Up-Regulation of Expression of Selected Genes in Human Bone Cells With Specific Capacitively Coupled Electric Fields

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Received 30 May 2013; accepted 17 January 2014
Published online 18 March 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22595

ABSTRACT: The objective of the described experiments was to determine the electrical parameters that lead to optimal expression of a number of bone-related genes in cultured human bone cells exposed to a capacitively coupled electric field. Human calvarial osteoblasts were grown in modified plastic Cooper dishes in which the cells could be exposed to various capacitively coupled electric fields. The optimal duration of stimulation and optimal duration of response to the electrical field, and the optimal amplitude, frequency and duty cycle were all determined for each of the genes analyzed. Results indicated that a capacitively coupled electric field of 60 kHz, 20 mV/cm, 50% duty cycle for 2 h duration per day significantly up-regulated mRNA expression of a number of transforming growth factor (TGF)-β family genes (bone morphogenetic proteins (BMP)-2 and -4, TGF-β1, - β2 and -β3) as well as fibroblast growth factor (FGF)-2, osteocalcin (BGP) and alkaline phosphatase (ALP). Protein levels of BMP-2 and -4, and TGF-β1 and - β2 were also elevated. The clinical relevance of these findings in the context of a noninvasive treatment modality for delayed union and nonunion fracture healing is discussed. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:894–903, 2014.

Keywords: human bone cells; electrical stimulation; capacitive coupling; gene up-regulation; fracture healing

Bone homeostasis is controlled by a wide variety of systemic and local stimuli—both biochemical and physical. On the biochemical side, a major source of active polypeptide agents is the TGF-β superfamily that includes, among others, TGF-β isotypes and the BMPs.[1] In concert, these multifunctional polypeptides can act to control many aspects of bone development, growth, repair, remodeling, and differentiation.[1,2] On the physical side, there are a number of forces that have been used as adjuncts to bone healing including direct mechanical stimulation[3] as well as low-intensity ultrasound and various modalities of electrical stimulation.[4] Depending upon the clinical situation, all of these methods have been successfully used to contribute to bone healing.

It is postulated that applied physical forces (e.g., weight bearing, distraction osteogenesis, shear flow) may be transduced in bone through mechano-receptors that open stretch-activated ion channels in osteocytes to allow calcium and other ions to enter the cell and activate a multitude of chemical signaling cascades.[3] The biological consequences of low-intensity ultrasound stimulation appear to mimic those of fluid-induced shear flow.[5] It is the load-induced flow of interstitial fluid that may provide a convergence feature between mechanical and electrical signals. To provide the latter, there are a number of electrical signaling modalities that have been used to stimulate tissues and/or cells both in vitro and in vivo.[6–8] They include direct current, inductive fields, capacitive coupling, biphasic electric current and pulsed electromagnetic fields (PEMFs). Due to major differences in the clinical situation/experimental design, signal parameters and underlying mechanisms, it is difficult to compare the advantages and/or disadvantages of each modality, but a brief validation for capacitive coupling is found in the Discussion Section. Capacitively coupled electric stimulation, has been successfully used in vivo in animals to treat healing fractures[9,10] and to promote osseointegration,[11] and used in humans to treat nonunions[12] and as an adjunct to surgery to effect spine fusions.[13,14]

Although all of these forces appear to work by different mechanisms, they all result in an increase in intracellular calcium that can ultimately lead to the up-regulation of specific second messenger pathways to promote gene expression. In this way, the biochemical and physical outcomes are eventually linked.

Nearly 10% of bone fractures fail to heal in a timely manner resulting in nonunion.[15,16] Since it has long been known that bone tissue manifests electrical potentials in both normal and pathological states,[17] electrical stimulation has been used clinically to initiate osteogenesis. The first recorded use of electricity to heal nonunion of a fracture in a human was in 1812 at Saint Thomas Hospital in London, England. A Mr. Birch, one of the surgeons at Saint Thomas, healed a nonunion of a tibia of 13 months with "shocks of electric fluid" applied daily for 6 weeks. The patient "was then able to walk and left the hospital.[18] Mott in 1820[19] and Lente in 1850[20] recorded healing a combined total of four nonunions with galvanic stimulation. The next recorded use of electricity to form bone occurred 103 years later when, in 1953, Yasuda[21] demonstrated new bone formation in the vicinity of a negative electrode, or cathode, in a rabbit tibia when a current in the microampere range was applied for 3 weeks. This paper ignited great interest in the use of electricity in bone formation and fracture healing such that by 1985, 97 articles—by this author's count—had been published in the world's literature on the effects

Grant sponsor: Sponsored Research Agreement between Innovative Clinical Solutions (New York, NY); Grant sponsor: University of Pennsylvania.
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of various forms of electrical and electromagnetic fields on bone formation and fracture healing.

Recently, using a murine cell culture system, we showed that application of a specifically defined capacitively coupled electric field (60 kHz, 20 mV/cm for 24 h at a 50% duty cycle) resulted in an up-regulation of the mRNA expression levels of a number of osteoinductive BMPs (e.g., BMP-2, -4, -5, -6 and -7) while concomitantly only moderately affecting other BMPs (e.g., BMP-3 and -8) and the BMP antagonists gremlin and noggin.[22] In addition, levels of BMP-2 protein were doubled, as was alkaline phosphatase (ALP) activity under the same stimulation conditions. Because of the bone inductive properties of the BMPs that were responsive to electric stimulation, it was concluded that in a therapeutic setting, "an appropriate electrical stimulation applied in vivo could cause the local up-regulation of a number of osteogenic BMPs in a safe, effective, inexpensive, non-invasive and repeatable manner."[22]

Because of the clinical successes of noted above, we hypothesized that capacitively coupled electrical stimulation may increase the expression of some bone repair-related genes. Therefore, the primary purpose of the experiments described in the present study was to systematically determine the conditions for optimal up-regulation of a variety of relevant bone cell gene expressions in human bone cell cultures using capacitively coupled electric fields. This study showed that a capacitively coupled electric field of 60 kHz, 20 mV/cm for 2 h at 50% duty cycle significantly up-regulated the expression of a number of human osteogenic genes and/or proteins.

METHODS

Cell Culture

Human calvarial osteoblasts were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured using their proprietary Osteoblast Medium according to the vendor's instructions; we further supplemented this medium with 10% fetal calf serum (FCS), 50 μg/ml sodium ascorbate and 4 mM β-glycerophosphate. Cells (4th passage) were seeded onto poly-L-lysine-coated quartz glass surface of modified Cooper dishes[23] at ~5,000 cells/cm^2 and grown to visual confluence (~7 days) prior to stimulation.

