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Full Length Article

A two phase regulation of bone regeneration: IL-17F mediates osteoblastogenesis via C/EBP-β *in vitro*

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ABSTRACT

T lymphocytes and pro-inflammatory cytokines, specifically interleukin-17F (IL-17F) have been identified as important regulators in bone regeneration during fracture repair. To better understand the molecular mechanisms of IL-17F-mediated osteoblastogenesis, a mouse pre-osteoblast cell line (MC3T3-E1) was utilized to characterize the intracellular signal transduction of IL-17F. Comparisons to the established canonical Wnt signaling pathway were made using Wnt3a ligand. Our results demonstrated greater bone marker gene expression in IL-17F-treated cells, compared to cells treated with Wnt3a. Western blot analysis confirmed degradation of β-catenin and up-regulation of two key proteins in osteoblast differentiation, Runx2 and C/EBP-β, in response to IL-17F treatment. RNA silencing of IL-17F receptors, IL-17Ra and IL-17Rc via siRNA transfection resulted in decreased expression of Act2, Runx2, and C/EBP-β, demonstrating the direct ligand-receptor interaction between IL-17F and IL-17Ra/c as an activator of osteoblastogenesis. Our findings suggest that IL-17F promotes osteoblast differentiation independent of the canonical Wnt pathway and β-catenin signaling, presenting new insights on modulating the adaptive immune response in the inflammatory phase, temporally distinct from the reparative and remodeling phases of fracture healing.

1. Introduction

Successful fracture healing requires proper spatiotemporal regulation of osteoblastogenesis. Injury resulting in skeletal fracture at time zero initiates various molecular processes and signaling cascades triggering the immune response both locally and systemically [1–3]. While many factors can contribute to and interfere with fracture healing, emerging data suggests that the inflammatory response is a crucial modulator in this regenerative process [1,4–7]. Animal studies have demonstrated the significance of a few hallmark pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) in promoting fracture healing [8–11]. Yet, knowledge of key immune mediators responsible for fracture healing still remains largely unknown.

IL-17F is a pro-inflammatory mediator for the adaptive immune response during the early phase of fracture healing [12]. T helper cell 17 (Th17)-mediated secretion of IL-17F has been shown to increase osteoblastogeneis in the mouse fracture healing process *in vivo* and rescued impaired bone formation *in vitro* [12]. This link between T-lymphocytes, IL-17F and osteoblastogenesis introduced a new therapeutic target that could potentially modulate fracture healing via immune-mediated approaches.

IL-17F's signal transduction via a specific ligand-receptor interaction with key downstream mediators is well known in immune cells, epithelial cells, astrocytes, and fibroblasts [13,14] (Fig. 1A). However, the signal transduction of IL-17F in osteoblasts has not been previously been studied. A heteromeric receptor complex (IL-17R) consisting of single-pass transmembrane receptor proteins, IL-17Ra and IL-17Rc, interacts with IL-17 cytokines, IL-17A and IL-17F, for proper signal transduction to be established [14]. Act1 is an immediate and essential transducer of intercellular signaling mediated by IL-17R [15–17]. Act1 contains a tumor necrosis factor receptor-associated factor 6 (TRAF6) binding site, and mediates TRAF6 ubiquitination upon recruitment [18]. This ubiquitination results in phosphorylation of downstream kinases including mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase 1 (ERK1) and ERK2.

