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Turning on stem cell cardiogenesis with extremely low frequency magnetic fields

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ABSTRACT

Modulation of stem cell differentiation is an important assignment for cellular engineering. Embryonic stem (ES) cells can differentiate into cardiomyocytes, but the efficiency is typically low. Here, we show that exposure of mouse ES cells to extremely low frequency magnetic fields triggered the expression of GATA-4 and Nkx-2.5, acting as cardiac lineage-promoting genes in different animal species, including humans. Magnetic fields also enhanced prodynorphin gene expression, and the synthesis and secretion of dynorphin B, an endorphin playing a major role in cardiogenesis. These effects occurred at the transcriptional level and ultimately ensued into a remarkable increase in the yield of ES-derived cardiomyocytes. These results demonstrate the potential use of magnetic fields for modifying the gene program of cardiac differentiation in ES cells without the aid of gene transfer technologies and may pave the way for novel approaches in tissue engineering and cell therapy.

Key words: gene expression • cardiac differentiation

Magnetic fields (MF) have been shown to affect proliferation and growth factor expression in cultured cells (1–3) and interfere with endorphinergic and cholinergic systems in intact organisms (4–6). Nevertheless, compelling evidence that MF may trigger a coordinate program of cell differentiation or may contribute to the specification of a cellular architectural plan is still lacking. We have previously shown that exposure to extremely low frequency MF increased the expression of the prodynorphin gene in adult rat ventricular cardiomyocytes (7). This effect was associated with the synthesis and secretion of dynorphin B (7), a bioactive gene product acting as a selective agonist of kappa opioid receptors coupled to phosphoinositide turnover, and regulation of cytosolic calcium homeostasis and contractility in the myocardial cell (8–10). We have recently provided evidence that dynorphin B primed cardiac

differentiation in embryonal carcinoma cells (11) and that a dynorphinergic system acted as a major conductor of cardiogenesis in multipotent mouse embryonic stem (ES) cells (12, 13).

Here, we sought whether MF may be able to trigger prodynorphin gene expression even in ES cells and whether, in the affirmative, MF may commit these cells to the specification of a cardiac lineage.

MATERIALS AND METHODS

ES cells

GTR1 cells, a derivative of R1 ES cells (14) bearing the puromycin-resistance gene driven by the cardiomyocyte-specific α-myosin heavy chain promoter, were kindly provided by Dr. William L. Stanford (University of Toronto and Centre for Modeling Human Disease, Canada). Cardiac differentiation and puromycin selection of ES-derived cardiomyocytes were performed as described previously in detail (12). ES cells were maintained in the undifferentiated state by culturing in DMEM containing 15% FBS, supplemented with a final concentration of 1000 U/ml ESGRO-LIF (LIF). To induce cardiac differentiation, cells were plated onto bacterial Petri dishes, containing DMEM lacking supplemental LIF. After 2 days of culture, the resulting embryoid bodies (EBs) were plated onto tissue culture dishes. When spontaneous contractile activity was noticed, puromycin (2 μg/ml) was added to eliminate noncardiomyocytes. After 2 days, puromycin-selected myocytes were transferred to new tissue culture dishes. As indicated in the legend of each figure, EBs, collected at several stages after plating, as well as puromycin-selected cells, were processed for gene expression analyses.

Exposure conditions

Following LIF removal, GTR1 cells were continuously exposed to a sinusoidal MF (50 Hz, 0.8 mTrms) up to the time of collection of EBs or puromycin-selected cardiomyocytes (3 or 10 days from LIF withdrawal, respectively). The exposure system consisted of two identical apparatuses each consisting of 4 coaxial circular coils placed horizontally in a commercial CO_2 incubator, and thus parallel with respect to the surface of the culture well plates. The geometry of the coil system was numerically calculated in order to optimize the extension of the magnetic field uniformity. Each coil was wound by a pair of parallel wires so that, according to different connections, the current could either flow in the same direction ("wound configuration"), generating the active exposure, or in the opposite direction ("counter-wound configuration"), allowing a sham exposure. The coils were powered by a home-made DC amplifier connected to a function generator (HP 3314A). During the experiments, performed in blind, the temperature was monitored by means of a thermoresistor. The local geomagnetic (45 μT) field was measured by a gaussmeter (F. W. Bell 7010), and the AC background magnetic field (0.05 μT) was measured by means of a sensitive probe (EMDEXII, Enertech).