Electrical Stimulation

The in vitro stimulation procedure has been described in detail previously.[24] Briefly, stainless steel electrodes were placed in contact with quartz cover slips on the top and bottom of the Cooper culture dish, and a uniform electric field was generated in the medium and cells between the electrodes. The duration, amplitude, frequency and duty cycle of this electrical field can be easily varied. All stimulation experiments were performed on cells at confluence. Control samples were treated exactly as above, side by side with the experimental samples, except that the electrodes were not connected to a power source. Using thermistor probes and a Digital Dual Channel Thermometer (Fisher Scientific, Pittsburgh, PA), simultaneous measurement of temperatures in the medium of unstimulated and electrical stimulated cultures showed that they were unchanged.

Histochemistry

Cells were stained in Cooper dishes for ALP activity[25] and mineral deposition.[26,27] For ALP staining, dishes at each time point were washed twice with PBS containing calcium and magnesium, fixed in cold 10% neutral buffered formalin for 15 min, rinsed with deionized water and not allowed to air dry. The fixed cells were immediately overlaid with 3 ml of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml fast red violet LB salt (Sigma Chemical Co., St. Louis, MO) for 45 min at room temperature in the dark. Culture dishes were rinsed with deionized water and were observed microscopically and photographed.

For mineral deposition, three dishes from each time point were washed and fixed as above. The fixed cells were stained with fresh 5% silver nitrate (Sigma Chemical Co.) in front of a 60-W lamp with foil placed behind the dishes to reflect the light for 60 min, and then washed at least three times with PBS. The unreacted silver was removed with 5% sodium thiosulfate (Sigma Chemical Co.) for 5 min, the dishes rinsed three times in distilled water, counterstained with nuclear fast red (Sigma Chemical Co.) for 5 min, and then rinsed in distilled water. After dehydration through graded alcohol and clearing in xylene, the dishes were coverslipped using permanent mounting medium. Samples were observed microscopically and photographed.

Assays

ALP activity was measured by a colorimetric assay.[28] Briefly, after culture for up to 21 days, cells were harvested at various times, homogenized in PBS and ALP activity was measured as the production of *p*-nitrophenol (PNP) from *p*-nitrophenyl phosphate (Sigma Chemical Co.); values were normalized to total DNA.[29]

RNA Isolation and Quantitative Polymerase Chain Reaction

At the end of the experiment, cultures were harvested in 2 ml Trizol Reagent (Life Technologies, Inc., Rockville, MD) and frozen at −80°C prior to total RNA isolation. The RNA was purified using elements of a one-step method combined with a column purification step (RNeasy Total RNA Kit, Qiagen, Inc., Valencia, CA) and subsequently digested with DNase I (DNA-*free* kit, Ambion, Austin, TX).[22]

Reverse transcription (RT) followed by quantitative real-time polymerase chain reaction (qPCR) (two-step) was performed as described previously.[22] Oligonucleotide primers for human ALP, BGP, BMP-2, and -4, FGF-2, TGF-β1, -β2, and -β3 and glyceraldehyde phosphate dehydrogenase (GAPDH, reference gene) were based on database sequences and were designed using PrimerExpress 2.0 (Applied Biosystems, Foster City, CA) software. To further ensure that the qPCR signal was generated from cDNA (as opposed to genomic DNA), primer pairs were placed in different exons or across an intron/exon boundary wherever possible. All primer pairs had melting temperatures of 58–60°C and yielded products 73–161 bp (Table 1). To verify that the expected products with respect to size and homogeneity were obtained, melting profiles of qPCR products were acquired after completion of the amplification process (Applied Biosystems), and aliquots were analyzed by electrophoresis on 2% agarose gels. Relative quantification of mRNA expression for each of the target genes was accomplished by normalizing to a reference gene, GAPDH, that is not affected by the stimulation conditions.[30] For each experiment, corrected ΔC_T values were obtained for control (unstimulated) cultures and experimental (stimulated) cultures whose RNA was isolated, reverse transcribed

Table 1. Oligonucleotide Primers Used for qPCR of Human Proteins

Gene	Genbank Number	Nucleotide Sequence (5′–3′)[a]	Amplicon Size (bp)
GAPDH	BC00160	5′-ATGGGGAAGGTGAAGGTCG-3′ 5′-TAAAAGCAGCCCTGGTGACC-3′	119
BMP-2	NM001200	5′-GTGGAATTGACTGGATTGTGGCT-3′ 5′-GGACACAGCATGCCTTAGGAAT-3′	161
BMP-4	NM130851	5′-TTCACCGTTTTCTCGACTCC-3′ 5′-AAACTTGCTGGAAAGGCTCA-3′	98
TGF-β1	NM000660	5′-TCCTGGCGATACCTCAGCAA-3′ 5′-GCCCTCAATTTCCCCTCCAC-3′	117
TGF-β2	NM001135599	5′-CTGTCCCTGCTGCACTTTTGTA-3′ 5′-TGTGGAGGTGCCATCAATACCT-3′	100
TGF-β3	NM003239	5′-CAGGGAGAAAATCCAGGTCA-3′ 5′-CTGCGTTCAGCATATCCAAA-3′	73
FGF-2	NM002006	5′-AGAAGAGCGACCCTCACATCAA-3′ 5′-TCCATCTTCCTTCATAGCCAGCT-3′	107
ALP	X14174	5′-GGGAACGAGGTCACCTCCAT-3′ 5′-TCGTGGTGGTCACAATGCC-3′	73
BGP	X53698	5′-TAGTGAAGAGACCCAGGCGC-3′ 5′-CACAGTCCGGATTGAGCTCA-3′	108

[a]Forward (sense) primer is listed first.

and analyzed by qPCR at the same time; for comparison within and between experiments, these values were expressed as the ratio of Treated to Control. To compare the relative expression levels of target genes, their qPCR values were directly normalized to GAPDH levels.

Protein Measurements by ELISA

Cultures were stimulated in the presence of 0.3% bovine serum albumin (instead of 10% FCS) for 2 h using the optimal signal, and 22 h later the cell layer and medium were separately analyzed for specific targets. Protein levels of BMP-2, BMP-4, FGF-2, TGF-β1, and -β2 were obtained by ELISA assays using commercially available kits (R&D Systems, Minneapolis, MN); the values were normalized to total DNA.

Statistical Analysis

Cell experiments were duplicated at least once with ≥ 3 dishes per group per experiment. In all RT-qPCR experiments, 3 aliquots of each sample (technical triplicates, $n = 1$) were analyzed; each reported value represents separate RNA isolations and RT reactions from at least three samples ($n \geq 3$) and analyzed as described above. For gene expression, the ratios of Treated/Control values were reported as the mean ± SD. The means among groups were compared by a one-way analysis of variance and the Tukey–Kramer multiple comparison test for considerable differences between groups. For protein measurements, the means between Control and Stimulated were compared by a Student's *t*-test. Statistical results were obtained using a graphing software package (KaleidaGraph 4.1, Synergy Software, Reading, PA); statistical significance was considered to be $p \leq 0.05$.

RESULTS

Characterization of Human Bone Cells

Since we could find nothing in the scientific literature or in the vendor's literature about the characteristics of these human calvarial bone cells in culture, the purpose of the initial histochemical, biochemical and expression analyses was to establish the suitability of these cells for our experiments.

