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Loss of transcription factor early growth response gene 1 results in impaired endochondral bone repair

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Abstract

Transcription factors that play a role in ossification during development are expected to participate in postnatal fracture repair since the endochondral bone formation that occurs in embryos is recapitulated during fracture repair. However, inherent differences exist between bone development and fracture repair, including a sudden disruption of tissue integrity followed by an inflammatory response. This raises the possibility that repair-specific transcription factors participate in bone healing. Here, we assessed the consequence of loss of early growth response gene 1 (EGR-1) on endochondral bone healing because this transcription factor has been shown to modulate repair in vascularized tissues. Model fractures were created in ribs of wild type (wt) and EGR-1^{-/-} mice. Differences in tissue morphology and composition between these two animal groups were followed over 28 post fracture days (PFDs). In wt mice, bone healing occurred in healing phases characteristic of endochondral bone repair. A similar healing sequence was observed in EGR-1^{-/-} mice but was impaired by alterations. A persistent accumulation of fibrin between the disconnected bones was observed on PFD7 and remained pronounced in the callus on PFD14. Additionally, the PFD14 callus was abnormally enlarged and showed increased deposition of mineralized tissue. Cartilage ossification in the callus was associated with hyper-vascularity and -proliferation. Moreover, cell deposits located in proximity to the callus within skeletal muscle were detected on PFD14. Despite these impairments, repair in EGR-1^{-/-} callus advanced on PFD28, suggesting EGR-1 is not essential for healing. Together, this study provides genetic evidence that EGR-1 is a pleiotropic regulator of endochondral fracture repair.

Keywords

Fracture repair; endochondral bone formation; transcription factor; early growth response gene

1. Introduction

The classical zinc finger transcription factor EGR-1, also known as NGFI-A [1], zif268 [2], krox24 [3], and TIS8 [4], was discovered as an immediate early gene [5]. It appears to mediate the cellular responses to mitogenic stimuli and stress signals such as growth factors and hypoxia, respectively [6–11]. The specific DNA binding site for EGR-1 is G/C rich and has been identified within the promoter regions of a large number of target genes important for proliferation, differentiation, apoptosis, growth control, and inflammation [12–25]. Even though EGR-1 regulates a broad array of genes *in vitro*, deficiency of EGR-1 in mice manifests only in a mild phenotype characterized by normal gross appearance, regular growth rate and cellular differentiation [20], however, female EGR-1^{-/-} mice are infertile due to a deficiency in luteinizing hormone [26]. The *in vivo* functions of EGR-1 are only partially elucidated. Evidence suggests a role for EGR-1 in the molecular response to tissue damage. For example, Yan et al. examined the role of EGR-1 gene deletion in an ischemic and reperfusion lung injury model [11]. Expression of multiple genes, including essential regulators of inflammation and vascular permeability, was diminished in EGR-1^{-/-} mice, thereby enhancing animal survival and organ function. In addition, Khachigian et al. observed in the endothelial wound edge after acute mechanical vessel injury that EGR-1 is a positive regulator of angiogenic growth factors [27]. Together, these studies suggest a crucial role for EGR-1 in the repair of vascularized tissue. Therefore, we hypothesized a potential role for EGR-1 in bone fracture repair, which is characterized by damage to multiple hard and soft tissues, including the vascular system.

In this study, we compared endochondral bone repair in a mouse rib fracture model between wt and EGR-1 deficient animals. Tissue morphology and composition of PFD7, 14 and 28 specimens were evaluated and revealed a series of profound healing abnormalities in EGR-1^{-/-} mice, including persistent fibrin accumulation, abnormal callus ossification, and muscular cell deposits. Thus, EGR-1 was discovered to be a pleiotropic regulator of bone repair.

2. Material and Methods

2.1. Mouse rib fracture model

Studies in mice were performed in accordance with the Institutional Animal Care and Use Committee (IACUC). The EGR-1 deficient (EGR-1^{-/-}) mouse model #2013 (B6 background) was originally purchased from Taconic (Hudson, NY). Wild type and EGR-1^{-/-} mice were derived from an in-house colony through a het/het breeding scheme. For the initial characterization of rib bone healing in wt mice, additional animals of matched genetic background were obtained from vendors (Taconic or The Jackson Laboratories, Bar Harbor, ME). The right 8th rib of mice 10–12 weeks of age (body weight of 24 ± 2 g) was subjected to an osteotomy as previously described by Hashimoto et al. [28]. Briefly, each mouse was anesthetized with ketamine (50 mg/kg, *i.p.*) and 2% isoflurane supplemented with 1% oxygen. A longitudinal incision was made along the thoracic spine and the superficial back muscles were retracted carefully to expose the dorsal aspect of the right ribs. Counting the ribs from caudal to cranial starting with the 13th rib, the 8th rib was identified and fixed mechanically with forceps. A ruler was used to place the osteotomy 1 cm lateral of the spine. The 8th rib was cut with scissors vertically to the axis of the ribs. The skin incision was closed with wound clips, no internal sutures were needed. On designated post-fracture days, fractured or intact 8th ribs were removed *en bloc* together with the neighboring 7th and 9th ribs as a rectangular specimen (approximately 8 mm horizontal and 6 mm vertical). A total of 8 wt mice were studied at each PFD1, 3, 5, 7, 14, 21 and 28, while at least 4 EGR1^{-/-} mice were examined histologically on each PFD7, 14, and 28. Extra intact 8th ribs from unfractured wt and EGR-1^{-/-} mice served as controls for histological

