Published in final edited form as:

J Bone Joint Surg Am. 2008 February ; 90(Suppl 1): 9–13. doi:10.2106/JBJS.G.01212.

Tamoxifen-Inducible CreER Mediated Gene Targeting in Periosteum Via Bone Graft Transplantation

Chao Xie¹, Xue Ming¹, Qun Wang¹, Edward M. Schwarz¹, Regis J. O’Keefe¹, and Xinping Zhang^{1,3}

¹Center for Musculoskeletal Research, University of Rochester, School of Medicine and Dentistry, Rochester, NY

Abstract

Periosteum plays a key role in bone repair via activation of residing stem/progenitor cells. The molecular signals regulating differentiation and expansion of periosteal stem cells during early repair are poorly understood. Understanding the molecular basis for initiation and completion of bone healing is vital for the success of bone tissue engineering and regeneration therapy for impaired bone healing. We have established a live bone graft transplantation model that allows us to quantitatively evaluate the periosteal cell fate and cell initiated endochondral bone healing using transgenic and knockout mouse model. By combining this live bone graft transplantation method with a tamoxifen-inducible CreER mediated gene recombination model (R26CreER), we developed a novel approach to efficiently delete genes in periosteal cells during initiation of skeletal repair. This approach allows us to use floxed mice to examine the function of genes whose germline deletion results in lethality during development.

Clinical relevance—Successful bone repair and regeneration therapies require a deeper understanding of the signals and signaling pathways that are critical for the morphogenesis of the repair tissues. Early lethality in genetically manipulated mice prohibit us from understanding the function of genes in adult repair process. Our current approach overcomes this encumbrance and enable us to examine gene function in a time dependent and repair tissue specific manner.

Introduction

Adult bone repair is a stem/progenitor cell-driven process that requires the precise temporal and spatial presentation of factors to control their proliferation and differentiation at injury sites. The molecular signals that control differentiation and expansion of stem/progenitor cells in early bone repair are poorly defined. Surprisingly, little data exist on the regulation and control of mesenchymal stem cell fate during adult bone repair and the conservation with the molecular and cellular events that govern embryonic skeletogenesis. A thorough understanding of the spatial and temporal presentation of factors directing adult stem cell mediated bone repair is vital for the improvement of repair and regenerative therapies.

Periosteum contains a significant population of mesenchymal stem/progenitor cells that play key roles in cortical bone repair¹⁻⁵. Using a live bone graft transplantation approach combined with a mouse model that constitutively expresses β -galactosidase (Rosa26A), we marked the early periosteal progenitor cells and tracked the fate of these progenitors during cortical bone graft healing. Our data strongly suggests that the initiation of bone repair requires activation,

³To whom correspondence and reprints should be addressed, Dr. Xinping Zhang, The Center for Musculoskeletal Research, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, Tel: 585-275-7928, Fax: 585-275-1121, E-mail: Xinping_Zhang@URMC.rochester.edu

differentiation and expansion of residing periosteal stem/progenitor cells. The differentiation of stem/periosteal cells induced robust chondrogenesis and osteogenesis, which serves as the initial template for host cell invasion. By removing periosteum, the robust periosteal bone formation on the bone graft surface was abolished, further indicating an indispensable role of the periosteal stem/progenitor cells on the initiation and progression of cortical bone healing⁶.

To further understand the role of these periosteal stem cells and the genes that mediate their effects during bone healing, we aimed to determine the feasibility of using a conditional inducible gene targeting system established by others⁷, in combination with our live femoral isograft model. This system utilizes a mutated estrogen receptor fused to Cre recombinase as a transgene (Cre-ER), which only become activated following injection of tamoxifen in adult mice and is insensitive to endogenous estrogen. Here we used this transgenic mouse model in which the CreER is driven by the constitutive mouse *Gt(ROSA)26Sor* promoter so that its expression is ubiquitous in nearly all cells. The CreER fusion protein does not bind natural ligand at physiological concentrations, but will bind the synthetic ligand, 4-hydroxytamoxifen (4-OHT) and gain access to the nuclear compartment to mediate recombination after exposure to exogenous Tamoxifen (TM)⁸. When crossed with a strain containing a *loxP* site-flanked sequence of interest, this mouse can generate Tamoxifen-induced, Cre-mediated targeted deletions in various cells (global inducible). To demonstrate the utility of this model we generated RosaCreER; Rosa26R double transgenic mice in which the expression of β -galactosidase is activated following injection of Tamoxifen. This study demonstrates a novel approach by which a selective deletion of genes in periosteal stem cells can be achieved using live bone graft transplantation. This method will allow us to examine the role of genes whose germline deletion results in lethality during early embryonic development.

Methods

Mouse strains

The breeding colonies Rosa 26R and RosaCreER mice were originally purchased from Jackson laboratory (Bar Harbor, Maine). Both were maintained as on a C57Bl6 background. All animal surgery procedures were approved by University Committee of Animal Resources (UCAR).

