisher Scientific) by isothermal single reaction assembly (New England Biolabs, Ipswich, MA). Serial dilutions of these constructs were used to estimate copy number of the hnRNA [Gibson et al., 2009]. Reactions were conducted in a CFX96 qRT-PCR detection system (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step. Presence of a single PCR product was documented by melting curve analysis carried out after the final amplification cycle. Data are expressed as copy number corrected for *Rpl38* [Tso et al., 1985].

Transient Transfections

To monitor RBPJ-mediated transcription, osteoblast-enriched cells were transfected with a plasmid containing 6 dimeric consensus sequences for *RBPJ*, previously referred to as core binding factor-1/suppressor of hairless/Lag-1 (CSL), cloned upstream of the β -globin basal promoter and *Luciferase* (12xCSL-Luc; L.J. Strobl, Munich, Germany) [Strobl et al., 1997]. The pGL2 plasmid alone was transfected to measure basal or non-specific expression of *Luciferase*. To induce Notch signaling, cells were either seeded on DLL1 or co-transfected with a 2.4 kilobase (kb) DNA fragment containing the coding sequence of the murine NOTCH1 NICD (J.S. Nye, New York, NY) cloned into pcDNA3.1(–) downstream of the cytomegalovirus (CMV) promoter (pcDNA-NICD) [Nye et al., 1994]. BSA or a pcDNA3.1(–) plasmid served as controls. Transfections were conducted in 70% confluent osteoblast-enriched cells with X-tremeGENE 9 (2 μ l X-tremeGENE 9/1 μ g DNA), according

to manufacturer's instructions (Roche, Indianapolis, IN). A plasmid where the CMV promoter directs the expression of β -galactosidase (Clontech, Mountain View, CA) was co-transfected to account for transfection efficiency. Cells were exposed to the X-tremeGENE 9-DNA mix for 16 h, transferred to DMEM without serum for 6 h, and exposed to cortisol 1 μ M or vehicle for 24 h. Cells were harvested in reporter lysis buffer (Promega) and lysed by freezing and thawing. Luciferase and β -galactosidase activity were measured with a Luciferase Assay System kit (Promega) and with galacton plus (Life Technologies, Grand Island, NY), respectively, in accordance with manufacturers' instructions on an Optocomp II luminometer (MGM Instruments, Hamden, CT).

Electrophoretic Mobility Shift Assay (EMSA)

Osteoblast-enriched cells were seeded on BSA or DLL1, allowed to achieve confluence, cultured for 24 h in the absence of serum, exposed to cortisol 1 μ M or vehicle for 6 h, and nuclear extracts obtained as described [Schreiber et al., 1989]. A double-stranded DNA oligonucleotide biotinylated at the 5'-end and containing an *RBPI* consensus sequence found in the *EBNA2* promoter (forward strand: 5'-GGAAACACGCCGTGGGAAAAAATTTGGG-3') was synthesized commercially (IDT) [Henkel et al., 1994]. Binding reactions of 1 μ M biotinylated DNA with 5 μ g of nuclear extracts were carried out with the LightShift Chemiluminescent EMSA Kit, as recommended by the manufacturer (Thermo Fisher Scientific). To determine the specificity of the binding reactions, unlabeled homologous DNA (IDT) was added in 200-fold excess. Nucleic acid/protein complexes were resolved on non-denaturing, non-reducing 4% polyacrylamide gels (Bio-Rad) for 45 min and then transferred to a nylon membrane with a 0.45 μ m pore size (MP Biomedicals, Solon, OH) in 45 mM Tris, 45 mM boric acid (both from Sigma-Aldrich), 1 mM EDTA at pH 8.3. Transfer was conducted for 30 min at 4°C, and complexes were cross-linked to the membrane by 1 min exposure to 120 mJ/cm² UV-light in a CL-1000 instrument (UVP, Upland, CA). The immobilized biotinylated DNA was detected with a streptavidin-horseradish peroxidase conjugate with the LightShift Chemiluminescent EMSA Kit detection module in accordance to manufacturers' instructions and chemiluminescent images were acquired with a Chemidoc XSR molecular imager (Bio-Rad).

