# REGULATION OF TRANSFORMING GROWTH FACTOR – BETA (TGF-β) GENE EXPRESSION IN LIVING CELLS VIA THE APPLICATION OF SPECIFIC AND SELECTIVE ELECTRIC AND ELECTROMAGNETIC FIELDS

## Cross-Reference to Related Applications

[0001] The present patent application is a continuation-in-part patent application of U.S. Patent Application Serial No. 10/257,126, filed October 8, 2002, which is the U.S. national phase patent application of PCT/US01/05991, filed February 23, 2001, which, in turn, claims the benefit of the filing date of U.S. Provisional Patent Application Serial No. 60/184,491, filed February 23, 2000.

## Field of the Invention

[0002] The present invention is directed to a method of up-regulating transforming growth factor-beta (TGF- β) gene expression in living cells via the application of electric and electromagnetic fields generated by specific and selective electric and electromagnetic signals for the treatment of injured or diseased tissues, as well as devices for generating such signals.

## Background of the Invention

[0003] The bioelectrical interactions and activity believed to be present in a variety of biological tissues and cells are one of the least understood of the physiological processes. However, there has recently been much research into these interactions and activity regarding the growth and repair of certain tissues and cells. In particular, there has been much research into stimulation by electric and electromagnetic fields and its effect on the growth and repair of bone, cartilage, and various growth factors. Researchers believe that such research may be useful in the development of new treatments for a variety of medical problems.

[0004] Transforming growth factor – beta (TGF-β) is a pleiotropic growth factor that is present in most tissues and is implicated in cell proliferation, migration, differentiation, and survival. Consequently, TGF-β has clinical applications in diverse conditions such as angiogenesis, autoimmunity, bone repair (fractures, delayed unions, nonunions) and bone maintenance (osteoporosis), cartilage maintenance (degenerative arthritis), tumor suppression, and wound healing (Kim et al., J of Biochemistry and Molecular Biology, 38: 1-8, (2005); Janssens et al., Endocrine Reviews, 26: 743-774, (2005).

[0005] In acute fractures, delayed union and nonunion of fractures, and in various defects of bone, the formation of new, healing bone is dependent upon the presence of bone morphogenetic proteins (BMPs) to induce bone formation and TGF-βs to induce cartilage formation. In PCT Patent Application Serial No. PCT/US2005/00793, filed January 11, 2005 (claiming priority from U.S. Provisional Patent Application No. 60/535,755, filed January 12, 2004), it was shown that the gene expression of BMPs could be up-regulated by specific and selective electric and electromagnetic fields for the treatment of injured or diseased bone. It is shown herein that the gene expression of TGF-βs can also be up-regulated by specific and selected electric and electromagnetic fields. It is also shown herein that the optimal signal for the gene expression of BMPs is slightly different from that of TGF-βs, and this difference allows one to design a device that delivers one signal that maximally up-regulates the BMPs during the bone phase of fracture healing and another signal that primarily up-regulates the TGF-βs during the cartilage phase of fracture healing. This would be very useful in fracture healing, for instance, where the fracture callus is initially composed of cartilage that gradually is replaced by bone. By maximally up-regulating the TGF-βs to form cartilage early in the healing process, and maximally up-regulating the BMPs to form bone later in the healing process, one is able to optimize the healing of acute fractures, accelerate the healing in delayed fracture healing, and restart the healing process in nonunion fractures.

[0006] Up-regulation of TGF-β may also be useful in the treatment of the disease commonly known as osteoporosis, where bone demineralizes and becomes abnormally rarefied. Bone comprises an organic component of cells and matrix as well as an inorganic or mineral component. The cells and matrix comprise a framework of collagenous fibers that is impregnated with the mineral component of calcium phosphate (85%) and calcium carbonate (10%) that imparts rigidity to the bone. In healthy bone, bone formation and bone resorption are in balance. In osteoporosis, bone resorption exceeds bone formation, leading to bone weakening and possible vertebral body fracture and collapse. While osteoporosis is generally thought as afflicting the elderly, certain types of osteoporosis may affect persons of all ages whose bones are not subject to functional stress. In such cases, patients may experience a significant loss of cortical and cancellous bone during prolonged periods of immobilization. Elderly patients are known to experience bone loss due to disuse when immobilized after fracture of a bone, and such bone loss may ultimately lead to a secondary fracture in an already osteoporotic skeleton.