and immunohistochemical analysis. Additional 5 mice for each the EGR-1^{-/-} and wt group were studied for microCT analysis, while RT-PCR was carried out on pooled samples from another 8 mice each for EGR-1^{-/-} and wt.

2.2. Histology and immunohistochemistry

All dissected rib samples were fixed in 10% formalin at 4°C. Fixation times of 24 hours and 2 – 4 hours were applied prior to histological/immunohistochemical and enzymatic stains, respectively. All samples were embedded in paraffin after decalcification in ethylenediamine tetraacetate (10% EDTA, pH 7.4). Serial longitudinal sections (6–7 µm thickness) were cut for each sample. Sections were deparaffinized in xylene and rehydrated in an ethanol gradient. Using standard procedures, histological stains were carried out for hematoxylin and eosin (H&E), alcian blue (AB), safranin-O (S-O), picosirius red (PR), and tartrate-resistant acid phosphatase (TRAP). For immunohistochemical analysis, antigens were retrieved by treatment in hot citrate buffer (20 min). Endogenous peroxidases were quenched subsequently through sequential exposure to 2% hydrogen peroxide (5 min), 2.5% periodic acid (5 min) and 0.02% sodium borohydride (5 min). Antibodies against fibrin(ogen) (1:1000, A0080, Dako, Carpinteria, CA), alpha smooth muscle actin (1:100 or 1:200, clone 1A4, Dako), and EGR-1 (1:1000 unless stated otherwise, sc-110, Santa Cruz Biotechnologies, Santa Cruz, CA) required blocking of endogenous biotin and avidin for 15 min (ABC system, Vectastain, Vector Laboratories, Burlingame, CA) followed by a blocking step with a mixture of 5% bovine serum albumin plus 10% goat serum and 1% mouse serum (for fibrin and EGR-1 staining) or 10% horse serum (for alpha smooth muscle actin staining) for 20 min in 1 × phosphate-buffered saline (PBS), while antibodies against type 1 procollagen (1:1000, Developmental Studies Hybridoma Bank, DSHB, University of Iowa, IA) and proliferating cell nuclear antigen (1:100, #sc-56, clone PC10, Santa Cruz Biotechnologies) only required conventional protein blocking (protein block solution, Dako). All primary antibodies were incubated over night at RT and subsequent washing steps were carried out three times with PBS at RT. Sections were washed and incubated in the corresponding biotinylated secondary antibody in PBS for 1 hour at RT. After additional washing, ABC solution (Vectastain ABC System) was applied for a 1 hour incubation. Then, sections were washed and the antibody visualized using DAB reagent (Sigma-Aldrich, St. Louis, MO). Mouse tibial and femoral growth plates served as positive controls. Digital bright field images from slides were captured using the Mirax Scan or an Axio 2 imaging system (Carl Zeiss, Thornwood, NY).

2.3. Micro computed tomography (microCT) analysis of fracture callus

Three-dimensional, quantitative morphological analysis of the calluses were performed on a microCT scanner (µCT 35, Scanco Medical, Bassersdorf, Switzerland). All bones were scanned in air. Images were acquired at 55 KVp, 0.36° rotation steps, 180° angular range, 400 ms exposure time and 3 frame averages per view with a 6 µm voxel size resolution. The Scanco microCT software (HP, DECwindows Motif 1.6) was used for 3D reconstruction, evaluation and viewing of images. After 3D reconstruction, the volumes of interest were segmented manually and analyzed using a global threshold of 0.4 g/ccm. Directly measured mineral densities (Apparent Mineral Density and TMD, both in mg/ml), total callus volume (TV in mm³), and bone volume within the callus (BV in mm³). In addition, the following parameters were calculated: fraction of bone contained within the callus (BV/TV in %), trabecular number (Tb.N in mm⁻¹), trabecular thickness (Tb.Th in mm), and trabecular separation (Tb.Sp in mm). Statistical analysis was carried out using a two-sample t-test assuming equal variance, p<0.05 was considered significant.