Statistical Analysis

Data are expressed as means $\pm$ standard deviation (SD). Statistical differences were determined by Student's *t*-test or two-way analysis of variance (ANOVA) with Holm-Šídák post-hoc analysis for pairwise or multiple comparisons, respectively. Differences in the slopes of mRNA decay were tested by analysis of covariance (ANCOVA) [Sokal and Rohlf, 1981].

RESULTS

Prednisolone Suppresses the Expression of Notch Target Genes In Vivo

To establish whether glucocorticoids regulate Notch signaling in cells of the osteoblastic lineage *in vivo*, wild-type male mice were administered prednisolone pellets or placebo for 72 h, and the expression of Notch target genes was measured in total RNA from femoral

bone fragments. Prednisolone induced the expression of *Tsc22d3*, a glucocorticoid target gene, and suppressed the transcript levels of *Hey1* and *HeyL*, indicating that glucocorticoids suppress Notch target genes in osteoblastic cells *in vivo* (Figure 1) [D'Adamio et al., 1997]. A tendency toward reduced *Hey2* expression also was noted in mice administered prednisolone, but the effect did not achieve statistical significance.

Glucocorticoids Suppress the Expression of Notch Target Genes in Osteoblasts

To test whether the inhibition of *Hey* expression occurs in osteoblasts, cultures enriched in osteoblasts were obtained from C57BL/6J mice and carried out on a DLL1-coated substrate to activate the Notch receptors, or on BSA as a control. Cells were subsequently exposed to cortisol or vehicle. Cortisol increased the expression of its target *Tsc22d3* either in the presence of DLL1 or under basal conditions, and DLL1 induced *Hey1*, *Hey2* and *HeyL*, confirming activation of Notch signaling. Cortisol opposed the induction of *Hey1*, *Hey2* and *HeyL* but had a non-significant effect on their basal expression (Figure 2).

Cortisol Represses Hey1, Hey2 and HeyL hnRNA in Osteoblasts

To determine whether glucocorticoids regulate the transcription of *Hey1*, *Hey2* or *HeyL*, the expression of their respective hnRNA was measured in osteoblast-enriched cells cultured on a DLL1-coated substrate or on control BSA, either in the presence or absence of cortisol. As reported for the effects of DLL1 and cortisol on mRNA expression, DLL1 induced the hnRNA for *Hey1*, *Hey2* and *HeyL* and cortisol opposed this effect, which is indicative of Notch signaling suppression through transcriptional mechanisms (Figure 3). To establish whether glucocorticoids regulate the stability of *Hey1*, *Hey2* and *HeyL* transcripts, osteoblast-enriched cells cultured on DLL1-coated substrates were exposed to either cortisol or vehicle and then transcriptionally arrested with actinomycin D. Cortisol had no effect on the half-life of *Hey1*, *Hey2* or *HeyL*, indicating that the inhibitory effects of cortisol on Notch signaling are primarily transcriptional (Figure 4).

Cortisol does not Inhibit RBPJ-mediated Notch Signaling

To establish whether the suppression of *Hey1*, *Hey2* and *HeyL* mRNA and hnRNA by cortisol is due to an effect on NICD/RBPJ/DNA interactions, osteoblasts were transiently transfected with the 12xCSL-Luc reporter, which contains multiple consensus sequences for CSL, the human RBPJ ortholog. To activate Notch signaling, cells were seeded on DLL1 or control BSA, or co-transfected with pcDNA-NICD or a pcDNA3.1(–) control vector, and treated with cortisol or vehicle. Exposure to DLL1 or overexpression of the NOTCH1 NICD enhanced the transactivation of 12xCSL-Luc, and cortisol did not oppose these effects (Figure 5). These observations suggest that cortisol does not affect RBPJ-mediated Notch signaling.