Analysis of mRNA expression

Expression of GATA-4, Nkx-2.5, prodynorphin, α-myosin heavy chain and myosin light chain-2V mRNA was assessed by RT-PCR as described previously (11), using GAPDH mRNA as a measure of equal loading and mRNA stability. GATA-4 mRNA levels were also quantitated by

RNase protection assay, as described elsewhere (13). Briefly, a fragment of the main exon of the mouse GATA-4 (292 bp) gene was inserted into pCRII-TOPO (Invitrogen, Carlsbad, CA). Transcription of the plasmid linearized with *BamHI* generated a sense strand of GATA-4 mRNA, which was used to construct a standard mRNA curve. Transcription in the presence of [^{32}P]CTP (800 Ci/mmol) (Amersham International, Didcot, United Kingdom) of plasmids linearized with *Xba*I produced an antisense strand of GATA-4 mRNA (radiolabeled cRNA probe). Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide nondenaturing gel. Autoradiographic exposure was performed for 48 h. The individual bands were counted for radioactivity by liquid scintillation spectrometry, and cpm values were translated to pg values on a correlated standard curve. Data were expressed as pg of mRNA/μg of total RNA.

Transcriptional analysis in isolated nuclei

Nuclear runoff was performed as detailed elsewhere (13, 15). Ninety microliters of nuclear suspension were added with 100 μl of 2 × reaction buffer (10 mmol/L Tris/HCl, pH 7.5, 5 mmol/L $MgCl_2$, 0.3 mol/L KCl, 5 mmol/L dithiothreitol, 1 mmol/L each of ATP, GTP, and CTP), and 5 μl of [α-^{32}P]UTP (3000 Ci/mmol), followed by incubation at room temperature for 15 min. DNA was digested by incubating the transcription mixture for 5 min at room temperature in the presence of 1 μl of 20,000 units/ml RNase-free DNase. Equal counts of ^{32}P-labeled nuclear RNA (~5 × 10^6 cpm) were then subjected to a solution hybridization RNase protection assay and were hybridized for 12 h at 55°C in the presence of unlabeled antisense GATA-4 mRNA. Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide nondenaturing gel. Autoradiographic exposure was for 48 h. ^{32}P-labeled nuclear RNA was also hybridized with unlabeled antisense cyclophilin mRNA synthesized from an *Nco*I-linearized pBS vector containing a 270-base pair fragment of plB15, a cDNA clone encoding for rat cyclophilin (13). Cyclophilin mRNA was used as a constant mRNA for control.

Identification of dynorphin B

Immunoreactive dynorphin B (ir-dyn B) was measured, as detailed (11, 12, 15), by the aid of the 13S antiserum recognizing the high molecular weight peptides cleaved from the prodynorphin precursor and containing dynorphin B in their sequence. Acetic acid extracts from undifferentiated or cardiac-lineage-committed cells, or pooled samples from their incubation media were processed by reverse-phase high-performance liquid chromatography (RP-HPLC). The collected fractions were radioimmunoassayed and immunoreactivity was attributed to authentic dynorphin B by comparison with the elution position of a synthetic standard (12, 15).

Immunofluorescence analyses

Puromycin-selected cells were fixed with 4% paraformaldehyde. MHC was assessed by the MF20 monoclonal antibody (12) with a Bio-Rad (Hercules, CA) Microradians confocal microscope. DNA was visualized with propidium iodide (1 μg/ml).

Data analysis

The statistical analysis of the data was performed by using a one-way ANOVA followed by Newman Keul's test and assuming a *P* value less than 0.05 as the limit of significance.