2.4 Semi-quantitative RT-PCR

Total RNA was extracted from PFD14 fracture calluses using Trizol (Invitrogen, Carlsbad, CA) and the RNeasy kit (Qiagen, Germantown, MD) as recommended by the product manuals. RNA was treated with DNase I (Qiagen) and cDNA was synthesized utilizing the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) as instructed by the manufacturer. We used the following primers: type 1 procollagen (COL1A1), F: 5'-ACGTCCTGGTGAAGTTGGTC-3' and R: 5'-CAGGGAAGCCTCTTTCTCCT-3' [29]; tissue non-specific alkaline phosphatase (TNAP), F: 5'-AAGGCTTCTTCTTGCTGGTG-3' and R: 5'-GCCTTACCCTCATGATGTCC-3' [30]; osteocalcin (OC), F: 5'-CGGGAGCAGTGTGAGCTTA-3' and R: 5'-AGGCGGTCTTCAAGCCATACT-3' [31]; tartrate-resistant acid phosphatase (TRAP), F: 5'-CCAATGCCAAGAGATCGCC-3' and R: 5'-TCTGTGCAGAGACGTTGCCAAG-3' [32]; cathepsin K (CTSK), F: 5'-AAAGCAGTACAACGGCAAGG-3' and R: 5'-GAGCTCATCTTGGGGAAG-3' [33]; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5'-TGCGACTTCAACAGCAACTC-3' and R: 5'-ATGTAGGCCATGAGGTCCAC-3' [34]. Amplifications were carried out in a total volume of 50 µl for 25–35 cycles with denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, and amplification at 72°C for 1 minute. Fifteen microliter aliquots of each reaction mixture were separated by size on a 1.5% agarose gel followed by photographic documentation under UV light in the presence of 0.2 µg/ml ethidium bromide. Gel band mean intensities relative to GAPDH were analyzed using the gel analyzing function in ImageJ (NIH, Bethesda, MD).

3. Results

3.1 A mouse rib osteotomy is a fracture model characterized by endochondral bone repair

To examine the consequences of loss of transcription factor EGR-1 on endochondral bone repair we explored a mouse rib fracture model. Figure 1 details the consecutive healing phases that were observed in wt mice utilizing this model. An intact control rib is shown as a reference (Fig. 1A). An osteotomy of the 8th rib resulted in temporary bleeding at the fracture site and subsequent blood clot formation. The fracture site between the damaged bone ends presented a typical morphology for hematoma formation, including absence of cohesive cellularity, and matrix embedded cells of hematopoietic appearance (Fig. 1B). During the inflammatory phase the hematoma gradually transitioned to a fibrous structure between the bone ends, and a pronounced periosteal increase in cellularity was visible (Fig. 1C and D). On PFD5, the morphology around the damaged bone changed with the invasion of significant amounts of mainly fibroblast-like cells (Fig. 1E). Concurrently, periosteal cartilage began to form (Fig. 1F). The onset of callus formation was seen on PFD7 and was discerned by a dense cellularity of fibroblast-like cells around the damage site that defined the periphery of the developing callus (Fig. 1G). In addition, the periosteal cartilage depositions increased (Fig. 1H). Mature callus was detected on PFD14. A cartilage bridge was seen between the edges of the bone ends and pronounced blood vessel invasion adjacent to periosteal cortical bone was observed (Fig. 1I and J). In agreement with previous reports [35–38], PFD21 was characterized by a network of trabecular bone which united the bone ends (Fig. 1K). Further, extended areas of bone marrow restoration were seen while cartilage deposits were still pronounced (Fig. 1K and L). Increased bone formation as well as remodeling of the trabecular bone was observed on PFD28 (Fig. 1M and N, Suppl. Fig. 1). Taken together, in wt mice the rib osteotomy repaired through a sequence of healing phases characteristic for endochondral bone repair. The onset of callus formation (PFD7), its maximal maturation (PFD14), and the stage of remodeling and continuing new bone formation (PFD28) defined the primary time-points that were chosen for comparison of endochondral bone formation between EGR-1 deficient and wt mice.

3.2. EGR-1 is expressed in the fracture callus

The potential expression of EGR-1 in the fracture callus was investigated by immunohistochemistry against EGR-1 on PFD14 samples. A majority of bone marrow cells expressed EGR-1, while no EGR-1 was detected in the bone-embedded osteocytes (Fig. 2A). Further, EGR-1 was detectable around blood vessels (Fig. 2B). In addition, cells in cartilaginous tissue stained positive for EGR-1 (Fig. 2C, Suppl. Fig. 2). Together, EGR-1 was detected in the mature wt callus in multiple cell types, including cells of the bone marrow and cartilage lineages.