To verify the findings from the transactivation experiments, EMSAs were carried out in osteoblast-enriched cells seeded on DLL1 or on BSA and exposed to cortisol or vehicle. A biotinylated oligonucleotide containing the consensus sequence for *RBPJ* was bound by nuclear protein extracts from cells cultured on BSA. The interaction was specific as it was competed away by an excess of unlabeled *RBPJ* oligonucleotides. Previous reports demonstrated that the binding of RBPJ to DNA decreases in the context of Notch activation;

accordingly, the formation of a nuclear protein/*RBPJ* consensus DNA complex was reduced in osteoblasts seeded on DLL1 [Oswald et al., 2001]. Cortisol modestly enhanced the formation of a nuclear protein/DNA complex under basal conditions and had no effect on DNA binding in the context of DLL1 exposure (Figure 6). These results are congruent with observations in cells transfected with the 12xCSL-Luc reporter and demonstrate that glucocorticoids do not inhibit *RBPJ*/DNA interactions.

DISCUSSION

The studies presented in this manuscript demonstrate that selected glucocorticoids, namely prednisolone and cortisol, suppress the expression of the Notch target genes *Hey1*, *Hey2* and *HeyL* in osteoblasts. Prednisolone suppressed *Hey1* and *HeyL* expression in the context of basal Notch activation *in vivo*, whereas stimulation of Notch receptors with DLL1 was required to observe the inhibitory effect of cortisol on *Hey* expression in osteoblast-enriched cultures. The effects of glucocorticoids are of a transcriptional nature, as cortisol opposed the stimulatory effects of DLL1 on *Hey1*, *Hey2* and *HeyL* hnRNA levels but did not affect the half-life of the respective mRNA. Transactivation experiments with the 12xCSL-Luc reporter, which contains multiple copies of the consensus sequence for the human ortholog of *RBPJ*, suggest that cortisol does not suppress *Hey* transcription by *RBPJ*-dependent mechanisms. This suggests that the expression of the *Hey* paralogs may be governed by glucocorticoid response elements (GRE) that oppose the transcriptional response to the Notch receptors. This possibility is substantiated by the observation that prednisolone- and cortisol-induced expression of *Tsc22d3*, a known target gene of the glucocorticoid receptor that harbors 4 GRE within the 2.5 kb upstream of the transcriptional start site [Wang et al., 2004]. Stimulation of *Tsc22d3* is compatible with suppression of *Hey1*, *Hey2* and *HeyL* as the sequence context of the GRE determines whether glucocorticoid receptors act as inducers or inhibitor of transcription [Wang et al., 2004].

EMSA confirmed that cortisol does not act by precluding *RBPJ*/DNA interactions. In fact, cortisol enhanced binding of nuclear proteins to the biotinylated *RBPJ* probes, possibly by increasing the expression of *RBPJ* or its affinity to cognate consensus sequences. The mechanisms of this effect remain to be determined. Enhanced binding of *RBPJ* to DNA does not necessarily imply an increase in Notch signaling, as *RBPJ* acts as a transcriptional inhibitor in the absence of the NICD [Kovall, 2008]. Accordingly, cortisol did not induce the activity of an acutely transfected 12xCSL-Luc reporter construct either in the context of DLL1 stimulation or overexpression of the NOTCH1 NICD [Strobl et al., 1997]. These observations demonstrate that despite the known stimulatory effects of cortisol on *Notch1* and *Notch2* expression in MC3T3 osteoblastic cell lines, glucocorticoids do not enhance the activity of the Notch receptors in osteoblasts. This is not necessarily unexpected, as cortisol did not appear to regulate the expression of Notch ligands in MC3T3 cells [Pereira et al., 2002].

Previous work in mice demonstrated that the misexpression of *Hey1* or the global deletion of *HeyL* have a minimal impact on skeletal structure, whereas the conditional inactivation of *Hey2* in osteoblasts leads to modest gains in bone mass [Canalis and Zanotti, 2017; Salie et al., 2010; Zanotti and Canalis, 2013]. The mild skeletal phenotypes of the individual *Hey1*,

Hey2 or *HeyL* deletion can be explained by genetic redundancy that may compensate for the loss of a single gene within a group of closely related paralogs. This possibility is substantiated by the ability of Hey proteins to form homodimers as well as heterodimers and thereby determine the specificity of DNA binding [Fischer et al., 2007; Iso et al., 2001b]. Accordingly, global *HeyL* null mice harboring a heterozygous *Hey1* deletion were reported to have increased bone mass, so that increased bone volume would be a plausible outcome of the *Hey1*, *Hey2* and *HeyL* downregulation [Tu et al., 2012]. Although this postulated effect is difficult to reconcile with the skeletal impact of glucocorticoids in excess, suppression of *Hey1*, *Hey2* and *HeyL* may contribute to the ability of glucocorticoids to maintain optimal osteoblastic function under physiological conditions [Rauch et al., 2010].