Bone graft preparation and transplantation

Prior to transplantation, the donor RosaCreER;RosaR mice were treated with 1mg TM (Sigma-Aldrich) in Ethanol via intraperitoneal injection daily for 3 times to induce gene recombination in all tissues. Following TM treatment, a 4mm diaphyseal femoral bone graft was harvested from the mice and transplanted into a non-TM treated RosaCreER+/- littermate. The surgical procedure for live bone graft transplantation was described previously⁹. Briefly, ten week-old donor and recipient mice were anesthetized. A 7-8mm long incision was made, and the mid-shaft femur was exposed by blunt dissection of muscles without disturbing the periosteum. A 4mm mid-diaphyseal segment was removed from the donor femur by osteotomizing the bone using a saw. The live bone graft was then inserted into the segmental defect created in the recipient mice. The graft was stabilized by a 22-gauge metal pin placed through intramedullary marrow cavity. The grafted femurs were harvested at day 7 and were fixed in 0.2% glutaraldehyde at 4°C for 4. All samples were decalcified in EDTA at 4°C for 14 days and then embedded in OCT medium for cryosectioning. Tissue sections were stained in X-gal solution (0.02% NP40, 10mM EDTA, 0.02% glutaraldehyde, 0.05% X-gal and 2mM MgCL2 in phosphate buffer, PH 7.5) for 24 hours. Beta-gal positive cells were visualized and photographed under light microscopy.

Fracture healing model

RosaCreER;RosaR mice were anesthetized. A stainless steel 25G spinal needle (BD, Franklin Lakes, NJ) was inserted into the intramedullary space of the femur, followed by three-point bending with an Einhorn device using a standardized force¹⁰. Following fracture, 1mg TM was administered ip. on day 1, 3, and 5. Fractured femurs were harvested and processed for histological analyses as previously described¹¹.

Results and Discussion

Targeted gene deletion specific to adult periosteum has not been realized due to poorly defined cell populations and the absence of specific markers. The live segmental bone graft transplantation approach allows us to indiscriminately analyze the residing progenitor cells via tracking donor cell fate and evaluating their contribution to healing. If combined with a TM inducible CreER mouse model, this approach could achieve an inducible gene targeting in early periosteal stem/progenitor cells. To obtain more efficient and ubiquitous deletion in periosteum, we choose to use R26CreER mice⁷. To determine the efficiency of TM-induced Cre-mediated recombination, we crossed R26CreER with a LacZ reporter R26R mouse. The R26R mice consist of a *loxP* flanked neo expression cassette upstream of the LacZ gene, which prevents transcriptional read-through of the LacZ gene. When crossed with a strain that expresses Cre recombinase, the stop sequence will be removed in Cre expressing cells thereby activating LacZ gene expression. As demonstrated in Fig. 1A-C, strong LacZ staining was found in the tailbone of R26CreER; R26R mice following TM ip. injection (Fig. 1B&C). In contrast, no LacZ positive staining was found in the control non-TM treated mice (Fig. 1A).

To determine whether a selective gene recombination in periosteal progenitor cells can be achieved via bone graft transplantation, R26CreER;R26R mice were treated with 1mg of TM for consecutive 3 days to induce global gene recombination. Following the treatment, the femoral bone graft from a RosaCreER;RosaR mouse was harvested and immediately implanted into a mid-diaphyseal defect created in non-TM treated RosaCreER littermates. Figure 2A schematically illustrates the approach as described. The grafted femoral samples were harvested on day 7 and processed for X-Gal staining. As shown in Fig. 2B&C, large numbers of LacZ positive cells were found at the cortical bone junctions, similar to what we have previously observed in R26A transplantation⁶. At the graft side, nearly 70% of mesenchymal cells and chondrocytes were stained as LacZ positive (Fig. 2B), confirming the effective gene targeting in periosteal progenitor cells.

To further characterize the efficiency of TM induced recombination in repair tissue, a mid-diaphyseal femur fracture was created in R26CreER;R26R mouse. TM was administered for one (day1), two (day 1 and 3) and three times (day 1, 3 and 5) post-fracture at a dose of 1mg/mouse. All samples were harvested at day10 post-fracture. As demonstrated in Fig. 3, two ip. injections of TM were sufficient to induce recombination in nearly 70% of the reparative tissues in fracture callus (Fig. 3I, J and K). And three-time injection of TM induced recombination in 80% of repair tissue in fracture callus (Fig. 3M, N and O). Remarkably, we found that the percentage of LacZ positive cells was much higher in the callus than in its surrounding tissue or in growth plate, particularly at low dosing treatment (Figure 3M-P).