3.3. Loss of EGR-1 results in persistent fibrin accumulation in the fracture gap

There was evidence for several distinct morphological abnormalities that impaired bone repair in EGR-1^{-/-} mice compared to wt mice. Initial comparisons between EGR-1^{-/-} and wt mice on PFD7 found in EGR-1^{-/-} animals amid the damaged bone ends a lack of cellularity accompanied by a fibrous build up (Fig. 3A and B). This abnormality was even more pronounced on PFD14 (Fig. 3C and D). At this time point, a similar size gap in wt mice was populated by cells (Fig. 3E and F). The fibrous build up stained negative for collagen (Fig. 3G) which is an abundant marker in callus of wt mice (Fig. 3H). This indicated that it did not originate from broken or devitalized bone. Because the histological appearance of the build up was reminiscent of the fibrin clot typical seen in the early phases of endochondral bone repair (Fig. 1C), it was probed specifically for fibrin which was demonstrated to compose entirely the observed build up (Fig. 3I and J). Fracture gaps of wt control mice did not show significant fibrin accumulation (Fig. 3K and L).

3.4. Abnormal callus ossification in EGR-1^{-/-} mice

In addition to the persistent fibrin accumulation, enlarged areas of cartilaginous and partially mineralized tissue appeared on PFD14 and gave the EGR-1^{-/-} a spherical overall shape (Fig. 4A) compared to wild type callus (Fig. 4B). Cartilage formation in EGR-1^{-/-} callus extended frequently perpendicular to the periosteal bone (Fig. 4C), while cartilage in wt callus formed adjacent to the bone surface (Fig. 4D). Morphological evidence for increased blood vessel density in the partially mineralized tissue of the enlarged EGR-1^{-/-} callus was seen (Fig. 4E), staining for alpha smooth muscle actin (Suppl. Fig. 3) confirmed the vessel-like structure as blood vessels. In comparison, wt callus presented with fewer, much larger blood vessels located in the proximity to the periosteal bone surface (Fig. 4F). Hyper-vascularized areas in EGR-1^{-/-} callus also stained positive for proliferating cell nuclear antigen (Fig. 4G and H). Proliferating cells were observed around blood vessels but also within the cartilaginous tissue. In comparison, proliferating cells in wt callus were largely restricted to bone marrow cells and were mostly seen in conjunction with blood vessels, their incidence in cartilaginous tissue was more limited. As callus ossification is driven by vascular invasion, the observation of hyper-vascularity and -proliferation in the callus of EGR-1^{-/-} mice was suggestive of an alteration in mineralized tissue formation within the callus. Therefore, we conducted a microCT analysis on a limited number of PFD14 calluses from EGR-1^{-/-} (n=5) and wt (n=5) mice. The EGR-1^{-/-} mouse callus showed a dumbbell-shaped, enlarged area of ossification (Fig. 4K) as compared to a typical callus of a wt animal (Fig. 4L). Quantitative analysis of the calluses (Suppl. Table 1) showed an overall 60% increase in total callus volume in EGR-1^{-/-} mice as compared to wt animals, however a considerable variability between mice was seen (Fig. 4M). A statistically significant 49% increase in bone volume within the EGR-1^{-/-} callus compared to wt calluses was observed (Fig. 4N). Combined, these changes resulted in a 10% decrease in the fraction of bone contained within the callus of EGR-1^{-/-} mice in comparison to wt animals (Fig. 4O). Together, this suggested that loss of EGR-1 leads to abnormal callus size and ossification characterized by hyper-vascularization and -proliferation.

In addition to PFD14, a comparison between EGR-1^{-/-} and wt calluses was made on PFD28, a time-point at which healing in the studied fracture model significantly progressed (Fig. 1). The bone ends united through formation of trabecular bone in the calluses of both EGR-1^{-/-} (P) and wt (Q) mice. Gross morphological appearance (Fig. 4P and Q) as well as cartilage (Fig. 4R and S) and collagen (Fig. 4T and U) amount and distributions were comparable between EGR-1^{-/-} and wt callus. These data indicate that healing advanced in both EGR-1^{-/-} and wt callus, suggesting EGR-1 is not essential for healing. However, collagen organization in EGR-1^{-/-} callus on PFD28 was sporadic (Fig. 4V and W) as compared to wt callus, which was more organized (Fig. 4X and Y). Thus, in comparison to wt callus, the impairment of bone healing in EGR-1 deficient callus did not fully resolve over the observation period of 28 PFDs.