Fourth passage fetal human bone cells reached visual confluence in 7 days. When grown for up to 21 days in the presence of vitamin C and β-glycerophosphate, the cells progressively accumulated an extracellular matrix and formed multilayer nodules that showed increasing amounts of ALP staining (Fig. 1A), mineral deposition (Fig. 1B) and ALP activity (Fig. 1C) characteristic of cultured human bone cells.[31,32]

To determine the baseline expression of the relevant genes in human calvarial bone cells in our standard culture conditions, qPCR was used to obtain relative levels. The data showed that these cells expressed relatively robust mRNA levels of the TGF-β isoforms and ALP, but lower levels of FGF-2 and BGP (Fig. 2). Surprisingly, the mRNA levels of BMP-2 and -4 were on the order of 1,000-fold less abundant (Fig. 2, *inset*) than the above factors.

Effect of Varying Capacitive Coupling Electrical Stimulation Parameters on mRNA Expression

Initial experiments to determine the duration of electric stimulation that gave the best overall response for the genes being studied employed a continuous (100% duty cycle) 60 kHz signal at a 20 mV/cm amplitude. These parameters had been previously shown to be effective in vivo.[12] Using durations ranging from 30 min to 24 h, the data showed that a 1 h stimulation period (at 100% duty cycle) was just as effective as longer time periods (Fig. 3). Using this stimulation period and keeping the other parameters fixed, the duty cycle was varied from 10% (viz., 1 min on, 9 min off) to 100% in the next set of experiments. Although there was no single duty cycle that was optimal for all

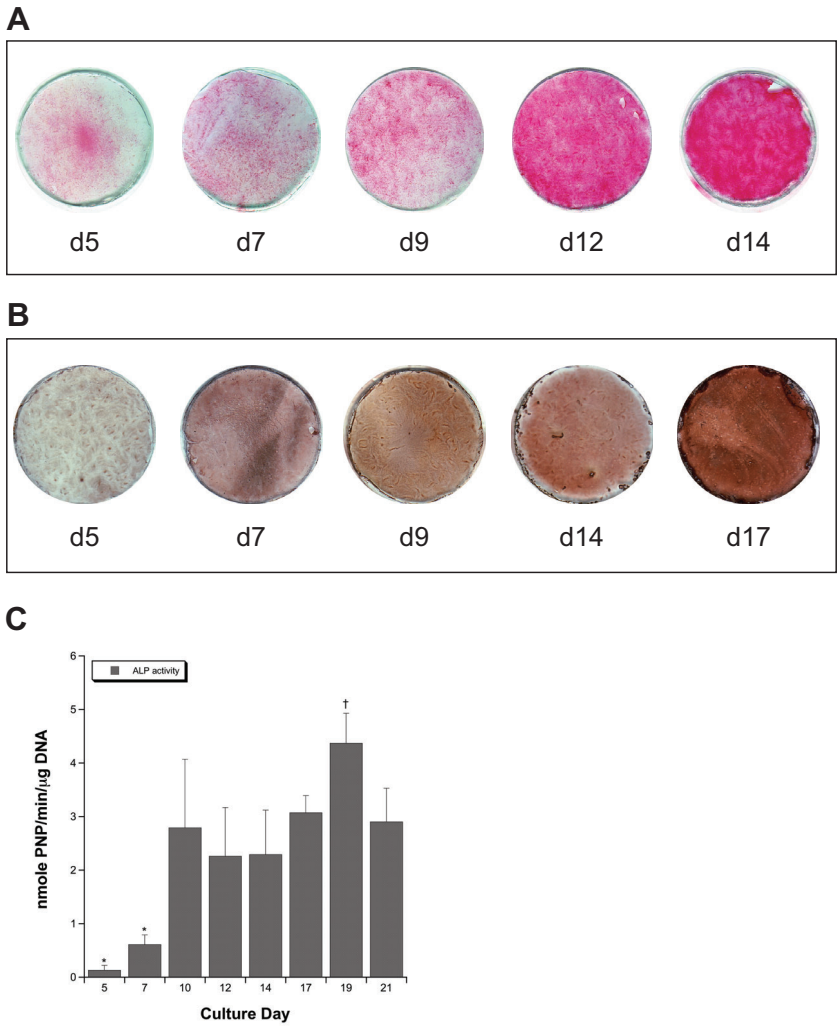

Figure 1. Histochemistry of human fetal calvarial bone cells in culture. (A) ALP staining; (B) von Kossa staining; (C) ALP activity (nmoles p-nitrophenol (PNP)/min/μg DNA). *Significantly different from all other days ($p < 0.01$); †significantly different from days 12, 14, and 17; n = 3 for ALP activity samples.

the genes studied (Fig. 4), we chose 50% (1 min on, 1 min off).

In the next set of experiments, the amplitude was varied keeping the duration, frequency and duty cycle fixed at 2 h, 60 kHz and 50% (power only on 1 h), respectively. It was clear that 20 mV/cm was the preferred amplitude for all genes studied (Fig. 5). Finally, the frequency was varied keeping the duration, amplitude and duty cycle fixed at 2 h, 20 mV/cm and 50%, respectively. The data showed that 60 kHz was the most effective for all the genes studied, although 30 and 45 kHz were sometimes equally effective for some genes (Fig. 6). Thus, the signal used for all subsequent experiments was 60 kHz, 20 mV/cm, 50% duty cycle for 2 h.

Duration of the Response to Electrical Stimulation

To determine how long the effect of electrical stimulation persisted, samples were stimulated under the conditions above and harvested immediately and after 1, 2, and 3 days without further stimulation. The results showed that the stimulatory effect for all genes diminished slowly during the first day, but then more rapidly thereafter (Fig. 7).

Effect of Electric Stimulation on Protein Synthesis

To determine whether protein levels of certain target protein were also elevated by electric stimulation using the optimal conditions described above, levels of BMP-2, BMP-4, FGF-2, TGF-β1, and -β2 were measured (Fig. 8). Similar to the gene expression results, levels of BMP-2 and -4 protein were very low in these cells. Nevertheless, there was an approximately two-fold increase in BMP protein after stimulation. For TGF-β1 and -β2, the protein increases were significant, but less than the corresponding increase in mRNA under the same conditions (Fig. 7C); FGF-2 protein levels were unchanged.

