Osteogenic, stem cell and molecular characterisation of the human induced membrane from extremity bone defects

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Objectives
The biomembrane (induced membrane) formed around polymethylmethacrylate (PMMA) spacers has value in clinical applications for bone defect reconstruction. Few studies have evaluated its cellular, molecular or stem cell features. Our objective was to characterise induced membrane morphology, molecular features and osteogenic stem cell characteristics.

Methods
Following Institutional Review Board approval, biomembrane specimens were obtained from 12 patient surgeries for management of segmental bony defects (mean patient age 40.7 years, standard deviation 14.4). Biomembranes from nine tibias and three femurs were processed for morphologic, molecular or stem cell analyses. Gene expression was determined using the Affymetrix GeneChip Operating Software (GCOS). Molecular analyses compared biomembrane gene expression patterns with a mineralising osteoblast culture, and gene expression in specimens with longer spacer duration (> 12 weeks) with specimens with shorter durations. Statistical analyses used the unpaired student t-test (two tailed; p < 0.05 was considered significant).

Results
Average PMMA spacer in vivo time was 11.9 weeks (six to 18). Trabecular bone was present in 33.3% of the biomembrane specimens; bone presence did not correlate with spacer duration. Biomembrane morphology showed high vascularity and collagen content and positive staining for the key bone forming regulators, bone morphogenetic protein 2 (BMP2) and runt-related transcription factor 2 (RUNX2). Positive differentiation of cultured biomembrane cells for osteogenesis was found in cells from patients with PMMA present for six to 17 weeks. Stem cell differentiation showed greater variability in pluripotency for osteogenic potential (70.0%) compared with chondrogenic or adipogenic potentials (100% and 90.0%, respectively). Significant upregulation of BMP2 and 6, numerous collagens, and bone gla protein was present in biomembrane compared with the cultured cell line. Biomembranes with longer resident PMMA spacer duration (vs those with shorter residence) showed significant upregulation of bone-related, stem cell, and vascular-related genes.

Conclusion
The biomembrane technique is gaining favour in the management of complicated bone defects. Novel data on biological mechanisms provide improved understanding of the biomembrane’s osteogenic potential and molecular properties.

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Keywords: induced biomembrane; Masquelet technique; Stem cells; Segmental defect

Article focus
The induced membrane technique is gaining importance in current bone defect reconstruction.
However, limited information is available on the cell biology of the human biomembrane.
Objectives were to evaluate the cell biology and stem cell content of the biomembrane formed during the Masquelet technique applied to treatment of segmental bone loss.

Key messages
Positive osteogenic differentiation was found in cells from biomembranes residing in the defect for six to 17 weeks.
Biomembrane morphology showed high vascularity and collagen content.

Biomembranes with longer maturation times showed upregulation of bone morphogenetic proteins, sonic hedgehog, and vascular and stem cell-related genes.

**Strengths and limitations**

- Although this study’s sample size was limited to 13 patients, data presented help to advance the understanding of cellular and stem cell properties of the human biomembrane.
- Greater understanding of the biological properties of the biomembrane will facilitate development of methods to optimise bone defect reconstruction strategies.
- Future research should focus on optimisation of ostegenic features of the cell population, and on ways to directly prevent stem cells towards the ostegenic lineage. These advances will help develop an optimal bone healing microenvironment.

**Introduction**

High-energy trauma often produces complex limb injuries and large segmental bone defects. While several techniques have been employed to manage large bone defects, there is controversy regarding the optimal treatment.

One promising approach, the two-stage Masquelet technique (the polymethylmethacrylate (PMMA)-induced biomembrane or ‘induced membrane’), has demonstrated moderate success in small clinical series.1-14 The first stage of the technique involves the development of an induced membrane layer of cells around surgically placed methacrylate spacers placed in a segmental bone defect. In the second stage, the spacers are removed, leaving behind the encasing biomembrane into which autologous cancellous bone grafts or other inductive and/or conductive materials may then be placed.

Few studies have attempted to characterise the biological properties of the human biomembrane,14 and the broad extent of its clinical potential in treatment of segmental bone defects remains to be fully explored. Aho et al14 examined histological properties of the biomembrane in 14 subjects and concluded that it consisted of mature vascularised fibrous tissue with some time-sensitive ostegenic and chondrogenic potential. The purpose of the present work was to evaluate the cell biology and stem cell content of the human biomembrane formed during the Masquelet technique for treatment of segmental bone loss. This study specifically explored the morphologic, stem cell, molecular and gene expression features. Improved understanding of the biological properties of the biomembrane will facilitate development of methods to optimise bone defect reconstruction strategies.

