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RESEARCH ARTICLE

Electromagnetic fields counteract IL-1β activity during chondrogenesis of bovine mesenchymal stem cells

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Abstract

Osteoarthritis (OA) is a common joint disease associated with articular cartilage degeneration. To improve the therapeutic options of OA, tissue engineering based on the use of mesenchymal stem cells (MSCs) has emerged. However, the presence of inflammatory cytokines, such as interleukin-1β (IL-1β), during chondrogenesis reduces the efficacy of cartilage engineering repair procedures by preventing chondrogenic differentiation. Previous studies have shown that electromagnetic fields (EMFs) stimulate anabolic processes in OA cartilage and limit IL-1β catabolic effects. We investigated the role of EMFs during chondrogenic differentiation of MSCs, isolated from bovine synovial fluid, in the absence and presence of IL-1β. Pellets of MSCs were differentiated for 3 and 5 weeks with transforming growth factor-β3 (TGFβ3), in the absence and presence of IL-1β and exposed or unexposed to EMFs. Biochemical, quantitative real-time RT–PCR and histological results showed that EMFs alone or in the presence of TGFβ3 play a limited role in promoting chondrogenic differentiation. Notably, in the presence of IL-1β and TGFβ3 a recovery of proteoglycan (PG) synthesis, PG content and aggrecan and type II collagen mRNA expression in the EMF-exposed compared to unexposed pellets was observed. Also, histological and immunohistochemical results showed an increase in staining for alcian blue, type II collagen and aggrecan in EMF-exposed pellets. In conclusion, this study shows a significant role of EMFs in counteracting the IL-1β-induced inhibition of chondrogenesis, suggesting EMFs as a therapeutic strategy for improving the clinical outcome of cartilage engineering repair procedures, based on the use of MSCs. Copyright © 2012 John Wiley & Sons, Ltd.

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1. Introduction

Osteoarthritis (OA) is a common disease of all the anatomical structures of the joint that is associated with articular cartilage loss, subchondral bone change and synovitis leading to progressive joint degeneration (Aigner *et al*., 2006). To date, cartilage damage is an irreversible process, because of the limited capacity of the adult articular chondrocytes to repair and regenerate the normal cartilage matrix architecture. Despite the large amount of active research to identify structure-modifying approaches to inhibit cartilage destruction in OA, there is an urgent need to improve the therapeutic options, because existing drug therapies reduce symptoms, primarily pain, but have a limited long-term efficacy (Goldring, 2006).

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In recent years, tissue engineering based on the use of mesenchymal stem cells (MSCs) has emerged as an alternative approach to current treatments for cartilage defects. Alternative cell sources with chondrogenic potential include MSCs derived from synovium, adipose tissue and periosteum, among others (De Bari *et al*., 2001; Guilak *et al*., 2004; Tuli *et al*., 2004). Although cartilage engineering repair strategies that rely on the *in situ* differentiation of MSCs are attractive, existing methods fail to provide a reliably successful, long-term clinical outcome (Richter, 2009). A limitation of these procedures may be due to the presence of the inflamed environment in which chondrogenesis takes place. Intra-articular inflammation may result from disease, such as arthritis, or trauma, including the trauma during cartilage repair surgery itself. In inflammatory conditions, pro-inflammatory and catabolic mediators released from chondrocytes, synovial fibroblasts and inflammatory cells have been identified at high levels in the joint's inflammatory environment. Among them, the pro-inflammatory cytokine interleukin-1β (IL-1β) plays a pivotal role in local inflammatory processes, and it has been shown that elevated IL-1β concentrations may be maintained in synovial fluid for a long time after injury (Catterall *et al*., 2010). It has been also reported that the IL-1β levels, not detectable in healthy knee joints, show an increasing trend in cartilage lesions and reach a significantly elevated peak following the surgical cartilage-regenerating intervention (Schmal *et al*., 2009). Furthermore, a frequent problem associated with cartilage repair strategies is the formation of fibrocartilaginous tissue. The formation of fibrotic tissue seems to be reduced by limiting the activity of inflammatory cytokines. In fact, it has been shown that a human MSC subpopulation expressing high levels of IL-1 receptor antagonist was able to inhibit bleomycin-induced inflammation and fibrosis within the lungs of mice (Ortiz *et al*., 2007). In addition, IL-1β has been shown to inhibit the chondrogenic differentiation of human MSCs (Wehling *et al*., 2009). Therefore, IL-1β activity might be strongly involved in the biological problems related to the regeneration of new cartilage tissue from MSCs.

Previous studies show that physical stimulation with electromagnetic fields (EMFs) represent a viable therapeutic approach to limiting cartilage degradation and to controlling inflammation associated with joint diseases, by stimulating anabolic chondrocyte functions and limiting the effects due to inflammatory activities of synovial fibroblasts (Ongaro *et al*., 2012; Sellam and Berenbaum, 2010). In bovine and human cartilage explants, EMFs prevent the catabolic effect of the pro-inflammatory cytokine IL-1β and act in synergy with insulin-like growth factor-I (IGF-I), the main anabolic factor of cartilage (De Mattei *et al*., 2003, 2004, 2007; Ongaro *et al*., 2011). Further, in IL-1β-stimulated human osteoarthritic synovial fibroblasts, EMFs counteract the catabolic effects induced by the cytokine (De Mattei *et al*., 2009), as EMFs reduce the synthesis of inflammatory mediators such as PGE_2 and pro-inflammatory cytokines (IL-6 and IL-8) (Ongaro *et al*., 2012). *In vivo*, EMFs preserve the morphology of articular cartilage and retard the development of osteoarthritic lesions in guinea pigs (Fini *et al*., 2005). Also, EMFs favour the integration of osteochondral autografts in sheep, by decreasing IL-1 and tumour necrosis factor-α concentration in the synovial fluid, (Benazzo *et al*., 2008a). In humans, EMFs show effectiveness in controlling joint inflammation after surgical procedures at the knee (Benazzo *et al*., 2008b; Zorzi *et al*., 2007).

More recently, several studies have shown that EMFs can also influence the behaviour of MSCs, particularly their differentiation into the osteogenic lineage (Saino *et al*., 2011; Schwartz *et al*., 2008; Tsai *et al*., 2009).

All of this evidence suggests that EMFs might have effects on MSC differentiation, thus limiting the catabolic activity of IL-1β, during cartilage-regenerating surgical interventions. Therefore, we first evaluated whether the chondrogenic differentiation of MSCs might be influenced by EMF exposure; then we investigated the role of EMFs during chondrogenic differentiation in the presence of IL-1β. As an experimental model we used MSCs isolated from bovine synovial fluid, since MSCs derived from the synovial membrane or the synovial fluid show higher chondrogenic potential than MSCs from other tissues (Sakaguchi *et al*., 2005; Shirasawa *et al*., 2006), suggesting that synovium is an excellent source of MSCs for cartilage regeneration. Our hypothesis was that EMFs might counteract the inhibition of the differentiation process induced by IL-1β during chondrogenesis.