DISCUSSION

In 1970 we began to look into the reaction of bone to varying amounts of direct current.[33] Shortly thereafter

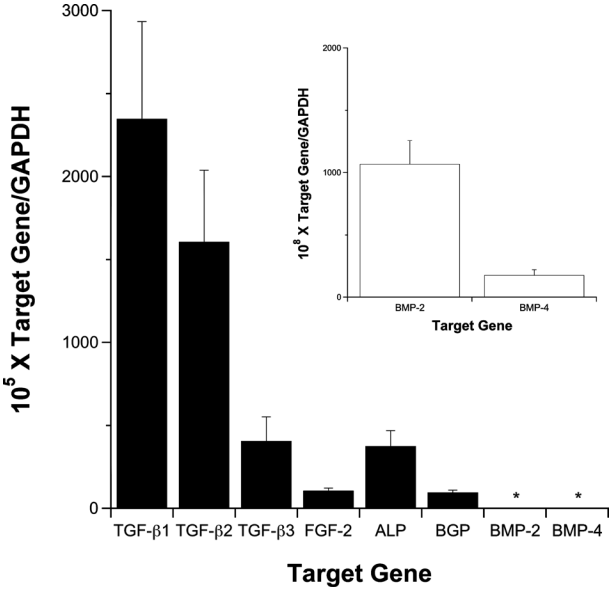

Figure 2. Relative mRNA expression in untreated human calvarial bone cells of a variety of relevant proteins. n = 6 for all samples. *See inset.

we treated a patient with nonunion of the medial malleolus of 15 months duration with direct current of 10 μA delivered via a stainless steel cathode inserted percutaneously across the nonunion site. In 9 weeks time the nonunion was healed. In the mid-1980s we began studying the effects of various capacitively coupled electric fields, first in the rabbit tibia,[9] and later in the treatment of recalcitrant nonunions in humans.[12] Today, capacitively coupled electrical stimulation is still used in the treatment of nonunions and delayed healing in fractures.[8]

According to Wikipedia, "in electronics, capacitive coupling is the transfer of energy within an electrical network by means of the capacitance between circuit nodes. Capacitive coupling is typically achieved by placing a capacitor in series with the signal to be coupled." In a biological context, electrodes are placed on opposite sides of a target tissue (e.g., fracture) and an external potential is applied. "The term capacitive is used since the arrangement of electrodes and the portion of the experimental [tissue target] to be stimulated usually resembles a capacitor, with tissue [or medium] constituting the interelectrode dielectric medium. [The term] coupling [is] in recognition of the fact that the electrodes, usually external, are placed at a distance away from the defect and that the stimulation is produced by a coupling between the electrode and the defect produced by an electric field." An external potential, usually between 1 and 10 V peak to peak at frequencies between 20 and 200 kHz, is applied (for technical reasons, the effective frequencies are usually 50–100 kHz). The relative high frequencies permit lower input voltages to be used. Within tissues, the local voltage gradients are 1–100 mV/cm, but no net current flows.[34] Besides being noninvasive, a

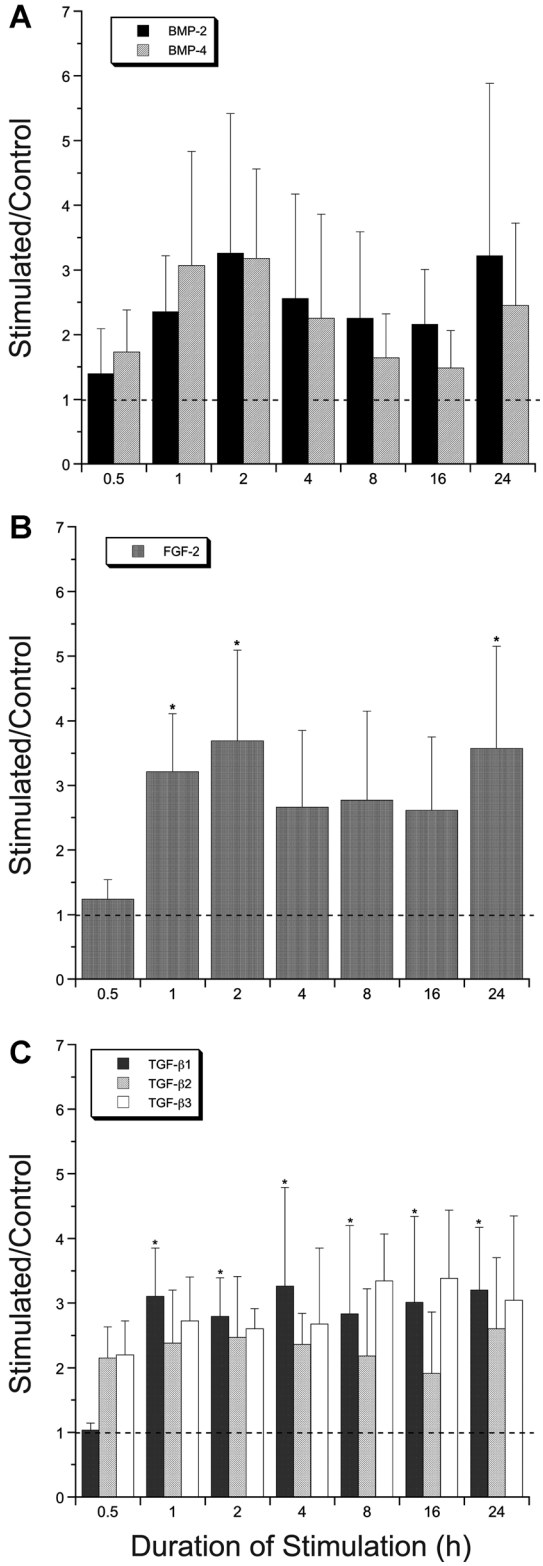

Figure 3. Effect of signal duration on bone cell gene expression. (A) BMP-2 and -4. All stimulated values were significantly different from unstimulated control values ($p < 0.04$). (B) FGF-2. *Significantly different from 0.5 h ($p < 0.02$); all stimulated values were significantly different from unstimulated control values ($p < 0.004$). (C) TGF-β1, -β2 and -β3. *Significantly different from TGF-β1 0.5 h ($p < 0.005$) but not each other; all stimulated values were significantly different from unstimulated control values ($p < 0.001$) except TGF-β1 at 0.5 h; n ≥ 5 for all samples. The *dashed line* represents the control value.

