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Role of Nanog in the maintenance of marrow stromal stem cells during post natal bone regeneration

Manish V. Bais [a], Zabrina M. Shabin [a], Megan Young [a], Thomas A. Einhorn [a], Darrell N. Kotton [b], Louis C. Gerstnefeld [a,*]

[a] Orthopaedic Research Laboratory, Department of Orthopedic Surgery, Boston University School of Medicine, Boston, MA 02118, USA
[b] Pulmonary Center, Boston University School of Medicine, Boston, MA 02118, USA

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ABSTRACT

Post natal bone repair elicits a regenerative mechanism that restores the injured tissue to its pre-injury cellular composition and structure and is believed to recapitulate the embryological processes of bone formation. Prior studies showed that Nanog, a central epigenetic regulator associated with the maintenance of embryonic stem cells (ESC) was transiently expressed during fracture healing, Bais et al. [1]. In this study, we show that murine bone marrow stromal cells (MSCs) before they are induced to undergo osteogenic differentiation express $\sim$50$\times$ the background levels of Nanog seen in murine embryonic fibroblasts (MEFs) and the W20-17 murine marrow stromal cell line stably expresses Nanog at $\sim$80$\times$ the MEF levels. Nanog expression in this cell line was inhibited by BMP7 treatment and Nanog lentivrial shRNA knockdown induced the expression of the terminal osteogenic gene osteocalcin. Lentivrial shRNA knockdown or lentiviral overexpression of Nanog in bone MSCs had inverse effects on proliferation, with knockdown decreasing and overexpression increasing MSC cell proliferation. Surgical marrow ablation of mouse tibia by medullary reaming led to a $\sim$3-fold increase in Nanog that preceded osteogenic differentiation during intramembranous bone formation. Lentiviral shRNA knockdown of Nanog after surgical ablation led to an initial overexpression of osteogenic gene expression with no initial effect on bone formation but during subsequent remodeling of the newly formed bone a $\sim$50% decrease was seen in the expression of terminal osteogenic gene expression and a $\sim$50% loss in trabecular bone mass. This loss of bone mass was accompanied by an increased $\sim$2- to 5-fold adipogenic gene expression and observed increase of fat cells in the marrow space. In summary these data show that Nanog is expressed during surgically induced marrow bone formation and is functionally involved in post natal marrow stromal cell maintenance and differentiation.

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1. Introduction

The homeotic gene Nanog is a key mediator of embryonic stem cell (ESC) maintenance [2,3]. BMP in combination with LIF has been shown to maintain ESCs in an undifferentiated state, which is achieved by BMP's up regulation of ID that blocks neural tissue differentiation, while LIF through the up regulation of Nanog blocks mesodermal/endodermal differentiation [4]. In subsequent studies Nanog's functions were further elucidated and shown to block BMP induced mesodermal differentiation of ESC, by interacting with Smad1 thereby interfering with its interaction with other activating Smads [5]. Thus Nanog acts like a rheostat to modulate BMP activities and the initial fate decisions of the ESC. While the global deletion of Nanog causes early embryonic lethality, conditional deletion after the blastocyst stage only affects spermatogenesis [4]. While Nanog has not been shown to have any functions in the post natal animals, a handful of studies have demonstrated that Nanog is expressed in various populations of mulitpotential post natal mesenchymal stem cells [6–10].

In previous studies [1], we identified that Nanog was upregulated during fracture healing. This observation led us to hypothesize that Nanog might play a functional role in the maintenance of skeletal stems populations during periods of post natal bone regeneration. Based on this circumstantial evidence, we examined the functional role of Nanog in post natal skeletal stem cell populations.

2. Materials and methods

2.1. Materials

W20-17 murine marrow stromal cells were from ATCC (CRL-2623™) and they were cultured as previously described [11]. Expression plasmids for Nanog, and Nanog promoter were from

* Corresponding author. Address: Orthopaedic Research Laboratory, Department of Orthopaedic Surgery, Boston University Medical Center, 715 Albany Street, R-205, Boston, MA 02118, USA. Fax: +1 617 414 1661.
E-mail address: lgersten@bu.edu (L.C. Gerstnefeld).

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Addgene, Cambridge, MA, USA (plasmid 18920 and 16337). All lentivirus based shRNA DNA clones and packaging cell lines that were used for making the viral transduction particles were from Sigma–Aldrich Inc., St. Louis, MO, USA.

2.2. Surgical models

Research was conducted in conformity with Federal and USDA guidelines, under an IACUC approved protocol. All studies were performed on male 8–10 weeks old C57 BL/6J (B6) mice. Surgical marrow ablation was carried out by reaming of the marrow space as described in Gerstenfeld et al. [12].

2.3. MSC culture and osteoinduction

Marrow stromal cell cultures were prepared from C57 BL/6J (B6) male mice of 8–10 weeks of age (Jackson Laboratories, Bar Harbor, ME) and osteoinduction was carried out as previously described [13].