RESULTS AND DISCUSSION

MF were applied to GTR1 cells, a derivative of R1 ES cells generating late-stage viable tetraploid embryos, and germ line transmitting chimeras at a remarkable rate (14). GTR1 cells contain a transgene encoding the cardiomyocyte-specific α-myosin heavy chain (MHC) promoter driving the puromycin-resistance gene, and afford genetic selection of a virtually pure population of ES-derived cardiomyocytes (12). RT-PCR analysis of targeted transcripts revealed that cell exposure to MF following removal of leukemia inhibitory factor (LIF) remarkably increased GATA-4 and Nkx-2.5 mRNA expression in both embryoid bodies (EBs) and puromycin-selected cardiomyocytes (Fig. 1). These responses were further inferred by the quantitative analysis of GATA-4 mRNA levels as shown in RNase protection experiments (Fig. 1). These mRNAs encode respectively for a zinc finger containing transcription factor and a homeodomain that have been shown to be essential for cardiogenesis in different animal species (16, 17), including humans (18). MF also enhanced prodynorphin mRNA expression and the levels of dynorphin B, a natural κ opioid receptor agonist, in both EBs and ES-derived cardiomyocytes, and in their incubation media (Fig. 1). This finding is particularly rewarding, since we have recently shown that the prodynorphin gene and dynorphin B primed GATA-4 and Nkx-2.5 transcription (11), and triggered protein kinase C (PKC) signaling through complex subcellular redistribution patterning of targeted PKC isozymes, another major requirement for ES cell commitment to the cardiac lineage (12). Additionally, dynorphin B was found to act as an agonist of nuclear opioid receptors coupling nuclear PKC activation to the transcription of cardiogenic genes, indicating that intracrine signals for cardiac differentiation may also be fashioned by the prodynorphin gene and its related peptides (13). Interestingly, nuclear runoff analyses of GATA-4 gene transcription indicated that the MF action occurred at the transcriptional level (Fig. 1). The activation of a program of cardiogenic gene transcription was also associated with an increase in the expression of the cardiac-specific transcripts α-myosin heavy chain and myosin light-chain 2V (Fig. 2).

Exposure of GTR1 ES cells to MF after LIF removal and throughout 4 days of puromycin selection for an overall period of 10 days from LIF withdrawal consistently increased the yield of ES-derived cardiomyocytes (Fig. 2). The number of beating colonies reached 180.38 ± 33.0% of the control value, estimated in cardiomyocytes selected from untreated cells (mean ± SEM of 4 separate experiments).

We finally investigated whether the transcriptional responses evoked by MF may encompass genes that are essential for the specification of nonmyocardial lineages. Noteworthy, the expression of *MyoD*, a gene involved in skeletal myogenesis was not affected in both EBs and puromycin-selected cells (Fig. 3). On the contrary, the expression of neurogenin1, a neuronal specification gene, was slightly enhanced only in EBs (Fig. 3). Failure of MF to affect the transcription of a gene promoting skeletal muscle determination and the faint effect on neuronal specification seem to exclude a generalized activation of repressed genes and suggests that

coupling of MF with GATA-4, Nkx-2.5 and prodynorphin gene expression may represent a mechanism pertaining to ES cell cardiogenesis.

Stem cells have been recently proposed as a renewable source of donor cells for the rescue of damaged tissues. Since the rescuing potential is limited by the fact that differentiating cells withdraw early from the cell cycle, the development of strategies affording high-throughput of targeted lineages from pluripotent cells would have obvious biomedical implications. However, overexpression of tissue-specific genes by vector-mediated gene transfer is a cumbersome approach that may perturb normal homeostasis in both stem cells and recipient tissues, and is not readily envisionable in humans.

The current findings open the new perspective of using MF to direct the differentiation processes of stem cells into a specific cellular phenotype without the aid of gene transfer technologies. Studies are in progress to shed additional light on the molecular events underlying the differentiating response primed by MF in ES cells and to assess whether such a response may be dependent on the field characteristics (intensity, frequency, and wave shape).