In view of the fact that mesenchymal stem cells come from different sources, the global inducible model holds a better chance to induce recombination in various adult progenitor cell pools. The efficient recombination in early stem/progenitor cells further results in an efficient removal of the targeted gene from all progeny following cortical bone fracture or osteotomy. The presented data represent a novel approach to achieve efficient global gene targeting in adult repair tissues. If combined with bone graft transplantation, a selectively targeted gene deletion can be achieved only in periosteal progenitor cells and their progeny around bone

graft. When incorporated with appropriate floxed mice, this approach can be used to examine the function of genes in controlling donor periosteal progenitor cell fate and cell dependent healing in adult repair. Another advantage of using a bone-grafting approach is that TM is administered to induce gene recombination in donor cells prior to transplantation. After transplantation, the mice will not receive any TM therefore completely eliminating its potential negative effects on bone graft healing.

Identifying essential signals for stem/progenitor dependent bone healing is of paramount importance. Our inability to treat patients with impaired bone healing is largely due to our limited understanding of the molecular signals or signaling that are essential for this process. The current study represent a novel approach among available technologies to examine the function of a single gene in adult bone and cartilage tissue repair. This approach is extremely useful in delineating the function of genes whose conventional deletion results lethality during development.

Acknowledgements

This study is supported by grants from the Musculoskeletal Transplant Foundation, the National Institute of Health (AR051469, AR46545).

References

1. Einhorn TA. The cell and molecular biology of fracture healing. *Clin Orthop* 1998;(355 Suppl):S7–21. [PubMed: 9917622]
2. Allen MR, Hock JM, Burr DB. Periosteum: biology, regulation, and response to osteoporosis therapies. *Bone* 2004;35(5):1003–12. [PubMed: 15542024]
3. Orwoll ES. Toward an expanded understanding of the role of the periosteum in skeletal health. *J Bone Miner Res* 2003;18(6):949–54. [PubMed: 12817746]
4. O'Driscoll SW, Fitzsimmons JS. The role of periosteum in cartilage repair. *Clin Orthop* 2001;(391 Suppl):S190–207. [PubMed: 11603704]
5. Wlodarski KH. Normal and heterotopic periosteum. *Clin Orthop* 1989;(241):265–77. [PubMed: 2647335]
6. Zhang X, Xie C, Lin AS, Ito H, Awad H, Lieberman JR, Rubery PT, Schwarz EM, O'Keefe RJ, Goldberg RE. Periosteal progenitor cell fate in segmental cortical bone graft transplantations: implications for functional tissue engineering. *J Bone Miner Res* 2005;20(12):2124–37. [PubMed: 16294266]
7. Badea TC, Wang Y, Nathans J. A noninvasive genetic/pharmacologic strategy for visualizing cell morphology and clonal relationships in the mouse. *J Neurosci* 2003;23(6):2314–22. [PubMed: 12657690]
8. Feil R, Brocard J, Mascres B, LeMeur M, Metzger D, Chambon P. Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci U S A* 1996;93(20):10887–90. [PubMed: 8855277]
9. Tiyyapatanaputi P, Rubery PT, Carmouche J, Schwarz EM, O'Keefe RJ, Zhang X. A novel murine segmental femoral graft model. *J Orthop Res* 2004;22(6):1254–60. [PubMed: 15475206]
10. Bonnarens F, Einhorn TA. Production of a standard closed fracture in laboratory animal bone. *J Orthop Res* 1984;2(1):97–101. [PubMed: 6491805]
11. Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest* 2002;109(11):1405–15. [PubMed: 12045254]

Figure 1.

A global Tamoxifen inducible model for targeted gene recombination. R26CreER;R26R mice were treated with TM three times at a dose of 1mg/day. X-Gal staining was performed in mouse tailbones. LacZ staining was observed in nearly all cells including chondrocytes, osteoblasts and osteocytes in the tamoxifen-treated group (B and C), whereas the vehicle-treated group showed no LacZ-positive cells (A).

Figure 2.

Tamoxifen-inducible CreER mediated gene targeting in early periosteal callus can be achieved via bone graft transplantation. Schematic illustration of the graft transplantation approach using R26CreER;R26R mice (A). Following TM treatment, bone grafts were harvested and transplanted into control non-TM treated mice. Grafted femur samples were stained by X-Gal at day 7 post transplantation. X-Gal staining shows efficient recombination in donor periosteal cells overlying the graft (larger box in B). Photograph (C) taken at higher magnification for small boxed region in B shows efficient gene recombination in chondrocytes (arrow in C) and surrounding periosteal progenitors (# in C).

Figure 3.

Efficient CreER-mediated gene recombination in the fracture repair tissues. A femoral fracture was created in R26CreER;R26R mice and TM was administered once (day1), twice (day 1 and 3) or three times (day 1,3 and 5) post-fracture at a dose of 1mg/day/mouse. X-Gal staining was performed on samples harvested on day 10 post-fracture. Photographs were taken at 4X (A-D) and 10X(E-H) and 40X (I-P). Vehicle treated group showed no LacZ staining in fracture callus (A, E, I). In contrast, increasing number of cells in the fracture callus stained LacZ positive following TM injections of one time (B, F, J), two times (C, G, K) or three times (D, H, L). Compared with fracture callus, the activation of LacZ gene in growth plate (M-P) was less efficient.