**Materials and Methods**

This study was performed following Institutional Review Board approval. The need for informed consent was waived by the ethical board as the biomembrane tissue was sampled for tissue culture as part of routine surgical practice. Biomembrane tissue was harvested during the routine surgical removal of PMMA spacers in the second stage of the Masquelet technique performed by one of the senior authors (MJB) and transported to the laboratory in sterile media where it was subdivided for studies described below.

**Histology and immunohistochemical analysis.** Biomembrane fragments were processed for routine histological studies using haematoxylin and eosin (H&E) and Masson trichrome staining. Trabecular bone was identified by direct visualisation at ×200 magnification, by two reviewers. Immunolocalisation for bone morphogenetic protein (BMP)2 and runt-related transcription factor (RUNX)2 used the anti-BMP2 antibody (Bioworld Technology, inc., Saint Louis Park, Minnesota) at a 1:50 dilution and the anti-RUNX2 antibody (anti-RUNX2 monoclonal antibody, Abnova Corporation, Taipei, Taiwan) at a 1:100 dilution. Endogenous peroxidase was blocked using 3% H2O2 (Sigma-Aldrich, St Louis, Missouri). Universal Rabbit Negative Control and Mouse IgG (both Dako, Carpinteria, California) were used as negative controls for BMP2 and RUNX2, respectively. The secondary reagent was Vector ImmPRESS Reagent, Rabbit (Vector Laboratories Inc., Burlingame, California) for 30 minutes, followed by diaminobenzidine (DAB) (Dako) for five minutes. Slides were rinsed in water, counterstained with light green, dehydrated, cleared and mounted with resinous mounting media.

**Cell differentiation analyses.** Cells were cultured in monolayer from biomembrane fragments and used to test for stem cell potency using defined media, which allow differentiation to osteogenic, chondrogenic and adipogenic phenotypes using methods previously reported by our laboratory.15,16 Differentiation of osteogenic cells was performed using the Osteogenesis Kit (Lonza Group AG, Basel, Switzerland)17 and assessed with positive alizarin red (Sigma-Aldrich) staining of mineralised matrix following 21 to 28 days of culture. Biomembrane cells were seeded at a density of 50 000 cells/well in a 24-well tissue culture plate, established in culture for between one and nine days, and then supplemented with the kit’s Osteogenic Differentiation Media. Biomembrane cells were differentiated to adipogenic cells using the Mesenchymal Stem Cell Adipogenic Differentiation medium (Lonza); differentiated cells were stained with oil red O (Sigma-Aldrich) to demonstrate fat droplets.

Demonstration of chondrogenic differentiation was based on micromass in vitro formation by cells cultured for two to 18 days in Chondrogenic Induction Medium (Lonza), supplemented with 5% foetal bovine serum (FBS) and 10 ng/mL transforming growth factor-β3 (TGF-β3). Cells were seeded at a density of 200 000 cells/well in a 24-well tissue culture plate and supplemented three times a week. Control cultures were supplemented...
with MSCBM (Mesenchymal Stem Cell Basal Medium, Lonza) basal media only. Control and differentiating cultures were supplemented three times per week. Digital images were used to document cell differentiations in vitro.

**Human foetal osteoblast culture.** The human osteoblast cell line hFOB 1.19 was obtained from the American Type Culture Collection (ATCC; Manassas, Virginia). Cells were grown in a 24-well tissue culture plate at 33.5° C, 95% humidity, and 5% CO2 with Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s F10 medium: 1:1 ratio DMEM/Ham’s F12 with L-glutamine (DMEM/F12; Life Technologies, Carlsbad, California), 10% FBS (Atlas Biologicals, Fort Collins, Colorado), 0.3 mg/ml G418 (Geneticin R; Life Technologies). Once confluent, the growth medium was switched to differentiation media which consisted of DMEM/Ham’s F10 with 0.1 mg/ml L-ascorbic acid, 10-8M menadione, 5 mM β-glycerol phosphate, and 10-7M 1α,25-Dihydroxyvitamin D3 (Sigma-Aldrich). This differentiation methodology and this cell line have been previously described.18,19 Cells were incubated at 39.5° C for seven days. To confirm osteoblast differentiation, wells were stained for bone mineralisation via alizarin red (data not shown).