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Fig. 1

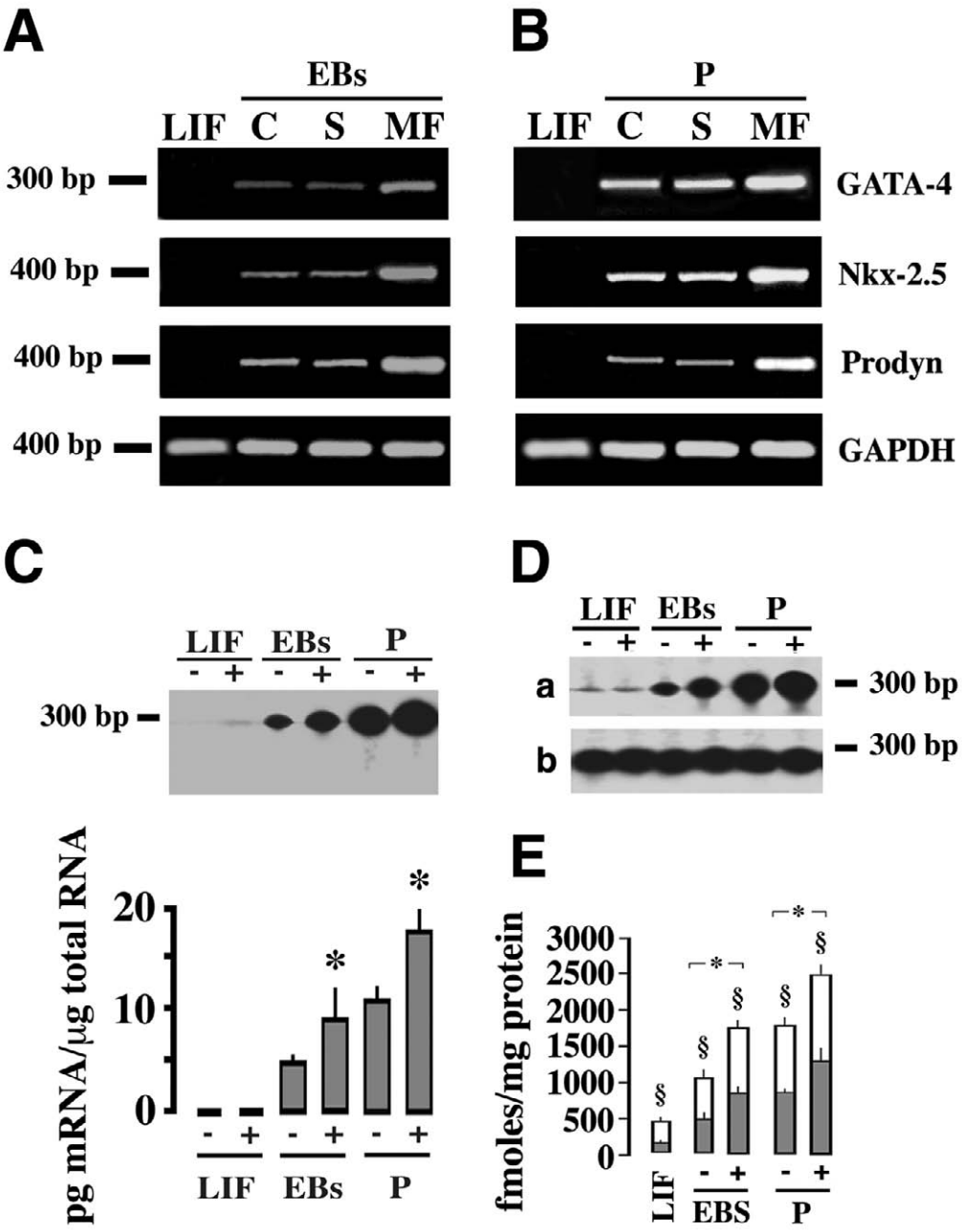

Figure 1. Effect of magnetic field on the expression of cardiac lineage-promoting genes. Magnetic field (MF) was applied from the time of LIF removal. EBs (***A***) or puromycin-selected cardiomyocytes (P, ***panel B***) were collected after 3 or 10 additional days, respectively, and processed for RT-PCR analysis of the indicated transcripts. LIF, undifferentiated cells; (***C***) unexposed controls; S, sham-exposed (counter-wound coils); MF, MF-exposed cells. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments). ***C***) RNase protection analysis of GATA-4 mRNA expression. GTR1 ES cells were cultured as described in the absence (–) or presence (+) of MF. *, significantly different from unexposed. ***D***) Nuclear runoff analysis of GATA-4 gene transcription in isolated ES cell nuclei. Nuclei were isolated from undifferentiated cells (LIF), EBs, or P collected 3 or 10 days after LIF removal, respectively. Each group of cells was exposed in the absence (–) or presence (+) of MF from the time of LIF withdrawal. ***Row a***) GATA-4 gene transcription. ***Row b***) Cyclophilin gene transcription. Autoradiograms are representative of 3 separate experiments. ***E***) Immunoreactive dynorphin B (ir-dyn B) in cells (gray bars) or medium (white bars), mean ± SE (n = 6). Asterisks with brackets, significant difference (one-way ANOVA, Newman Keul's test). §, significantly different from the values of the gray bars.