Diminished bone density may lead not only to vertebrae collapse, but also to fractures of hips, lower arms, wrists, ankles as well as incapacitating pains. Alternative non-surgical therapies for such diseases are needed.

[0007] Pulsed electromagnetic fields (PEMF) and capacitive coupling (CC) have been used widely to treat nonhealing fractures (nonunion) and related problems in bone healing since approval by the Food and Drug Administration in 1979. The original basis for the trial of this form of therapy was the observation that physical stress on bone causes the appearance of tiny electric currents that, along with mechanical strain, were thought to be the mechanisms underlying transduction of the physical stresses into a signal that promotes bone formation. Along with direct electric field stimulation that was successful in the treatment of nonunion, noninvasive technologies using PEMF and capacitive coupling (where the electrodes are placed on the skin in the treatment zone) were also found to be effective. PEMFs generate small, induced currents (Faraday currents) in the highly-conductive extracellular fluid, while capacitive coupling directly causes currents in the tissues; both PEMFs and CC thereby mimic endogenous electrical currents.

[0008] The endogenous electrical currents, originally thought to be due to phenomena occurring at the surface of crystals in the bone, have been shown to be due primarily to movement of fluid containing electrolytes in channels of the bone containing organic constituents with fixed negative charges, generating what are called "streaming potentials." Studies of electrical phenomena in bone have demonstrated a mechanical-electrical transduction mechanism that appears when bone is mechanically compressed, causing movement of fluid and electrolytes over the surface of fixed negative charges in the proteoglycans and collagen in the bone matrix. These streaming potentials serve a purpose in bone, and, along with mechanical strain, lead to signal transduction that is capable of stimulating bone cell synthesis of a calcifiable matrix, and, hence, the formation of bone.

[0009] The main application of direct current, capacitive coupling, and PEMFs has been in orthopedics in healing of nonunion bone fractures (Brighton et al., *J. Bone Joint Surg.* 63: 2–13, 1981; Brighton and Pollack, *J. Bone Joint Surg.* 67: 577–585, 1985; Bassett et al., *Crit. Rev. Biomed. Eng.* 17: 451–529, 1989; Bassett et al., *JAMA* 247: 623–628, 1982). Clinical responses

have been reported in avascular necrosis of hips in adults and Legg-Perthes's disease in children (Bassett et al., *Clin. Orthop.* 246: 172–176, 1989; Aaron et al., *Clin. Orthop.* 249: 209–218, 1989; Harrison et al., *J. Pediatr. Orthop.* 4: 579–584, 1984). It has also been shown that PEMFs (Mooney, *Spine* 15: 708–712, 1990) and capacitive coupling (Goodwin, Brighton et al., *Spine* 24: 1349–1356, 1999) can significantly increase the success rate of lumbar fusions. There are also reports of augmentation of peripheral nerve regeneration and function and promotion of angiogenesis (Bassett, *Bioessays* 6: 36–42, 1987). Patients with persistent rotator cuff tendonitis refractory to steroid injection and other conventional measures, showed significant benefit compared with placebo-treated patients (Binder et al., *Lancet* 695–698, 1984). Finally, Brighton et al. have shown in rats the ability of an appropriate capacitive coupling electric field to both prevent and reverse vertebral osteoporosis in the lumbar spine (Brighton et al., *J. Orthop. Res.* 6: 676–684, 1988; Brighton et al., *J. Bone Joint Surg.* 71: 228–236, 1989).

