Objective: Regeneration of alveolar bone lost to disease is often compromised by the systemic health of the patient. Clinical strategies to mitigate the effects of these complicating systemic disorders are currently limited, and new therapeutic targets are necessary to provide the optimal care to these patients. The primary hypothesis of this work is that NE signaling promotes regeneration of alveolar bone through direct action on the osteoblasts and indirectly through resolution of inflammation. Methods: The effect of GAS6 on normal bone development was explored though the creation of mice lacking GAS6 expression. Histology and qPCR were completed using Gas6(-/-) and Gas6(+/+) mice. Further, differentiation assays were completed through isolation of bone marrow MSCs from Gas6(-/-) and Gas6(+/+) mice and differentiation in the presence or absence of NE for 21 days. To identify the effects of adrenergic signaling on paracrine factors secreted by OBs, a dot blot was completed to elucidate significant alterations in cytokine expression in response to adrenergic signaling in MC3T3 preosteoblasts. Results: GAS6 over-expressing MC3T3s showed increased expression of bone differentiation markers alkaline phosphatase, collagen 1A1 and osteopontin. Soluble GAS6 promoted matrix deposition and expression of OB maturation markers in MC3T3s. In WT differentiation media, a parallel reporter assay identified OB specific pathways RUNX1, BMP and AT4 as transcriptional targets activated by GAS6 signaling. Together these results suggested GAS6 can activate OB maturation pathways and promote the development of mature bone. Paracrine signals were elucidated using a dot blot analysis showing downregulated cytokines: GAS6, M-CSF, and osteopontin. Confirming the effects of GAS6 on our model system, preosteoblasts isolated from GAS6-/- mice showed no significant change in alizarin red staining in response to NE signaling during maturation. Conclusions: We have identified GAS6 as a novel mediator of bone development that is dysregulated due to systemic conditions. These studies are the first preclinical evidence that targeting the GAS6 pathway may be a viable route to promote bone regeneration in both healthy and medically compromised patients.

BACKGROUND

Regeneration of bone defects and associated structures remains one of the primary challenges related to periodontal surgery. Stress-related disorders are contributing factors in the development of bone defects through periodontal disease progression and clinically impaired regeneration/healing of these bone defects. The periodontal compartment is richly innervated and this innervation contributes to sustained adrenergic signaling in chronically stressed individuals. Adrenergic signaling and psychosocial stress are well known to significantly delay wound healing, limit regenerative capacity, and detrimentally affect tissues in the periodontium, yet the mechanisms behind these clinical observations are poorly understood.

Figure 1: NE affects osteoblast function through downregulation of GAS6.

(A, B) Treatment with 2.5 μM NE downregulated GAS6 mRNA in MC3T3 osteoblasts (A) and GAS6 protein in three different cell lines by ELISA (B) (murine osteosarcoma line MG63, murine osteoblast line MC3T3-E1, primary derived OBs from C57BL6/J mice).

(C) Histology of bones from wildtype and GAS6(-/-) mice revealed important differences in morphology. Specifically, bones from GAS6(-/-) mice showed increased adiposity compared with wildtype mice both through hematoxylin and eosin staining as well as immunohistochemistry, indicating GAS6 is necessary for normal bone development.

(D) Osteoblasts isolated from wildtype (WT) and GAS6(-/-) (GKO) mice were exposed to OB differentiation cocktail both with and without NE. OBs from WT mice showed a significant decrease in alizarin red staining as a result of NE treatment, while OBs from GAS6(-/-) mice were unaffected, indicating GAS6 is necessary for the normal response of OBs to adrenergic stimulation.

CONCLUSIONS

1. NE downregulates GAS6 mRNA and protein in osteoblasts.
2. GAS6 deficiency leads to increased adiposity in bone by suppressing GAS6 expression.
3. NE suppresses osteoblast differentiation in vitro in a GAS6 dependent manner.
4. Individual knockdown of adrenergic receptors was insufficient to eliminate the effects of NE on GAS6.
5. Dynamic transcriptional factor analysis identified CREB family proteins as mediating the NE response. ATF4, ATFS and CREB1 were the most highly expressed in MC3T3s, and inhibition of CREB significantly impacted the effects of NE on GAS6, confirming the effects of this family of factors on GAS6 expression.

We hypothesize that suppression of GAS6, through NE delivery, can impact bone matrix formation and subsequently impact regeneration.

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ABSTRACT

Stress Impairs Regulator of Bone Homeostasis and Inflammation Resolution

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METHODS & RESULTS

Figure 2: Downregulation of adrenergic receptors affects NE response

(A,B) siRNA (A) or pharmacological (B) blockade of adrenergic signaling mitigates, but doesn’t eliminate, effects of NE on GAS6

Figure 5. Transcription factor profiling reveals CREB family factors mediating NE/GAS6 axis

(A) A Transcription Activity Cell Array (TRACER) identifies central transcriptional mediators of the NE response in MC3T3s. ATF factors (2,3,4 and 6) and LHX8 were identified as central to adrenergic response; of these, only the ATF family factors were significant

(B) gPCR identifies three factors (ATF4, ATFS and CREB1) as significantly expressed in MC3T3s

(C) A CREB inhibitor (CREBi) both increased basal GAS6 expression as well as eliminated the effects of NE on GAS6 in MC3T3s

OVERVIEW

• Norepinephrine Effect: NE downregulated GAS6 in the periodontal compartment through sympathetic signaling mediator, CREB, leading to associated disruptions in osteoblast function

• Effects on Bone regeneration: Decreased GAS6 expression reduces bone formation.

• Norepinephrine: NE downregulated GAS6 in the periodontal compartment through sympathetic signaling mediator, CREB, leading to associated disruptions in osteoblast function

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