3.5 Differential reduction of expression of bone formation and resorption markers in EGR-1^{-/-} callus

To provide first clues about potential molecular changes underlying the observed abnormality in callus ossification, expression of markers of bone formation and resorption were compared semi-quantitatively by RT-PCR between wt (n=8) and EGR-1^{-/-} (n=8) callus (Fig. 5). Expression of the early osteoblastic marker type 1 procollagen was similar in EGR-1^{-/-} as compared to wt callus. Likewise, no difference between EGR-1^{-/-} and wt callus was detected for alkaline phosphatase, an osteoblastic marker of extracellular matrix maturation. For the osteoblast marker osteocalcin, an indicator of matrix mineralization, a decrease in EGR-1^{-/-} compared to wt callus was observed. In comparison to the bone formation markers, a more pronounced difference between knock out and wt animals was seen for bone resorption markers. Both tartrate-resistant acid phosphatase and cathepsin K displayed lower levels of mRNA expression in EGR-1^{-/-} compared to wt callus. These observations were confirmed by calculation of changes in target gene expression using signal densitometry on independent RT-PCR reactions (n=3). We found in KO callus reductions of 0.1%, 2%, 9%, 4%, and 17% for type 1 procollagen, alkaline phosphatase, osteocalcin, tartrate-resistant acid phosphatase, and cathepsin K, respectively. Together, the differential reduction in expression of bone formation and resorption genes in the PFD14 callus of EGR-1^{-/-} mice was most significant for bone resorption markers.

3.6. EGR-1 deficiency results in cell deposits in skeletal muscle

Cell deposits in proximity to the callus were detected at PFD14 in EGR-1^{-/-} mice (Fig. 6A and B), but not in wt animals (Fig. 6C). The majority of these structures were located within the muscle surrounding the severed bones. The cells associated with the deposits displayed a morphology reminiscent of hematopoietic cells found within bone marrow, however, the origin or identity of these cells warrants further investigation. In an effort to better understand the biological state of the cell deposits, we tested against expression of the cell proliferation marker proliferating cell nuclear antigen and observed staining of the cells composing the ectopic deposits (Fig. 6D). This suggested that the cell deposits were compiled by viable, dividing cells. To exclude the possibility that the cell deposits were triggered by an unspecific response due to surgical intervention, we conducted sham operations in which the osteotomy scissors were put in position and pressed against the rib, but the bone was not clipped. Although a periosteal response was frequently seen in PFD14 sham operated ribs, no cell deposits were detected (data not shown).

4. Discussion

This loss-of-function study was conducted to assess the effect of deficiency in EGR-1 on bone repair. As a starting point, we decided to carry out a detailed morphological analysis in a mouse bone repair model. Our criteria for selection of the model were based on relevant

data on EGR-1 biology. First, *in vitro* studies have shown that EGR-1 is a regulator of expression of type II collagen [39], a chondrocyte marker. To be able to assess a potential cartilage abnormality due to loss of EGR-1, we aimed at utilizing a model that would repair through endochondral healing via a cartilage intermediate. Secondly, EGR-1 was recently shown to be critical for hematopoietic stem cell proliferation and migration post stress [40]. Thus, we intended to choose a model without the need for intramedullary stabilization; thereby excluding additional disturbance of the bone marrow compartment. Thirdly, EGR-1 is likely to respond to damage in tissues other than bone and bone marrow [6, 7]. Therefore, we strived to select a model with limited soft tissue damage in order to permit more specific observation of the bone/bone marrow compartment. A model that served all of the above requirements is a rib fracture model which was first described in rats by Hashimoto et al. [28]. It is based on the creation of an osteotomy, which is an established method for production of model fractures [41]. The work by Hashimoto et al. led to the adaptation of the model to mice [35–38, 42]. Since then, it has been frequently employed for fracture healing studies as it produces unstabilized, reproducible fractures that heal through an endochondral mechanism and unite around PFD21 via formation of trabecular bone [35–38, 42]. A limitation of the model, however, is that over 3–4 weeks post fracture no complete restoration of the rib occurs and additional long-term data will be needed to determine if the ribs completely restore. Therefore, the currently validated applications of the model are not well suited for the detection of abnormalities of complete bone restoration after fracture.

Figure 1 demonstrates that over an observation period of 28 PFDs the disrupted rib healed in discrete phases defined histologically as hemorrhage formation, inflammatory response, initiation of the callus, development of a mature callus, and onset of bone remodeling. This sequence is considered characteristic for endochondral fracture repair [43]. However, we cannot rule out the possibility that at a later time-point differential healing between this model and other fracture models may occur. Loss of EGR-1, which is normally expressed in the fracture callus (Fig. 2), resulted in three major alterations which we defined as persistent fibrin accumulation (Fig. 3), abnormal ossification (Fig. 4 and 5), and muscular cell deposits (Fig. 6).