**Microarray gene expression studies.** mRNA was harvested from biomembrane specimens following homogenisation; mRNA from the biomembrane and from cultured osteoblasts using Trizol (Life Technologies). Affymetrix microarray analyses were used to compare gene expression patterns of the biomembrane with cultured osteoblast cells (HG-U133 + PM strips; Affymetrix Inc., Santa Clara, California). Affymetrix ‘.cel’ files were uploaded to GeneSifter web-based software (VizX Labs, Seattle, Washington), normalised, and statistical significance determined (p < 0.05) using the unpaired student t-test (two-tailed). Data were corrected for false discovery rates using the Benjamini-Hochberg test and results are expressed as fold changes (2.0 and greater only). Ontology searches (which allow one to avoid searching gene by gene and also provide a controlled vocabulary of search terms for gene characteristics) were used for the following bone-related ontology groupings: angiogenesis and related vascularity categories; collagen; bone development; bone remodeling; negative regulation of bone remodeling; positive regulation of bone remodeling; regulation of bone remodeling; bone resorption; BMP signaling pathway; ossification; regulation of osteoblast differentiation; positive regulation of osteoblast differentiation; osteoblast differentiation; osteoblast development; negative regulation of osteoclast differentiation; positive regulation of osteoclast differentiation; regulation of osteoclast differentiation; osteoclast differentiation; chondrocyte differentiation; chondrocyte development; cartilage development, and the following ontologies for stem cells: differentiation, division, regulation of stem cell division, maintenance, and canonical WNT receptor signaling pathway involved in mesenchymal stem cell differentiation.

An additional analysis of gene expression patterns was carried out, which used the ontology groups described above to test for differences in major bone-, cartilage- and vascular-related genes in specimens with longer spacer duration periods (> 12 weeks) versus specimens with shorter spacer durations.

**Statistical analysis.** For non-microarray data analysis, standard statistical methods were used employing InStat (GraphPad Software Inc., San Diego, California). Means and standard deviation (sd) were calculated, and p < 0.05 was set as the significance level. Spearman’s correlation was used to test for linear relationships between the presence (scored as one) or absence (scored as two) of trabecular bone within the biomembrane histological specimen and the duration of the PMMA spacer in the surgical site.

**Results**

**Subjects.** Demographic and clinical features of the study population are presented in Table I. Biomembrane specimens were obtained from 12 surgeries for complex fractures (mean age 40.7 years, SD 14.4; four women, eight men) resulting from six motor vehicle or motorcycle accidents, four falls, and two gunshot wounds. Table I presents data on the surgical site, low- or high-energy causes for the trauma, the presence or absence of infection, the length of the bony defect, the volume of the PMMA spacer, and Arbeitsgemeinschaft für Osteosynthesefragen/American Orthopaedic Trauma Association (AO/OTA) classification.20 The mean duration of the PMMA spacers within the patient fracture sites was 11.9 weeks (six to 18). Radiographic measurements (in cm) were performed using AP and lateral radiographs of fractures at the time of fixation during PMMA spacer placement. Lengths, widths and depths were obtained and data used to calculate the approximate PMMA spacer volume (in cm³) (Table I). In total, 50% of the subjects had bone infections at the time of PMMA spacer placement (Table I).

**Morphological features of the human biomembrane.** Biomembrane tissues examined with routine histology showed the presence of trabecular bone in four out of 12 (33.3%) of the biomembrane specimens (Fig. 1). There was no correlation between the presence of trabecular bone in the specimens and the duration of the PMMA spacer in the surgical site. Morphological analysis showed the presence of high collagen content and extensive vascularity (Fig. 2). Immunohistochemistry showed that the osteoinductive factor BMP2 was present in osteoblasts, osteocytes and in cells within the biomembrane stroma (Fig. 3). RUNX2 (also called CBFA1, a regulator of osteoblast differentiation) was also found to be present within the biomembrane (Fig. 4).

**Stem cell capacity of the biomembrane.** Cells cultured from the biomembrane grew well and displayed a
spindle-shaped morphology in monolayer culture. Positive osteogenic capacity was demonstrated in seven out of ten (70%) tested cultures (Fig. 5). Chondrogenic capacity was demonstrated by formation of micromasses (Fig. 6) with the strong presence of chondroitin sulfate on immunohistochemistry (Fig. 7). Chondrogenic differentiation was seen in 90.9% of specimens (11 out of 12 tested specimens). Adipogenic differentiation was demonstrated by the presence of fat droplets (Fig. 8). Adipogenic differentiation was seen in 90% of the specimens (nine out of ten tested specimens). Additional data were gained on the stem cell features of the