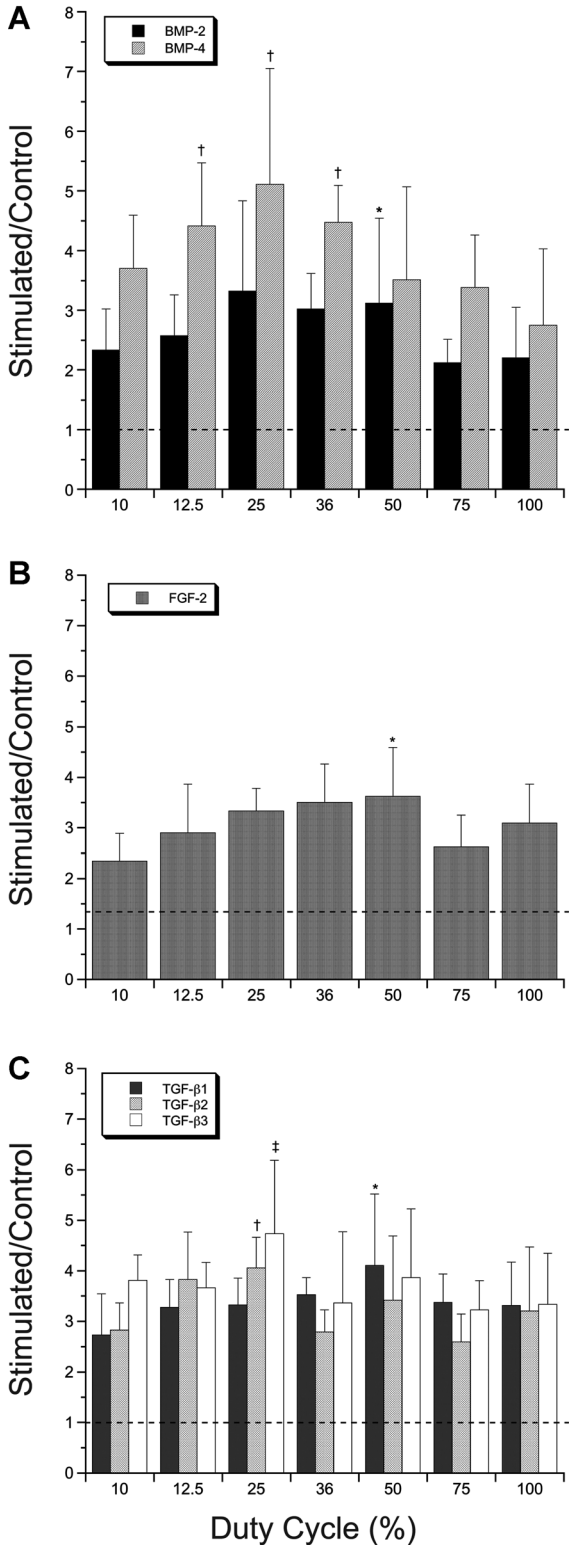

Figure 4. Effect of signal duty cycle on bone cell gene expression. (A) BMP-2 and -4. *Significantly different from BMP-2 at 100% ($p < 0.02$); †significantly different from BMP-4 at 100% ($p < 0.03$). (B) FGF-2. *Significantly different from 10% and 75% ($p < 0.03$). (C) TGF-β1, -β2 and -β3. *Significantly different from TGF-β1 at 10% and 100% ($p < 0.04$), †significantly different from TGF-β2 at 10%, 36%, and 75% ($p < 0.05$), ‡significantly different from TGF-β3 at 75% and 100%; $n \geq 8$ for all samples. For all samples, all stimulated values were significantly different from unstimulated control values ($p < 0.0001$). The *dashed line* in all panels represents the control value.

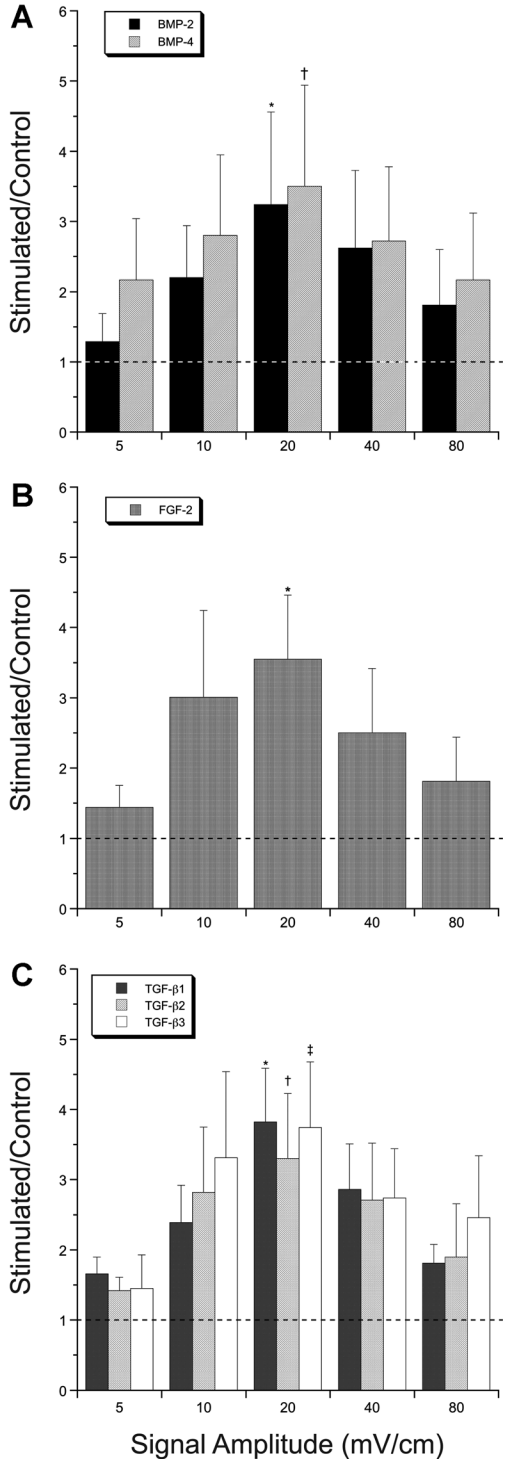

Figure 5. Effect of signal amplitude on bone cell gene expression. (A) BMP-2 and -4. *Significantly different from other BMP-2 groups ($p < 0.02$); †significantly different from other BMP-4 groups ($p < 0.03$); all stimulated values were significantly different from unstimulated control values except BMP-2 at 5 and 80 mV/cm ($p < 0.008$). (B) FGF-2. *Significantly different from other FGF-2 groups ($p < 0.05$); all stimulated values were significantly different from unstimulated control values ($p < 0.005$). (C) TGF-β1, -β2 and -β3. *Significantly different from other TGF-β1 groups ($p < 0.0001$); †significantly different from other TGF-β2 groups except 10 mV/cm ($p < 0.009$); ‡significantly different from other TGF-β3 groups except 10 mV/cm ($p < 0.0001$); all stimulated values were significantly different from unstimulated control values ($p < 0.0003$); $n \geq 6$ for all samples. The *dashed line* represents the control value.

