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CONNECTIVE TISSUE GROWTH FACTOR IS A TARGET OF NOTCH SIGNALING IN CELLS OF THE OSTEOBLASTIC LINEAGE

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Abstract

Connective tissue growth factor (Ctgf) or CCN2 is a protein synthesized by osteoblasts necessary for skeletal homeostasis, although its overexpression inhibits osteogenic signals and bone formation. Ctgf is induced by bone morphogenetic proteins, transforming growth factor β and Wnt; and in the present studies, we explored whether Notch regulated Ctgf expression in osteoblasts. We employed $Rosa^{Notch}$ mice, where the Notch intracellular domain (NICD) is expressed following the excision of a STOP cassette, placed between the *Rosa26* promoter and NICD. Notch was activated by transduction of adenoviral vectors expressing Cre recombinase (Ad-CMV-Cre). Notch induced *Ctgf* mRNA levels in a time dependent manner and increased *Ctgf* heterogeneous nuclear RNA. Notch also destabilized *Ctgf* mRNA shortening its half-life from 13 h to 3 h. The effect of Notch on Ctgf expression was lost following Rbpjκ downregulation, demonstrating that it was mediated by Notch canonical signaling. However, downregulation of the classic Notch target genes *Hes1, Hey1* and *Hey2* did not modify the effect of Notch on Ctgf expression. Wild type osteoblasts exposed to immobilized Delta-like 1 displayed enhanced Notch signaling and increased Ctgf expression. In addition to the effects of Notch *in vitro*, Notch induced Ctgf *in vivo*, and calvariae and femurs from $Rosa^{Notch}$ mice mated with transgenics expressing the Cre recombinase in cells of the osteoblastic lineage exhibited increased expression of Ctgf. In conclusion, Ctgf is a target of Notch canonical signaling in osteoblasts, and may act in concert with Notch to regulate skeletal homeostasis.

Keywords

Notch; CCN proteins; connective tissue growth factor; osteoblasts; transcription

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1. INTRODUCTION

The fate of mesenchymal cells and their differentiation toward cells of the osteoblastic lineage is tightly controlled by extracellular and intracellular signals [1–6]. A critical regulatory component of cell differentiation and function is provided by families of proteins that modulate the extracellular signals that target cells of the osteoblastic lineage. These regulatory proteins can bind growth factors directly or modify growth factor-receptor interactions, and frequently act as growth factor antagonists. These proteins include insulin-like growth factor binding proteins (IGFBP), bone morphogenetic protein (BMP) and Wnt antagonists, as well as members of the Cyr61, connective tissue growth factor (Ctgf) and nephroblastoma overexpressed (Nov) (CCN) family of proteins [1, 4, 7, 8]. CCN proteins are highly conserved, and are structurally related to IGFBPs, and to certain BMP antagonists, such as twisted gastrulation and chordin, and can interact with regulators of osteoblast cell growth and differentiation [4, 9, 10].

Ctgf or CCN2 is a protein synthesized by chondrocytes, osteoblasts and osteocytes. In osteoblasts, Ctgf expression is induced by BMP, transforming growth factor β and Wnt [11–13]. Ctgf regulates different cellular functions, including cell adhesion, proliferation, migration and differentiation [14, 15]. The effects of Ctgf on osteoblast differentiation and function depend on its interactions with local regulatory signals, the concentration of Ctgf in the bone environment and the stage of osteoblast differentiation [16–19]. Ctgf is necessary for chondrogenesis and osteoblastogenesis, but when in excess Ctgf is inhibitory since it tempers the effects of osteogenic signals in the skeleton [16, 17, 20, 21]. Studies performed by our laboratory revealed that the overexpression of Ctgf under the control of the osteocalcin/bone gamma carboxyglutamate protein (*Bglap*) promoter causes osteopenia by decreasing bone formation, an effect attributed to suppressed BMP, Wnt and IGFI signaling [17]. Similarly, Ctgf overexpression in chondrocytes causes bone loss [22]. Targeted disruption of *Ctgf* in mice leads to severe skeletal developmental abnormalities, as a result of impaired cartilage/bone development [21, 23]. We demonstrated that the conditional inactivation of *Ctgf* in the limb bud or in differentiated osteoblasts results in osteopenia, confirming its direct role in skeletal development, and demonstrating that Ctgf is necessary for adult skeletal homeostasis [20].

Notch signaling plays a critical role in osteoblast cell fate and function, and is activated following interactions with specific ligands of the Delta-like (Dll) and Jagged families [3, 6]. Notch-ligand interactions result in the proteolytic cleavage of the Notch receptor and the release and translocation of the Notch intracellular domain (NICD) to the nucleus, where it forms a complex with CSL (for CBF1, suppressor of hairless and Lag1), also termed Rbpjκ, and with Mastermind [24, 25]. This is known as the Notch canonical signaling pathway and results in the expression of the classic Notch target genes Hairy and Enhancer of Split (*Hes*) and Hes-related with an YRPW motif (*Hey*) [26]. However, it is not known whether other genes are targeted by Notch signaling in osteoblasts.

The purpose of this study was to investigate the direct effects of Notch signaling on Ctgf expression in osteoblasts from the *Rosa*^{Notch} mouse model, where a STOP cassette, placed

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between the *Rosa26* promoter and the *Notch1* NICD coding sequence, is flanked by *loxP* sites [27, 28]. Notch was activated in *Rosa*^{Notch} osteoblasts by the transduction of adenoviral vectors expressing the Cre recombinase [29, 30]. In addition, Ctgf expression was studied *in vivo* by obtaining calvariae and femurs from *Rosa*^{Notch} mice crossed with transgenics expressing the Cre recombinase under the control of the *Osterix* (*Osx*), the *Bglap* (*Osteocalcin*), the 2.3 kb fragment of *Col1a1* (*Col2.3*) or the *Dentin matrix protein1* (*Dmp1*) promoter [31–34].

2. MATERIALS AND METHODS

2.1 Rosa^{Notch} Conditional Mice

Rosa^{Notch} mice were obtained from Jackson Laboratory (Bar Harbor, ME) in a 129SvJ/C57BL/6 genetic background [27, 28]. Homozygous *Rosa*^{Notch} mice were used as a source of calvarial osteoblasts or were bred with heterozygous mice expressing Cre under the control of the *Osx* (*Osx-Cre*), the *Bglap* (*Bglap-Cre*), the *Col1a1* (*Col2.3-Cre*) or the *Dmp1* promoter (*Dmp1-Cre*) [33, 35–37]. All transgenics were in a C57BL/6 genetic background, but the *Col2.3-Cre*, which were in a tropism to friend leukemia virus type B (FVB) background. All mating schemes created *Cre*$^{+/-}$*;Rosa*^{Notch} experimental and *Rosa*^{Notch} littermate controls, as described [38]. In the *Osx-Cre* transgenics, the expression of Cre is under the control of a tet-off cassette, and *Rosa*^{Notch} pregnant dams were treated with a diet containing 625 mg of doxycycline hyclate/kg of chow to deliver 2 to 3 mg of doxycycline daily from the time of conception to delivery (Harlan Laboratories, Indianapolis, IN). *Osx-Cre, Bglap-Cre, Col2.3-Cre* and *Dmp1-Cre* were obtained from the Jackson Laboratory, T. Clemens (Baltimore, MD), the Mutated Mouse Regional Resource Center (Davis, CA) and J. Fang (Dallas, TX), respectively [33, 35–37]. Genotyping was carried out by polymerase chain reaction (PCR) in tail DNA extracts, and deletion of the *loxP* flanked STOP cassette by the Cre recombinase was documented by PCR in DNA from tibiae, as previously reported [38]. The induction of Notch in the skeleton was confirmed by documenting enhanced *Notch1* NICD*Hes1, Hey1* and *Hey2* mRNA expression in calvarial extracts by quantitative reverse transcription (qRT)-PCR, as reported previously [38]. All animal experiments were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