The molecular mechanisms that lead to the observed abnormalities are presently not known. However, previous *in vitro* work has pointed to relevant *in vivo* targets of EGR-1. Transcriptional activation through EGR-1 has been shown for key regulators of coagulation, including tissue factor (TF) [44, 45], plasminogen activator inhibitor 1 (PAI-1) [46], and urokinase-type plasminogen activator (uPA) [27, 47]. Reduction of expression of the latter is likely to cause fibrin excess due to decreased plasmin production. Indeed, work by Lucerna et al. depleted free EGR-1 through expression of the EGR-1 co-repressor NAB2 and demonstrated that this leads to a complete loss of growth factor-induced uPA expression in endothelial cells [47]. On the contrary, reduced induction of TF or PAI-1 due to loss of EGR-1 would produce decreased amounts of fibrin. However, work by Cui et al. showed that the magnitude of TF promoter activation through the EGR-1 binding site is significantly dependent on the type of cellular stimulus [48]. Additionally, studies by Liao et al. demonstrated that specific mutation of the EGR-1 binding site resulted in only a 50% reduction of hypoxia-induced promoter stimulation which is still a 6-fold elevation when compared to normoxic controls [46]. Thus, a decrease in fibrin production as a result of reduction in TF expression might be limited and outweighed by the increase of fibrin that results from decreased plasmin production. Our *in vivo* observations reported here support the view that EGR-1 control of the uPA promoter may be relevant for proper fibrinolysis during endochondral bone repair. However, we cannot rule out the possibility that EGR-1 target genes are regulated indirectly and that other previously undefined genes may be involved. Further, we note that prior *in vitro* work has been carried out mainly in transformed cells and differences to the *in vivo* healing model presented here may exist.

We anticipate that the observed abnormal ossification is caused by alterations of a different set of EGR-1 targets. The hyper-vascularization of the hypertrophic cartilage may suggest an uncontrolled angiogenesis or vasculogenesis. The promoter of the principal angiogenic mitogen vascular endothelial growth factor A contains EGR-1 binding sites, however, activation of the promoter is complex and might be critically dependent on the mode of induction [49–51]. Further, expression of both PDGF-A/B [27, 52] and fibroblast growth factor 2 [16] is positively regulated by EGR-1. Thus, it is less likely that an EGR-1 deficiency directly provokes overactivity of these primary angiogenic growth factors. Interestingly, constitutive expression of the potent angiogenic inhibitor thrombospondin-1 is positively regulated by EGR-1 [53], and EGR-1 deficiency may result in reduced anti-angiogenic activity. Perhaps more importantly, recent work by Shin et al. and Bouchard et al. raises the possibility that EGR-1 is an inhibitor of both constitutive and induced matrix metalloproteinase 9 (MMP-9) expression [54, 55]. As previous work has demonstrated the critical importance of MMP-9 in vascularization of callus cartilage during endochondral fracture repair [56], it is possible that MMP-9 overproduction is associated with the observed hyper-vascularization.

Currently, we are in the process of determining potential changes in expression levels of EGR-1 targets thought to be involved in endochondral fracture repair, including, but not limited to, all the target genes discussed above. Presented in this study is an analysis of expression of bone formation and resorption markers. Utilization of RT-PCR permitted examination of samples from whole callus in a semi-quantitative fashion, however, a general limitation of RT-PCR is the inability to discern between expression levels per cell and changes in gene expression due to cell composition. In line with the detected ossification abnormality which is characterized by an excess of mineralized tissue in mature PFD14 callus of EGR-1^{-/-} mice, our RT-PCR experiment indicated a significant decrease in the bone resorption markers tartrate-resistant acid phosphatase and cathepsin K in the EGR-1^{-/-} compared to wt callus. In the bone compartment, both these markers are typically associated with osteoclast activity, suggesting a potential defect in osteoclast maturation or activity due to loss of EGR-1. Previous findings which have uncovered EGR-1 as a negative regulator of stromal cell expression of macrophage colony-stimulating factor [57–59], a cytokine essential for osteoclastogenesis, do not provide a direct explanation for the findings made in this study and thus further work on the potential functions of EGR-1 in osteoclast biology is warranted.

In contrast to the observed fibrin accumulation and abnormal ossification, interpretation of the detected cell deposits is more restricted. Our limited experimental data suggests that the cell deposits constitute viable, proliferating cells that are embedded within skeletal muscle. To our knowledge, no similar abnormality has been reported before. However, we cannot exclude the possibility that the observed cell deposits are part of the normal healing process and that loss of EGR-1 just provoked an increase in cell number, size, or persistence. Prior to any extended analysis we will attempt to identify the relevant cell types within the cellular deposits through electron microscopy. It is notable that all the discussed abnormalities occurred spatially separated in tissues of presumably different origins. In addition, all three alterations appear to produce a pathological excess of tissue that is normally not present. In future studies, it will be valuable to address the role of EGR-1 deficiency in bone repair after (1) stabilized long bone fracture to assess full bone reconstitution over time and (2) bone defect which heals predominantly through intramembranous bone formation. In addition, it will be important to define potential changes of fracture biomechanics due to loss of EGR-1.