2. Materials and methods

2.1. Collection of MSCs from synovial fluid and cell cultures

MSCs were isolated as previously reported by other authors, with minor changes in the experimental practice (Jones *et al*., 2008; Jones *et al*., 2004). Briefly, synovial fluid was aspirated from the metacarpophalangeal joints from eight bovines (females, 14–18 months old) using a syringe. For isolating cells, fresh synovial fluid (2 ± 0.6 ml for each animal) was diluted 1:4 with complete medium [Dulbecco's modified Eagle's/Ham's F12 (1:1) medium (DMEM/F12; Life Technologies, Paisley, UK)] containing 10% fetal bovine serum (FBS), centrifuged at 2000 rpm for 10 min and plated in 25 cm^2 culture flasks (Falcon, Becton-Dickinson, Franklin Lakes, NJ, USA). After 3 h, the medium was removed and fresh complete medium was added to the flasks. Immediately after cell isolation, a conventional colony-forming unit–fibroblast (CFU-F) assay was performed to evaluate the number of clonogenic cells (Jones *et al*., 2004). At the beginning, cells isolated for the differentiation experiments were plated at about 500 cells/cm^2, maintained in culture and then subcultured at 5000/cm^2 (Sakaguchi *et al*., 2005). MSCs at passage 3 were used in chondrogenic differentiation experiments, according to previous studies on synovial MSCs (De Bari *et al*., 2001; Jones *et al*., 2004, 2008).

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2.2. Chondrogenic differentiation

Cells (400 000) were centrifuged at 1000 rpm for 10 min in 15 ml tubes to form a pellet. The pellets were cultured in 0.5 ml chondrogenic differentiation basal medium containing dexamethasone, ascorbate, insulin, transferrin, selenium, penicillin/streptomycin, proline, sodium pyruvate and L-glutamine (Lonza Walkersville, Walkersville, MD, USA) at 37 °C, 5% CO_2, alone (control) or supplemented with 10 ng/ml TGFβ3 (Lonza), according to the manufacturer's protocol of chondrogenic differentiation. In a subset of pellets, 50 ng/ml IL-1β (PeproTech, London, UK) were added to the chondrogenic medium to evaluate the effects of the cytokine on chondrogenic differentiation. The IL-1β dose was chosen on the basis of our previous study performed on bovine cartilage explants (De Mattei *et al.*, 2003). In a parallel setting, the pellets were exposed to EMFs (75 Hz, 1.5 mT) inside an incubator during the whole period of culture. The medium was changed every 3 days. The pellets were harvested after 3 and 5 weeks of culture.

To evaluate chondrogenic differentiation, two series of pellets were used, one for biochemical assays and the other for quantitative real-time RT–PCR and histological analysis. Each series of pellets was derived from cells harvested from the same animal. Based on cell availability, when possible, cells from the same animal were used for all the experimental assays (biochemical, quantitative real-time RT–PCR and histological analysis). Furthermore, three pellets were prepared for each experimental condition tested. Four experiments for biochemical analysis and three for quantitative real-time RT–PCR and histological analysis were carried out.

2.3. Characteristics of EMF and exposure conditions

The EMF generator system was the same as used in previous studies (De Mattei *et al.*, 2003, 2004, 2009; Varani *et al.*, 2008). It consisted of a pair of circular Helmoltz coils of copper wire placed opposite to each other and in a signal generator (IGEA S.p.A., Carpi, Italy). The pellets were placed between the pair of Helmoltz coils. The power generator produced a pulsed signal with pulse duration of 1.3 ms and frequency of 75 Hz, yielding a duty cycle of 1/10. The intensity peak of the magnetic field was 1.5 mT and was detected between two coils from one side to the other, by the Hall probe of a Gaussmeter (LE, Gaussmeter DG500, USA) with a reading sensitivity of 0.2%. The shape of the induced electric field and its impulse length were kept constant. The intensity value of the magnetic field measured between two coils was uniform in the whole area in which the pellets were placed. In EMF-exposed pellets, the EMF exposure was maintained for the whole differentiation time (3 or 5 weeks). Control pellets were maintained in the same incubator, placed at a distance from the coils where no difference from background magnetic field was observed when the EMF generator was turned on.

2.4. Proteoglycan (PG) synthesis

PG synthesis was determined by radioactive sulphate incorporation into glycosaminoglycans (GAGs), which are biochemical components of PGs, after 3 or 5 weeks of culture, as previously reported (De Mattei *et al.*, 2004). Briefly, 5 μCi/ml Na_2-$^{35}SO_4$ (2.2 mCi/ml; Amersham Pharmacia Biotech, Buckinghamshire, UK) was added to the culture media of both EMF-unexposed and -exposed pellets at time 0 and when the medium was replaced (every 3 days). After the radiolabelling, at the end of the culture period, the pellets were rinsed and digested in 20 mM phosphate buffer, pH 6.8, containing 2 mg/ml papain (Sigma-Aldrich, Milan, Italy) at 60 °C for 12 h. The content of ^{35}S-labelled newly synthesized PGs (^{35}S-PGs) was measured following precipitation of the ^{35}S-PGs with cetylpyridinium chloride (Sigma-Aldrich) and filtration onto glass fibre filters (Whatman GF/C). The filters were dried and the radioactivity quantified by liquid scintillation counting. Data were normalized to the DNA content, measured fluorometrically by binding to Hoechst 33258 dye.

2.5. PG content

Total PG content within pellets was measured, using a spectrophotometer (Jenway, Zetalab Padova, Italy), as total sulphated GAGs, using the dimethylmethylene blue (DMMB) assay, with bovine chondroitin sulphate (Sigma-Aldrich Chemie, Steinheim, Germany) as the standard (De Mattei *et al.*, 2003), after digestion in papain as indicated above. The results were normalized to the pellet DNA content.

2.6. RNA isolation and quantitative real-time reverse transcription with polymerase chain reaction (RT–PCR)

Total RNA was extracted from formalin-fixed, paraffin-embedded pellets cultured for 3 and 5 weeks. For each sample, tissue slices were deparaffinized by two repeated incubations in xylene, followed by three repeated incubations in 100% ethanol, air-dried and homogenized in Qiazol lysis reagent in the RNeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA was purified according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). 75 ng total RNA was reverse-transcribed using the SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) and amplified using the PerfeCTa SYBR Green SuperMix, Rox (Quanta, 95055-02 K), with an initial 3 min incubation at 95 °C

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followed by 40 cycles of amplification (95 °C for 15 s and 60 °C for 1 min), and examined on a 7500 Fast Real-Time PCR system (Applied Biosystems) (Rizzo *et al.*, 2008). Real-time RT–PCR was performed using primers for bovine collagen type II [α1(II)] and for aggrecan previously reported (Shintani and Hunziker, 2007). The real-time PCR data were analysed using the relative gene expression ($^{\Delta\Delta}C_T$) method, as described in Applied Biosystems User Bulletin No. 2. Data are presented as the fold change in gene expression normalized to the endogenous reference gene (*18S* rRNA) and calculated relative to those of unexposed untreated pellets (control).

2.7. Histological analysis

At weeks 3 and 5 of culture, the pellets were fixed in a neutral-buffered, isotonic formalin solution at 4 °C overnight, embedded in paraffin and serially sectioned at 5 μm, using a hard-cutting microtome (Leica RM2025, Leica Instuments GmbH, Nussloch, Germany). The sections were stained with haematoxylin and eosin (H&E) for morphological observations and with Alcian blue (Sigma-Aldrich) to assess for sulphated GAG content. Non-consecutive sections were immunostained with monoclonal antibodies against collagen type II and aggrecan (Abcam, Cambridge, MA, USA) individually. Immunohistochemical sections were deparaffinized, rehydrated and incubated for 1 h with 1 mg/ml collagenase from *Clostridium hystoliticum* type VII (Sigma-Aldrich) in Tris-buffered saline (TBS), pH 7.2, at 37 °C. Then the sections were treated with 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase and incubated for 5 min with Ultra V Block at room temperature to block non-specific background staining. The primary antibody was applied to the sections and incubated overnight at 4 °C; then the sections were washed in buffer and incubated with primary antibody enhancer for 10 min at room temperature. This was followed by 15 min with horseradish peroxidase (HRP) polymer (Ultravision LP Large Volume Detection System HRP polymer, Thermo Scientific, Runcorn, UK). Finally, the sections were counterstained with haematoxylin. Normal bovine articular cartilage was used as the positive control. Twelve sections/treatment, i.e. three pellets/treatment and four non-consecutive sections/pellet, were examined.

2.8. Statistical analysis

Biological data shown in the Figures represent mean standard deviation (SD). Statistical analysis was assessed by one-way analysis of variance (ANOVA). Comparisons between groups were performed using Student's *t*-test. For statistical validation of the data, four independent experiments for biochemical analysis and three for quantitative real-time RT–PCR and histological analysis were carried out. In all experiments, each experimental condition was tested in triplicate. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Synovial fluid-derived MSC cultures

It has been previously reported that during the first week of culture, MSCs derived from the synovial fluid undergo several rounds of cell division, resulting in fibroblastic colonies clearly detectable by day 7 (Jones *et al.*, 2004, 2008). Accordingly, in our cultures we observed a progressive growth of cells derived from the synovial fluid. The cells appeared very sparse 24 h after seeding, then colony formation was detectable by days 3–5. Data obtained from the CFU-F assay ($n = 5$) showed that CFU-F represented a mean ± SD = 0.32 ± 0.08% of total synovial fluid cells (about 240 cells/ml of synovial fluid). Under our experimental culture conditions, the expansion of synovial fluid MSC-derived colonies yielded typical spindle-shaped fibroblast-like cells, which were used at passage 3 for chondrogenic differentiation experiments (data not shown).

3.2. Effects of EMFs on chondrogenesis

To investigate the effects of EMFs on chondrogenic differentiation of synovium-derived MSCs, the pellets were cultured for 3 and 5 weeks in the absence and presence of 10 ng/ml TGFβ3 and exposed or unexposed to EMFs for the whole period in culture.

Biochemical results concerning pellet PG synthesis and PG content obtained after 3 and 5 weeks of differentiation time are shown in Figure 1. In the absence of TGFβ3, control pellets showed a very low PG synthesis and PG content. Pellets exposed to EMFs had behaviour similar to that of unexposed control pellets, suggesting that EMF exposure alone does not influence pellet culture differentiation. In the presence of the chondrogenic growth factor TGFβ3, a significant increase in PG synthesis and PG content was observed in EMF-exposed and -unexposed pellets compared with TGFβ3-untreated and the EMF-unexposed control at both differentiation times, in a time-dependent fashion. The enhancement observed in the TGFβ3-treated pellets compared with control was 305% at 3 weeks and 547% at 5 weeks in PG synthesis, and 50% at 3 weeks and 254% at 5 weeks in PG content. Similarly, pellets cultured in the presence of TGFβ3 and exposed to EMFs showed a time-dependent increase in PG synthesis (274% at 3 weeks and 469% at 5 weeks, compared to control) and in PG content (28% at 3 weeks and 201% at 5 weeks, compared to control) and no significant differences were observed between EMF-exposed and -unexposed pellets in the presence of TGFβ3.

Type II collagen and aggrecan gene expressions were measured by quantitative real-time RT–PCR to assess the expression of chondrocyte-specific genes in pellets cultured under the same experimental conditions. As shown in Figure 2, TGFβ3-treated pellets showed 55- and 262-fold increases in type II collagen gene expression, compared to control, at 3 and 5 weeks, respectively.

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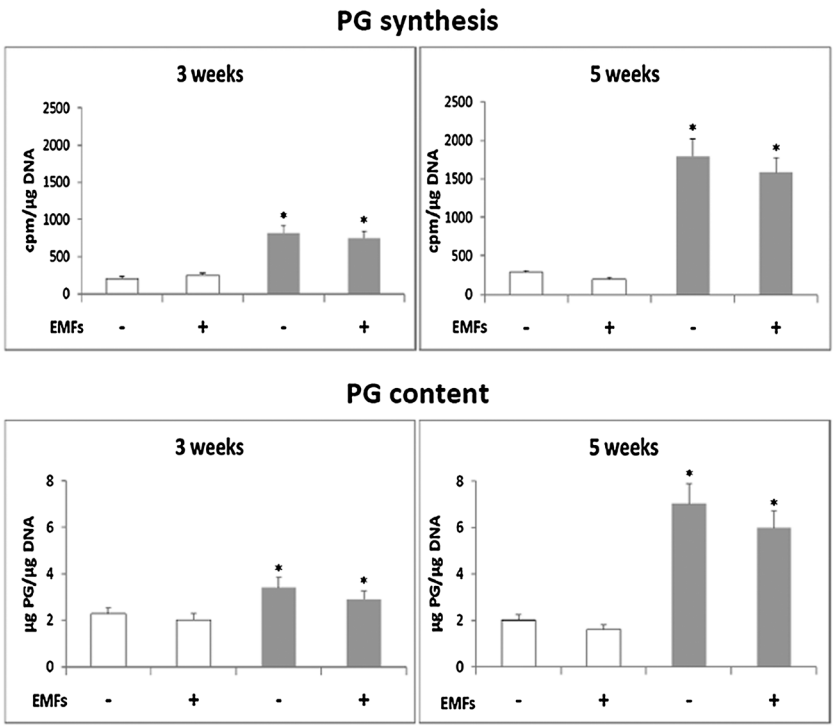

Figure 1. Effects of EMFs on chondrogenic differentiation of MSCs: biochemical data ($n = 4$). PG synthesis and PG content in pellets cultured in the absence (white bar) and presence (grey bar) of 10 ng/ml TGFβ3 for 3 and 5 weeks and exposed or unexposed to EMFs. All data are expressed as mean ± SD. *Statistical significance vs TGFβ3-untreated and EMF-unexposed pellets (control)

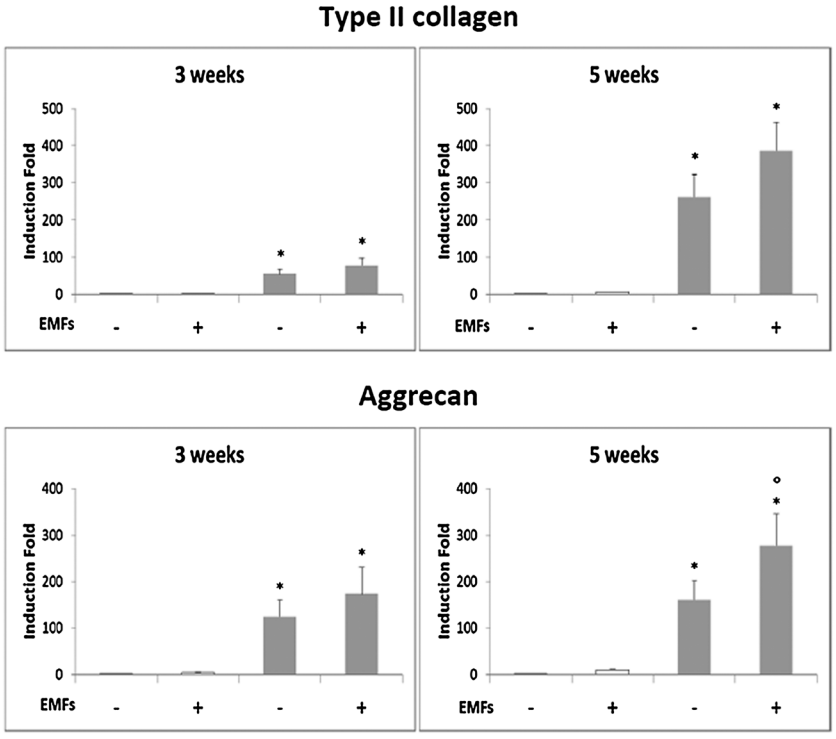

Figure 2. Effects of EMFs on chondrogenic differentiation of MSCs: quantitative real-time RT–PCR data ($n = 3$). Type II collagen and aggrecan gene expression in pellets cultured in the absence (white bar) and presence (grey bar) of 10 ng/ml TGFβ3 for 3 and 5 weeks and exposed or unexposed to EMFs. All data are expressed as mean ± SD. *Statistical significance vs TGFβ3-untreated and EMF-unexposed pellets (control); °statistical significance vs TGFβ3-treated and EMF-unexposed pellets

In TGFβ3-treated pellets exposed to EMFs, a further induction in type II collagen expression compared to control was observed (78- and 387-fold at 3 and 5 weeks, respectively), although differences between EMF-exposed and -unexposed pellets were not significant. A similar trend was found on aggrecan gene expression. TGFβ3-treated pellets showed

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124- and 161-fold increases, compared to control, at 3 and 5 weeks, respectively. In TGFβ3-treated pellets, a further significant increase of 72.7% in aggrecan gene expression was induced by EMFs at 5 weeks.

In line with quantitative results showing that TGFβ3 enhances cartilage extracellular matrix production and chondrocyte-specific gene expression, histological data supported that supplementation with the growth factor stimulated chondrogenic differentiation. Histological results obtained at 5 weeks of differentiation are shown in Figure 3. The pellets cultured without TGFβ3 appeared smaller than those cultured with TGFβ3 and they had no visible staining for Alcian blue, type II collagen or aggrecan, regardless of the differentiation time considered. In contrast, pellets cultured in the presence of TGFβ3 exhibited a rounded shape and positive staining for Alcian blue, type II collagen and aggrecan compared to controls. In the pellets, a difference in the distribution of type II collagen and aggrecan was observed because the first preferentially accumulated of at the edges of the pellets, whilst the second was distributed within the pellets. Pellets cultured in the presence of TGFβ3 and exposed to EMFs did not show significant differences in morphology and staining for Alcian blue and type II collagen compared with EMF-unexposed pellets. Only a little more intense staining for aggrecan was observed in EMF exposed TGFβ3-treated pellets compared with unexposed pellets.

3.3. Effects of EMFs on chondrogenesis in IL-1β-treated pellets

In order to evaluate whether EMFs can counteract the action of IL-1β during chondrogenesis, an untreated set and a TGFβ3-treated set of pellets were cultured for 3 and 5 weeks in the presence of 50 ng/ml IL-1β and exposed or unexposed to EMFs. In the absence of TGFβ3, the pellets cultured with IL-1β presented quantitative and histological results similar to the control pellets (data not shown).

In the presence of IL-1β and TGFβ3 into the culture medium, significant time-dependent decreases in PG synthesis (–52% and –74% at 3 and 5 weeks, respectively) and PG content (–35% and –71% at 3 and 5 weeks, respectively) were observed compared to TGFβ3-treated pellets (Figure 4). Interestingly, in the presence of both TGFβ3 and IL-1β, EMF-exposed pellets showed a recovery of 40% at 3 weeks and of 88% at 5 weeks in PG synthesis, when compared to EMF-unexposed pellets. In addition, at 5 weeks a recovery of 138% in PG content was observed in the EMF-exposed TGFβ3- and IL-1β-treated pellets compared to EMF-unexposed pellets. This suggests that the pro-inflammatory cytokine inhibited PG biosynthesis in our pellet cultures and EMF exposure was able to partially reverse this inhibition.

Quantitative real-time RT–PCR analysis showed that IL-1β induced a significant inhibition in both type II

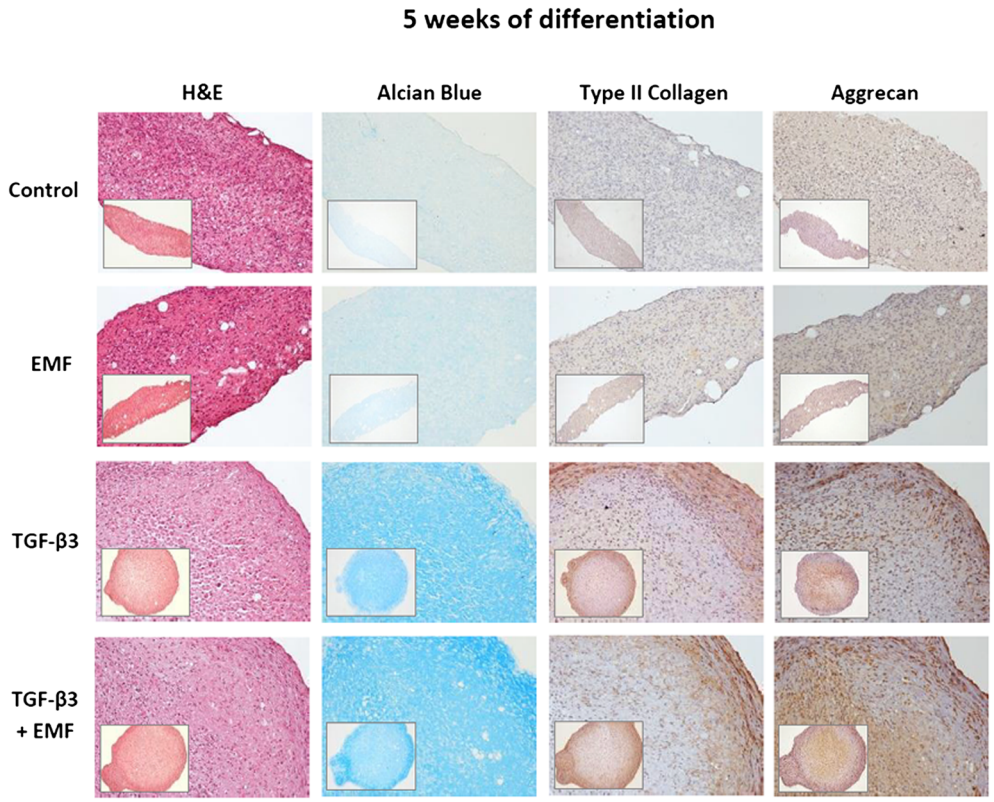

Figure 3. Effects of EMFs on chondrogenic differentiation of MSCs: histological data ($n = 3$). Haematoxylin and eosin (H&E) and alcian blue staining and immunohistochemistry for type II collagen and aggrecan in pellets cultured in the absence and presence of 10 ng/ml TGFβ3, for 5 weeks and exposed or unexposed to EMFs. Higher-magnification images (×200) of the boxed areas (original magnification × 100) are shown

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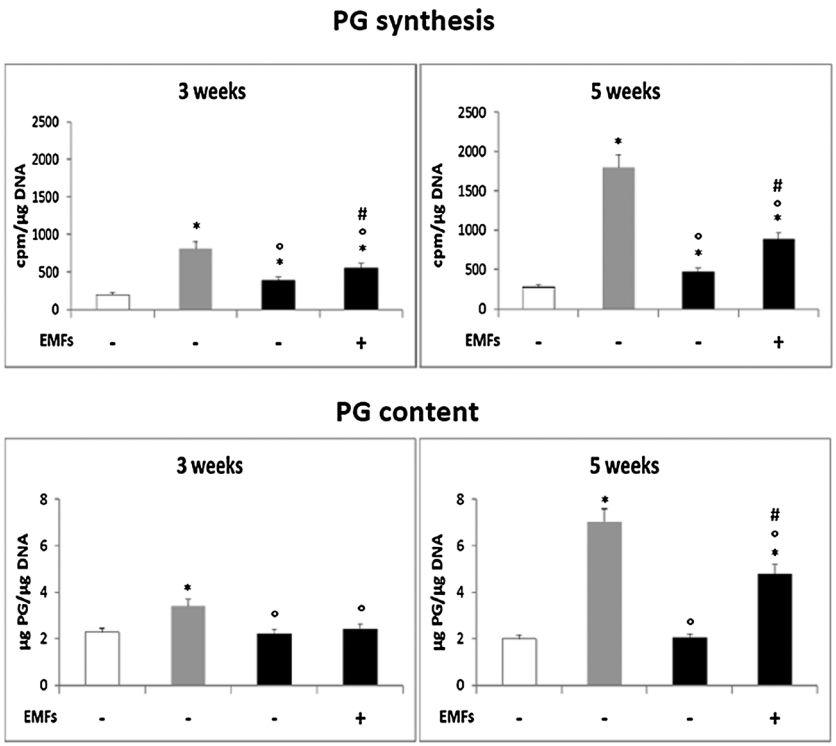

Figure 4. Effects of EMFs on chondrogenic differentiation of MSCs in the presence of the pro-inflammatory cytokine IL-1β: biochemical data ($n = 4$). PG synthesis and PG content in pellets cultured in the absence (white bar) and presence (grey bar) of 10 ng/ml TGFβ3 and 50 ng/ml IL-1β (black bar), for 3 and 5 weeks and exposed or unexposed to EMFs. All data are expressed as mean $\pm$ SD. *Statistical significance vs TGFβ3-untreated and EMF unexposed pellets (control); °statistical significance vs TGFβ3-treated and EMF unexposed pellets; #statistical significance vs unexposed pellets cultured in the presence of TGFβ3 and IL-1β

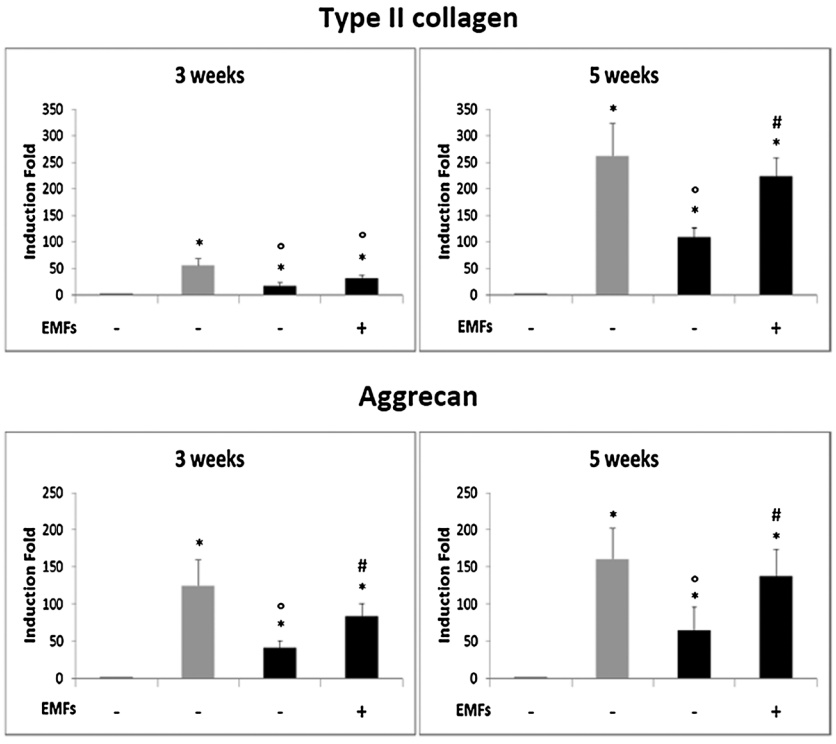

Figure 5. Effects of EMFs on chondrogenic differentiation of MSCs in the presence of the pro-inflammatory cytokine IL-1β: quantitative real-time RT–PCR data ($n = 3$). Type II collagen and aggrecan gene expression in pellets cultured in the absence (white bar) and presence (grey bar) of 10 ng/ml TGFβ3 and 50 ng/ml IL-1β (black bar), for 3 and 5 weeks and exposed or unexposed to EMFs. All data are expressed as mean $\pm$ SD. *Statistical significance vs TGFβ3-untreated and EMF-unexposed pellets (control); °statistical significance vs TGFβ3-treated and EMF-unexposed pellets; #statistical significance vs unexposed pellets cultured in the presence of TGFβ3 and IL-1β

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collagen and aggrecan expression (Figure 5). Specifically, when compared with TGFβ3-treated pellets, the decrease in type II collagen expression was 68% and 59% at 3 and 5 weeks, respectively, and in aggrecan expression 67% and 60% at 3 and 5 weeks, respectively. These reductions were in part counteracted by EMF exposure. In particular, a total recovery in type II collagen gene expression was induced by EMFs at 5 weeks (107% increase compared to EMF-unexposed TGFβ3- and IL-1β-treated pellets) and in aggrecan expression at both differentiation times (105% and 111% increase at 3 and 5 weeks, respectively, compared to EMF-unexposed TGFβ3- and IL-1β-treated pellets).

These findings were confirmed by histological and immunohistochemical results showing a reduction in staining for Alcian blue, type II collagen and aggrecan in pellets cultured in the presence of IL-1β and TGFβ3, compared with pellets cultured with TGFβ3 alone. Interestingly, at 5 weeks of differentiation, in EMF-exposed pellets an appreciable recovery of the histological parameters analysed was observed compared to unexposed pellets (Figure 6).

4. Discussion

In this study we employed the pellet culture system of synovial fluid derived-MSCs to evaluate the effects of EMF exposure on chondrogenesis. MSCs used for cartilage engineering repair strategies have been identified in various adult tissues: bone marrow (Jiang *et al.*, 2002), periosteum, fat, synovial membrane (Pei *et al.*, 2008) and synovial fluid (Jones *et al.*, 2004, 2008). Furthermore, a chondrocyte progenitor population isolated from the surface zone of articular cartilage appears to be a promising cell source for cell-based cartilage repair (McCarthy *et al.*, 2012; Williams *et al.*, 2010).

The choice of our experimental model is due to the observation that MSCs isolated from the synovium and synovial fluid of animals or humans have been shown to have a higher chondrogenic potential than MSCs derived from other tissues (De Bari *et al.*, 2001; Jones *et al.*, 2004, 2008; Park *et al.*, 2005; Shirasawa *et al.*, 2006), thus making them a suitable source for studies on chondrogenesis (Sakaguchi *et al.*, 2005).

Several growth factors, including TGFβ1, TGFβ3, insulin-like growth factor-1 (IGF-1) (Longobardi *et al.*, 2006) and bone morphogenetic proteins (BMPs) (Park *et al.*, 2005), are commonly used to induce chondrogenesis in synovial-derived MSCs or MSCs from other tissues. In our study, we stimulated chondrogenic differentiation by adding 10 ng/ml TGFβ3 to the culture medium. It has been reported that the TGFβ subtypes are equally active chondrogenic factors (Richter, 2009), and both TGFβ1 and TGFβ3 stimulate *in vitro* chondrogenesis (Fortier *et al.*, 2011). For synovium-derived stem cells-based chondrogenesis, TGFβ1 (De Bari *et al.*, 2001; Pei *et al.*, 2008)

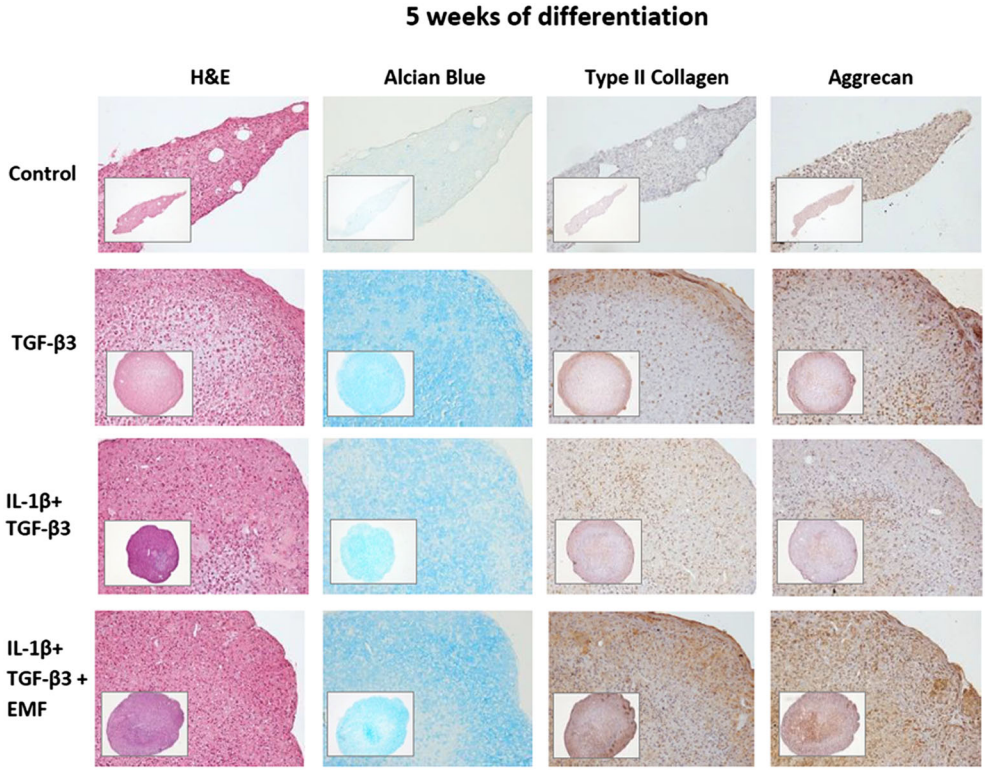

Figure 6. Effects of EMFs on chondrogenic differentiation of MSCs in the presence of the pro-inflammatory cytokine IL-1β: histological data ($n = 3$). H&E and alcian blue staining and immunohistochemistry for type II collagen and aggrecan in pellets cultured in the absence and presence of 10 ng/ml TGFβ3and 50 ng/ml IL-1β, for 5 weeks and exposed or unexposed to EMFs. Higher-magnification images ($\times$200) of the boxed areas (original magnification $\times$ 100) are shown

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and TGFβ3 (Jones *et al*., 2004; Sakaguchi *et al*., 2005; Shirasawa *et al*., 2006) have both been used successfully as chondrogenic factors in different experimental models. In line with these studies, in our TGFβ3-treated pellets the MSCs assumed chondrocyte-like characteristics by synthesizing PGs, type II collagen and aggrecan, as shown by biochemical, quantitative gene-expression and histological results. Regarding treatment times in *in vitro* differentiation experiments, several times of chondrogenic differentiation are reported in the literature, from 2 to 6 weeks (Sakaguchi *et al*., 2005; Shintani and Hunziker, 2007; Shirasawa *et al*., 2006). Specifically, in bovine synovial explants a time-dependent chondrogenic differentiation was observed (Shintani and Hunziker, 2007). Therefore, to analyse chondrogenic differentiation in our experimental model during an early and a later differentiation time, we maintained the pellets in culture for 3 and 5 weeks. We observed a time-dependent increase in the parameters investigated, showing that TGFβ3 induces progressive differentiation with increasing culture time. Regarding the analysis of the EMF effects, the results indicate that under our experimental conditions EMF exposure had limited effects on TGFβ3-induced chondrogenesis, as shown by quantitative and histological data, although it could increase aggrecan gene expression, at least at 5 weeks. Although there is growing evidence showing the impact of EMF exposure on human MSCs during *in vitro* osteogenic differentiation (Schwartz *et al*., 2008; Tsai *et al*., 2009), conversely, there is a lack of research addressing whether the chondrogenic potential of human MSCs can be increased *in vitro* by the application of low-frequency EMFs. However, the study of Mayer-Wagner *et al*. (2011) focused on this aim and analysed, by real-time PCR, the expression of chondrogenic differentiation-related genes. They found that low-frequency electromagnetic stimulation (15 Hz, 5 mT) led to a significant increase in type II collagen mRNA expression in human MSCs at passage 6, but it did not modify aggrecan, SOX 9 and type X collagen mRNA expression. In our study, in the pellets exposed to EMFs, we did not find a significant increase of type II collagen expression analysed by real-time PCR and immunostaining, whilst we observed an increase in aggrecan gene expression. These apparently different results may be related to the tissue source of MSCs or the different cell passage number, as well as to differences in the characteristics of the electromagnetic field.

Regarding the efficacy of cartilage-regenerative procedures, a relevant problem is the presence in the site of the lesion of several pro-inflammatory and catabolic mediators. Among these factors, the cytokine IL-1β plays a crucial role, not only in the inflammatory process but also in the inhibition of *in vitro* chondrogenic differentiation of MSCs (Wehling *et al*., 2009). Accordingly, in our experimental model IL-1β also strongly inhibited chondrogenesis induced by TGFβ3, by reducing PG synthesis and PG content accumulation, type II collagen and aggrecan expression. Notably, in pellets treated with TGFβ3 in the presence of the cytokine, EMF exposure could counteract the inhibitory IL-1β effects on chondrogenesis by sustaining the expression of type II collagen, aggrecan and PGs, although EMFs were not able to completely re-establish the level of chondrogenic differentiation observed in the absence of IL-1β.

These data indicate that synovial MSCs are sensitive to EMFs, in agreement with the results of previous studies on MSCs obtained from different tissues (Mayer-Wagner *et al*., 2011; Schwartz *et al*., 2008). The ability of EMFs to counteract IL-1β activity is in line with previous data showing similar effects in differentiated cartilage (De Mattei *et al*., 2003; Ongaro *et al*., 2011). In fact, in both bovine (De Mattei *et al*., 2003) and human cartilage explants (Ongaro *et al*., 2011) cultured in the presence of IL-1β, EMF exposure counteracted the inhibitory effect on PG synthesis and PG content induced by the cytokine, suggesting an EMF chondroprotective role under inflammatory conditions. Similar anti-inflammatory effects have been also observed in bovine and human synovial fibroblasts, in which EMFs reduced the production of pro-inflammatory mediators and cytokines induced by IL-1β (De Mattei *et al*., 2009; Ongaro *et al*., 2012). In agreement with these previous studies, the results of this study show that EMFs also maintain the capability to inhibit the catabolic activity of IL-1β in MSCs. Although the mechanisms through which EMFs may counteract the inhibitory effects of IL-1β on chondrogenesis remain to be elucidated, these effects might be mediated by an EMF-induced increase of anti-inflammatory adenosine receptors. Indeed, it has been previously reported that EMFs stimulate an increase of A_{2A} and A_3 adenosine receptors, which are involved in the regulation of inflammatory processes in both chondrocytes and synovial fibroblasts (De Mattei *et al*., 2009; Ongaro *et al*., 2012; Varani *et al*., 2008, 2010).

In conclusion, the results of this study support the hypothesis that EMF therapy may maintain and favour chondrogenic differentiation under inflammatory conditions. Low-frequency EMFs are a non-invasive therapy that has not been linked with any side-effects (Benazzo *et al*., 2008b; Zorzi *et al*., 2007). EMFs can be applied during cell culture and beyond under *in vivo* conditions. In regenerative medicine, after implantation of the tissue substitute, physical stimuli could have a primary role, by preventing the catabolic effects of the inflammatory microenvironment and instead favouring the anabolic activities of the implanted cells and surrounding tissues.

Taken together, these results suggest EMF exposure as a possible strategy for improving the clinical outcome of cartilage repair procedures, based on tissue engineering and the use of MSCs.

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Conflicts of interest

Stefania Setti is an employee of IGEA S.p.A. (Carpi, Italy).

References

Aigner T, Sachse A, Gebhard PM, *et al*. 2006; Osteoarthritis: pathobiology – targets and ways for therapeutic intervention. *Adv Drug Deliv Rev* **58**: 128–149.

Benazzo F, Cadossi M, Cavani F, *et al*. 2008a; Cartilage repair with osteochondral autografts in sheep: effect of biophysical stimulation with pulsed electromagnetic fields. *J Orthop Res* **26**: 631–642.

Benazzo F, Zanon G, Pederzini L, *et al*. 2008b; Effects of biophysical stimulation in patients undergoing arthroscopic reconstruction of anterior cruciate ligament: prospective, randomized and double blind study. *Knee Surg Sports Traumatol Arthrosc* **16**: 595–601.

Catterall JB, Stabler TV, Flannery CR, *et al*. 2010; Changes in serum and synovial fluid biomarkers after acute injury (NCT00332254). *Arthritis Res Therapy* **12**: R229.

De Bari C, Dell'Accio F, Tylzanowski P, *et al*. 2001; Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* **44**: 1928–1942.

De Mattei M, Fini M, Setti S, *et al*. 2007; Proteoglycan synthesis in bovine articular cartilage explants exposed to different low-frequency low-energy pulsed electromagnetic fields. *Osteoarthr Cartilage* **15**: 163–168.

De Mattei M, Pasello M, Pellati A, *et al*. 2003; Effects of electromagnetic fields on proteoglycan metabolism of bovine articular cartilage explants. *Connect Tissue Res* **44**: 154–159.

De Mattei M, Pellati A, Pasello M, *et al*. 2004; Effects of physical stimulation with electromagnetic field and insulin growth factor-I treatment on proteoglycan synthesis of bovine articular cartilage. *Osteoarthr Cartilage* **12**: 793–800.

De Mattei M, Varani K, Masieri FF, *et al*. 2009; Adenosine analogs and electromagnetic fields inhibit prostaglandin E2 release in bovine synovial fibroblasts. *Osteoarthr Cartilage* **17**: 252–262.

Fini M, Giavaresi G, Torricelli P, *et al*. 2005; Pulsed electromagnetic fields reduce knee osteoarthritic lesion progression in the aged Dunkin Hartley guinea pig. *J Orthop Res* **23**: 899–908.

Fortier LA, Barker JU, Strauss EJ, *et al*. 2011; The role of growth factors in cartilage repair. *Clin Orthop Relat Res* **469**: 2706–2715.

Goldring MB. 2006; Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. *Best Pract Res Clin Rheumatol* **20**: 1003–1025.

Guilak F, Awad HA, Fermor B, *et al*. 2004; Adipose-derived adult stem cells for cartilage tissue engineering. *Biorheology* **41**: 389–399.

Jiang Y, Jahagirdar BN, Reinhardt RL, *et al*. 2002; Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**: 41–49.

Jones EA, Crawford A, English A, *et al*. 2008; Synovial fluid mesenchymal stem cells in health and early osteoarthritis: detection and functional evaluation at the single-cell level. *Arthritis Rheum* **58**: 1731–1740.

Jones EA, English A, Henshaw K, *et al*. 2004; Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor cells in inflammatory and degenerative arthritis. *Arthritis Rheum* **50**: 817–827.

Longobardi L, O'Rear L, Aakula S, *et al*. 2006; Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-β signaling. *J Bone Miner Res* **21**: 626–636.

Mayer-Wagner S, Passberger A, Sievers B, *et al*. 2011; Effects of low frequency electromagnetic fields on the chondrogenic differentiation of human mesenchymal stem cells. *Bioelectromagnetics* **32**: 283–290.

McCarthy HE, Bara JJ, Brakspear K, *et al*. 2012; The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse. *Vet J* **192**: 345–351.

Ongaro A, Pellati A, Masieri FF, *et al*. 2011; Chondroprotective effects of pulsed electromagnetic fields on human cartilage explants. *Bioelectromagnetics* **32**: 543–551.

Ongaro A, Varani K, Masieri FF, *et al*. 2012; Electromagnetic fields (EMFs) and adenosine receptors modulate prostaglandin E (2) and cytokine release in human osteoarthritic synovial fibroblasts. *J Cell Physiol* **227**: 2461–2469.

Ortiz LA, Dutreil M, Fattman C, *et al*. 2007; Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci USA* **104**: 11002–11007.

Park Y, Sugimoto M, Watrin A, *et al*. 2005; BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel. *Osteoarthr Cartilage* **13**: 527–536.

Pei M, He F, Vunjak-Novakovic G. 2008; Synovium-derived stem cell-based chondrogenesis. *Differentiation* **76**: 1044–1056.

Richter W. 2009; Mesenchymal stem cells and cartilage *in situ* regeneration. *J Intern Med* **266**: 390–405.

Rizzo P, Miao H, D'Souza G, *et al*. 2008; Cross-talk between notch and the estrogen receptor in breast cancer suggests novel therapeutic approaches. *Cancer Res* **68**: 5226–5235.

Saino E, Fassina L, Van Vlierberghe S, *et al*. 2011; Effects of electromagnetic stimulation on osteogenic differentiation of human mesenchymal stromal cells seeded onto gelatin cryogel. *Int J Immunopathol Pharmacol* **24**: 1–6.

Sakaguchi Y, Sekiya I, Yagishita K, *et al*. 2005; Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum* **52**: 2521–2529.

Schmal H, Mehlhorn A, Stoffel F, *et al*. 2009; *In vivo* quantification of intraarticular cytokines in knees during natural and surgically induced cartilage repair. *Cytotherapy* **11**: 1065–1075.

Schwartz Z, Simon BJ, Duran MA, *et al*. 2008; Pulsed electromagnetic fields enhance BMP-2 dependent osteoblastic differentiation of human mesenchymal stem cells. *J Orthop Res* **26**: 1250–1255.

Sellam J, Berenbaum F. 2010; The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nat Rev Rheumatol* **6**: 625–635.

Shintani N, Hunziker EB. 2007; Chondrogenic differentiation of bovine synovium: bone morphogenetic proteins 2 and 7 and transforming growth factor-β1 induce the formation of different types of cartilaginous tissue. *Arthritis Rheum* **56**: 1869–1879.

Shirasawa S, Sekiya I, Sakaguchi Y, *et al*. 2006; In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells. *J Cell Biochem* **97**: 84–97.

Tsai MT, Li WJ, Tuan RS, *et al*. 2009; Modulation of osteogenesis in human mesenchymal stem cells by specific pulsed electromagnetic field stimulation. *J Orthop Res* **27**: 1169–1174.

Tuli R, Nandi S, Li WJ, *et al*. 2004; Human mesenchymal progenitor cell-based tissue engineering of a single-unit osteochondral construct. *Tissue Eng* **10**: 1169–1179.

Varani K, De Mattei M, Vincenzi F, *et al*. 2008; Characterization of adenosine receptors in bovine chondrocytes and fibroblast-like synoviocytes exposed to low frequency low energy pulsed electromagnetic fields. *Osteoarthr Cartilage* **16**: 292–304.

Varani K, Vincenzi F, Tosi A, *et al*. 2010; Expression and functional role of adenosine receptors in regulating inflammatory responses in human synoviocytes. *Br J Pharmacol* **160**: 101–115.

Wehling N, Palmer GD, Pilapil C, *et al*. 2009; Interleukin-1β and tumor necrosis factor-α inhibit chondrogenesis by human mesenchymal stem cells through NF-κB-dependent pathways. *Arthritis Rheum* **60**: 801–812.

Williams R, Khan IM, Richardson K, *et al*. 2010; Identification and clonal characterisation of a progenitor cell sub-population in normal human articular cartilage. *PLoS One* **5**: e13246.

Zorzi C, Dall'Oca C, Cadossi R, *et al*. 2007; Effects of pulsed electromagnetic fields on patients' recovery after arthroscopic surgery: prospective, randomized and double-blind study. *Knee Surg Sports Traumatol Arthrosc* **15**: 830–834.