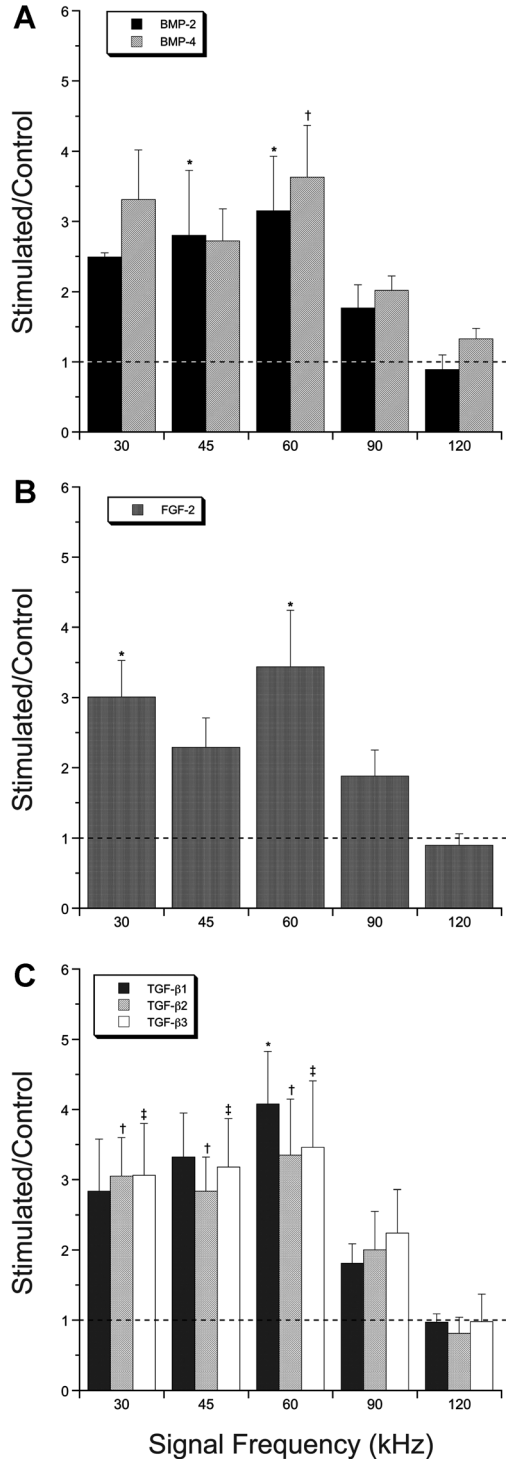

Figure 6. Effect of signal frequency on bone cell gene expression. (A) BMP-2 and -4. *Significantly different from BMP-2 at 30, 90, and 120 kHz ($p < 0.04$); †significantly different from other BMP-4 groups ($p < 0.05$); all stimulated values were significantly different from unstimulated control values except at 120 kHz ($p < 0.004$). (B) FGF-2. *Significantly different from 45, 90, and 120 kHz (<0.02); all stimulated values were significantly different from unstimulated control values ($p \leq 0.0004$) except at 120 kHz; (C) TGF-β1, -β2, and -β3. *Significantly different from other TGF-β1 groups ($p < 0.002$); †significantly different from TGF-β2 at 90 and 120 kHz ($p < 0.009$); ‡significantly different from TGF-β3 at 90 and 120 kHz ($p < 0.05$); all stimulated values were significantly different from unstimulated control values ($p \leq 0.0004$) except at 120 kHz; n ≥ 6 for all samples. The *dashed line* in all panels represents the control value.

major clinical advantage of this modality is that the power source can be small and portable and the electrodes can be incorporated into apparel and worn by the patient undergoing therapy.

Because of its clinical efficacy, the mechanism(s) by which capacitively coupled electrical stimulation initiates or enhances fracture healing were investigated by a series of laboratory studies. Using murine MC3T3-E1 cells, we found that a specific electric field with defined frequency, amplitude, duration, and duty cycle up-regulated a number of osteogenic BMP genes 4–6 fold[22] via the activation of voltage-gated calcium channels. Using a series of specific metabolic inhibitors, it was determined that the resulting influx of extracellular calcium leads to the stimulation of a downstream pathway involving calmodulin, calcineurin, and phosphorylated Nuclear Factor of Activated T-cells. The latter is transported to the nucleus, dephosphorylated and initiates a transcription process for relevant genes.[35,36] The activation of a calcium-dependent pathway is not unique to capacitive coupling, but appears to be a common cellular response to electric signals.[37–39] At the same time, it is also not unique to bone cells since we have found the same pathway active in human cartilage cells.[40] However, the Ca^{2+} source varies—inductive and combined fields stimulation release Ca^{2+} from intracellular stores that soon become depleted, whereas capacitive stimulation promotes the continued influx of extracellular Ca^{2+} via voltage-gated calcium channels as long as the signal is applied.[7,35]

In light of studies that have shown differences in physiological responses between murine and human bone cell culture models,[41] we chose to look at the effects of electrical stimulation on human calvarial osteoblasts—a proven model system.[42] Experiments were performed to determine the optimal stimulation duration, amplitude, frequency, duty cycle, and the duration of the response. In previous studies with chondrocytes and bone cells, we have found that the parameters of signal duration and duty cycle seem to be the most important.[22,43] The results reported here clearly show that the appropriate capacitively coupled electric signal (60 kHz, 20 mV/cm, 50% duty cycle for 2 h) significantly (~2.2–3.5 fold) up-regulates gene expression of ALP, BGP, FGF-2, and members of the TGF-β superfamily, and this up-regulation persists for at least 24 h. ALP activity did not appear to be affected perhaps due to the relatively short stimulation period (data not presented). It should be noted, that the overall results are different from those obtained with murine cells where a 24-h stimulation was required using the same signal parameters, but the up-regulation of BMPs was somewhat higher (FGF-2 and TGF-β isoforms were not measured).[22] Protein levels of most of the measured bone cell proteins were also significantly elevated after electrical stimulation.

All of the genes studied are intimately involved in osteogenesis and, hence, fracture healing. For exam-