In conclusion, glucocorticoids suppress *Hey1*, *Hey2* and *HeyL* expression in osteoblasts without inhibiting the activity of the Notch receptors or RBPJ/DNA interactions.

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ABBREVIATIONS

ANCOVA	analysis of covariance
ANOVA	analysis of variance
BSA	bovine serum albumin
Cebp	CCAAT-enhancer binding protein
CMV	cytomegalovirus
CSL	core binding factor-1/suppressor of hairless/Lag-1
Dll	delta-like
EMSA	electrophoretic mobility shift assay
Fwd	forward
GRE	glucocorticoid response element
hn	heterogeneous nuclear
IDT	Integrated DNA Technologies
Jag	Jagged
kb	kilobase
NICD	Notch intracellular domain

PCR	polymerase chain reaction
Ppar	peroxisome proliferator-activated receptor
qRT-PCR	quantitative reverse transcription-PCR
RANKL	receptor activator of nuclear factor kB ligand
RBPJ	recombination signal binding protein Jk
Rev	reverse
SD	standard deviation

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Figure 1. Prednisolone suppresses expression of *Hey1* and *HeyL* in bone.

Pellets containing placebo (white bars) or prednisolone (black bars) were implanted in 1 month-old wild-type C57BL/6J male mice. Mice were sacrificed after 72 h and total RNA extracted from femoral bone fragment. Transcript levels were measured by qRT-PCR and data are presented as *Tsc22d3*, *Hey1*, *Hey2* and *HeyL* copy number corrected for *Rpl38* expression. Values are means $\pm$ SD, $n = 4$ for placebo; $n = 3$ for prednisolone. *Significant difference, $p < 0.05$ by unpaired Student's *t*-test.

Figure 2. Cortisol prevents the induction *Hey1*, *Hey2* and *HeyL* by DLL1 in osteoblasts. Primary osteoblast-enriched cells from the parietal bones of 3 to 5 day old wild-type C57BL/6J mice were seeded on BSA (white bars) or DLL1 (black bars). Cells were allowed to recover for 24 h, subsequently cultured for 24 h in the absence of serum and then exposed to vehicle or cortisol 1 μ M for 6 h. Total RNA was extracted and mRNA levels quantified by qRT-PCR; data are expressed as *Hey1*, *Hey2* and *HeyL* copy number corrected for *Rpl38* expression. Values are means $\pm$ SD; $n = 4$ for all groups and genes with the exception of *Hey1* expression in osteoblasts exposed to cortisol and BSA, $n = 3$. *Significantly different between Control and Notch, $p < 0.05$; +significantly different between vehicle and cortisol, $p < 0.05$; two-way ANOVA with Holm-Šídák post-hoc analysis.

Figure 3. Cortisol suppresses *Hey1*, *Hey2* and *HeyL* expression in osteoblasts by transcriptional mechanisms.

Primary osteoblast-enriched cells from the parietal bones of 3 to 5 day old wild-type C57BL/6J mice were seeded on BSA (white bars) or DLL1 (black bars). Cells were allowed to recover for 24 h, subsequently cultured for 24 h in the absence of serum and then exposed to vehicle or cortisol 1 μ M for 6 h. Total RNA was extracted and hnRNA levels quantified by qRT-PCR; data are expressed as *Hey1*, *Hey2* and *HeyL* hnRNA copy number corrected for *Rpl38* expression. Values are means $\pm$ SD; n = 4. *Significantly different between Control and Notch, $p < 0.05$; +significantly different between vehicle and cortisol, $p < 0.05$; two-way ANOVA with Holm-Šídák post-hoc analysis.

Figure 4. Cortisol does not affect the stability of *Hey1*, *Hey2* or *HeyL* mRNA.