**[0010]** More recently, research in this area has focused on the effects stimulation has on tissues and cells. For example, it has been conjectured that direct currents do not penetrate cellular membranes, and that control is achieved via extracellular matrix differentiation (Grodzinsky, *Crit. Rev. Biomed. Eng.* 9:133–199, 1983). In contrast to direct currents, it has been reported that PEMFs can penetrate cell membranes and either stimulate them or directly affect intracellular organelles. An examination of the effect of PEMFs on extracellular matrices and *in vivo* endochondral ossification found increased synthesis of cartilage molecules and maturation of bone trabeculae (Aaron et al., *J. Bone Miner. Res.* 4: 227–233, 1989). More recently, Lorich et al. (*Clin. Orthop. Related Res.* 350: 246–256, 1998) and Brighton et al. (*J. Bone Joint Surg.* 83-A, 1514–1523, 2001) reported that signal transduction of a capacitively coupled electric signal is via voltage gated calcium channels, whereas signal transduction of PEMFs or combined electromagnetic fields is via the release of calcium from intracellular stores. In all three types of electrical stimulation there is an increase in cytosolic calcium with a subsequent increase in activated (cytoskeletal) calmodulin.

**[0011]** It was reported in 1996 by the present inventors that a cyclic biaxial 0.17% mechanical strain produces a significant increase in TGF-$\beta_1$ mRNA in cultured MC3T3-E1 bone cells in a cooper dish (Brighton et al., *Biochem. Biophys. Res. Commun.* 229: 449–453, 1996). Several significant studies followed in 1997. In one study it was reported that the same cyclic

biaxial 0.17% mechanical strain produced a significant increase in PDGF-A mRNA in similar bone cells (Brighton et al., *Biochem. Biophys. Res. Commun.* 43: 339–346, 1997). It was also reported that a 60 kHz capacitively coupled electric field of 20 mV/cm produced a significant increase in TGF-$\beta_1$ in similar bone cells in a cooper dish (Brighton et al., *Biochem. Biophys. Res. Commun.* 237: 225–229, 1997). However, the effect such a field would have on other genes within the body has not been reported in the literature.

**[0012]** In the above-referenced parent patent application, entitled *Regulation of Genes Via Application of Specific and Selective Electrical and Electromagnetic Signals*, methods were disclosed for determining the specific and selective electrical and electromagnetic signals for use in creating fields for regulating target genes of diseased or injured tissues. The present invention builds upon the technique described therein by describing the method of regulating expression of one targeted gene family, namely, TGF-β's gene expression, through application of a field generated by a specific and selective electrical and electromagnetic signal, for the treatment of fresh fractures, fractures at risk, delayed unions, nonunion of fractures, bone defects, spine fusions, osteonecrosis or avascular necrosis, as an adjunct to other therapies in the treatment of one or all of the above, and in the treatment of osteoporosis..

## Summary of the Invention

**[0013]** The present invention relates to regulating transforming growth factor-beta (TGF-β) gene expression in bone cells (as an example) via the application of specific and selective electric and/or electromagnetic fields generated by specific and selective electric and/or electromagnetic signals applied to electrodes. By performing sequential dose-response curves on the electric signal duration, amplitude, frequency, and duty cycle in which the effects of the resultant electric field are measured, the optimal signal for up-regulating TGF-β mRNA in bone cells was discovered. The optimal signal generated a capacitively coupled electric field with an amplitude of 20-40 mV/cm, a duration of 24 hours, a frequency of 60 kHz, and a duty cycle of 50%. In particular, the present invention relates to up-regulating TGF-β 1, 2, and 3 gene expression in bone cells via the application of fields generated by such signals.

**[0014]** In an exemplary embodiment of the invention, methods are provided to specifically and selectively up-regulate the gene expression (as measured by mRNA) of TGF-β1,

TGF-β2, and TGF-β3 with capacitively coupled electric fields, electromagnetic fields, or combined fields. Fresh fractures, fractures at risk, delayed unions, nonunion fractures, bone defects, osteonecrosis, osteoporosis, and the like are treated with a capacitively coupled electric field of about 20-40 mV/cm with an electric field duration of about 24 hours, a frequency of 60 kHz, a duty cycle of about 50%, and a sine wave configuration that causes the expression of TGF-βs 1, 2, and 3 to be up-regulated. In accordance with the method of the invention, a "specific and selective" signal is a signal that has predetermined characteristics of amplitude, duration, duty-cycle, frequency, and waveform that up-regulates the expression of the TGF-β genes (specificity). This allows one to choose different signals to up-regulate TGF-β gene expressions in order to achieve a given biological or therapeutic response (selectivity). The invention further relates to devices employing the methods described herein to generate specific and selective signals that create electric fields to up-regulate the expression of TGF-β genes.

