Bone 64 (2014) 273–280

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Connective Tissue Growth Factor is a Target of Notch Signaling in Cells of the Osteoblastic Lineage☆,☆☆

Ernesto Canalis [a,b,*], Stefano Zanotti [a,b], Anna Smerdel-Ramoya [a]

[a] Department of Research, Saint Francis Hospital and Medical Center, Hartford, CT, 06105
[b] The University of Connecticut School of Medicine, Farmington, CT, 06030

ARTICLE INFO

Article history:
Received 30 December 2013
Revised 3 April 2014
Accepted 23 April 2014
Available online 2 May 2014

Edited by Shu Takeda

Keywords:
Notch
CCN proteins
connective tissue growth factor
osteoblasts
transcription

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.

1. Introduction

The fate of mesenchymal cells and their differentiation toward cells of the osteoblastic lineage are 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 (IGFBPs), 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,

Abbreviations: ATCC, American Type Culture Collection; *Bglap*, bone gamma carboxyglutamate protein; BMP, bone morphogenetic protein; BSA, bovine serum albumin; CCN, Cyr61, connective tissue growth factor and Nov; CMV, cytomegalovirus; 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.

☆ Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, under Award Numbers AR021707 and AR063049 (EC) and the National Institute of Diabetes and Digestive and Kidney Diseases, under award number DK045227 (EC). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

☆☆ Disclosure Statement: The authors have nothing to disclose.

* Corresponding author at: Department of Research, Saint Francis Hospital and Medical Center, 114 Woodland Street, Hartford, CT 06105-1299.

E-mail addresses: ECanalis@stfranciscare.org (E. Canalis), SZanott@stfranciscare.org (S. Zanotti), ASRamoya@stfranciscare.org (A. Smerdel-Ramoya).

http://dx.doi.org/10.1016/j.bone.2014.04.028
8756-3282/

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 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. 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 non-essential 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 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 number 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 *Hey2* 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