2.4. Micro computer assisted tomography (μCT)

Specimens were scanned at 16 μm resolution using a Scanco μCT 40 system (Scanco Medical, Basserdorf, Switzerland) using a region of interest (ROI) as defined in studies by Bais et al. [13]. Total Bone volume and average mineral density were each compared across groups using a Kruskal–Wallis test (analysis of variance by ranks) [14].

2.5. Demineralized histology

For histological assessment, the tibiae were fixed, decalcified and sectioned as previously described [13]. Serial sections were generated and slides were taken every 100 μM. Slides were stained with either hematoxylin and eosin or Goldener Trichrome.

2.6. Lentivirus preparation and particle transduction

All work with lentiviruses was performed under BL2 conditions. All procedures for viral preparation and transduction of either primary MSC or after surgical marrow ablation transduction were as previously described [13].

2.7. Murine embryonic fibroblast cultures

MEFs were obtained using standard culture methods [15] for their isolation and expansion, from E13.5 mouse embryos (Charles River; strain #023; CF-1). All results shown are from passage 5 MEFs.

2.8. Messenger RNA analysis

Marrow ablation specimens were prepared by removing the distal cartilage condylar surfaces of the operated tibia and cutting approximately at the center of the mid-diaphyseal region. All bone tissues were collected into liquid nitrogen and stored at −80 °C until used for RNA extraction. RNA was prepared from both cell cultures and from bone tissues and qRT-PCR was carried out as previous described [13].

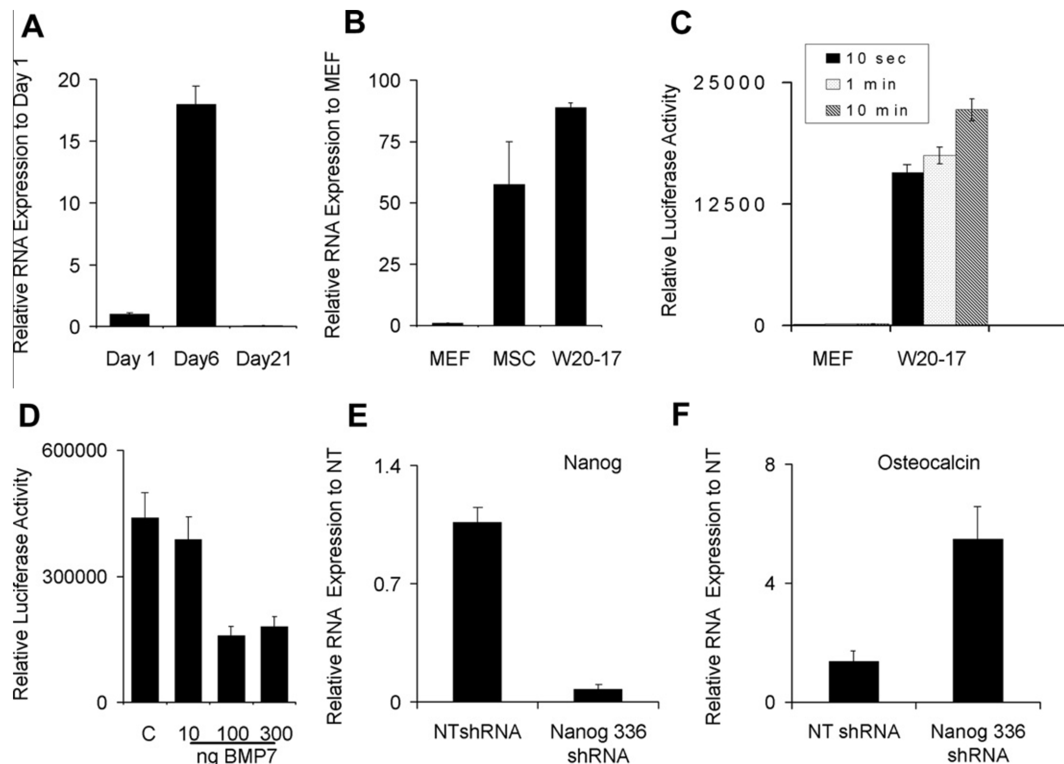

Fig. 1. Characterization of Nanog expression in bone marrow stromal cells. (A) The relative mRNA expression in adherent mouse marrow stromal cells isolated one day after plating in tissue culture, 6 days after plating but before switching to osteoinductive media and at 21 days in culture (15 days in osteoinductive media) when they are fully differentiated. (B) Comparison of undifferentiated marrow stromal cells (MSCs) to W20-17 marrow stromal cell line. Levels are expressed relative to murine embryonic fibroblasts that have been used as a null expressing reference to which ESC are compared. (C) Evaluation of relative 2.5 Kb Nanog promoter activities in MEFs and W20-17 cell line. (D) BMP-7 down regulation of Nanog promoter activity with exogenous addition of BMP7 protein at 10, 100 and 300 ng concentration compared to control in W20-17 cell line. (E) Effect of lentiviral Nanog shRNA particle transduction on endogenous Nanog mRNA within W20-17 cells. (F) Functional effect of Nanog shRNA mediated knockdown on Osteocalcin mRNA expression. NT = transduced with non target virus. Error bars represent standard deviation from replicate measurements from three experiments.

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