Primary osteoblast-enriched cells from the parietal bones of 3 to 5 day old wild-type C57BL/6J mice were seeded on DLL1. Cells were allowed to recover for 24 h and subsequently cultured for 24 h in the absence of serum and then exposed to vehicle or cortisol 1 μ M for 6 h. Cells were transcriptionally arrested with actinomycin D (Time 0) and harvested at the indicated times. Total RNA was extracted and mRNA levels quantified by qRT-PCR. Data are expressed as percent of *Hey1*, *Hey2* and *HeyL* mRNA levels corrected for *Rpl38* copy number prior to exposure to actinomycin D, plotted versus time. Values are means $\pm$ SD; n = 4. No differences between cortisol and control slopes of decay by ANCOVA.

Figure 5. Cortisol does not affect RBPJ-mediated Notch signaling in osteoblasts.

Primary osteoblast-enriched cells were harvested from the parietal bones of 3 to 5 day old wild-type C57BL/6J mice. In panel A, cells were seeded on BSA (white bars) or DLL1 (black bars) and transiently transfected with the 12xCSL-Luc reporter. In panel B, osteoblasts were transiently transfected with pcDNA3.1(−) (pcDNA) or with pcDNA-NICD (NICD) and co-transfected with the 12xCSL-Luc reporter. A CMV/β-galactosidase expression vector was co-transfected to test for transfection efficiency. Cells were allowed to recover for 16 h and subsequently cultured for 6 h in the absence of serum, exposed to vehicle or cortisol 1 μM, and harvested after 24 h. Data represent luciferase/β-galactosidase activity. Values are means ± SD, panel A n = 4; panel B n = 6. *Significantly different between BSA and DLL1 or pcDNA and NICD, $p < 0.05$; two-way ANOVA with Holm-Šídák post-hoc analysis.

Figure 6. Cortisol has a modest impact on RBPJ/DNA interactions.

Primary osteoblast-enriched cells were seeded on BSA or DLL1, confluent cultures maintained for 24 h in the absence of serum and then exposed to vehicle or cortisol 1 μ M for 6 h. Nuclear proteins were extracted and binding reactions carried out with a biotinylated oligonucleotide containing an *RBPJ* consensus sequence from the *EBNA2* promoter. Competition of binding reactions was performed in the presence of unlabeled oligonucleotides containing homologous *RBPJ* consensus sequences in 200-fold excess. DNA-nuclear protein complexes were resolved by gel electrophoresis, transferred to a nylon membrane, cross-linked with UV-light, exposed to a streptavidin-horseradish peroxidase conjugate and visualized by chemiluminescence. The arrows indicate the position of the DNA/protein complexes.

Table 1.**Primers for qRT-PCR.**

Forward (Fwd) and reverse (Rev) primers used for detection of mRNA and hnRNA by qRT-PCR. GenBank accession numbers indicate transcript variants with homologous sequences to primers.

Gene	RNA species	Strand	Primer Sequence 5' to 3'	GenBank Accession Numbers
<i>Hey1</i>	mRNA	Fwd Rev	ATCTCAACAACACTACGCATCCCAGC GTGTGGGTGATGTCCGAAGG	NM_010423
	hnRNA	Fwd Rev	CCCAACGACATCGTCCCAGGTTT GGGTACTCACGCCTCTCCGTCTTT	
<i>Hey2</i>	mRNA	Fwd Rev	AGCGAGAACAATTACCCTGGGCAC GGTAGTTGTCGGTGAATTGGACCT	NM_013904
	hnRNA	Fwd Rev	CGCCCTTGTGAGGAAACG CGCTCACCCAGGTAATTG	
<i>HeyL</i>	mRNA	Fwd Rev	CAGTAGCCTTTCTGAATTGCGAC CCCAGCACAACCTCCTCCCTA	NM_013905
	hnRNA	Fwd Rev	GAGAACGATCTTAGGTAAGACAGG AACTTGAGTGTGCTGGACTT	
<i>Rpl38</i>	mRNA	Fwd Rev	AGAACAAGGATAATGTGAAGTTCAAGGTTT CTGCTTCAGCTTCTCTGCCCTTT	NM_001048057 NM_023372 NM_001048058
<i>Tsc22d3</i>	mRNA	Fwd Rev	GCGTCATCTCATGTGGAGAACTT CTCTGGCACCAGCTATGTTAGG	NM_010286 NM_001077364