**[0015]** In related aspects, the invention relates to methods and devices for the treatment of fresh fractures, fractures at risk, delayed unions, nonunions, bone defects, spine fusion, osteonecrosis, as an adjunct to other therapies treating one or more of the above, and in the treatment of osteoporosis. The method of the invention also includes the methodology for determining the "specific and selective" signal for TGF-β gene expression by methodically varying the duration of a starting signal known to increase, or suspected to increase, cellular production of TGF-βs. After finding the optimal duration, the amplitude of the signal is varied for the optimal duration of time as determined by the gene expression of TGF-β 1, 2, and 3. The duty cycle, frequency, and waveform are varied methodically in the same dose response manner as above while keeping the other signal characteristics constant. This process is repeated until the optimal signal is determined that produces the greatest increase in the expression of TGF-βs.

**[0016]** These and other aspects of the present invention will be elucidated in the following detailed description of the invention.

## Brief Description of the Drawings

**[0017]** **Figure 1** is a graphic representation of the mRNA expression of TGF-βs 1, 2, and 3 when bone cells are exposed to a 20 mV/cm capacitively coupled electric field for various time

durations. As indicated, the maximum expression for the various TGF-β mRNAs occurred with a signal of 24 hours duration.

[0018] **Figure 2** is a graphic representation of the mRNA expression of TGF-β 1, 2, and 3 when bone cells are exposed to various capacitively coupled electric field amplitudes with a duration of 24 hours. As indicated, the maximum expression for the various TGF-β mRNAs occurred with a field amplitude of 20-40 mV/cm.

[0019] **Figure 3** is a graphic representation of the mRNA expression of TGF-β 1, 2, and 3 when bone cells are exposed to various capacitively coupled electric field frequencies with a field amplitude of 20-40 mV/cm and a signal duration of 24 hours. As indicated, the maximum expression for the various TGF-β mRNAs occurred with a frequency of 60 kHz.

[0020] **Figure 4** is a graphic representation of the mRNA expression of TGF-β 1, 2, and 3 when bone cells are exposed to various capacitively coupled electric field duty cycles with a frequency of 60 kHz, a field amplitude of 20 mV/cm, and a signal duration of 24 hours. As indicated, the maximum expression for the various TGF-β mRNAs occurred with a 50% to 100% duty cycle with a sine wave configuration.

[0021] **Figure 5** is a graphic representation of the TGF-β1 protein when bone cells are exposed 24 hours to a capacitively coupled electric field of a 50% duty cycle with a field amplitude of 20 mV/cm or 40mV/cm, a frequency of 60 kHz, and a sine wave configuration. As indicated, the amount of TGF-β1 protein increase was the same with either 20 or 40 mV/cm.

[0022] **Figure 6** is a graphic representation of BMP-2 protein when bone cells are exposed 24 hours to a capacitively coupled electric field of a 50% duty cycle with a field amplitude of 20 mV/cm or 40 mV/cm, a frequency of 60 kHz, and a sine wave configuration. As indicated, unlike the TGF-β1 response shown in Figure 5, there was no significant increase in BMP-2 protein production at a field of 40 mV/cm as compared to that occurring at 20mV/cm.

[0023] **Figure 7** is a graphic representation of BMP mRNA expression when bone cells are exposed to a 50% duty cycle, capacitively coupled electric field (20 mV/cm, 60 kHz, sine wave) for 24 hours. Comparing this figure to Figure 2 shows the clear distinction between the

lack of response of the bone cell production of BMP protein in a 40 mV/cm field versus the very significant increase of the bone cell production of TGF-β protein in a 40 mV/cm field.

[0024] **Figure 8** illustrates the BMP gene expression of Figure 7 on the same graph as the TGF-β gene expression of Figure 2.