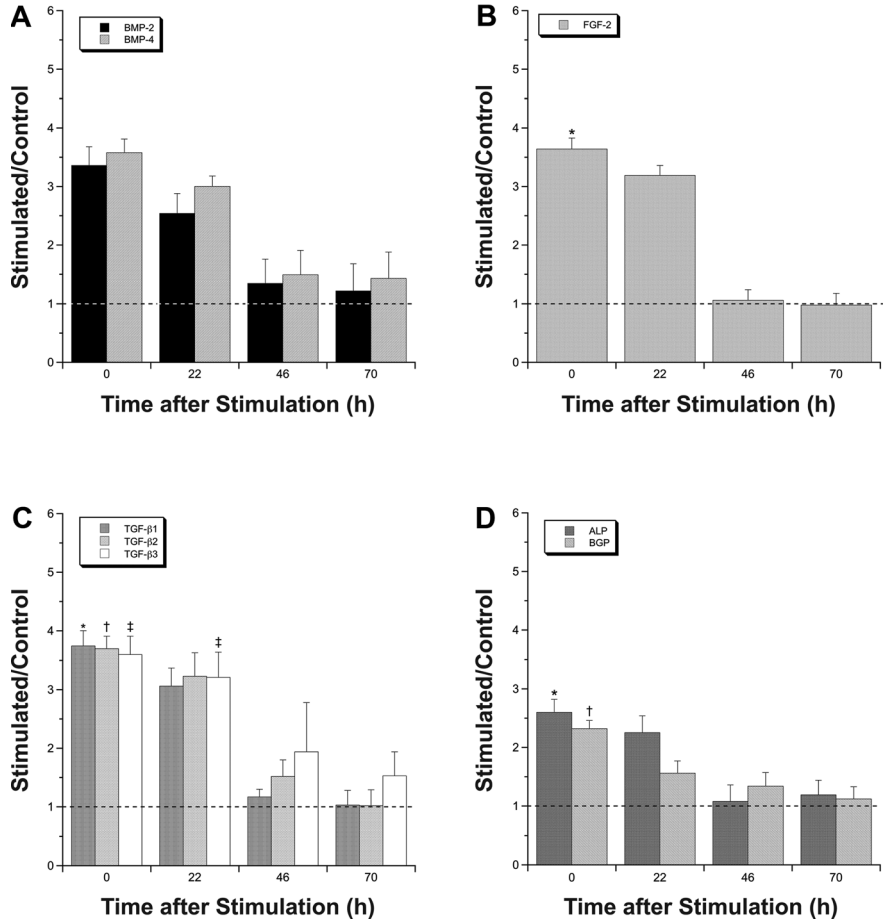

Figure 7. Duration of response of bone cell gene expression to electrical stimulation. Cells were stimulated at 60 kHz, 20 mV/cm for 2 h at 50% duty cycle at harvested at various times afterward. (A) BMP-2 and -4. *Significantly different from all other BMP-2 values ($p < 0.002$); †significantly different from all other BMP-4 values ($p < 0.009$). (B) FGF-2. *Significantly different from all other FGF-2 values ($p < 0.004$). (C) TGF-β1, -β2 and -β3. *Significantly different from all other TGF-β1 values ($p < 0.0001$), †significantly different from all other TGF-β2 values ($p < 0.013$), ‡significantly different from 46 and 70 h ($p < 0.0006$). (D) ALP and BGP. *Significantly different from all other ALP values ($p < 0.035$); †significantly different from all other BGP values ($p < 0.0001$). The *dashed line* in all panels represents the control value; n = 6 for all samples.

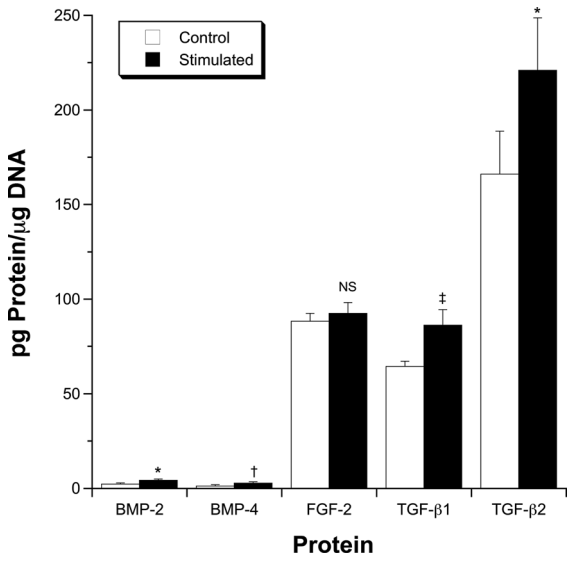

Figure 8. Effect of electric stimulation on bone cell protein expression. Cells were stimulated at 60 kHz, 20 mV/cm for 2 h at 50% duty cycle. *Significantly different from control values ($p < 0.04$); †significantly different from control values ($p < 0.006$); ‡significantly different from control values ($p < 0.003$).

ple, preclinical and clinical studies have shown that BMP-2 can be utilized in the treatment of fracture nonunions.[44,45] In other studies, FGF-2 accelerated fracture healing and prevented nonunions in nonhuman primates,[46] and TGF-β1[47] and BMP[48] levels were found to be lower in nonunions. It has also been shown that levels of another group of unrelated genes—the matrix metalloproteinases (MMPs)—are elevated in human nonunion patients.[49] Since capacitive coupling has been shown to down-regulate a number of MMPs in osteoarthritic cartilage,[24] it is possible that this noninvasive mode of treatment could promote fracture healing both by up-regulating the expression of anabolic proteins and down-regulating the expression of catabolic proteins.

CONCLUSION

This study showed that a capacitively coupled electric field up-regulated the expression of a number of osteogenic genes and/or proteins—including ALP, BGB, BMP-2, and -4, FGF-2 and TGF-β1, β2, and β3—in cultures of human calvarial bone cells. Since

the proteins encoded by these genes are critical factors in fracture healing, the results provide evidence for a mechanism whereby this noninvasive modality of treatment has been effective in nonunion fracture healing and spinal fusion. Although it is difficult to compare efficacy among different stimulation modalities because of the heterogeneity in clinical trial designs and the diversity in device specifications, a recent review[8] nevertheless recommended capacitive coupling as preferable to either direct current, PEMFs or ultrasound for the treatment of nonunions.

ACKNOWLEDGMENTS

This work was supported by a Sponsored Research Agreement between Innovative Clinical Solutions (New York, NY) and the University of Pennsylvania. Innovative Clinical Solutions had no role in the study design, in collection, analysis or interpretation of the data, or in preparation of the manuscript. C.C.C. and W.W. received compensation from the Sponsored Research Agreement.