The transcription factor EGR-1 is an immediate early gene, such as AP-1, and a series of studies has demonstrated that this class of transcription factors is crucial for bone formation, particularly during early development [60, 61]. Consequently, deficiency in immediate early

gene transcription factors is frequently lethal. In contrast, EGR-1^{-/-} mice are viable and data indicates that loss of EGR-1 affects skeletal development only mildly [20]. In adult EGR-1^{-/-} mice, decreased bone mass in limb bones has been reported [57], but data on EGR-1 deficient bone is sparse and further in-depth analysis is warranted. The study presented here is the first to report a role for EGR-1 in skeletal damage repair. In addition to being an immediate early gene, EGR-1 shares features with transcription factors such as HIF-1 α , which mediates a prompt response to physiological distress such as low oxygen tension [62]. Similar to HIF-1 α , EGR-1 has been reported to be increased under hypoxic conditions [6, 7, 63]. Intriguingly, EGR-1 also responds to inflammatory signals [11, 39, 64, 65] and mechanical stress in bone cells [66, 67], both of which are essential signals in postnatal bone repair. Taken together, EGR-1 might not only be a pleiotropic regulator but a coordinator of adult fracture healing with a unique function in the control of tissue formation and destruction during skeletal repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. EGR-1 deficiency was studied in a fracture model.
2. Loss of EGR-1 resulted in persistent fibrin accumulation in the callus.
3. Loss of EGR-1 led to altered bone formation in the callus.
4. Loss of EGR-1 was associated with callus hyper-vascularity and -proliferation.
5. Loss of EGR-1 caused cell deposits located within skeletal muscle.

Figure 1. Mouse rib fractures heal through endochondral fracture repair

(A) H&E staining of an intact wt mouse 8th rib. (B – N) Time course analysis of the bone healing pattern between PFD1 and PFD28. (B) An 8th rib on PFD1 after placement of an osteotomy. Arrows indicate the disconnected bone ends. (C) On PFD3, coagulation residue was seen between the damaged bone ends. The white frame in (C) is represented as a high magnification view in (D); showing typical characteristics of an inflammatory response, including a thickened periosteum along regions of non-viable cortical bone (arrows). (E) A characteristic change in cellularity due to invasion of mainly fibroblast-like cells from surrounding tissues was observed on PFD5 (arrows). (F) Further analysis revealed the initiation of chondrocyte formation in areas adjacent to the thickened periosteum. (G) On PFD7, increased organization of mainly fibroblast-like cells around the damaged bone was seen along with the onset of alignment of the developing callus (arrows). (H) Concurrently, large areas of cartilage chondrocytes near the damage site were detected on PFD7. (I and J) Adjacent sections of PFD14 showed a mature callus characterized by vascular ingrowth, extended areas of hypertrophic chondrocytes and cartilage bridging the disrupted bone ends. (K) Callus on PFD21 showed that the bone ends united through formation of a trabecular bone network. In addition, the bone marrow compartment expanded significantly. (L) PFD21 still had a marked cartilage intermediate. (M and N) Callus healing progressed on PFD28 with an increase in bone bridging and resorption of the cartilage tissue. (P) Callus on PFD28. Abbreviations: AB, alcian blue; BM, bone marrow; CB, cortical bone; Ch, chondrocytes; H&E, hematoxylin and eosin; M, muscle; PFD, post fracture day; Tb, trabecular bone; wt, wild type.

Figure 2. EGR-1 expression in mature callus of wt mice

All sections depicted show wt fracture callus on PFD14. (A–C) Samples were probed for EGR-1 by immunohistochemistry. (A) EGR-1 was detected in cells the bone marrow compartment but not in cells of cortical bone. (B) Cells associated with vascular structures probed positive for EGR-1. (C) EGR-1 expressing cells were also detected in the cartilage intermediate. (D–E) Staining controls omitting the primary antibody are shown for anatomically matched sections. Abbreviations: BM, bone marrow; CB, cortical bone; Ch, chondrocytes; PFD, post fracture day; wt, wild type.

Figure 3. Persistent fibrin accumulation at the fracture gap in $EGR-1^{-/-}$ mice

Shown is a comparison of endochondral bone healing between $EGR-1$ deficient and wt mice with a focus on PFD7 (A, B) and PFD14 (C–L). (A) On PFD7, $EGR-1^{-/-}$ mice presented with a gap between the disconnected bones that was filled with dense areas of fibrous build up (arrows). (B) In comparison, the disrupted bone ends in wt mice presented with a normal amount of fibrous tissue. (C and D) The abnormal fibrous build up (arrows) observed on PFD7 remained entopic and was pronounced on PFD14. (E and F) In comparison, a similar size gap in wt mice was occupied by cells. (G) The fibrous build up (arrows) did not react with picrosirius red, a stain for collagen. (H) Wild type PFD14 controls for picrosirius red. (I and J) Immunohistochemistry positively identified the fibrous deposits as fibrin accumulation (arrows). (K and L) Wild type PFD14 controls for anti-fibrin immunohistochemistry. Abbreviations: BM, bone marrow; CB, cortical bone; Ch,

chondrocytes; EGR-1, early growth response gene 1; H&E, hematoxylin and eosin; KO, knock out; M, muscle; PFD, post fracture day; PR, picrosirius red; wt, wild type.