Fig. 2

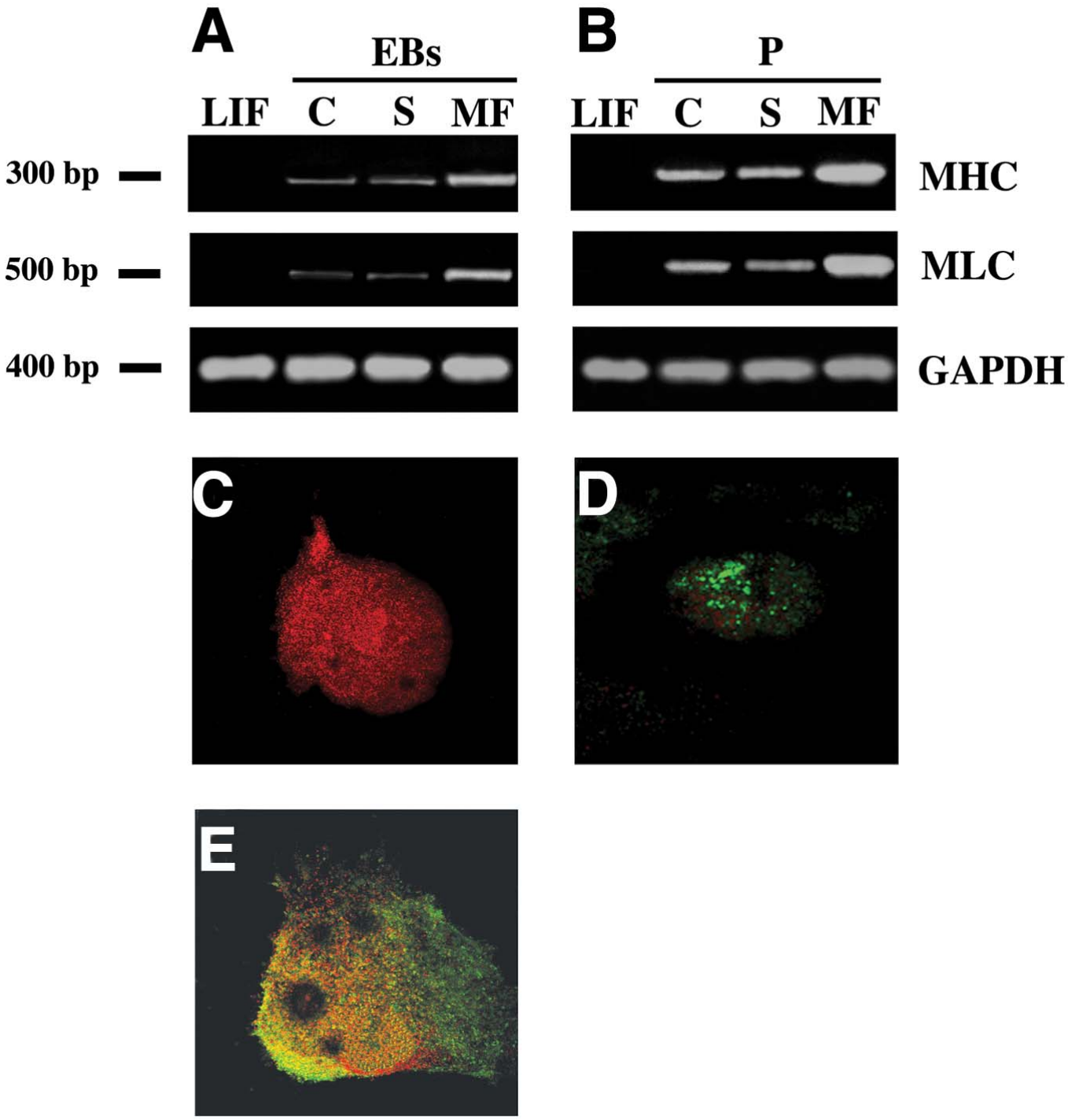

Figure 2. MF primes the expression of cardiac-specific genes and enhances the yield of ES-derived cardiomyocytes. MF was applied from the time of LIF removal. EBs or P were collected after 3 or 10 additional days, respectively. ***A***, ***B***) RT-PCR analysis of α-myosin heavy chain (MHC) and myosin light chain-2V (MLC). LIF, undifferentiated cells. ***C***) Unexposed controls; S, sham-exposed (counter-wound coils); MF, MF-exposed cells. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments). MHC immunostaining (×20 objective) was assessed in undifferentiated cells (***C***), and in cardiomyocytes derived from cells cultured in the absence (***D***) or presence (***E***) of MF.