REFERENCES

1. Centrella M, Horowitz MC, Wozney JM, et al. 1994. Transforming growth factor-β gene family members and bone. Endocr Rev 15:27–39.
2. Bonewald LF, Mundy GR. 1990. Role of transforming growth factor-beta in bone remodeling. Clin Orthop 250:261–276.
3. Klein-Nulend J, Bacabac RG, Bakker AD. 2012. Mechanical loading and how it affects bone cells: the role of the osteocyte cytoskeleton in maintaining our skeleton. Eur Cells Mater 24:278–291.
4. Nelson FRT, Brighton CT, Ryaby J, et al. 2003. Uses of physical forces in bone healing. J Am Acad Orthop Surg 11:344–354.
5. Khan Y, Laurencin CT. 2008. Fracture repair with ultrasound: clinical and cell-based evaluation. J Bone Joint Surg 90-A:138–144.
6. Aaron RK, Boyan BD, Ciombor DM, et al. 2004. Stimulation of growth factor synthesis by electric and electromagnetic fields. Clin Orthop 419:30–37.
7. Balint R, Cassidy NJ, Cartmell SH. 2013. Electrical stimulation: a novel tool for tissue engineering. Tissue Eng Part B 19:48–57.
8. Behrens SB, Deren ME, Monchik KO. 2013. A review of bone growth stimulation for fracture treatment. Curr Orthop Pract 24:84–91.
9. Brighton CT, Hozack WJ, Brager MD, et al. 1985. Fracture healing in the rabbit fibula when subjected to various capacitively coupled electrical fields. J Orthop Res 3:331–340.
10. Chang WH, Hwang I-M, Liu H-C. 1991. Enhancement of fracture healing by specific pulsed capacitively-coupled electric field stimulation. Front Med Biol Eng 3:57–64.
11. Shigino T, Ochi M, Hirose Y, et al. 2001. Enhancing osseointegration by capacitively coupled electric field: a pilot study on early occlusal loading in the dog mandible. Int J Oral Maxillofac Implants 16:841–850.
12. Brighton CT, Pollack SR. 1985. Treatment of recalcitrant non-union with a capacitively coupled electrical field. J Bone Joint Surg 67-A:577–585.
13. Bush JL, Vaccaro AR. 2000. Electrical stimulation in lumbar spinal fusion. Orthopedics 23:737–744.
14. Goodwin CB, Brighton CT, Guyer RD, et al. 1999. A double-blind study of capacitively coupled electrical stimulation as an adjunct to lumbar spinal fusions. Spine 24:1349–1357.
15. Court-Brown CT, McQueen MM, Tornetta PI. 2006. Nonunions and bone defects. In: Tornetta PI, Einhorn T, editors. *Trauma*. Philadelphia: Lippincott, Williams & Wilkins. p 503–519.
16. Kanakaris NK, Giannoudis PV. 2007. The health economics of the treatment of long-bone non-unions. Injury 38:S77–S84.
17. Friedenberg ZB, Brighton CT. 1966. Bioelectric potentials of bone. J Bone Joint Surg 48-A:915–923.
18. Peltier LF, Miller SH. 1981. A brief historical note on the use of electricity in the treatment of fractures. Clin Orthop Rel Res 181:4–7.
19. Mott V. 1820. Two cases of ununited fractures successfully treated by Seton. Med Surg Reg 1:375.
20. Lente FD. 1850. Cases of ununited fractures treated by electricity. NY J Med 5:317.
21. Yasuda I. 1953. Fundamental aspects of fracture treatment. J Kyoto Med Soc 4:395–406.
22. Wang Z, Clark CC, Brighton CT. 2006. Up-regulation of bone morphogenetic proteins (BMPs) in cultured murine bone cells using specific electric fields. J Bone Joint Surg 88:1053–1065.
23. Brighton CT, Okereke E, Pollack SR, et al. 1992. *In vitro* bone-cell response to a capacitively coupled electric field. The role of field strength, pulse pattern, and duty cycle. Clin Orthop Relat Res 285:255–262.
24. Brighton CT, Wang W, Clark CC. 2008. The effect of electric fields on gene and protein expression in human osteoarthritic cartilage explants. J Bone Joint Surg 90:833–848.
25. Yamauchi M, Yamaguchi T, Kaji H, et al. 2005. Involvement of calcium-sensing receptor in osteoblastic differentiation of mouse MC3T3-E1 cells. Am J Physiol Endocrinol Metab 288: E608–E616.
26. Luna L. 1968. *Manual of histologic staining methods of the armed forces institute of pathology*. New York: McGraw-Hill Book Company, Blakiston Division. p 176–177.
27. Mallory FB. 1961. *Pathological technique*. New York: Hafner Publishing Company. p 144.
28. Lowry OH, Roberts NR, Wu M, et al. 1954. The quantitative histochemistry of brain enzyme measurements. J Biol Chem 207:19–137.
29. Labarca C, Paigen K. 1980. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 120:344–352.
30. Zhuang H, Wang W, Seldes RM, et al. 1997. Electrical stimulation induces the level of TGF-β1 mRNA in osteoblastic cells by a mechanism involving calcium/calmodulin pathway. Biochem Biophys Res Commun 237:225–229.
31. Toesca A, Pagnotta A, Specchia N. 2001. Characterization of human bone cells in culture. It J Anat Embryol 106:13–26.
32. Kells AF, Coats SR, Schwartz HS, et al. 1995. TGF-β and PDGF act synergistically in affecting the growth of human osteoblast-enriched cultures. Connect Tissue Res 31:117–124.
33. Friedenberg ZB, Andrews ET, Smolenski BI, et al. 1970. Bone reaction to varying amounts of direct current. Surg Gynecol Obstet 131:894–899.
34. Black J. 1987. *Electrical stimulation. Its role in growth, repair, and remodeling of the musculoskeletal system*. Westport, CT: Praeger Publishers. p 225.
35. Brighton CT, Wang W, Seldes R, et al. 2001. Signal transduction in electrically stimulated bone cells. J Bone Joint Surg 83-A:1514–1523.
36. Lorich DG, Brighton CT, Gupta R, et al. 1998. Biochemical pathway mediating the response of bone cells to capacitive coupling. Clin Orthop Relat Res 350:246–256.
37. Ozawa H, Abe E, Shibasaki Y, et al. 1989. Electric fields stimulate DNA synthesis of mouse osteoblast-like cells (MC3T3-E1) by a mechanism involving calcium ions. J Cell Physiol 138:477–483.

38. Wang Q, Zhong SZ, Ouyang J, et al. 1998. Osteogenesis of electric stimulated bone cells mediated in part by calcium ions. Clin Orthop Relat Res 348:259–268.
39. Kim IS, Song JK, Song YM, et al. 2009. Novel effect of biphasic electric current on *in vitro* osteogenesis and cytokine production in human mesenchymal stromal cells. Tissue Eng Part A 15:2411–2422.
40. Xu J, Wang W, Clark CC, et al. 2009. Signal transduction in electrically stimulated articular chondrocytes involves transduction of extracellular calcium through voltage-gated channels. Osteoarthritis Cartilage 17:397–405.
41. Siggelkow H, Rebenstorff K, Kurre W, et al. 1999. Development of the osteoblast phenotype in primary human osteoblasts in culture: comparison with rat calvarial cells in osteoblast differentiation. J Cell Biochem 75:22–35.
42. Marie PJ, Debiais F, Hay E. 2002. Regulation of human cranial osteoblast phenotype by FGF-2, FGFR-2 and BMP-2 signaling. Histol Histopathol 17:877–885.
43. Wang W, Wang Z, Zhang G, et al. 2004. Up-regulation of cartilage matrix genes and products by electric fields. Clin Orthop Relat Res 427S:S163–S173.
44. Ronga M, Fagetti A, Canton G, et al. 2013. Clinical applications of growth factors in bone injuries: experience with BMPs. Injury 44:S34–S39.
45. Starman JS, Bosse MJ, Cates CA, et al. 2012. Recombinant human bone morphogenetic protein-2 use in the off-label treatment of nonunions and acute fractures: a retrospective review. J Trauma Acute Care Surg 72:676–681.
46. Kawaguchi H, Nakamura K, Tabata Y, et al. 2001. Acceleration of fracture healing in nonhuman primates by fibroblast growth factor-2. J Clin Endocrinol Metab 86:875–880.
47. Zimmermann G, Henle P, Kusswetter M, et al. 2005. TGF-beta1 as a marker of delayed fracture healing. Bone 36: 779–785.
48. Niikura T, Hak DJ, Reddi AH. 2006. Global gene profiling reveals a downregulation of BMP gene expression in experimental atrophic nonunions compared to standard healing fractures. J Orthop Res 24:1463–1471.
49. Henle P, Zimmermann G, Weiss S. 2005. Matrix metalloproteinases and failed fracture healing. Bone 37: 791–798.