Figure 4. Abnormal callus ossification in EGR-1^{-/-} mice

(A–O) EGR-1^{-/-} callus characterization on PFD14. (A) EGR-1 knockout mice exhibited a callus morphology characterized by large areas of mineralizing matrix, which gave the callus a spherical shape as compared to wt mice (B). The enlarged EGR-1^{-/-} callus constituted areas (A, area 1) with cartilaginous appearance and little H&E staining as well as adjacent areas (A, area 2) which presented with a typical appearance of mineralizing tissue after staining with H&E. (C) Extended cartilage formation, often perpendicular to the periosteal bone, in EGR-1^{-/-} callus was confirmed by safranin-O staining. (D) In wt callus, cartilage areas formed mostly adjacent to the periosteal bone surface. (E) Higher magnification view of an area similar to the one marked as area 2 in (A) indicated hyper-vascularization (arrows) and cartilage ossification in proximity to vessel ingrowth. (F) In wt callus, vessels were larger (arrows) and ingrowth occurred adjacent to the periosteal bone surface. (G) Areas similar to area 2 in (A) showed extended regions that stained positive for the proliferation marker proliferating cell nuclear antigen. (H) High magnification view demonstrating proliferating cells both as part of blood vessels (triangles) and the mineralizing cartilage (arrows). (I) In comparison, proliferating cells in wt callus included bone marrow cells (arrows) and were mostly seen in conjunction with blood vessels. (J) High magnification view from I. (K) MicroCT image of a pronounced EGR-1^{-/-} callus mineralization abnormality. (L) MicroCT image of a corresponding wt callus. (M) Total callus volume as measured by microCT. (N) Bone volume within the callus as measured by microCT. (O) Fraction of bone contained within the callus as measured by microCT. (P–Y) EGR-1^{-/-} callus characterization on PFD28. Gross morphological appearance was comparable between EGR-1^{-/-} (P) and wt (Q) callus. The bone ends united through formation of trabecular bone in the calluses of both EGR-1^{-/-} (P) and wt (Q) mice. Cartilage amount and distribution were also comparable between EGR-1^{-/-} (R) and wt (S) callus. Similarly, collagen quantity and distribution were equivalent between EGR-1^{-/-} (T) and wt (U) callus, however, EGR-1^{-/-} callus frequently appeared larger. (V) Collagen in the EGR-1^{-/-} callus was disorganized. (W) Higher magnification view of V. Arrows indicate collagen structures in trabeculae. (X) In contrast, wt callus showed a normal, more parallel organization. (Y) Higher magnification view of X. Arrows indicate collagen structures in trabeculae and triangles mark cortical bone fragments. Abbreviations: BM, bone marrow; BV, bone volume within the callus; BV/TV, fraction of bone contained within the callus; CB, cortical bone; Ch, chondrocytes; EGR-1, early growth response gene 1; H&E, hematoxylin and eosin; KO, knock out; n.s., statistically not significant; PCNA, proliferating cell nuclear antigen; PFD, post fracture day; PR, picrosirius red; S–O, safranin-O; Tb, trabecular bone; TV, total callus volume; wt, wild type.

Figure 5. Distinct reductions in expression of bone formation and resorption markers in $EGR-1^{-/-}$ callus

Semi-quantitative RT-PCR was utilized for gene expression analysis at three different amplification cycles. Samples were derived from PFD14 calluses of wt and $EGR-1^{-/-}$ (KO) mice. Shown is a representative image taken after chromatographic separation and DNA staining. Abbreviations: C, no template control; COL1A1, type 1 procollagen; CTSK, cathepsin K; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KO, knock out; OC, osteocalcin; TNAP, tissue non-specific alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; wt, wild type.

Figure 6. Ectopic cell deposits in skeletal muscle in *EGR-1*^{-/-} mice

(A and B) The *EGR-1*^{-/-} mice on PFD14 showed the appearance of ectopic cell deposits distant from the damaged bone site and located in surrounding muscle (arrows), while the wt skeletal muscle surrounding the damage site was completely undisturbed (C). (D) At varying intensities, all cells of the deposits stained positive for proliferating cell nuclear antigen, indicating viable, dividing cells as constituent of the cell deposits. Abbreviations: BM, bone marrow; CB, cortical bone; *EGR-1*, early growth response gene 1; H&E, hematoxylin and eosin; KO, knock out; M, muscle; PFD, post fracture day; PCNA, proliferating cell nuclear antigen; wt, wild type.