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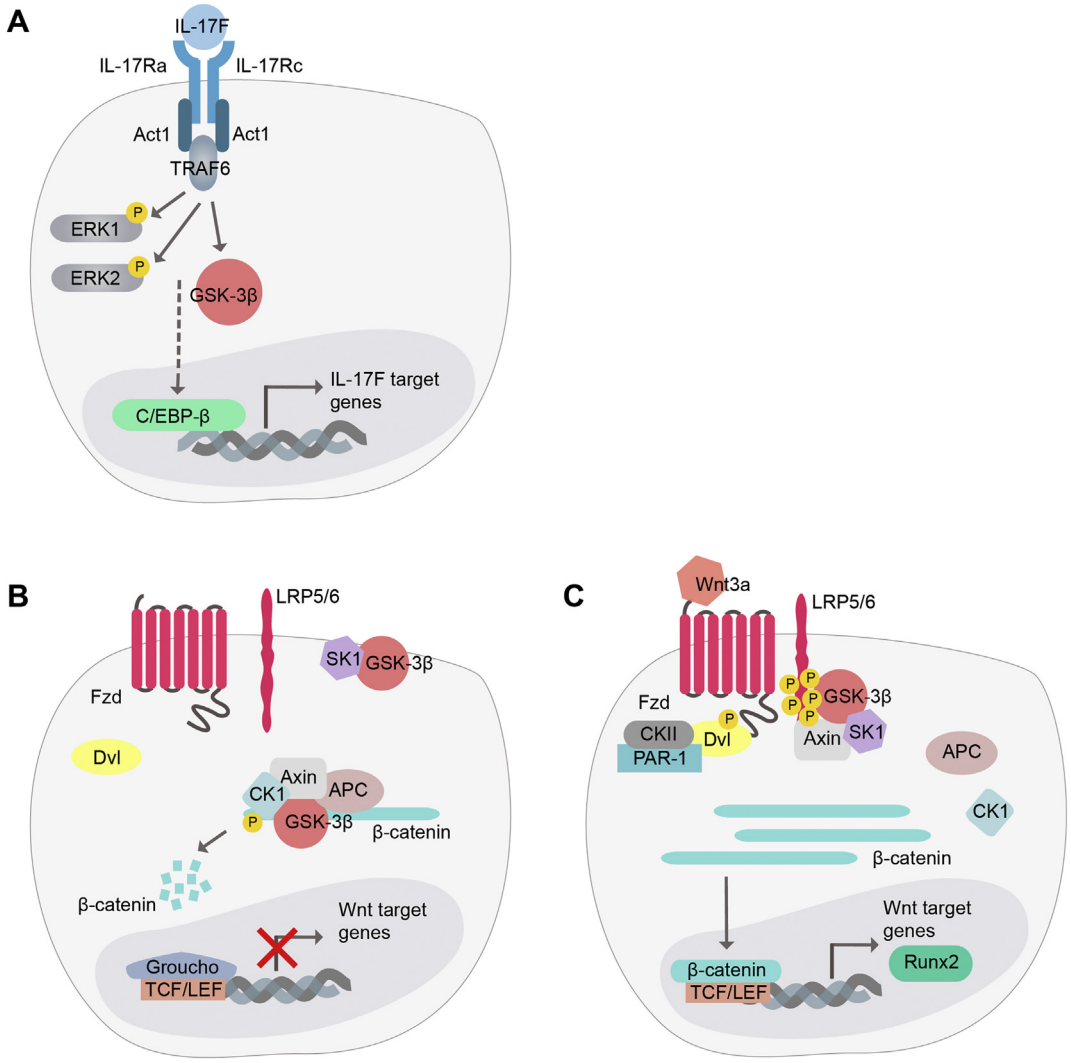

Fig. 1. IL-17F signaling pathway and the canonical Wnt/β-catenin signaling pathway:
(A) IL-17F signaling pathway identified in non-osteoblasts for IL-17F target gene activation;
(B) Canonical Wnt signaling pathway inhibited by GSK-3β activity;
(C) Canonical Wnt signaling pathway activated by Wnt agonist inhibiting GSK-3β activity.

Within fibroblasts, the phosphorylated ERK1 and ERK2, along with glycogen synthase kinase-3β (GSK-3β), regulate downstream intranuclear CCAAT/Enhancer binding protein-β (C/EBP-β), which in turn induces in IL-17 target gene expressions [19]. Osteoblastogenesis is ultimately mediated by Runt-related transcription factor 2 (Runx2), and a synergistic relationship between Runx2 and C/EBP-β for osteoblastogenesis has been demonstrated in other studies [20–22]. However, whether IL-17F directly regulates the interaction between C/EBP-β and Runx2 via the conserved signaling pathway to induce osteoblastogenesis remains to be investigated.

GSK-3β is a hallmark mediator of the canonical Wnt signaling pathway, a pathway crucial in osteoblastogenesis [23,24]. The phosphorylation of GSK-3β results in degradation of β-catenin when the pathway is inactive (Fig. 1B). Once an agonist such as Wnt3a activates the pathway, GSK-3β activity is inhibited and subsequent nuclear translocation of intact β-catenin activates transcription of downstream targets for osteoblastogenesis including Runx2 (Fig. 1C). In particular, β-catenin activity has been reported to be crucial in the reparative and remodeling phases of fracture healing and bone regeneration [25–29].

Shared mediators between the IL-17F signaling pathway and the canonical Wnt signaling pathway for Runx2 activation and osteoblastogenesis indicate conserved mechanisms may exist between the inflammatory, reparative and remodeling phases of fracture healing. In this study, we employed MC3T3-E1, a mouse pre-osteoblast cell line, to compare signal transduction rates and downstream targets between IL-17F and the canonical Wnt signaling pathways using Wnt3a. Our aim is to systematically characterize and delineate whether a distinct IL-17F signaling pathway independent of GSK-3β/β-catenin activity exists in osteoblast activation and to provide mechanistic insights on osteoblast progenitors that access the immune system during the inflammatory phase of fracture healing.

2. Materials and methods

2.1. Cell culture

The mouse pre-osteoblast cell line, MC3T3-E1 (ATCC, Manassa, VA, USA), was maintained in αMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA, USA) and 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2 atmosphere. Prior to Wnt3a, IL-17F, and IL-17A treatments, MC3T3-E1 seeded at 1.0×10^5 cells/well in a 6-well plate were maintained in αMEM without FBS for 24 h of serum starvation. Subsequently, 20 ng/mL of Wnt3a (R&D systems, Minneapolis, MN, USA), 20 ng/mL of IL-17F (R&