Fig. 3

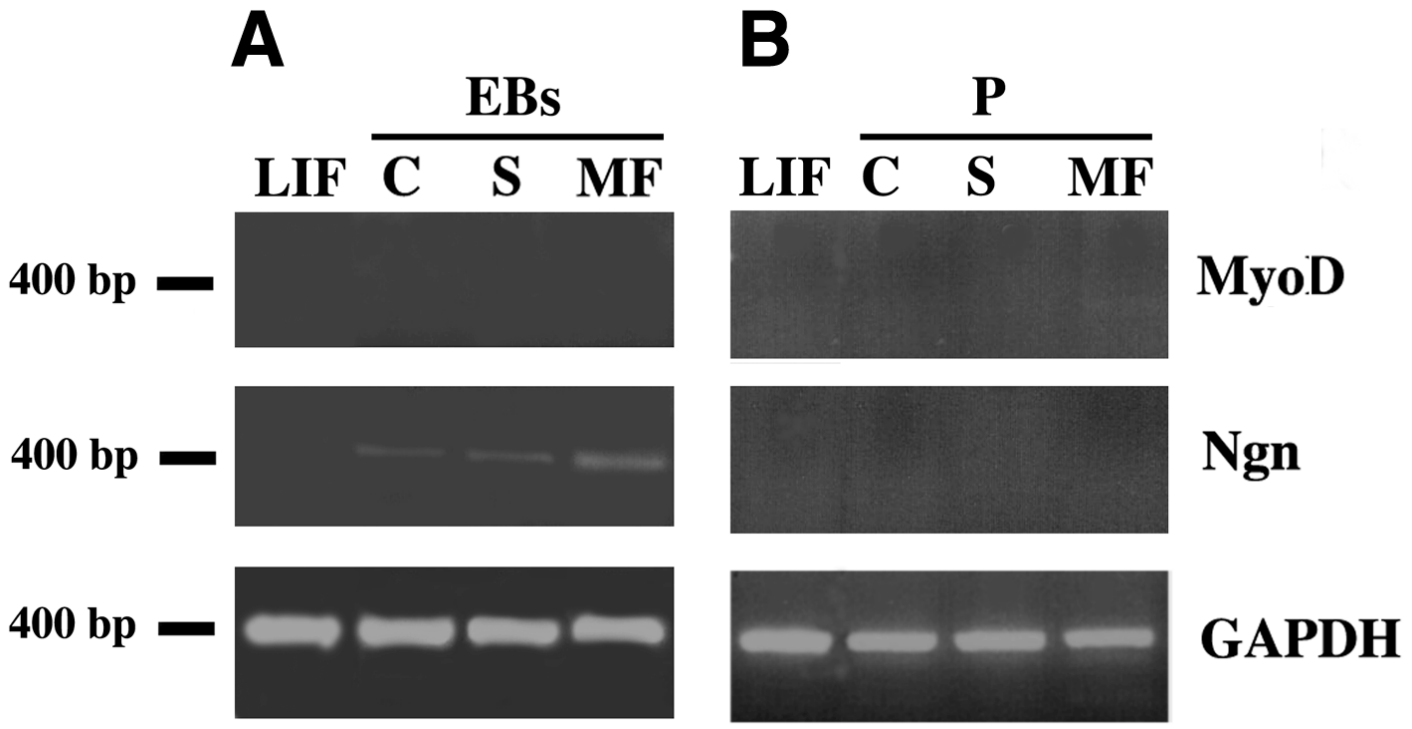

Figure 3. Effect of MF on the expression of genes promoting nonmyocardial lineages. MF was applied from the time of LIF removal. EBs (***A***) or P (***B***) were collected after 3 or 10 additional days, respectively, and processed for RT-PCR analysis of MyoD or neurogenin1 (Ngn). LIF, undifferentiated cells; C, unexposed controls; S, sham-exposed (counter-wound coils); MF, MF-exposed cells. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments).