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Adenoviral BMP-2 Gene Transfer in Mesenchymal Stem Cells: *In Vitro* and *in Vivo* Bone Formation on Biodegradable Polymer Scaffolds

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The aim of this study was to determine the feasibility of adenoviral gene transfer into primary human bone marrow osteoprogenitor cells in combination with biodegradeable scaffolds to tissue-engineer bone. Osteoprogenitors were infected with AxCAOBMP-2, a vector carrying the human BMP-2 gene. Alkaline phosphatase activity was induced in C2C12 cells following culture with conditioned media from BMP-2 expressing cells, confirming successful secretion of active BMP-2. Expression of alkaline phosphatase activity, type I collagen and mineralisation confirmed bone cell differentiation and maintenance of the osteoblast phenotype in extended culture for up to 6 weeks on PLGA porous scaffolds. *In vivo* implantation of adenoviral osteoprogenitor constructs on PLGA biodegradeable scaffolds, using diffusion chambers, also demonstrated bone cell differentiation and production of bone tissue. The maintenance of the osteoblast phenotype in extended culture and generation of mineralised 3-D scaffolds containing such constructs indicate the potential of such bone tissue engineering approaches in bone repair.

Key Words: **osteoprogenitor; adenoviral gene-transfer; biodegradable polymer; poly(DL-lactic-*co*-÷glycolic acid); PLGA; tissue engineering.**

The replacement of bone is a major clinical and socioeconomic need. Approaches include the use of autologous and allogeneic bone to restore the function of traumatised or degenerated bone tissue. However, a lack of sufficient material precludes the universal use of autogenous bone while the use of allogenic bone for transplantation carries potential risks of immune responses, pathogen transmission and the necessary immunosuppression. Tissue engineering has emerged as a possible alternative strategy to regenerate bone. Three components are essential: isolation and expansion of osteoprogenitors or mesenchymal stem cells, provision of appropriate osteoinductive factors and an appropriately designed scaffold that mimics the structural environment to promote bone regeneration.

Bone marrow contains multipotential stromal stem cells which can differentiate into fibroblastic, osteogenic, adipogenic and reticular cells (1–3). A number of studies have shown that human bone marrow osteoprogenitors can be isolated using selective markers and are readily expanded while retaining their differentiation ability, indicating their potential for marrow repopulation (4–7). Individual fibroblastic colonies have been shown to give rise to an osteogenic tissue within diffusion chambers while studies in a variety of animal species and more recently in preclinical trials in humans, indicate marrow tissue is capable of extensive osteogenesis (8–15).

The osteoinductive factors of choice are the bone morphogenetic proteins (BMPs), pivotal in the process of bone formation (16, reviewed in 17, 18). Over 30 distinct forms of the BMPs exist although the most widely studied are BMP-1 through 7. The BMPs induce differentiation of multipotential mesenchymal cells (19), pluripotent murine stem cell cultures and rat bone marrow stromal cells as well as proliferation and maturation in osteoblast populations (19–23).

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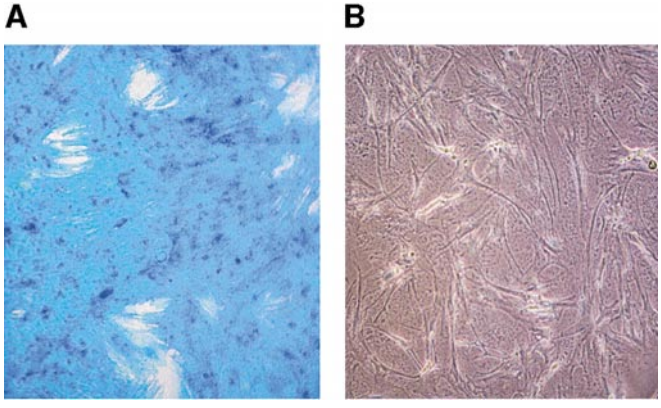

FIG. 1. Cultured primary human bone marrow cells were grown to confluence and transduced with AxCALacZ at increasing MOI or mock infection. After 3 days the cells were fixed and stained for β-galactosidase activity. (A) MOI 20. (B) Mock infection.

Although BMPs can induce bone formation, the inability to identify a suitable delivery system has, to date, limited their clinical use. The use of a cell delivery vehicle (gene therapy) offers a simple solution and a number of studies in recent years have examined the potential of adenoviral delivery of BMP-2 in target cell populations (24–27). These studies demonstrate bone formation *in vivo;* however, a number of issues regarding the use of adenovirus remain, including potential immune response, clearance, and safety of the virus, quantification and duration of protein production.

Synthetic materials, such as poly(lactic acid) (PLA), poly(DL-lactic- *co*-÷glycolic acid) (PLGA), polyglycolic acid (PGA) have emerged as potential scaffolds for cell transplantation and tissue growth. These materials have the advantage of FDA approval and are currently used as suture materials and in drug delivery vehicles. Moreover, protocols for the generation of these materials with defined porosity using super critical fluid mixing and procedures for the surface modification of these materials with biological agents, have been developed (reviewed in 28, 29). The resultant scaffolds generate attractive biomimetic scaffolds for cell growth and differentiation including isolated human osteoprogenitors (30–32).

Thus, an alternative attractive approach for skeletal repair is the selection and genetic modulation of primary human osteoprogenitor cells in combination with biodegradable polymer scaffolds, which interact and promote osteoblast differentiation and osteogenesis. These studies demonstrate bone formation, *in vitro* and *in vivo,* using human osteoprogenitors secreting active BMP-2 on a biodegradeable polymer scaffolds. The formation of mineralised bone tissue within diffusion chambers in nude mice highlight the potential to tissue- engineer bone for orthopaedic use.

MATERIALS AND METHODS

Materials. Tissue culture reagents were obtained from Gibco/BRL (Paisley, Scotland). Fetal Calf Serum was from Meldrum Ltd (Bourne End, UK). Resin support was purchased from Novabiochem (Calbiochem-Novabiochem (UK) Ltd, Beeston, Nottingham). Dexamethasone, alkaline phosphatase kits and all other biochemical reagents were of analytical grade from Sigma Chemical Company (Poole, Dorset) unless otherwise stated.

Cell culture. Bone marrows were obtained from haematologically normal patients undergoing routine total hip replacement surgery. Only tissue, which would have been discarded, was used with the approval of the Southampton General Hospital Ethics Committee. Primary cultures of bone marrow cells were established as previously described (33). In brief, marrow cells were harvested using Minimal Essential Medium - alpha modification (αMEM) from trabecular bone marrow samples and pelleted by centrifugation at 500 *g* for 5 min at 4°C. The cell pellet was resuspended in 10 ml αMEM and passed through nylon mesh (70 μm pore size; Becton–Dickinson, UK). Cells were maintained in 10% FCS in αMEM. C2C12 and HEK293 cells were grown in Dulbecco's modified Eagle medium with 10% FCS. For cell growth on PLGA scaffold, following trypsinization and resuspension, in serum-free αMEM, 1×10^6 cells were then added to individual wells of 24-well plates containing PLGA scaffolds After 24 h, the media was removed and cultures maintained in αMEM supplemented with 10% FCS for up to 6 weeks.

Infection of cells with adenovirus expressing BMP-2. Cell lines were transduced with AxCAOBMP-2, a replication-deficient adenoviral vector carrying the human BMP-2 gene or AxCALacZ (hereafter termed AdBMP2 or AdLacZ, respectively), a control vector carrying the *Escherichia coli* (*E. coli*) *LacZ* gene. The construction of AdBMP2 has been described previously (34). Virus was amplified in 293 cells, purified through a CsCl cushion and titrated as previously described (35). Cells were infected with the adenovirus once confluence had been reached and maintained for several days. Virus was added to the cells at various multiplicities of infection (MOI) in media containing 5% FCS. Flasks were rotated every 30 min for 1.5 h before addition of the same volume of fresh 5% FCS αMEM.

X-gal histochemistry and immunohistochemistry. Expression of β-galactosidase was visualised by staining with X-gal. Following 72 h exposure to AdLacZ at various MOI cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were stained for

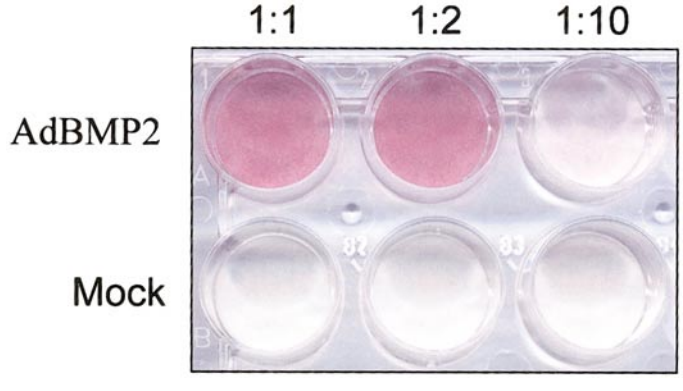

FIG. 2. C2C12 cells stained for alkaline phosphatase activity after exposure to conditioned media from AdBMP2 transduced cells or Mock transduced cells. Virus was added to confluent human bone marrow cells, washed after 24 h and given fresh medium. After 3 days the medium was removed, filtered, and applied to confluent C2C12 cells for 3 days. After fixation, the cells were stained for alkaline phosphatase activity.

1–3 h at 37°C using a solution containing X-gal (20 mg/ml), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride in PBS.

C2C12 alkaline phosphatase assay. BMP-2 activity was screened by conditioned media transfer to promyoblastic C2C12 cells as previously described (36). Briefly, following 24 h exposure to AdBMP-2, the media was removed and replaced with fresh. At various time intervals conditioned media was filtered and transferred to confluent C2C12 cells in a 96-well microtitre plate. Dilutions, 1:1, 1:2, or 1:10, were prepared with fresh 2% DMEM. Cells were incubated for a further 72 h before assaying for alkaline phosphatase activity. The effect of recombinant BMP-2 was measured in a range from 10–200 nM over 72 h in DMEM supplemented with 2% FCS. Cells were washed twice with PBS then fixed with cold 95% ethanol. Cell layers were washed with phosphate-buffered saline (PBS) and stored at −70°C until assayed for alkaline phosphatase activity. For assay, the cell layer from each well was scraped into 0.2 ml 0.05% (v/v) Triton X-100. Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (1.5 M, pH 10.3 at 25°C). DNA content was measured using PicoGreen as per manufacturer's instructions (Molecular Probes, Leiden, The Netherlands). Alkaline phosphatase specific activity was expressed as nanomoles of p-nitrophenol/min/ng DNA

Scaffolds. Poly(DL-lactic- *co*-÷glycolic acid) (PLGA 75:25) (Mw 22K) porous (200 μm) scaffolds were used in all studies. The scaffolds were produced by a supercritical carbon dioxide method in which the polymer is plasticised at 35°C under a pressure of 1500 psig (29). On release of the pressure, pores are formed in the polymer by the escape of the carbon dioxide gas. The PLGA used in this study will dissolve in the body over 10–12 weeks and was selected on the ability of incorporated glycolic acid to allow sufficiently rapid degradation of the poly lactic acid component. Porous scaffolds were sterilized using 70% ethanol for 3 h and coated with αMEM supplemented with 20% FCS for 3 h.

Alkaline phosphatase expression. Following 72 h exposure to media conditioned by AdBMP-2-infected cells, C2C12 cells were rinsed twice with PBS then fixed for 10 min in 95% ethanol at room temperature. Staining was with a Sigma alkaline phosphatase kit (No. 85) used according to the manufacturer's instructions.

Cell viability. Adenovirally transduced human bone marrow cells were incubated with Cell Tracker green (5-chloromethylfluorescein diacetate, CFMDA) (Molecular Probes, Leiden, The Netherlands) and Ethidium Homodimer-1 (EH-I) (Molecular Probes, Leiden, The Netherlands) for 45 min to label viable and necrotic cells respectively. The medium was then replaced and the cells incubated for a further hour.

Microscopy and image analysis. Images from PLGA porous scaffolds were taken using an inverted microscope (Leica DMIRB/E), equipped with a fluorescence filter enabling fluorescent imaging. Cells labeled with CFMDA and EH-1 were recorded on a Leica Leitz DM RBE with an X50 water immersion objective. Electron microscopy was undertaken using a Hitachi S-800.

Histochemistry and immunohistochemistry. Prior to immunocytochemical and histochemical analysis, PLGA scaffold samples were fixed with 4% Paraformaldehyde or 95% ethanol, dependent on the staining protocol and, as appropriate, processed to paraffin wax and 5-μm sections were prepared. Controls (omission of primary antibody) were included in all studies.

Alcian blue/Sirius red staining. Sections were stained using Weigert haematoxylin solutions prior to staining with 0.5% Alcian blue. After treatment with 1% molybdophosphoric acid, samples were stained using 0.1% Sirius red.

Toluidine blue and von Kossa staining. Samples were stained with 1% $AgNO_3$ under UV light for 20 min until black deposits were visible and after air drying, slides were counterstained with toluidine blue.

Type I collagen. Reactivity to Type I collagen antibody (LF 67, Dr. Larry Fisher, NIH, U.S.A.) was assessed after fixation in 4% Paraformaldehyde for 3 h. Endogenous peroxidase activity was blocked using 3% H_2O_2 prior to incubation with LF 67 (1:300 in PBS) for 3 h at 4°C. Samples were incubated with peroxidase-conjugated anti-rabbit IgG (1:30 in PBS) and peroxidase activity was detected using 3-amino-9-ethyl-carbazole in acetate buffer containing H_2O_2. Samples were counterstained with Mayer's haematoxylin.

In vivo studies. Adenovirally labeled human bone marrow cells were cultured in αMEM medium containing 5% (v/v) FCS prior to intraperitoneal implantation (2×10^6 cells/chamber) using diffusion chambers (130 μl capacity; Millipore, UK) in MF1-nu/nu mice. The diffusion chamber model provides an enclosed environment within a host animal to study the osteogenic capacity of skeletally derived cell populations which resolves the problems of host versus donor bone tissue generation. Cells were recovered by collagenase (*Clostridium histolyticum*, type VII; 25 U/ml) and trypsin (0.05% trypsin and 0.2% ethylenediaminetetraacetic acid in PBS, pH 7.4) digestion. Diffusion chambers were implanted intraperitoneally into athymic MF1-nu/nu mice (Harlan UK Ltd; 20–24 g, 4–5 weeks old, Harlan UK Ltd). Control chambers contained human bone marrow samples on PLGA scaffolds alone. After 6–10 weeks the mice were killed, chambers removed, examined by X-ray analysis and fixed in 95% ethanol at 4°C. Samples were processed undecalcified and sectioned at 5 μm and stained using toluidine blue and for type I collagen expression as well as mineralization by von Kossa.

RESULTS

Adenoviral Infection of Primary Human Bone Marrow Cells

The effectiveness of gene transfer into osteoprogenitor cells was examined using primary human bone marrow cells infected with AdLacZ at an MOI of 6.25, 12.5, 25, 50 and 100. After 72 h, the cells were stained for β-galactosidase activity. Efficiency of transduction was observed to be >98% and independent of MOI. X-Gal staining intensity was not affected by increasing MOI. Mock infected cells showed negligible β-galactosidase activity (Fig. 1).

Secretion of BMP-2 Following Adenoviral Infection

Having confirmed that osteoprogenitor cells could be infected with adenoviral constructs, human bone marrow cells were transduced with AdBMP-2 using an MOI of 20 (titre 4×10^9). The adenoviral vector AdBMP-2 has been shown previously by one of us to produce active BMP-2 protein by Western Blot and biochemical assay (34). To confirm secretion of active BMP-2 by the marrow stromal cells, media from transduced cultures were transferred to confluent promyoblastic C2C12 cells and alkaline phosphatase activity examined by histochemical and biochemical analysis. C2C12 cells exposed to the conditioned media from transduced cell lines showed strong staining for alkaline phosphatase while C2C12 cells exposed to conditioned media from mock infected cells showed no stain-

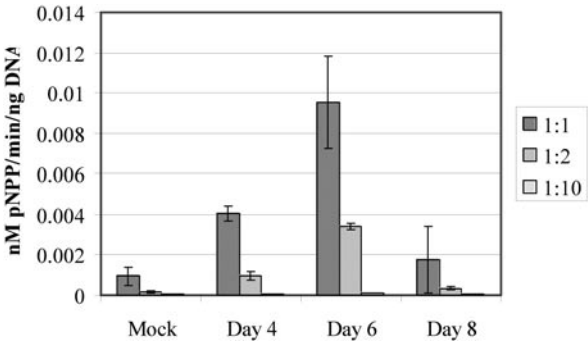

FIG. 3. Quantification of BMP2 activity in the medium of Ad-BMP2 transduced human bone marrow cells. After 24 h exposure to the virus, the cells were washed and given fresh medium. At the time points indicated all the media were removed and fresh medium was added. Alkaline phosphatase activity was measured as described in the C2C12 assay. Values indicate mean ± standard deviation.

ing (Fig. 2) confirming the secretion of active BMP-2 by osteoprogenitor cells transduced with AdBMP-2.

The concentration of BMP-2 produced by the human bone marrow cells transduced with AdBMP-2 was determined from quantitative alkaline phosphatase activity assays in C2C12 cells using standard curves prepared with rhBMP2 at concentrations of 10–200 nM (data not shown). Calculation of the concentration of BMP-2 in the media of the infected cells from 4 patients ranging in age from 14–72 give values of 10, 11.8, 109, 165.4 nM although no correlation with patient age was observed. A range is to be expected given the inherent variability in human bone marrow samples.

Secretion of active BMP2 was followed over 8 days. Flasks of identically prepared cells were transduced with AdBMP2 or mock. Virus was washed from the cells after 24 h and fresh media applied (Day 0). At 4, 6, and 8 days, the media was removed, filtered and fresh media provided. BMP2 activity was measured as for rhBMP2 (Fig. 3). Mock transduced cells displayed low alkaline phosphatase activity reflecting low constitutive expression. The peak of protein production was observed between days 4 and 6 when levels were higher over this 2 day period than from days 0–4. By day 8, production had decreased to control levels.

Growth of Adenoviral-Infected Osteoprogenitors on PLGA Scaffolds

Human bone marrow cells transduced with AdBMP2 were seeded onto biodegradable porous PLGA (75:25) (200 μm) scaffolds in basal media (media supplemented with 10%fetal calf serum alone). Cell adhesion and extensive cell in-growth were observed following culture for 4 weeks as observed by scanning electron microscopy (Fig. 4A). Cell viability was examined using Cell Tracker green and EH-1 (Fig. 4B). The presence of intense fluorescent staining of the human osteoprogenitor cells as a consequence of label incorporation confirmed cell viability of transduced cells after 4 weeks on the porous scaffolds. The lack of cell necrosis was evidenced by the absence of ethidium homodimer-1 staining. These results indicate that adenovirally transduced cells are capable of adhering and proliferating on PLGA scaffold *in vitro.*

Differentiation of the cells to the osteoblast phenotype was confirmed by histochemical staining for type I collagen, Alcian blue/Sirius red and evidence of extensive mineralisation (Fig. 5). Immunostaining for Type I collagen was observed after extended *in vitro* culture for 4 weeks (Fig. 5A) and extracellular matrix formation was observed by Sirius red and alcian blue staining (Fig. 5B). No immunostaining was observed in type I collagen) controls (omission of primary antibody-data not shown). Further evidence of matrix formation and mineralisation of the porous scaffolds was confirmed by von Kossa staining (Figs. 5C and 5D). Mineralisation was not observed on control bone marrow cells cultured on tissue culture plastic alone (data not shown). The results are consistent with osteoblastic differentiation of the transduced bone marrow cells.

Differentiation of Adenovirally Transduced Human Bone Marrow Cells in Vivo

Adenovirally transduced human bone marrow cells expressing BMP2 cultured in basal media only, in the absence of ascorbate and dexamethasone, were injected into diffusion chambers containing PLGA scaffolds and implanted intraperitoneally in 5 nude mice. The chambers were removed after 4 and 7 weeks and examined for bone formation by X-ray analysis and histochemical analysis. X-ray analysis indicated the presence of bone tissue (Fig. 6A) after only 4 weeks in 3 of 5 mice. On histological analysis, extensive cell growth on the PLGA scaffolds was observed and morphological evidence for the formation of cartilage and bone tissue by adenovirally transduced human bone marrow cells expressing BMP-2 (Figs. 6B–6I). Immunolocalisation and histochemical analysis showed expression of alkaline phosphatase (Fig. 6C), type I collagen and extensive matrix formation (Figs. 6D–6H). Metachromatic staining with toluidine blue in combination with von Kossa staining indicated the presence of bone and cartilage respectively (Figs. 6B–C and 6F–H). New bone formation was confirmed using polarising light microscopy to demonstrate, by birefringence, new collagen formation (Figs. 6E and 6H). In diffusion chambers containing human bone marrow cells alone, in the absence of BMP-2, no evidence of bone and cartilage formation was observed (data not shown). These results indicate osteoblastic differentiation and bone formation in cells transduced with an adenoviral vector expressing human BMP2 *in vivo.*

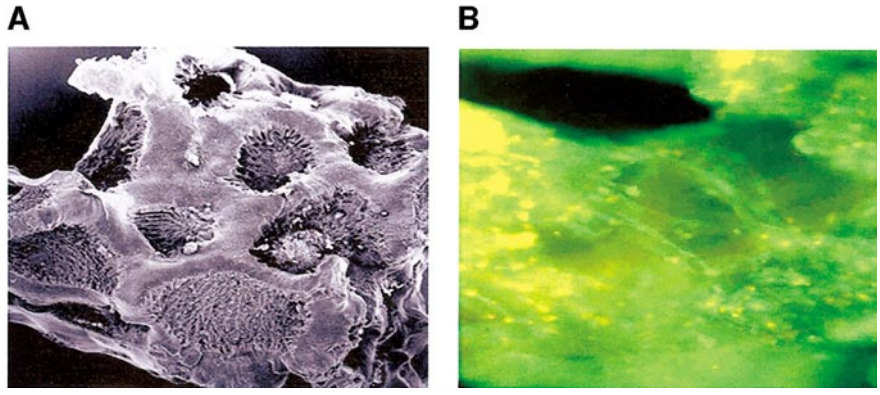

FIG. 4. Cultured primary human bone marrow cells were transduced with AdBMP2, seeded onto PLGA scaffolds and maintained for 4 weeks. (A) Confocal image of scaffold alone. (B) Confocal image of cells stained with Cell Tracker green and EH-1 showing adhesion and in growth of the cells onto the scaffold.

DISCUSSION

The current study indicates the potential to engineer bone tissue using selected human bone marrow mesenchymal cells transduced with an adenoviral vector expressing BMP-2 on designed porous biodegradeable polymer scaffolds. *In vitro,* active BMP-2 was secreted in the 10–100 nM range, the transduced cells expressed an osteoblast phenotype and formed mineralised 3-D structures on porous PLGA scaffolds following culture for 4 weeks. All studies were performed using basal media (media supplemented with 10% fetal calf serum alone) in the absence of dexamethasone or ascorbate. Evidence for the transduced constructs to generate bone tissue was confirmed using a diffusion chamber assay, which allows unequivocal demonstration of new bone tissue formation by the implanted cells within a closed environment. New bone formation in primary human bone marrow cells expressing human BMP2 on a porous PLGA scaffold was observed.

The ability to infect a wide variety of cell types including dividing and nondividing cells combined with ease of manipulation, and high efficiency of gene transfer in the absence of integration into the host genome,

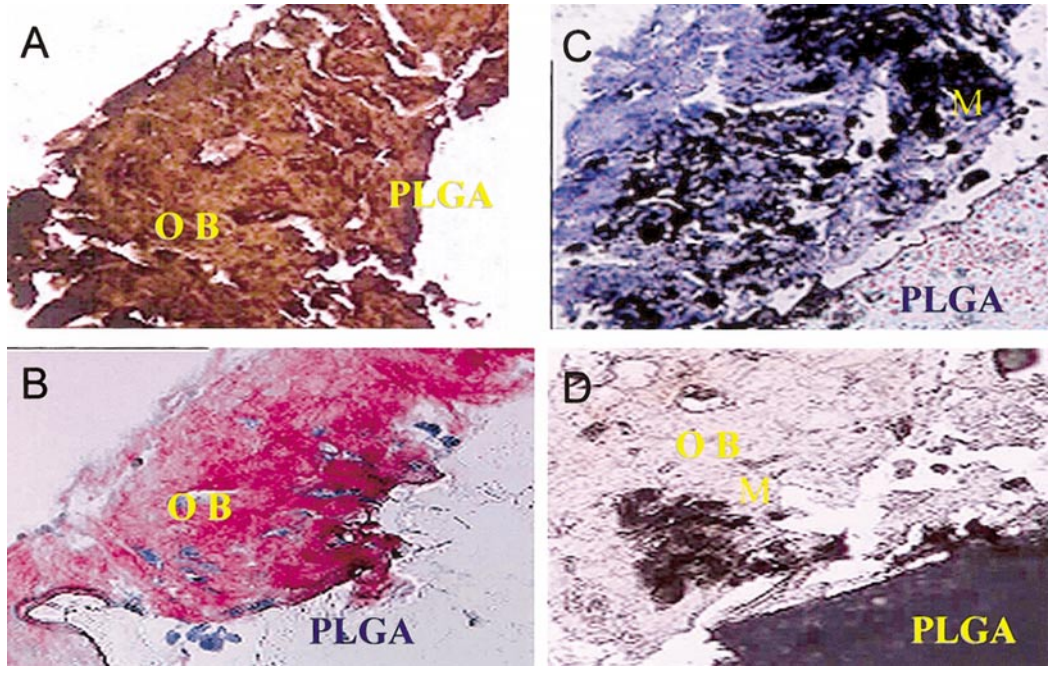

FIG. 5. Cultured primary human bone marrow cells were transduced with AdBMP2, seeded on PLGA scaffold and maintained in basal medium for 4 weeks. Samples were embedded in paraffin and sectioned. (A) Staining with Alcian blue and Sirius red demonstrating extensive matrix formation; (B) Type I collagen; (C) toluidine blue and von Kossa; (D) Von Kossa alone. Magnification ×100.

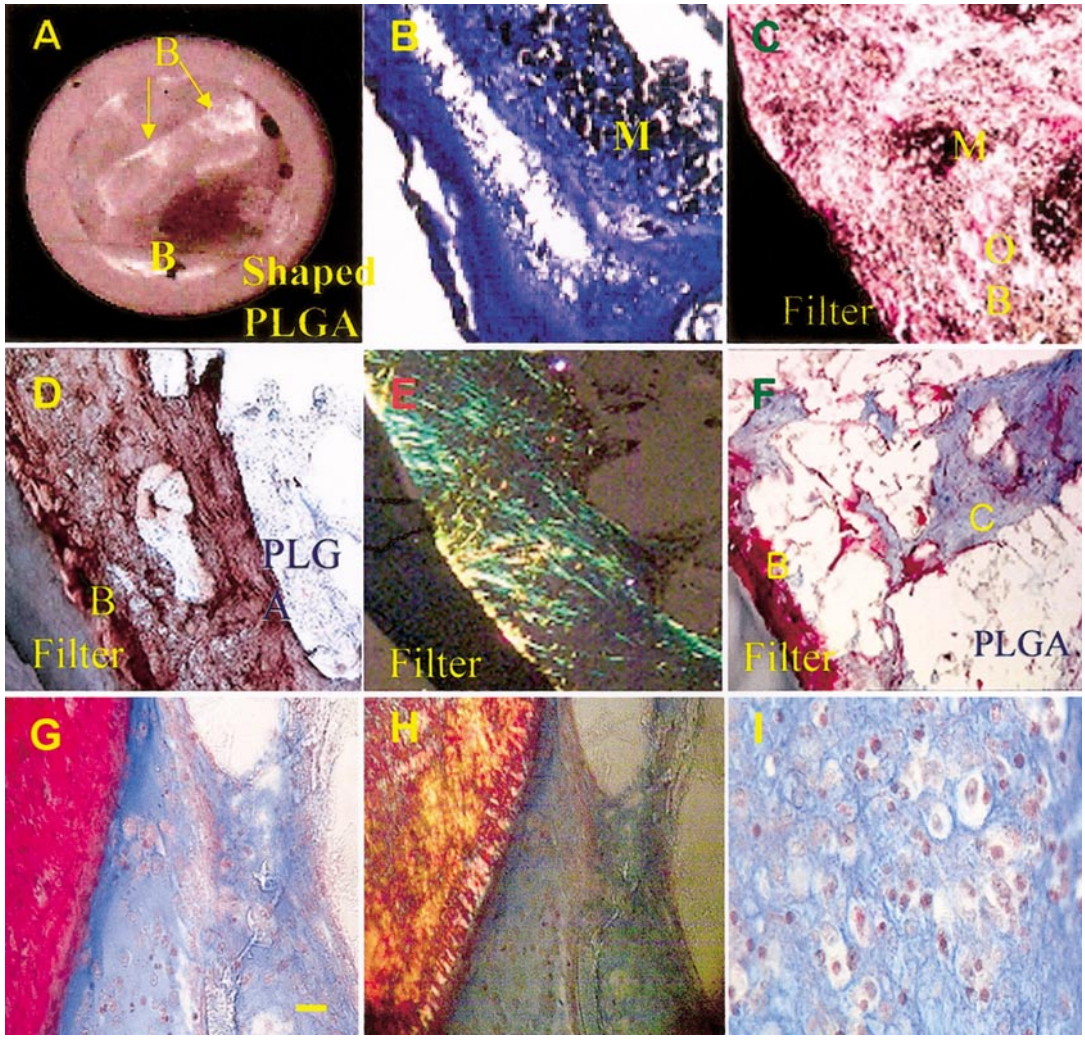

FIG. 6. Cartilage and bone formation within diffusion chambers after 4 and 7 weeks by cultured primary human bone marrow cells in basal media transduced with AdBMP2, seeded on PLGA scaffold, and implanted intraperitoneally into MF1nu/nu mice. (A) X-ray analysis of new bone formation within shaped PLGA scaffolds, (B) mineralisation as observed by von Kossa and (C) alkaline phosphatase with von Kossa staining, (D) Type I collagen immunocytochemistry and (E) parallel section viewed using polarised light to demonstrate new collagen sheet formation in woven bone by birefringence, (F and G) islands of cartilage and bone within the PLGA scaffold as viewed by Alcian blue and Sirius red staining respectively, (H) parallel section from H, viewed using polarised light and, (I) cartilage formation as demonstrated by Alcian blue staining.

makes recombinant adenoviruses an attractive choice for gene therapy. In this study the high level of infection of the heterogeneous target cell population containing osteoprogenitor cells at different stages of differentiation confirms the selective advantage of adenoviral vectors for gene therapy. However adenoviral infection has several key limitations (i) immunogenicity *in vivo* is a significant issue, (ii) long infection times are deleterious, (iii) delivery of cells requires expansion in culture prior to viral infection and reimplantation and, (iv) fate of adenoviral cells is unclear. A number of these issues have been circumvented by using a facile and reproducible technique to generate high titre of virus enabling infections of target cell populations at low MOI.

Previous studies indicate a dose of 10^7 particle units or an MOI of >50 will provoke an immune response (37, 38). In this study, a low MOI in comparison to the work of others has been used (25, 39–41) which may help to reduce these complications. The observation of over 98% efficiency of transduction with AdLacZ with low MOI differs from some reports (42); however, Baltzer *et al.* (24) report 100% AdLacZ transduction in an osteoblast cell line. The importance of the relationship between efficiency of transduction and levels of protein secretion remains to be determined, although, in this system sufficient protein was expressed to induce differentiation while using a low MOI.

Another factor that may contribute to a reduction in immunogenicity is highlighted by Cheng *et al.* (25) who

found that increasing the time to 7 days between transduction and implantation increased bone formation (3 of 5 positive by X-ray). In the current study, cells were implanted 3-7 days after transduction with 3 of 5 positive for bone formation by X-ray and 5 of 5 positive for bone markers histologically. This delay may allow for differentiation to begin before implantation when immune response may clear the virus. To eliminate the problems associated with delivery of BMP-2 to the required site Musgrove and co-workers reported on the use of direct adenoviral mediated gene therapy to deliver active BMP-2 and produce bone in skeletal muscle. Direct adenoviral mediated gene therapy, although attractive, produced little bone probably due to rapid immune clearance of the virus (27) and raises issues of pathogenicity and safety. In contrast to Turgeman (40) who allowed the virus to remain in the media, this study demonstrates that an exposure of the virus for 24 h followed by washing and provision of fresh medium was sufficient for transduction. In combination with the low MOI, delay in implantation and removal of the virus, these approaches may ameliorate the problem of immunogenicity. The balance between osteoblastic differentiation and induction of an immune response will need to be determined before progress toward preclinical models can be anticipated.

Interestingly, Olmsted and co-workers (43) were unable to demonstrate BMP2 secretion from primary bone marrow stromal cells that had reached confluence and were transduced with an adenoviral vector expressing BMP2. In the current system, cells were allowed to become confluent before transduction and subsequently expressed BMP2. This discrepancy may be explained by the different manner in which the cells were handled prior to confluence. For instance, the use of antibiotics and antifungals were avoided and cells were used at primary and first passage only. Clearly differences in experimental approaches have the potential to subtly alter the phenotype of primary cells, potentially either enabling or prohibiting adenoviral transduction.

The exact levels of BMP2 present *in vivo* are not clear. Active BMP2 was measured at 10–165 nM in comparison to rhBMP2 and was secreted for up to 8 days. These levels were sufficient to induce osteoblastic differentiation without toxicity to the cells. The time scale of secretion is in agreement with others measurements of adenoviral BMP2 production in cell lines (39, 43) with a peak at approximately 6 days followed by a rapid decrease.

This study expands on the tissue engineering theme by seeding transduced cells on a biodegradable scaffold which should avoid the potential for unwanted migration of cells. The generation of 3-D porous biodegradeable constructs *in vitro* is attractive in the development of skeletal repair strategies providing a scaffold/filler for bone regeneration. The development of supercritical fluid mixing technology to generate porous polymer scaffolds of defined porosity and degradation characteristics by Howdle and colleagues has provided new platform technologies for osteoprogenitor differentiation and mineralisation. These scaffolds resorb by hydrolysis and have found relevance for use as tissue engineering scaffolds as their resorption results in a natural replacement tissue without the long-term complications associated with foreign implants (44). These scaffolds can be coupled with adhesion motifs (32) or growth factors (45). The demonstration of extensive in growth and cell proliferation and differentiation *in vitro* as well as mineralisation *in vivo* using transduced cells alone (in the absence of osteogenic media such as ascorbate and dexamethasone) confirms the efficacy not only of the transduced BMP-2 secreting osteoprogenitor cells but the conductivity of the porous scaffold (pore size 50–200 μm).

The diffusion chamber model provides an enclosed environment within a host animal to study the osteogenic capacity of skeletally derived cell populations which resolves the problems of host versus donor bone tissue generation. In this situation, human osteoprogenitors adenovirally transduced with BMP-2 generated bone and cartilage tissue. If the cells and scaffold were implanted into the mice, that is the equivalent of a subcutaneous model, bone formation would occur. However, the bone formed may be by implanted human osteoprogenitor cells as well as recruited mouse progenitor cells.

In summary, these studies demonstrate the successful delivery of active BMP-2 using bone osteoprogenitors on porous biodegradeable scaffolds. The generation of mineralised 3-D structures *in vitro* and the subsequent demonstration of bone formation *in vivo* using the diffusion chamber assay, with such constructs indicates the potential to tissue-engineer bone for orthopaedic application. The challenge will be to demonstrate bone repair in preclinical models and subsequent absence of any immunological reaction. However, these current studies indicate tissue engineering of bone is a realistic target and offer a multidisciplinary approach to resolve a longstanding clinical need.

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REFERENCES

1. Bianco, P., and Robey, P. G. (2001) Stem cells in tissue engineering. *Nature* **414,** 118–121.

2. Friedenstein, A. J., Chailakhyan, R. K., and Gerasimov, U. V. (1987) Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet.* **20,** 263–272.
3. Triffitt, J. T., and Oreffo, R. O. C. (1998) The osteoblast lineage. *In* Molecular and Cellular Biology of Bone: Advances in Organ Biology Series (Zaidi, M., Ed.), pp. 429–451, JAI, London.
4. Beresford, J. N., Bennett, J. H., Devlin, C., Lebory, P. S., and Owen, M. E. (1992) Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *J. Cell Sci.* **102,** 341–351.
5. Bruder, S. P., Jaiswal, N., and Haynesworth, S. E. (1997). Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J. Cell Biochem.* **64,** 278–294.
6. Jaiswal, N., Haynesworth, S. E., Caplan, A. I., and Bruder, S. P. (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro. J. Cell Biochem.* **64,** 295–312.
7. Joyner, C. J., Bennett, A., and Triffitt, J. T. (1997) Identification and enrichment of human osteoprogenitor cells by using differentiation stage-specific monoclonal antibodies. *Bone* **21,** 1–6.
8. Ashton, B. A., Allen, T. D., Howlett, C. R. Eaglesom, C. C., Hattori, A., and Owen, M. (1980) Formation of bone and cartilage by marrow stromal cells in diffusion chambers *in vivo. Clin. Orthop.* **151,** 294–307.
9. Gundle, R., Joyner, C. J., and Triffitt, J. T. (1995) Human bone tissue formation in diffusion chamber culture *in vivo* by bone-derived cells and marrow stromal fibroblastic cells. *Bone* **16,** 597–601.
10. Kuznetzov, S. A., Mankani, M. H., Gronthos, S., Satomura, K., Bionco, P., and Robey, P. G. (2001) Circulating skeletal stem cells. *J. Cell Biol.* **153,** 1133–1139.
11. Nakahara. H., Bruder, S. P., Goldberg, V. M., and Caplan, A. I. (1990) *In vivo* osteochondrogenic potential of cultured cells derived from the periosteum. *Clin. Orthop. Rel. Res.* **259,** 223–232.
12. Ohgushi, H., Goldberg, V. M., and Caplan, A. I. (1989) Heterotopic osteogenesis in porous ceramics induced by marrow cells. *J. Orthop. Res.* **7,** 568–578.
13. Okumura, M., Ohgushi, H., and Tamai, S. (1991) Bonding osteogenesis in coralline hydroxyapatite combined with bone marrow cells. *Biomaterials* **12,** 411–416.
14. Petite, H. (2001) Tissue-engineered bone regeneration. *Med. Sci.* **17,** 128–130.
15. Quarto, R., Mastrogiacomo, M., Cancedda R., Kutepov, S. M., Mukhashev, V., Lavroukov, A., Kon, E., and Maracci, M. (2001) Repair of large bone defects with the use of autologous bone marrow stromal cells. *N. Engl. J. Med.* **344,** 385–386.
16. Urist, M. R. (1965) Bone: Formation by autoinduction. *Science* **150,** 893–899.
17. Wozney, J. M., and Rosen, V. (1998) Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. *Clin Orthop* **346,** 26–37.
18. Reddi, A. H. BMPs: Actions in flesh and bone. *Nat. Med.* **3,** 837–839.
19. Ahrens, M., Ankenbauer, T., Schoder, D., Hollnagel, A., Mayer, H., and Gross, G. (1993) Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. *DNA Cell Biol.* **12,** 871–880.
20. Katagiri, T., Yamagushi, A., Ikeda, T., Yoshiki, S., Wozney, J. M., Rosen, V., Wang, E. A., Tanaka, H., Omura, S., and Suda, T. (1990) The nonosteogenic pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. *Biochem. Biophys. Res. Commun.* **172,** 295–299.
21. Rickard, D. J., Sullivan, T. A., Shenker, B. J., Lebov, P. S., and Kazhdan, I. (1994) Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev Biol 161, 218–222.
22. Wang, E. A., Rose, V., D'Alessandro J. S., Bauduy, M., Cordes, P., Harada, T., Israel, D. I., Hewick, R. M., Kerns, K. M., LaPan, P., Luxenberg, D. P., McQuaid, D., Moutsatsos, I. K., Nove, J., and Wozney, J. M. (1990) Recombinant human bone morphogenetic protein induces bone formation. *Proc. Natl. Acad. Sci. USA* **87,** 2220–2224.
23. Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J. M., Rosen, V., Wang, E. A., Kahn, A. J., Suda, T., and Yashiki, S. (1991) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation *in vitro. J. Cell Biol.* **113,** 681–687.
24. Baltzer, A. W. A., Whalen, J. D., Stefanovic-Racic, M., Ziran, B., Robbines, P. D., and Evans, C. H. (1999) Adenoviral transduction of human osteoblastic cell cultures. *Acta Orthop. Scand.* **70,** 419–424.
25. Cheng, S.-L., Lou, J., Wright, N. M., Lai, C.-F., Avioli, L. V., and Riew, K. D. (2001) *In vitro* and *in vivo* induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene. *Calcif. Tissue Int.* **68,** 87–94.
26. Lieberman, J. R., Daluiski, A., Stevenson, S., Wu, L., McAllister, P., Lee, Y. P., Kabo, M., Finerman, G. A. M., Berk, A. J., and Witte, O. N. (1999) The effect of regional gene therapy with bone morphogenetic protein-2-producing bone-marrow cells on the repair of segmental femoral defects in rats. *J. Bone Joint Surg.* **81-A,** 905–917.
27. Musgrave, D. S., Bosch, P., Ghivizzani, S., Robbins, P. D., Evans. C. H., and Huard, J. (1999) Adenovirus-mediated direct gene therapy with bone morphogenetic protein-2 produces bone. *Bone* **24,** 541–547.
28. Shakesheff, K. M., Cannizaro, S. M., and Langer, R. (2000) Creating biomimetic micro-environments with synthetic polymer-peptide hybrid molecules. *J. Biomater. Sci. Polym. Ed.* **9,** 597–518.
29. Howdle, S. M., Watson, M. S., Whitaker, M. J., Popov, V. K., Davies, M. C., Mandel, F. S., Wang, J. D., and Shakesheff, K. M. (2001) Supercritical fluid mixing: Preparation of thermally sensitive polymer composites containing bioactive materials. *Chem. Commun.* **7,** 109–110.
30. Patel, N., Padera, R., Sanders, G. H., Cannizzaro, S. M., Davies, M. C., Langer, R., Roberts, C. J., Tendler, S. J., Williams, P. M., and Shakesheff, K. M. (1998) Spatially controlled cell engineering on biodegradable polymer surfaces. *FASEB J.* **12,** 1447–1454.
31. Quirk, R. A., Chan, W. C., Davies, M. C., Tendler, S. J. B., and Shakesheff, K. M. (2001) Poly(L-lysine)-GRGDS as a biomimetic surface modifier for poly(lactic acid). *Biomaterials* **22,** 865–872.
32. Yang, S. B., Roach, H. I., Clarke, N. M. P., Howdle, S. M., Shakesheff, K. M., and Oreffo, R. O. C. (2001) Human osteoprogenitor growth and differentiation on synthetic biodegradable structures after surface modification. *Bone* **29,** 523–531.
33. Oreffo, R. O. C., and Triffitt, J. T. (1999) *In vitro* and *in vivo* methods to determine the interactions of osteogenic cells with biomaterials. *J. Mater. Sci. Mater. Med.* **10,** 607–611.
34. Okubo, Y., Bessho, K., Fujimura, K., Iizuka, T., and Miyatake, S.-I. (1999) Expression of bone morphogenetic protein-2 via adenoviral vector in C2C12 myoblasts induces differentiation in to the osteoblast lineage. *Biochem. Biophys. Res. Commun.* **262,** 739–743.
35. Kanegae, Y., Makimura, M., and Saito, I. (1994) A simple and

efficient method for purification of infectious recombinant adenovirus. *Jpn. J. Med. Sci. Biol.* **47,** 157–166.

36. Kirsch, T., Nickel, J., and Sebald, W. (2000) BMP-2 antagonists emerge from alterations in the low-affinity binding epitope for receptor BMPR-II. *EMBO J.* **19,** 3314–3324.
37. Chirmule, N., Propert, K., Magosin, S., Qian, Y., and Wilson, J. (1999) Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther.* **6,** 1574–1583.
38. Harvey, B.-G., Worgall, S., Ely, S., Leopold, P. L., and Crystal, R. G. (1999) Cellular immune responses of healthy individuals to intradermal administration of an $E1^{-}E3^{-}$ adenovirus gene transfer vector. *Hum. Gene Ther.* **10,** 2823–2837.
39. Lieberman, J. R., Le, L. Q., Wu, L., Finerman, G. A. M., Berk, A., Witte, O. N., and Stevenson, S. (1998) Regional gene therapy with a BMP-2-porducing murine stromal cell line induces heterotopic and orthotopic bone formation in rodents. *J. Orthop. Res.* **16,** 330–339.
40. Turgeman, G., Pittman, D. D., Müller R., Kurkalli, B. G., Zhou, S., Pelled, G., Peyser, A., Zilberman Y., Moutsatsos, I. K., and Gazit, D. (2001) Engineered human mesenchymal stem cells: A novel platform for skeletal cell mediated gene therapy. *J. Gene Med.* **3,** 240–251.
41. Viggeswarapu, M., Boden, S. D., Liu, Y., Hair, G. A., Louis-Ugbo, J., Murakami, H., Kim, H. S., Mayr, M. T., Hutton, W. C., and Titus, L. (2001) Adenoviral delivery of LIM mineralization protein-1 induces new-bone formation *in vitro* and *in vivo*. *J. Bone Joint Surg.* **83A,** 364–376.
42. Conget, P. A., and Minguell, J. J. (2000) Adenoviral-mediated gene transfer into *ex vivo* expanded human bone marrow mesenchymal progenitor cells. *Exp. Hematol.* **28,** 382–390.
43. Olmsted, E. A., Blum, J. S., Rill, D., Yotnda, P., Gugala, Z., Lindsey, R. W., and Davis, A. R. (2001) Adenovirus-mediated BMP2 expression in human bone marrow stromal cells. *J. Cell Biochem.* **82,** 11–21.
44. Anderson, J. M., and Langone, J. J. (1999) Issues and perspectives on the biocompatibility and immunotoxicity evaluation of implanted controlled release systems. *J. Control Rel.* **57,** 107–113.
45. Whitaker, M. J., Quirk, R. A., Howdle, S. M., and Shakesheff, K. M. (2001) Growth factor release from tissue engineering scaffolds. *J. Pharm. Pharmacol.* **53,** 1427–1437.