2.2 Cell Cultures

Osteoblast-enriched cells were isolated by sequential collagenase digestion from parietal bones of 3–5 day old *Rosa*^{Notch} mice or wild-type C57BL/6 mice, as described [39]. Osteoblasts from homozygous *Rosa*^{Notch} mice were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY), supplemented with nonessential amino acids (Life Technologies), 20 mM HEPES, 100 μg/ml ascorbic acid (both from Sigma-Aldrich, St. Louis, MO) and 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) at 37°C in a humidified 5% CO_2 incubator. When *Rosa*^{Notch} osteoblast cultures reached 70% confluence, they were transferred to medium containing 2% FBS for 1 h and exposed overnight to 100 multiplicity of infection of replication defective recombinant adenoviruses. An adenoviral vector expressing Cre recombinase under the control of the cytomegalovirus (CMV) promoter (Ad-CMV-Cre, Vector Biolabs, Philadelphia, PA) was

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delivered to *Rosa*^{Notch} cells to induce recombination of the *loxP* sequences and NICD expression [40]. An adenoviral vector expressing green fluorescent protein (GFP) under the control of the CMV promoter (Ad-CMV-GFP, Vector Biolabs) was used as control. In one experiment, osteoblast enriched cells were obtained from *Bglap-Cre*$^{+/-}$*;Rosa*^{Notch} mice, to induce *loxP* recombination and excision of the STOP cassette *in vivo*, and *Rosa*^{Notch} controls and cultured as described. Notch receptors can be activated by Notch ligands adherent to the cell culture substrate [41]. For this purpose, cell culture plates were exposed to the Notch ligand Dll1 (R&D Systems, Minneapolis, MN) in phosphate-buffered saline (PBS) for 1 h at room temperature to immobilized Dll1. Bovine serum albumin (BSA, Sigma-Aldrich) in PBS at a concentration of 500 ng/ml was used as a control. Wild type C57BL/6 osteoblasts were seeded on immobilized Dll1 or BSA and cultured in DMEM as described for osteoblasts from *Rosa*^{Notch} mice.

2.3 RNA Decay Experiments

The effects of Notch on the stability of *Ctgf* mRNA were assessed in *Rosa*^{Notch} osteoblasts transduced with Ad-CMV-Cre or Ad-CMV-GFP, grown for 72 h after reaching confluence and exposed to 75 μm 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, BioMol, Plymouth Meeting, PA) to arrest transcription [42]. Total RNA was extracted and subjected to qRT-PCR analysis to determine *Ctgf* mRNA levels following different times of exposure to DRB. To establish the slopes of *Ctgf* mRNA decay, *Ctgf* copy numbers corrected for *Rpl38* transcript levels, expressed as a percentage of the corrected *Ctgf* mRNA levels measured before exposure to DRB, were transformed by a base 10 logarithmic function and fitted against time by linear regression.

2.4 RNA Interference (RNAi)

To downregulate *Rbpjκ, Hes1, Hey1* and *Hey*2 in *Rosa*^{Notch} osteoblasts transduced with Ad-CMV-Cre or Ad-CMV-GFP, 19-mer double-stranded small interfering (si) RNAs targeted to the murine *Rbpjκ* (siRNA Id: S72811), *Hes1* (siRNA Id: 158034), *Hey1* (siRNA Id: 158942) or *Hey2* (siRNA Id: 159333) mRNA sequences were obtained commercially (Life Technologies) [43]. A scrambled 19-mer siRNA with no homology to known mouse sequences was used as control. Rbpjκ, Hes1, Hey1 and Hey2 or scrambled siRNA at 20 nM were transfected into 60–70% confluent osteoblasts using siLentFect lipid reagent, in accordance with manufacturer's instructions (Bio-Rad, Hercules, CA). To test for the effects of Rbpjκ, Hes or Hey downregulation on Ctgf expression, *Ctgf* mRNA or heterogeneous nuclear (hnRNA) were determined by qRT-PCR 72 h following the transfection of siRNAs. To ensure adequate downregulation, *Rbpjκ, Hes1, Hey1* and *Hey2* mRNA levels were determined.

2.5 Reverse Transcription – Polymerase Chain Reaction

Total RNA was extracted from cell layers, calvariae or femurs, following removal of bone marrow stromal cells by centrifugation, with the RNeasy mini kit, according to manufacturer's instructions (Qiagen, Valencia, CA). Changes in mRNA and hnRNA levels were determined by qRT-PCR [44, 45]. 0.5–1 μg of total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions and

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amplified in the presence of specific primers (Table 1) and iQ SYBR Green supermix (Bio-Rad) at 60°C for 35 cycles. cDNA copy number was estimated by comparison with a standard curve constructed using *Ctgf* (from Rolf-Peter Rysek, Princeton, NJ), *Hes1* (from American Type Culture Collection, ATCC, Manassas, VA), *Hey1, Hey2* (both from T. Iso, Los Angeles, CA) and *Rbpjκ* (from Thermo Scientific, Lafayette, CO) cDNAs and corrected for ribosomal protein l38 (*Rpl38*) or glyceraldehydes-3-phosphate dehydrogenase (*Gapdh*) expression, estimated by comparison with serial dilutions of cDNA for either *Rpl38* (ATCC) or *Gapdh* (R. Wu, Ithaca, NY) [46–50]. Amplification reactions were conducted in a CFX96 real time system (Bio-Rad). To assess *Ctgf* hnRNA levels, 0.5μg of total RNA were reverse-transcribed in the presence of a specific antisense primer targeted to the junction between intron 2 and exon 3 of *Ctgf* and amplified, as described for mRNA. Amplification efficiency was estimated by comparison with a standard curve generated by parallel amplification of a dilution series of genomic murine DNA, and *Ctgf* hnRNA was normalized to *Rpl38* expression [51]. Fluorescence was monitored during every PCR cycle at the annealing step, and specificity of the reaction was confirmed by the presence of a single peak in the melt curve analysis of PCR products.

2.6 Constructs and Transfections

To study effects of Notch on *Notch* transactivation and *Ctgf* promoter activity, a construct containing six multimerized dimeric CSL binding sites, linked to the β-globin basal promoter (12xCSL-Luc; L. J. Strobl, Munich, Germany) or a 3.8 kilobase (kb) fragment of the *Ctgf* promoter (*Ctgf-Luc*; Bruce Kone, Houston, TX) cloned upstream of luciferase were transfected into $Rosa^{Notch}$ cells transduced with Ad-CMV-Cre or Ad-CMV-GFP vectors [52, 53]. To verify these effects, osteoblasts from wild type C57BL/6 mice were co-transfected with the described constructs and a construct expressing the Notch1 cloned into pcDNA3.1 (pcDNA-NICD) or control vector [54]. To determine whether the 3' untranslated region (3'UTR) of *Ctgf* was a target of Notch, the *Ctgf* 3'UTR was cloned into the CMV promoter driven luciferase reporter pMIR.Target (Blue Heron Biotech, Bothel, WA). pMIR.Target and pMIR-Ctgf 3'UTR were transfected into Ad-CMV-Cre and Ad-CMV-GFP control transduced $Rosa^{Notch}$ cells. All transfections were conducted in cells cultured to 70% confluence using X-tremeGENE 9 DNA Transfection Reagent (3 μl X-tremeGENE 9/2 μg of DNA), according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN). A CMV-directed β-galactosidase expression construct (Clontech, Mountain View, CA) was used to control for transfection efficiency. All cells were exposed to the X-tremeGENE 9/DNA mixture for 16 h, transferred to fresh medium for 24 h, and harvested. Luciferase and β-galactosidase activities were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT). Luciferase activity was corrected for β-galactosidase activity.

2.7 Ctgf Enzyme-linked Immune Absorbent Assay (ELISA)

Murine Ctgf was measured by ELISA in culture medium from $Rosa^{Notch}$ osteoblasts and from wild type osteoblasts plated on BSA or Dll1 coated plates and in serum from $Cre^{+/-}$;$Rosa^{Notch}$ and control mice using a commercially available kit in accordance with manufacturer's instructions (Uscn Life Science Inc., Wuhan, Hubei, China)

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2.8 Statistical Analysis

Data are expressed as means ± SEM. Statistical differences were determined by Student's *t* test or analysis of variance with Schaffés post hoc analysis for pairwise or multiple comparisons. Statistical differences for the slopes of mRNA decay were analyzed by analysis of covariance [55].

3. RESULTS

3.1 Effects of Notch on the Expression of Ctgf in Osteoblasts

The effects of Notch on the expression of Ctgf were tested in primary calvarial osteoblast cultures from *Rosa*^{Notch} mice transduced with Ad-CMV-Cre, to excise the *loxP* flanked STOP cassette and allow NICD expression under the control of the *Rosa26* promoter, or with control Ad-CMV-GFP. Notch activation was previously confirmed by demonstration of increased *Notch1* NICD, *Hey1* and *Hey2* mRNA expression and enhanced transactivation of Notch reporter constructs [40]. Notch increased *Ctgf* mRNA levels in a time dependent manner. Notch induced *Ctgf* transcripts 3 days after the cells reached confluence and the effect was sustained for 2 weeks (Figure 1, upper left panel). Notch increased *Ctgf* hnRNA levels by 2.7 fold, indicating that Notch enhances *Ctgf* transcription (Figure 1, upper middle panel). The induction of *Ctgf* transcripts by Notch was translated into a ~2 fold increase in Ctgf protein levels (Figure 1, upper right panel). The induction of Ctgf expression was concomitant with the induction of the canonical Notch target genes *Hey1* and *Hey2* (Figure 1, lower panel). Consistent with the known decline of adenoviral vector activity, the induction of *Hey1* and *Hey 2* was more prominent in the early phases in the culture. There was minimal effect of the transduced adenoviral vector on *Ctgf* mRNA levels. In an experiment where Notch activation induced *Ctgf* mRNA 7.5 fold, there was little difference in *Ctgf* mRNA levels between non-transduced *Rosa*^{Notch} osteoblasts, Ad-CMV-GFP-transduced *Rosa*^{Notch} cells and wild type osteoblasts. *Ctgf/Rpl38* copy number (values means ± SEM; n = 4) was 0.14 ± 0.1 in wild type osteoblasts; 0.24 ± 0.1 in non-transduced *Rosa*^{Notch} osteoblasts; 0.20 ± 0.1 in Ad-CMV-GFP-transduced; and 1.5 ± 0.1 in Ad-CMV-Cre-transduced *Rosa*^{Notch} cells.

Although Notch induced *Ctgf* mRNA and hnRNA levels, the effect occurred 3 days after the cells reached confluence, suggesting possible indirect effects on *Ctgf* transcription. Indeed, in acute transfection experiments conducted in transduced *Rosa*^{Notch} osteoblasts, Notch enhanced the transactivation of the Notch reporter construct 12xCSL-Luc, but failed to enhance the activity of a 3.8 kb fragment of the *Ctgf* promoter directing luciferase activity (Table 2A). Similar results were observed when wild type osteoblasts were co-transfected with pcDNA-NICD expression constructs and either 12xCSL-Luc or the *Ctgf-Luc* promoter fragment, confirming that Notch did not enhance the transactivation of the *Ctgf* promoter fragment tested (Table 2B). In both experiments, a modest decrease in *Ctgf* promoter activity was noted. To determine whether Notch also regulated Ctgf expression by post-transcriptional mechanisms, the decay of *Ctgf* transcripts was assessed in *Rosa*^{Notch} osteoblasts transcriptionally arrested with DRB 3 days after the cells reached confluence. The half-life of *Ctgf* mRNA was 13 h in control cultures, and unexpectedly Notch shortened the half-life of *Ctgf* mRNA to 3 h, demonstrating that Notch destabilizes *Ctgf* transcripts,

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and confirming that Notch increased *Ctgf* mRNA exclusively by transcriptional mechanisms (Figure 2). To explore further the effect of Notch on *Ctgf* mRNA stability, pMIR-Ctgf 3'UTR constructs were transfected into *Rosa*^{Notch} osteoblasts transduced with Ad-CMV-Cre or control vector. Notch decreased the activity of the pMIR-Ctgf 3'UTR reporter by 85% indicating that the 3'UTR, a region containing sequences that often confer transcript stability, is a target of Notch, offering a potential mechanism for the destabilization of *Ctgf* mRNA by Notch signaling (Table 2C) [56].

3.2 Mechanisms Responsible for the Induction of Ctgf by Notch

To determine whether or not the induction of Ctgf by Notch was mediated by canonical signaling, the effect of Notch on Ctgf expression was tested in the context of Rbpjκ downregulation by RNAi. Transfection of siRNAs targeting Rbpjκ into *Rosa*^{Notch} osteoblasts transduced with Ad-CMV-Cre precluded the induction of *Ctgf* mRNA and hnRNA by Notch, so that *Ctgf* mRNA and hnRNA levels were not different than those found in control *Rosa*^{Notch} osteoblasts transduced with Ad-CMV-GFP (Figure 3). To determine whether one of the classic Notch canonical target genes was responsible for the induction of Ctgf by Notch, the effect of Notch was tested in the context of the downregulation of *Hes1, Hey1* or *Hey2* expression by RNAi. Transfection of siHes1, siHey1 or siHey2 resulted in the downregulation of their respective transcripts by 30 to 80%, but did not modify the induction of Ctgf achieved by the activation of Notch, suggesting that the effect of Notch on Ctgf expression was not mediated by Hes1, Hey1 or Hey2 (Figure 4).

3.3 Effects of Notch on the Expression of Ctgf Under Physiological and In Vivo Conditions

To verify the results obtained following the activation of Notch *in vitro* by the transduction of Ad-CMV-Cre vectors, calvarial osteoblasts were obtained from *Bglap-Cre*$^{+/-}$;*Rosa*^{Notch} and *Rosa*^{Notch} control mice, and cultured. Three days after confluence, osteoblasts from *Bglap-Cre*$^{+/-}$;*Rosa*^{Notch} mice expressed 10 fold higher *Hey2* mRNA levels than control cultures, documenting activation of Notch signaling, and 2.5 fold higher *Ctgf* mRNA levels, confirming the induction of Ctgf by Notch. Copy number of *Ctgf/Rpl38* was (means ± SEM; n = 4) 1.3 ± 0.1 in control cultures and 3.1 ± 0.1 ($p < 0.05$) in cultures from *Bglap-Cre*$^{+/-}$;*Rosa*^{Notch} mice.

To confirm that Notch activation induced *Ctgf* mRNA levels under physiological conditions, wild type C57BL/6 osteoblasts were exposed to immobilized Dll1 to induce Notch signaling, or to BSA, as control. Following 3 days of culture, osteoblasts exposed to Dll1 exhibited increased *Hey2* mRNA expression in comparison to cells exposed to BSA, confirming activation of Notch signaling by Dll1. In agreement with the stimulatory effects of NICD overexpression on *Ctgf* transcripts in *Rosa*^{Notch} osteoblasts, Dll1 increased *Ctgf* mRNA and hnRNA levels by ~1.5 - 2 fold (Figure 5), confirming that Notch induces *Ctgf* transcription in osteoblasts. However, Ctgf protein levels were not increased in the culture medium possibly due to the limited induction of *Ctgf* mRNA. Ctgf concentrations in the medium of cultures plated on BSA (values means ± SEM; n = 4) were 1.0 ± 0.1 ng/ml and in cultures plated on Dll1 were 1.0 ± 0.1 ng/ml.

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To establish whether Notch induced Ctgf *in vivo*, calvariae and femurs from *Osx-Cre*$^{+/-}$*;Rosa*^{Notch}, *Bglap-Cre*$^{+/-}$*;Rosa*^{Notch}, *Col2.3-Cre*$^{+/-}$*;Rosa*^{Notch} and *Dmp1-Cre*$^{+/-}$*;Rosa*^{Notch} mice and *Rosa*^{Notch} controls were analyzed for *Ctgf* mRNA expression. Notch activation was documented by demonstrating increased expression of *Notch1* NICD, *Hey1* and *Hey2* transcripts, as previously published [38]. Notch induced *Ctgf* mRNA in the four *Rosa*^{Notch} models tested by 2 to 9 fold, demonstrating that *Ctgf* is a Notch target gene *in vitro* and *in vivo* (Figure 6).

To determine whether the induction of Ctgf by Notch in the skeleton may have a systemic effect in addition to a local function, serum levels of Ctgf were measured in 1 month old *Rosa*^{Notch} mice. Because *Osx-Cre*$^{+/-}$*;Rosa*^{Notch} dams were exposed to doxycycline throughout their pregnancy and the induction of *Ctgf* mRNA was modest in their progeny at 1 month of age, serum levels in this model were obtained in 3 month old mice. Activation of Notch in cells of the osteoblastic lineage resulted in an increase in serum levels of Ctgf in the four models studied, although the effect did not reach statistically significance in *Col2.3*$^{+/-}$*;Rosa*^{Notch} mice (Table 3).

4. DISCUSSION

The present studies demonstrate that Notch signaling causes a time dependent induction of Ctgf expression in osteoblasts by transcriptional mechanisms. The effect of Notch on Ctgf expression is mediated by the canonical signaling pathway since it is abrogated by the downregulation of Rbpjκ by RNAi. The induction of Ctgf by Notch was observed in the *Rosa*^{Notch} model, where Notch is activated following the deletion of the *loxP* flanked STOP cassette placed downstream the *Rosa26* promoter and upstream sequences coding for the NICD. It is of interest that Notch activation itself caused a modest but reproducible downregulation of *Rbpjk* mRNA. This may be a protective mechanism to reduce canonical effects of Notch signaling but not sufficient to prevent the induction of Ctgf by Notch, which may require nearly complete obliteration of Rbpjk expression. Notch also induced Ctgf under more "physiological" conditions in wild type osteoblasts cultured on plates pre-coated with immobilized Notch ligand Dll1. *Ctgf* hnRNA was induced at lower concentrations of Dll1 than *Ctgf* mRNA, but there is no immediate explanation for this different level of sensitivity in the response observed. The induction of Ctgf by Notch occurred *in vitro* and *in vivo* and was observed in calvariae and femurs from *Rosa*^{Notch} mice crossed with transgenics expressing Cre in cells of the osteoblastic lineage at various stages of differentiation and in osteocytes. There was a greater induction of Ctgf when Notch was activated by crossing *Rosa*^{Notch} mice with *Col2.3-Cre* transgenics expressing Cre in mature osteoblasts and osteocytes, and a lesser induction when *Rosa*^{Notch} mice were crossed with *Osx-Cre* transgenics expressing Cre in osteoblast precursors. This may be related to differences in the activity of the promoter used to direct Cre expression as well as differences in the genetic background of the transgenic lines expressing Cre. *Col2.3-Cre* transgenics are in an FVB background whereas all other lines are in a 129SvJ/C57BL/6 background. The expression of Cre in *Osx-Cre* transgenics is under the control of the tet-off cassette; and Cre expression was suppressed prenatally by administering doxycycline to dams throughout the pregnancy [27, 28]. This may account for the limited induction of Ctgf in 1 month old *Osx-Cre;Rosa*^{Notch} mice. It is important to note that skeletal derived Ctgf

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may be effective at the local level as well as in distant tissues since Notch activation in cells of the osteoblastic lineage *in vivo* resulted in an increase in serum levels of Ctgf. However, there was not a good correlation between the degree of mRNA induction in skeletal tissue and changes in serum levels. The greatest increase in serum Ctgf levels was observed in *Dmp1-Cre;Rosa^{Notch}* mice activating Notch in osteocytes. These cells communicate signals to other cells via a canalicular network that could make the secretion of the protein to the circulation more efficient than when induced in osteoblast precursors and mature osteoblasts.

The induction of Ctgf by Notch consistently occurred 3 days after cells reached confluence, suggesting that the effect was indirect and not due to direct interactions of the Notch transcriptional complex and the *Ctgf* promoter. Confirming this possibility, Notch failed to enhance the activity of a *Ctgf* promoter fragment acutely transfected into *Rosa^{Notch}* osteoblasts. We do recognize that this may also represent an absence of elements required for the activation of transcription in the promoter fragment tested. Members of the *Hes* and *Hey* families are classic Notch target genes and are thought to mediate most of the cellular effects of Notch in bone. However, the induction of Ctgf by Notch does not appear to be mediated by the products of classic canonical Notch target genes *Hes1, Hey1* or *Hey2* since their downregulation did not preclude the induction of Ctgf by Notch. It is noteworthy that the downregulation of each one of these target genes (30 to 80%) may have been insufficient or that the actions of a downregulated gene may have been compensated by a related gene since there is known redundancy in the biological functions of the products of *Hes* and *Hey* genes [3, 26]. However, Hes and Heys are mostly inhibitors of transcription; therefore, are not likely to be responsible for the induction of Ctgf by Notch [3].

It is conceivable that genes other than *Hes* and *Heys* are affected by Notch signaling to regulate selected cellular events. *Ctgf* may be among these genes since Ctgf has important effects on cell adhesion, proliferation, migration and differentiation [57]. The effects of Notch on cells of the osteoblastic lineage are cell-context dependent. When Notch is expressed in differentiated osteoblasts or in osteoblast precursors, it suppresses osteoblastic gene markers *in vitro* and causes osteopenia *in vivo* [38, 40, 58, 59]. Similarly, transgenic overexpression of Ctgf (*Bglap-Ctgf*) causes osteopenia and osteoblasts from these mice express suppressed osteocalcin and alkaline phosphatase mRNA levels [17]. These observations suggest that Ctgf may contribute to the effects of Notch or act in concert with Notch signaling in the skeleton [17]. Ctgf plays an important role in tissue fibrosis, and activation of Notch signaling has been implicated in the development of interstitial fibrosis in the kidney and in hepatic fibrosis [60–63]. The mechanism of action of Ctgf involves important interactions with other regulatory signals, such as Wnt, BMP and IGF, acting by binding either the peptide or its receptor [17–19]. Similarly, Notch has important interactions with Wnt signaling in cells of the osteoblastic lineage [54, 64].

In previous work, we documented that Ctgf decreases Notch signaling and that the effect is reversed by inhibitors of proteasome degradation [16]. The induction of Ctgf by Notch may lead to a decrease in Notch signaling and serve as a negative feedback mechanism to temper Notch activity in skeletal cells. It is of interest that Notch can destabilize *Ctgf* transcripts in transcriptionally arrested osteoblasts, and this effect may reduce steady state *Ctgf* mRNA

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levels. However, the net effect observed is an increase in *Ctgf* mRNA indicating that the prevailing effect of Notch is the transcriptional induction of *Ctgf*. The mechanisms involved in the destabilization of *Ctgf* transcripts by Notch were only partially explored, and we did not test whether the effect was due to activation of Notch canonical signaling. Reporter assays revealed that the 3'UTR of *Ctgf* is targeted by Notch and may be responsible for the effect on transcript destabilization. This is not unexpected since 3'UTRs frequently modulate mRNA stability. A well studied family of RNA stability motifs consists of adenosine-uridine (AU) rich elements and in previous work we demonstrated that they play a critical role in the stabilization of matrix metalloproteinase 13 in osteoblasts [56]. Similar motifs are present in the 3'UTR of *Ctgf* and may regulate the stability of *Ctgf* transcripts. It should not be surprising that Notch regulates *Ctgf* transcription and transcript stability since often the same regulatory elements and proteins regulate both events and could be controlled by Notch signaling [65, 66]. The destabilization of *Ctgf* mRNA may serve as a protective mechanism to prevent the excessive accumulation of *Ctgf* transcripts and protein.

Nov (CCN3) has been shown to have important interactions with Notch signaling; and in previous work, we demonstrated that Nov downregulates Notch signaling in cells of the osteoblastic lineage [67]. The effects of Nov are cell-context dependent and in myogenic cells Nov was found to upregulate Notch signaling [68]. We also tested whether Nov regulated Ctgf expression in osteoblastic ST-2 cells. In accordance with work by others in different cells, Nov suppressed *Ctgf* mRNA expression by 50% in ST-2 cells (E. Canalis unpublished). These results indicate that in cells of the osteoblastic lineage Nov inhibits Notch signaling and *Ctgf* expression.

In conclusion, Notch induces *Ctgf* expression in osteoblasts, and Ctgf and Notch may act in concert to regulate skeletal homeostasis.

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ABBREVIATIONS

ATCC	American Type Culture Collectin
Bglap	bone gamma carboxyglutamate protein
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CCN	Cyr61, connective tissue growth factor and Nov
CMV	cytomegalovirus

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Col2.3	2.3 kb fragment of Col1a1
CSL	CBF1, suppressor of hairless and Lag1
Ctgf	connective tissue growth factor
Dll1	Delta like 1
DMEM	Dulbecco's modified Eagle's medium
Dmp1	Dentin matrix protein 1
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
FBS	fetal bovine serum
FVB	friend leukemia virus type B
Gapdh	glyceraldehydes-3-phosphate dehydrogenase
GFP	green fluorescent protein
Hes	Hairy and Enhancer of Split
Hey	Hes-related with an YRPW motif
hnRNA	heterogeneous nuclear RNA
IGFBP	insulin-like growth factor binding protein
NICD	Notch intracellular domain
PBS	phosphate-buffered saline
Nov	nephroblastoma overexpressed
Oc	osteocalcin
Osx	osterix
PCR	polymerase chain reaction
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RNAi	RNA interference
Rpl38	ribosomal protein l38
siRNA	small interfering RNA

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HIGHLIGHTS

1. We examined the effects of Notch on Ctgf expression in osteoblasts *in vitro* and *in vivo*.
2. Notch induces Ctgf mRNA and protein levels in osteoblasts by transcriptional mechanisms.
3. Notch canonical signaling is responsible for the induction of Ctgf.
4. Ctgf is a novel target of Notch signaling, and could mediate selected effects of Notch in the skeleton.

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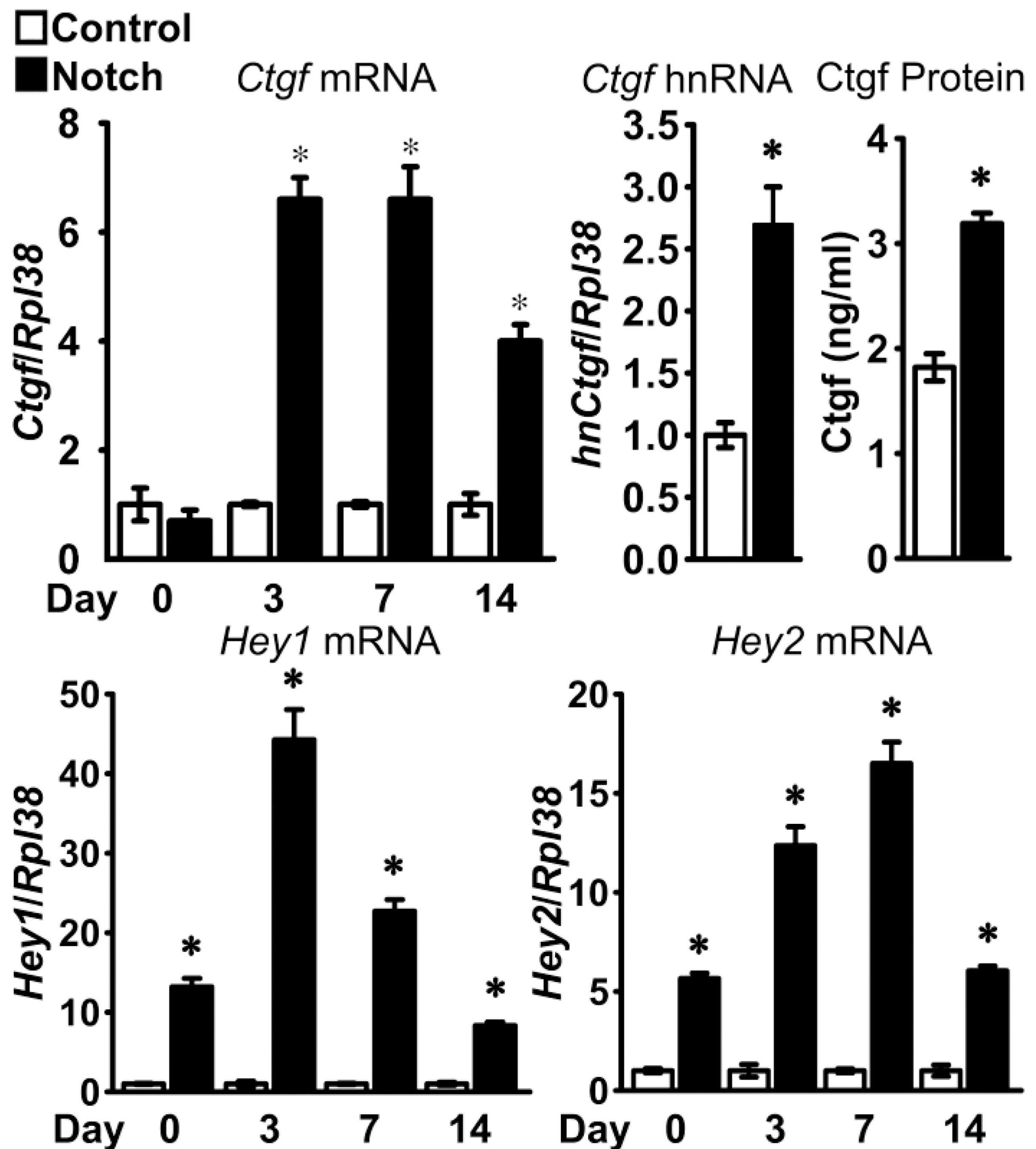

Figure 1.

Effect of Notch on *Hey1, Hey2* (lower panel) and *Ctgf* (upper left panel) mRNA, *Ctgf* hnRNA (upper middle panel) and Ctgf protein (upper right panel) expression in osteoblasts. Calvarial osteoblasts isolated from $Rosa^{Notch}$ mice were transduced with Ad-CMV-Cre, to activate Notch (black bars), or control Ad-CMV-GFP (white bars) and cultured to confluence (day 0) or up to 2 weeks following confluence. Samples for hnRNA and protein determination were obtained 3 days post-confluence. Total RNA was extracted, reversed transcribed and amplified by qRT-PCR. Data for mRNA and hnRNA are expressed as *Ctgf,*

Hey1 and *Hey2* copy number corrected for *Rpl38* expression relative to the mRNA or to the *Ctgf* hnRNA expression in Ad-CMV-GFP control cells, arbitrarily set at a value of 1. Data for Ctgf protein, measured by ELISA, are expressed as ng/ml of culture medium. Data for *Ctgf, Hey1* and *Hey2* mRNA were pooled from 2 experiments. Values are means ± SEM; n = 4. *Significantly different between Ad-CMV-Cre Notch activated cells and control, $p < 0.05$.

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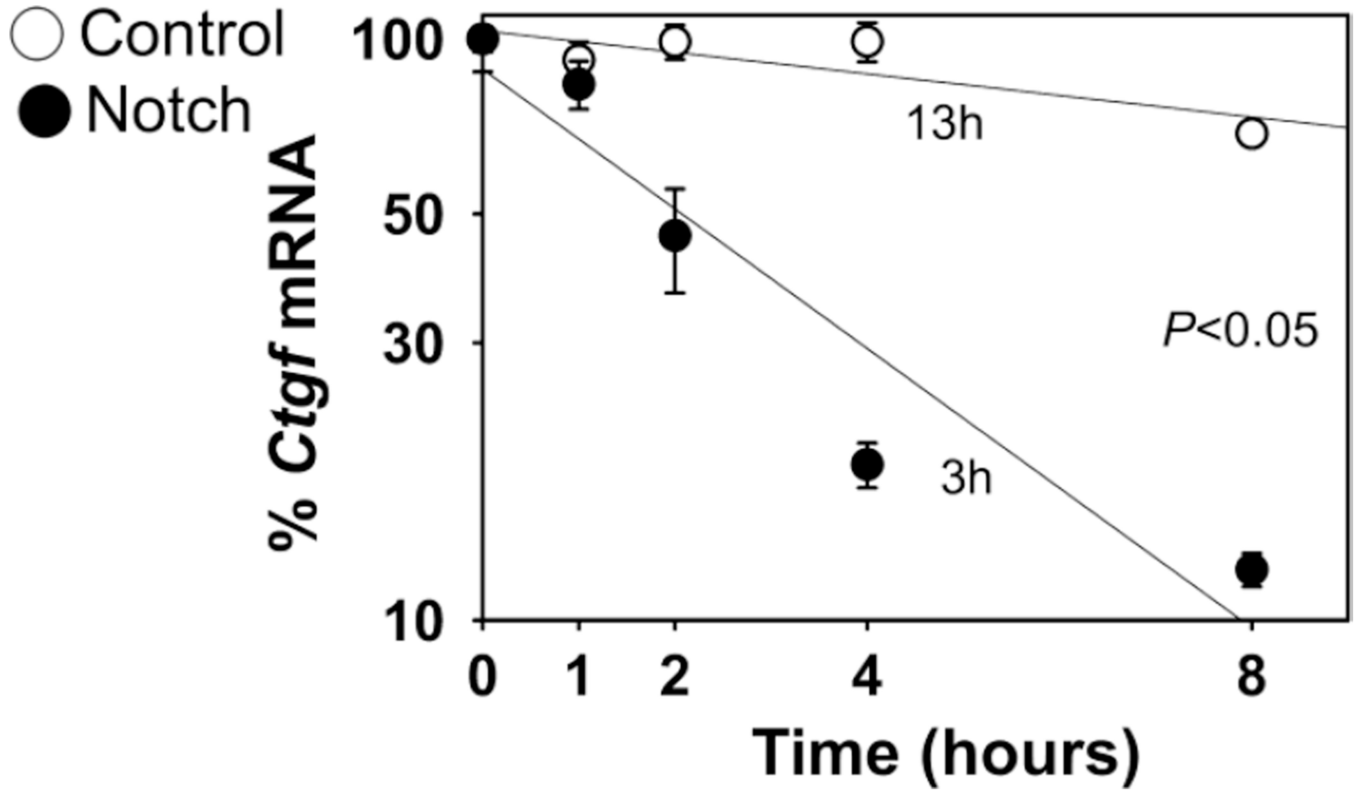

Figure 2.

Effect of Notch on *Ctgf* transcript stability in osteoblasts. Calvarial osteoblasts isolated from $Rosa^{Notch}$ mice were transduced with Ad-CMV-Cre, to activate Notch (filled circles), or control Ad-CMV-GFP (open circles) and cultured. Seventy-two h after confluence, cells were transcriptionally arrested by the addition of DRB (time 0), and harvested at the indicated times after DRB. Total RNA was extracted, reversed transcribed and amplified by qRT-PCR. Values are means ± SEM; n = 11 to 12. Data are expressed as percent of *Ctgf* mRNA corrected for Rpl38 expression, relative to the time of DRB addition and plotted versus time, and were pooled from 3 independent experiments. Slopes from Notch activated and control cells are significantly different, $p < 0.05$.

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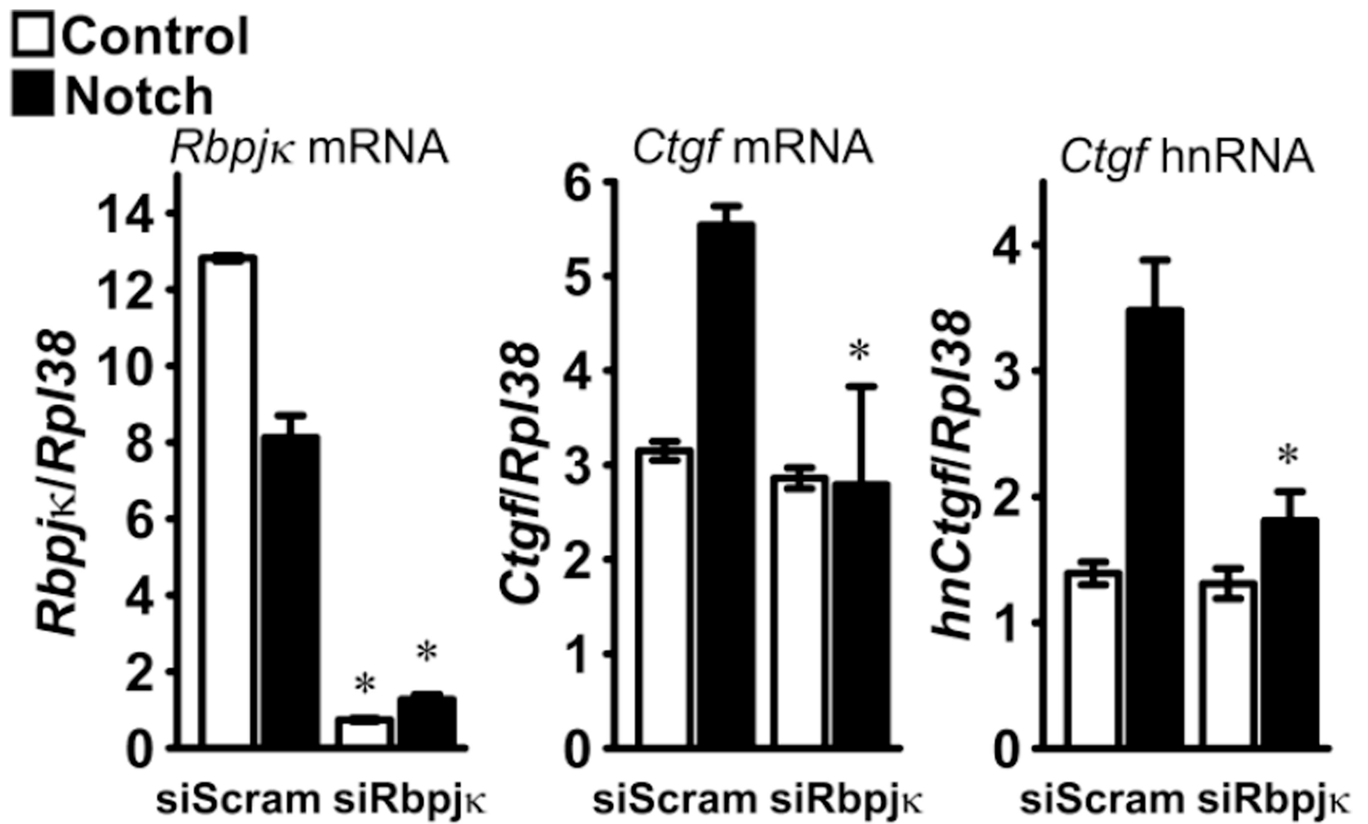

Figure 3.

Effect of Notch on Ctgf expression in the context of Rbpjκ downregulation. Calvarial osteoblasts isolated from *Rosa*^{Notch} mice were transduced with Ad-CMV-Cre, to activate Notch (black bars), or with control Ad-CMV-GFP (white bars), transfected with Rbpjκ small interfering RNA (siRbpjκ) or scrambled siRNA (siScram) and cultured for 72 h. Total RNA was extracted, reverse transcribed and amplified by qRT-PCR. Data are expressed as copy number of *Rbpjκ* mRNA, *Ctgf* mRNA and *Ctgf* hnRNA, corrected for *Rpl38*. Values are means ± SEM; n = 4. * Significantly different between siRbpjκ and siScram, $p < 0.05$.

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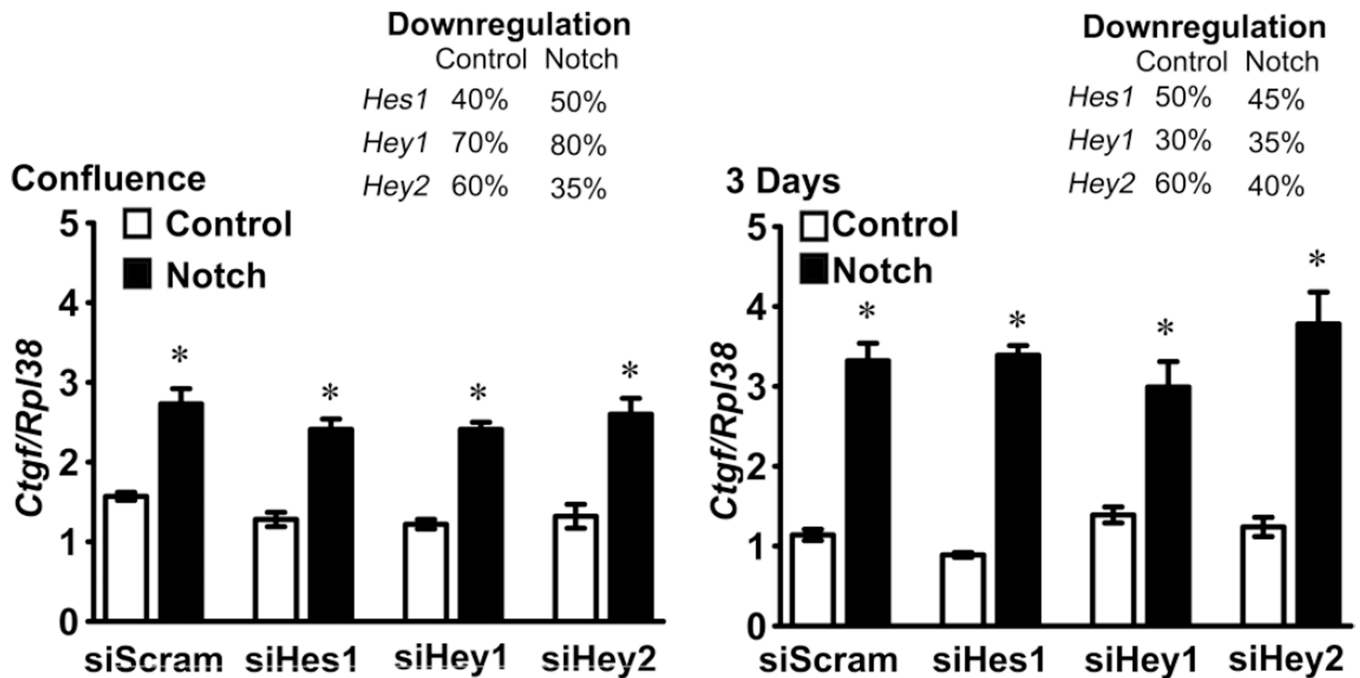

Figure 4.

Effect of Notch on *Ctgf* expression in the context of Hes1, Hey1 or Hey2 downregulation. Calvarial osteoblasts isolated from $Rosa^{Notch}$ mice were transduced with Ad-CMV-Cre, to activate Notch (black bars), or with control Ad-CMV-GFP (white bars), transfected with Hes1, Hey1 or Hey2 small interfering RNA (si) or scrambled siRNA (siScram) and cultured to confluence or for 3 days after confluence. Total RNA was extracted, reverse transcribed and amplified by qRT-PCR. Data are expressed as copy number of *Ctgf* mRNA corrected for *Rpl38*. Downregulation of *Hes1, Hey1* and *Hey2* mRNA in control (Ad-CMV-GFP) and Notch activated (Ad-CMV-Cre) cells, expressed as the mean % of suppression relative to the mRNA expression in siScram cells is indicated in the right upper corners of both panels. Values are means ± SEM; n = 4. * Significantly different between siHes1, Hey1 or Hey2 and siScram, $p < 0.05$.

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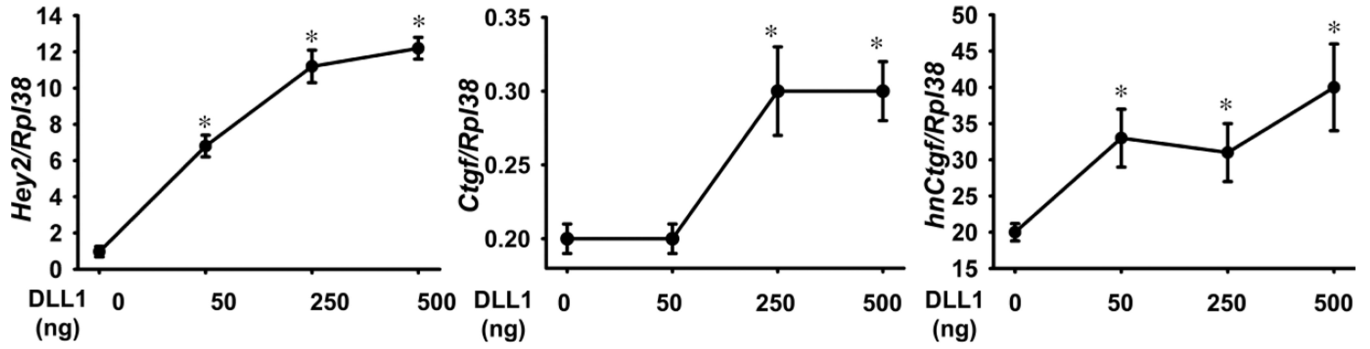

Figure 5.

Effect of Notch on *Hey2* mRNA, *Ctgf* mRNA and *Ctgf* hnRNA expression in osteoblasts. Wild type calvarial osteoblasts were cultured on plates coated with the Notch ligand Delta like 1 (Dll1) at the indicated doses for 72 h following confluence. Total RNA was extracted, reverse transcribed and amplified by qRT-PCR. Data are expressed as copy number of *Hey2* mRNA, *Ctgf* mRNA and *Ctgf* hnRNA corrected for *Rpl38* expression. Values are means ± SEM; n = 4. *Significantly different from control, $p < 0.05$.

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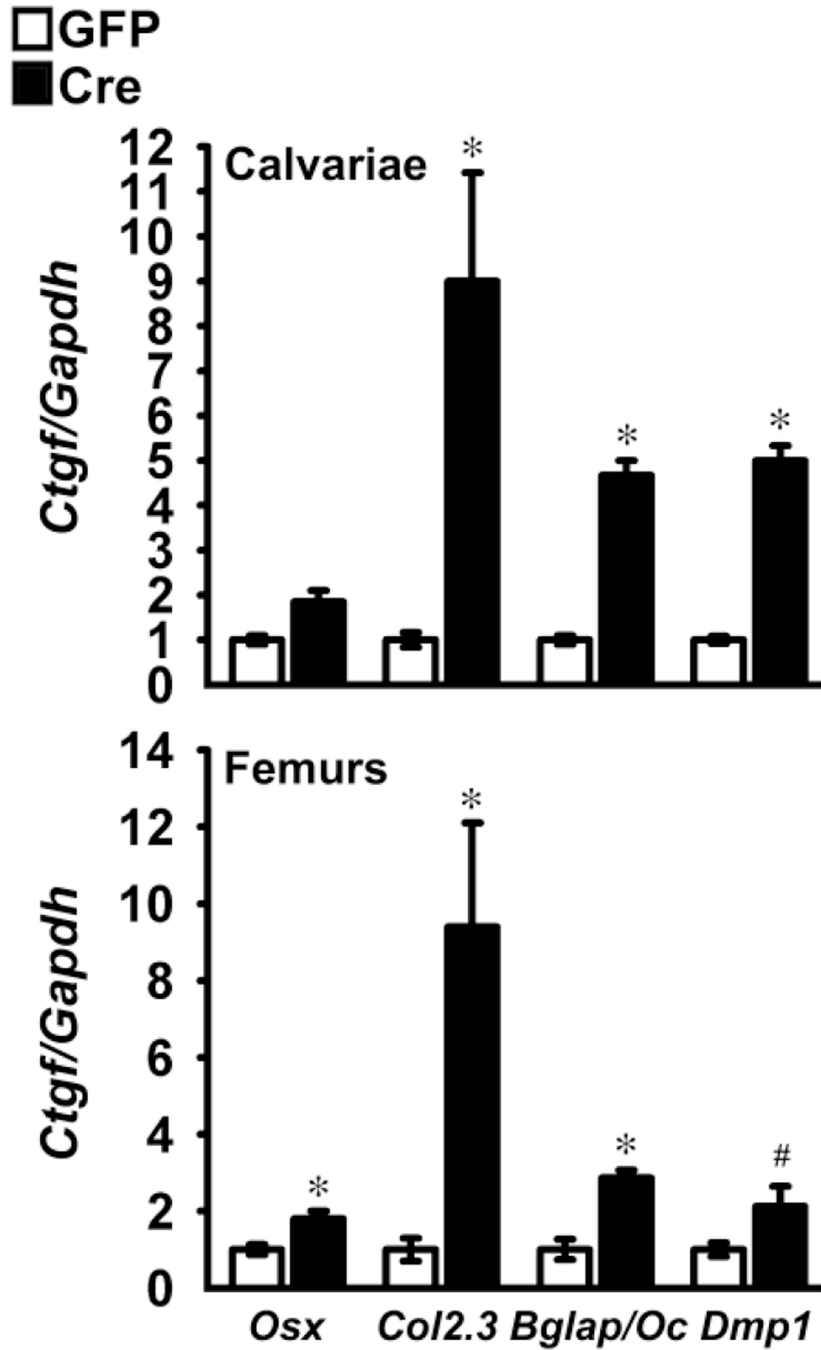

Figure 6.

Effect of Notch on *Ctgf* expression in calvarial and femoral extracts. Total RNA extracted from calvariae of male and female mice and femurs from male *Osx-Cre*$^{+/-}$;*Rosa*^{Notch}, *Col2.3-Cre*$^{+/-}$;*Rosa*^{Notch}; *Bglap/Oc-Cre*$^{+/-}$;*Rosa*^{Notch} and *Dmp1-Cre*$^{+/-}$;*Rosa*^{Notch} (black bars) and respective control *Rosa*^{Notch} littermates of the same sex (white bars) was reverse transcribed and amplified by qRT-PCR. Data are expressed as copy number of *Ctgf* corrected for *Gapdh* and expressed as the relative ratio with respect to controls (ratio 1.0).

Values are means ± SEM; n = 3 to 10. *Significantly different between $Rosa^{Notch}$ and control littermates, $p < 0.05$; # $p < 0.07$.

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Table 1

Primers used for qRT-PCR assay determinations. GenBank accession number identifies transcript recognized by primer pairs.

qRT-PCR

Gene	Strand	Sequence 5'–3'	GenBank Accession Number
Ctgf mRNA	Forward	CTGCCTGGGAAATGCTGCGAGGAGT	NM_010217
	Reverse	GTTGGGTCTTGGGCCAAATGT	
Ctgf hnRNA	Forward	ACCCTACGCCTGACCTACAA	NC_000076
	Reverse	CATCTTTGGCTGCGGGAGAG	
Gapdh	Forward	CCCCTCTGGAAAGCTGTGGCGT	NM_008084
	Reverse	AGCTTCCCGTTCAGCTCTGG	
Hes1	Forward	ACCAAAGACGGCCTCTGAGCACAGAAAGT	NM_008235
	Reverse	ATTCTTGCCCTTCGCCTCTT	
Hey1	Forward	ATCTCAACAACTACGCATCCCAGC	NM_010423
	Reverse	GTGTGGGTGATGTCCGAAGG	
Hey2	Forward	AGCGAGAACAATTACCCTGGGCAC	NM_013904
	Reverse	GGTAGTTGTCGGTGAATTGGACCT	
Rbpjκ	Forward	ACAGACAAGGCAGAATACAC	NM_001080928
			NM_009035
	Reverse	CAACTGAAGACTTTCTACGA	NM_001080927
Rpl38	Forward	AGAACAAGGATAATGTGAAGTTCAAGGTTC	NM_001048057
			NM_023372
	Reverse	CTGCTTCAGCTTCTCTGCCTTT	NM_001048058

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Table 2

Effect of Notch on the activity of *Ctgf* promoter and pMIR-Ctgf 3'UTR constructs.

	12xCSL-Luc	**3.8 kb *Ctgf-Luc***
	Luciferase/β-galactosidase	
A.		
Control	0.4 ± 0.4	1854 ± 250
Notch	19.7 ± 7.5*	1489 ± 393
B.		
Control	0.4 ± 0.1	190 ± 9
pcDNA-NICD	127 ± 15	141 ± 18*
	pMIR-Ctgf 3'UTR Luciferase/β-galactosidase	
C.		
Control	1637 ± 219	
Notch	232 ± 22*	

Calvarial osteoblasts isolated from $Rosa^{Notch}$ mice were transduced with Ad-CMV-Cre (Notch) or Ad-CMV-GFP (Control) and transfected in A. with a 12xCSL-Luc reporter or a 3.8 kb *Ctgf-Luc* promoter construct and in C. with pMIR-Ctgf 3'UTR reporter. In B. wild type osteoblasts were co-transfected with pcDNA-NICD expression construct or control pcDNA3.1 and 12xCSL-Luc or *Ctgf-Luc* promoter fragment. Values are means ± SEM; n = 6 of luciferase activity corrected for β-galactosidase activity.

*Significantly different from control, $p < 0.05$

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Table 3

Effect of Notch activation in the skeleton on Ctgf serum levels.

	Control	***Rosa^{Notch}***
	Ctgf pg/ml	
Osx-Cre;Rosa^{Notch}	318 ± 26	448 ± 40*
Col2.3-Cre;Rosa^{Notch}	833 ± 101	1448 ± 359
Bglap/Oc-Cre;Rosa^{Notch}	1011 ± 177	2055 ± 263*
Dmp1-Cre;Rosa^{Notch}	240 ± 51	2047 ± 235*

Serum levels of Ctgf were determined by ELISA in 3 month old *Osx-Cre$^{+/-}$;Rosa^{Notch}* and 1 month old *Bglap/Oc-Cre$^{+/-}$;Rosa^{Notch}*; *Col2.3-Cre$^{+/-}$;Rosa^{Notch}* and *Dmp1-Cre$^{+/-}$;Rosa^{Notch}* male mice and control male littermates. Values are means ± SEM; n = 3 – 4.

*Significantly different between *Rosa^{Notch}* and control littermates, $p < 0.05$.