[0025] **Figure 9** is a diagram illustrating a device for the treatment of osteoarthritis of the knee, in accordance with a preferred embodiment of the present invention.

## Detailed Description of Preferred Embodiments of the Invention

[0026] The invention will be described in detail below with reference to **Figures 1–9** Those skilled in the art will appreciate that the description given herein with respect to those figures is for exemplary purposes only and is not intended in any way to limit the scope of the invention. All questions regarding the scope of the invention may be resolved by referring to the appended claims.

[0027] The present invention is based on the discovery that the expression of certain genes can be regulated by the application of fields generated by specific and selective electric and/or electromagnetic signals. In other words, it has been discovered by the present inventor that there is a specific electric and/or electromagnetic signal that generates a field for regulating each gene in bone, cartilage and other tissue cells and that these specific signals are capable of specifically and selectively regulating the genes in such cells. In particular, gene expression governing the growth, maintenance, repair, and degeneration or deterioration of tissues or cells can be regulated in accordance with the invention via the application of fields generated by specific and selective electric and/or electromagnetic signals so as to produce a salutary clinical effect. Such discoveries are useful in the development of treatment methods that target certain medical conditions including fresh bone fractures, fractures at risk, delayed union, nonunion, bone defects, spine fusion, osteonecrosis, as an adjunct in the treatment of any one or more of the above, and in the treatment of osteoporosis.

[0028] As used herein, the phrase "signal" is used to refer to a variety of signals including mechanical signals, ultrasound signals, electromagnetic signals and electric signals output by a device. It is to be understood that the term "field" as used herein refers to an

electrical field within targeted tissue, whether it is a combined field or a pulsed electromagnetic field or generated by direct current, capacitive coupling or inductive coupling.

[0029] The phrase "remote" is used to mean acting, acted on or controlled from a distance. "Remote" regulation refers to controlling the expression of a gene from a distance. To provide "remotely" refers to providing from a distance. For example, providing a specific and selective signal from a remote source can refer to providing the signal from a source at a distance from a tissue or a cell, or from a source outside of or external to the body.

[0030] The phrase "specific and selective" signal means a signal that produces an electric field that has predetermined characteristics of amplitude, duration, duty cycle, frequency, and waveform that up-regulate or down-regulate a targeted gene or targeted functionally complementary genes (specificity). This allows one to choose different "specific and selective" signals to up-regulate or down-regulate expression of various genes in order to achieve a given biological or therapeutic response (selectivity).

[0031] The term "regulate" means to control gene expression. Regulate is understood to include both up-regulate and down-regulate. Up-regulate means to increase expression of a gene, while down-regulate means to inhibit or prevent expression of a gene.

[0032] "Functionally complementary" refers to two or more genes whose expressions are complementary or synergistic in a given cell or tissue.

[0033] "Tissue" refers to an aggregate of cells together with their extracellular substances that form one of the structural materials of a patient. As used herein, the term "tissue" is intended to include any tissue of the body including muscle and organ tissue, tumor tissue as well as bone or cartilage tissue. Also, the term "tissue" as used herein may also refer to an individual cell.

[0034] "Patient" refers to an animal, preferably a mammal, more preferably a human.

[0035] The present invention provides treatment methods and devices that target certain tissues, cells or diseases. In particular, the gene expression associated with the repair process in injured or diseased tissues or cells can be regulated by the application of fields generated by

electric signals that are specific and selective for the genes to be regulated in the target tissues or cells. Gene expression can be up-regulated or down-regulated by the application of signals that are specific and selective for each gene or each set of complementary genes so as to produce a beneficial clinical effect. For example, a particular specific and selective signal may create an electric field that up-regulates a certain desirable gene expression, while the same or another particular specific and selective signal may create an electric field that down-regulates a certain undesirable gene expression. A certain gene may be up-regulated by a field generated by one particular specific and selective signal and down-regulated by a field generated by another specific and selective signal. Those skilled in the art will understand that certain diseased or injured tissues can be targeted for treatment by regulating those genes governing the growth, maintenance, repair, and degeneration or deterioration of the tissues.

**[0036]** The methods and devices of the present invention are based on identifying those signals that generate fields that are specific and selective for the gene expression associated with certain targeted diseased or injured tissue. For example, electricity in its various forms (e.g., capacitive coupling, inductive coupling, combined fields) can specifically and selectively regulate gene expression in targeted tissues or cells in a patient's body by varying the frequency, amplitude, waveform or duty cycle of the applied field for each selected gene. The duration of time exposed to electricity can also influence the capability of electricity to specifically and selectively regulate gene expression in targeted tissues or cells in a patient's body. Specific and selective signals may generate electric fields for application to each gene systematically until the proper combination of frequency, amplitude, waveform, duty cycle, and duration is found that provides the desired effect on gene expression.

**[0037]** It is to be understood that a variety of diseased or injured tissues or disease states can be targeted for treatment because the specificity and selectivity of an electric field for a certain gene expression can be influenced by several factors. In particular, an electrical field of appropriate frequency, amplitude, waveform and/or duty cycle can be specific and selective for the expression of certain genes and thus provide for targeted treatments. Temporal factors (e.g., duration of time exposed to the electrical field) can also influence the specificity and selectivity of an electric field for a particular gene expression. The regulation of gene expression may be more effective (or made possible) via the application of an electrical field for a particular

duration of time. Therefore, those skilled in the art will understand that the present invention provides for varying the frequency, amplitude, waveform, duty cycle and/or duration of application of an electric field until the electric field is found to be specific and selective for certain gene expressions in order to provide for treatments targeting a variety of diseased or injured tissue or diseases.

[0038] Thus, the present invention can provide for targeted treatments because it is possible to regulate expression of certain genes associated with a particular diseased or injured tissue via the application of electric fields generated by specific and selective signals of appropriate frequency, amplitude, waveform and/or duty cycle for an appropriate duration of time. The specificity and selectivity of a signal generating an electrical field may thus be influenced so as to regulate the expression of certain genes in order to target certain diseased or injured tissue or disease states for treatment. In particular, the present invention provides for the targeted treatment of fresh bone fractures, fractures at risk, nonunion, bone defects, spine fusion, osteonecrosis, as an adjunct in the treatment of one or any of the above, and in the treatment of osteoporosis.

[0039] The devices of the present invention are capable of applying a field generated by specific and selective signals directly to diseased or injured tissue and/or to the skin of a patient. The devices of the present invention may also provide for the remote application of specific and selective fields (*e.g.*, application of a field at a distance from diseased or injured tissue yet which yields the desired effect within the targeted cells), although it will be appreciated that capacitively coupled devices must touch the subject's skin. The devices of the present invention may include means for attaching the electrodes to the body of a patient in the vicinity of injured or diseased tissue in the case of capacitive coupling. For example, self-adherent conductive electrodes may be attached to the skin of the patient on both sides of a fractured bone. As shown in **Figure 9**, the device 10 of the present invention may include self-adherent electrodes 12 for attaching the device to the body of a patient. For example, the device 10 of the present invention may include electrodes attached to a power unit 14 that has a VELCRO® patch 16 on the reverse side such that the power unit 14 can be attached to a VELCRO® strap (not shown) fitted around a cast on the patient. In the case of inductive coupling, the device of the present invention may include coils attached to a power unit in place of electrodes.

**[0040]** The device 10 of the present invention can be employed in a variety of ways. The device 10 may be portable or may be temporarily or permanently attached to a patient's body. The device 10 of the present invention is preferably non-invasive. For example, the device 10 of the present invention may be applied to the skin of a patient by application of electrodes adapted for contact with the skin of a patient for the application of electric fields generated by the predetermined specific and selective electric signals. Such signals may also be applied via coils in which time varying currents flow, thus producing specific and selective electromagnetic fields that penetrate the tissue and create the specific and selective electric fields in the target tissue. The device 10 of the present invention may also be capable of implantation in a patient, including implantation under the skin of a patient.

**[0041]** Those skilled in the art will further understand that the devices of the present invention can be provided in a variety of forms including a capacitively coupled power unit with programmed, multiple, switchable, specific and selective signals for application to one pair or to multiple pairs of electrodes, electromagnetic coils or a solenoid attached to a power unit with switchable, multiple, specific and selective signals, and an ultrasound stimulator with a power supply for generating specific and selective signals. Generally speaking, device preference is based on patient acceptance and patient compliance. The smallest and most portable unit available in the art at the present time is a capacitive coupling unit; however, patients with extremely sensitive skin may prefer to use inductive coupling units. On the other hand, ultrasound units require the most patient cooperation, but may be desirable for use by certain patients.

## Example

**[0042]** The invention is demonstrated in the following example, which is for purposes of illustration and is not intended to limit the scope of the present invention.

***Materials and Methods***

**[0043]** MC3T3-E1 bone cells ($5 \times 10^5$ cells/cm$^2$) were plated onto specially-modified Cooper dishes. The cells were grown for seven days with the medium changed just prior to beginning of the experimental condition. The experimental cell cultures throughout these studies were subjected to a capacitively coupled 60 kHz sine wave signal electric field with an output of

44.81 V peak-to-peak. This produced a calculated-field strength in the culture medium in the dishes of 20 mV/cm with a current density of 300 $\mu A/cm^2$. Control cell culture dishes were identical to those of the stimulated dishes except that the electrodes were not connected to a function generator.

**[0044]** At the end of the experiment, total RNA was isolated using TRIzol, according to the manufacturer's instructions, and reversed transcription (RT) using SuperScript II reverse transcriptase was performed. Oligonucleotide primers to be used in the real time RT-PCR technique were selected from published cDNA sequences or designed using the Primer Express software program. Quantitative real-time analysis of RT-PCR products was performed using an ABI Prism® 7000 Sequence Detection System.

**[0045]** The optimal signal for the desired up-regulation of (TGF) genes—including genes for TGF-β1, TGF-β2, TGF-β3, among others—was found systematically as follows. An electrical signal known to cause creation of an electric field that increases (or even just suspected to increase) cellular production of a given protein is taken as the starting signal for determining the specific signal for generating the electric field for the gene expression (mRNA) of that protein. A dose-response curve is first performed by varying the duration of the signal while holding all the other signal characteristics constant (amplitude, duty cycle, frequency, and waveform) (**Figure 1**). This determines the optimal duration of the starting signal for the gene expression of that protein. A second dose-response curve is then performed, this time varying the amplitude of the electric field (in mV/cm) while holding the optimal duration and other signal characteristics constant (**Figure 2**). A third dose response is performed, this time varying the signal frequency while holding constant the optimal duration and optimal amplitude as found previously (**Figure 3**). A fourth dose-response is performed varying the duty cycle from 100% (constant) to 10% or less while holding constant the optimal duration, amplitude, and frequency as found previously (**Figure 4**). By this method, an optimal signal is determined for producing the greatest increase in the gene expression of each of the various TGF-betas.

**[0046]** A fifth experiment is performed using a continuous 50% duty cycle (capacitive coupling, 60 kHz, sine wave) to compare a 20mV/cm field to a 40mV/cm field in the production of the TGF-β1 protein. As indicated, the TGF-β1 protein increased significantly and equally in

the two fields. **(Figure 5).** A sixth experiment is performed to demonstrate an increase in production of the BMP-2 protein in the same two fields as described in Figure 5 (20mV/cm and 40mV/cm). After 24 hours of stimulation with a 50% duty cycle (capacitively coupled, 60 kHz, sine wave) field, there was a significant increase in BMP-2 protein in the 20mV/cm field but no significant increase in the BMP-2 protein in the 40mV/cm field **(Figure 6.)** Thus, and this is very important, one can separate the expressions of the TGF-β genes from the BMP genes by stimulating cells with a 40m v/cm field, even though the genes all belong to the same TGF super family. This is clearly shown when one compares Figure 2 with **Figure 7.** In Figure 7, a field of 20mV/cm clearly has a much greater response in up-regulating BMP gene expression than any other field. By contrast, in Figure 2 it is shown that 20mV/cm and 40mV/cm fields are equally effective in up-regulating TGF-β gene expression. **Figure 8** illustrates the BMP gene expression of Figure 7 on the same graph as the TGF-β gene expression of Figure 2. Thus, in fracture healing, one has the option of stimulating bone and cartilage formation together during the osteogenetic phase of fracture healing or of cartilage only during the cartilage phase of fracture healing.

**[0047]** The present invention clearly shows that the optimal electric field described in the example can very significantly up-regulate TGF-β 1,2, and 3 mRNA and, hence, increase bone formation in fracture healing, delayed healing, nonunion, bone defects, spine fusions, and in osteoporosis. Those skilled in the art will appreciate that an appropriate electric field, as described herein with capacitive coupling, is also equally effective with inductive coupling and all electromagnetic systems that produce equivalent, or nearly equivalent, electric field characteristics. Those skilled in the art will also appreciate that more unique signal characteristics may be discovered through more experimentation with more data points (*e.g.*, a 100 ± 3% duty cycle for 30 ± 3 min), but such relatively minor variations in each of the signal characteristics are believed to be within the level of those skilled in the art given the teachings herein.

**[0048]** Those skilled in the art will also appreciate that numerous other modifications to the invention are possible within the scope of the invention. For example, the optimal field described herein can be applied to any bone via two or more appropriate surface electrodes, in pairs or strips, incorporated in braces, wraps, or casts, and delivered by means of capacitive

coupling. Also, the optimal field described here can be applied to any bone via coil(s) or solenoid incorporated into braces, wraps, or casts, and delivered by means of inductive coupling. Accordingly, the scope of the invention is not intended to be limited to the preferred embodiment described above, but only by the appended claims.

What is claimed is:

1. A device for the treatment of a bone fracture, fracture at risk, delayed union, nonunion, bone defect, spine fusion, osteonecrosis, osteoporosis, and/or other conditions in which Transforming Growth Factor (TGF)- β1, β2, and/or β3 protein has been implicated in a patient, comprising a signal source that generates at least one specific and selective signal having a frequency from 30 kHz to 120 kHz and a field generating device connected to the signal source so as to receive said at least one specific and selective signal and that is operatively disposed with respect to targeted tissue, said field generating device upon receipt of said at least one specific and selective signal causing the generation of an electric field having an amplitude of about 0.2 to 80 mV/cm in the targeted tissue that is specific and selective for up-regulating the gene expression of TGF-β1, β2, and/or β3 protein in said targeted tissue as measured by mRNA, said signal source controlling and varying the duration of time of application of said at least one specific and selective signal for a duration of time from approximately ½ hour to 24 hours per 24 hour period and controlling and varying the duty cycle of said at least one specific and selective signal applied to said field generating device from approximately 10% to 100% so as to selectively up-regulate gene expression of TGF-β1, β2, and/or β3 protein in said targeted tissue as a result of application of said specific and selective electric field thereto and a portable power unit that drives said signal source in first and second modes, an output signal in the first mode causing the generation of a 20mV/cm field in the targeted tissue for a selected period of time and an output signal in the second mode causing the generation of a 40mV/cm field in the targeted tissue for another selected period of time.

2. The device of claim 1 wherein the portable power unit is programmable such that fields generated during said first and second modes can be sequentially applied to the targeted tissue for various periods of time and in various orders.

3. The device of claim 1 further comprising means for attaching the field generating device to the body of a patient in the vicinity of bone tissue.

4. The device of claim 1 further comprising means for attaching the signal source to the body of a patient.

5. The device of claim 1 wherein the electric field generated by application of said at least one specific and selective signal to the field generating device is applied to said targeted tissue via capacitive coupling or inductive coupling.

6. The device of claim 5 wherein the specific and selective signal has a sine wave configuration, a duty cycle of approximately 50%, and a frequency of approximately 60kHz, and the resultant specific and selective electric field has an amplitude of about 20 mV/cm-40mV/cm in the targeted tissue.

7. The device of claim 1 wherein the field generating device comprises an electrode or one or more coils.