Stress Impairs Regulator of Bone Homeostasis and Inflammation Resolution

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ABSTRACT

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 GAS6 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 µCT were completed using Gas6 (+/+) and Gas6(-/-) mouse femurs. Differentiation assays were completed through isolation of bone marrow MSCs from Gas6 (+/+) and Gas6 (-/-) mice and differentiated 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 osteocalcin. Soluble GAS6 promoted matrix deposition and expression of OB maturation markers in MC3T3 cells in response to OB differentiation media. A parallel reporter assay identified OB specific pathways RUNX1, BMP and ATF4 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 effect 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. µCT of femurs in these mice revealed decreased trabecular bone volume and histological sections showed an increase in adipocyte formation in the bone marrow. Conclusion: 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.

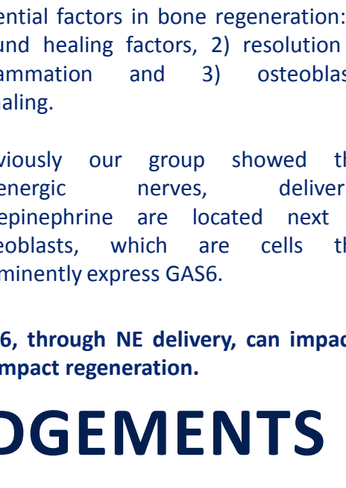


Figure 1: *Wound healing, bone homeostasis and inflammatory resolution must be achieved for optimal alveolar regeneration.*

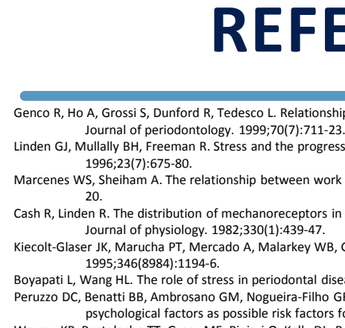


Figure 2. *IHC of long bone section (mouse) demonstrating co-localization of adrenergic neurons (Green arrow) and Osteoblasts (Red arrow).*

- *GAS6 is involved in all three aspects of bone regeneration*. Growth arrest specific 6 (GAS6), a vitamin K dependent cytokine, is released by a number of different cell types, including endothelial cells, vascular smooth muscle cells, and bone marrow osteoblasts.
- GAS6 has been implicated in three essential factors in bone regeneration: 1) wound healing factors, 2) resolution of inflammation and 3) osteoblastic signaling.
- Previously our group showed that adrenergic nerves, delivering norepinephrine are located next to osteoblasts, which are cells that prominently express GAS6.

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

METHODS & RESULTS

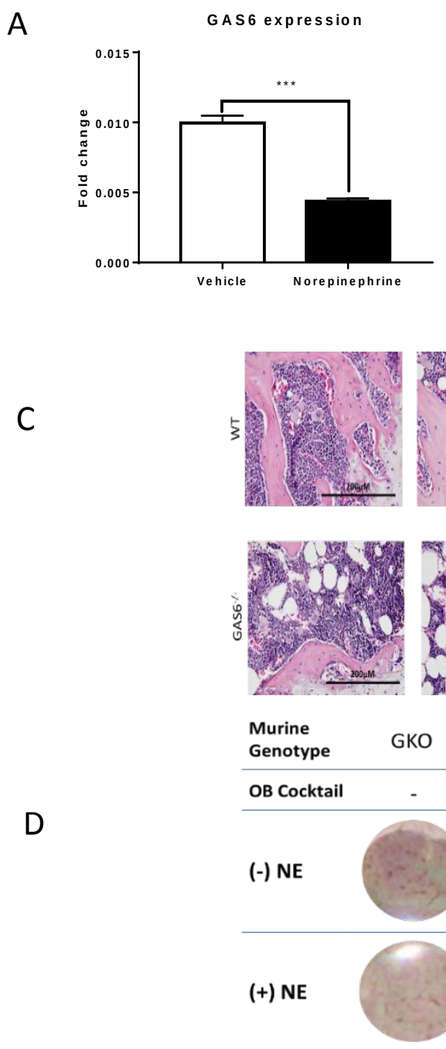


Figure 1. NE affects osteoblast function through downregulation of GAS6.

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

(C) Histology of bones from wildtype and GAS6$^{-\backslash-}$ mice revealed important differences in morphology. Specifically, bones from GAS6 deficient mice showed increased adiposity compared with bones from 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$^{-\backslash-}$ (GKO) mice were exposed to OB differentiation cocktail both with and without NE. OBs from WT mice showed a significant decrease in alaizarin red staining as a result of NE treatment, while Obs from GAS6$^{-\backslash-}$ mice were unaffected, indicating GAS6 is necessary for the normal response of OBs to adrenergic stimulation.

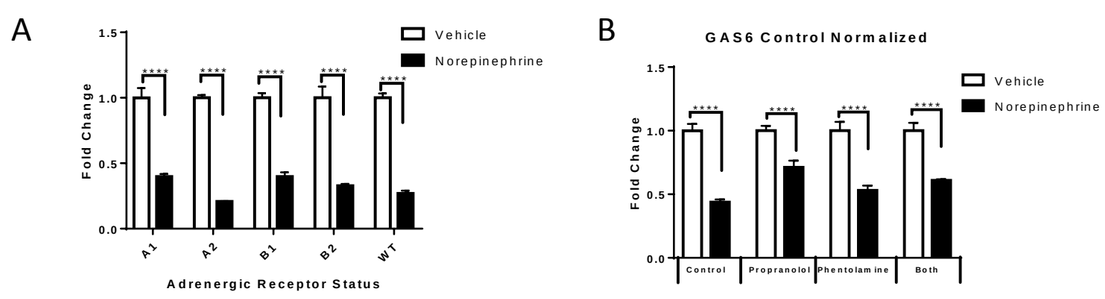


Figure 2: Downregulation of adrenergic receptors affects NE response

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

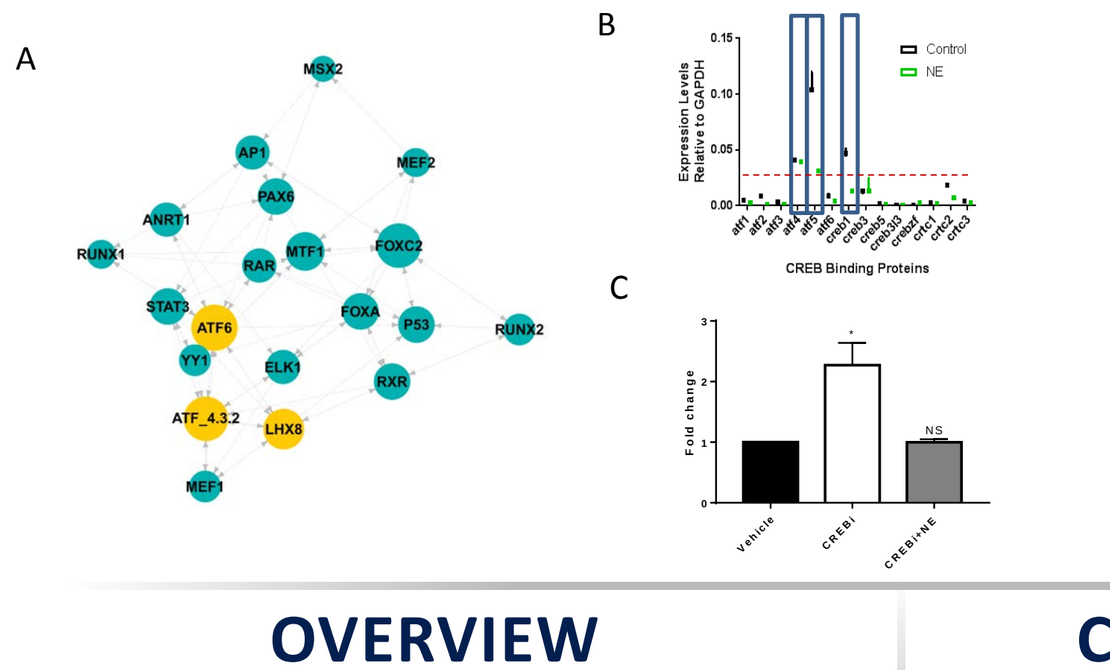


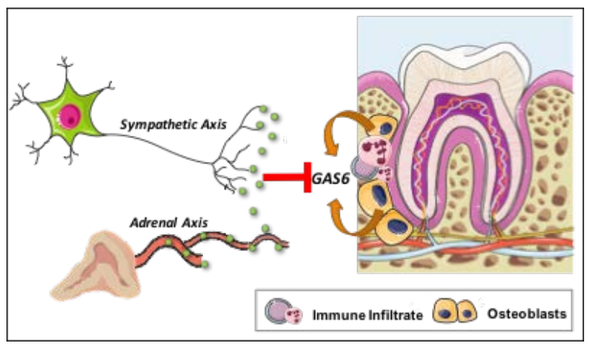
Figure 5. Transcription factor profiling reveals CREB family factors mediating NE/GAS6 axis

(A) A Transcriptional 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 factors were significant

(B) qPCR identifies three factors (ATF4, ATF5 and CREB1) as significantly expressed in MC3T3s

(C) A CREB inhibitor (CREBi) both increased baseline 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.

CONCLUSIONS

1. NE downregulates GAS6 mRNA and protein in osteoblasts
2. GAS6 deficiency leads to increased adiposity in bone by histology
3. NE suppresses osteoblast differentiation *in vitro* in a GA6 dependent manner
4. Individual knockdown of adrenergic receptors was insufficient to eliminate the effects of NE on GAS6. Pharmacological inhibitors were significantly more effective and mitigating this effect
5. Dynamic transcriptional factor analysis identified CREB family proteins as mediating the NE response. ATF4, ATF5 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.

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