| Age (yrs)/gender | Fracture location | Cause of trauma | AO/OTA classification | Gustilo classification | Fracture fixation during cement spacer | Bone infection (Y/N) | Bony defect length (cm) | Volume of PMMA spacer (cm³) | Duration (weeks) of spacer in site | Experimental studies performed |
|------------------|------------------|-----------------|------------------------|------------------------|--------------------------------------|---------------------|------------------------|------------------------------|----------------------------------|----------------------------------|
| 18/F             | Distal tibia     | Vehicle crash into tree | 43-A3                 | 3A                     | ORIF tibia                          | N                   | 5                      | 23                           | 6                                | H, O, C, A, G                     |
| 59/M             | Distal femur†    | Low-energy fall (fell while standing; periprosthetic fracture) | 33-A3                 | N/A                    | ORIF distal femur                   | Y                   | 12                     | 293                          | 6                                | H, O, C, A, G                     |
| 33/F             | Distal femur     | Motorcycle/vehicle accident | 33-C3                 | 3A                     | ORIF distal femur                   | N                   | 10                     | 220                          | 7                                | H, O, C, A, G                     |
| 28/M             | Diaphyseal femur | GSW              | 32-C3                 | 3A                     | IM nail femur                       | Y                   | 8                      | 185                          | 9                                | H, O, C, A, G                     |
| 48/F             | Distal tibia     | MVC              | 43-C3                 | 3B                     | ORIF distal tibia/IM fixation fibula | N                   | 9                      | 80                           | 9                                | H, O, C, A, G                     |
| 56/M             | Distal tibia     | Low energy fall (fell from stool) | 43-A3                 | 2                      | Ilizarov external fixation fibula    | Y                   | 6                      | 57                           | 11                               | H, O, C, A, G                     |
| 52/M             | Distal tibia     | Fell out of vehicle and hit by following vehicle | 43-A3                 | 3B                     | IM nail tibia                       | N                   | 6                      | 17                           | 13                               | H, C, G                           |
| 27/F             | Distal tibia     | MVC              | 43-C3                 | 3B                     | External fixation and ORIF fibula    | Y                   | 4                      | 37                           | 15                               | H, O, C, A, G                     |
| 42/M             | Diaphyseal tibia | Motorcycle/vehicle accident | 42-C2                 | 3B                     | IM nail tibia                       | N                   | 13                     | 141                          | 16                               | H, G                              |
| 62/M             | Distal tibia     | Crush injury (tree limb fell on leg) | 43-A3                 | 3B                     | External fixation tibia              | Y                   | 7                      | 49                           | 16                               | H, O, C, A, G                     |
| 30/M             | Diaphyseal tibia | GSW              | 42-C3                 | 3A                     | Ilizarov external fixation tibia     | N                   | 14                     | 112                          | 17                               | H, O, C, A, G                     |
| 34/M             | Distal tibia     | High-energy fall (jumped from balcony) | 43-C2                 | 3B                     | ORIF tibia/fibula                    | Y                   | 7                      | 84                           | 18                               | H, O, C, A                        |

PMMA, polymethylmethacrylate; M, male; F, female; MVC, motor vehicle collision; GSW, gunshot wound; H, histology and immunohistochemistry; O, osteogenesis determination; C, chondrogenesis; A, adipogenesis determination; G, microarray gene expression analysis; AO/OTA, Arbeitsgemeinschaft für Osteosynthesefragen/American Orthopaedic Trauma Association; ORIF, open reduction and internal fixation; IM, intramedullary

*Closed fracture (all other cases were open)
biomembrane by microarray analyses which compared the biomembrane to cultured osteoblasts (Table II). Gene expression was stronger in the biomembrane for secreted frizzled-related protein (SFRP) 2 (upregulated 77-fold), an important factor which interacts with Wnt signaling to enhance mesenchymal stem cell engraftment and myocardial repair.22,23

**Molecular characterisation of bone-, cartilage- and vasculature-related gene expression: comparison of biomembrane expression patterns with osteoblast cell culture.** Table II shows findings regarding the upregulation of genes in the biomembrane (in comparison with expression patterns in cultured osteoblasts) for genes related to bone and cartilage (BMP2, upregulated three-fold; BMP6, upregulated three-fold; matrix gla protein, upregulated 158-fold; and RUNX2, upregulated six-fold), and many collagen genes, including collagen type I, alpha 2. Also present were several genes related to vasculature with strong expression patterns, including angiopoietin 2 (upregulated 12-fold), and endothelial cell-specific chemotaxis regulator (upregulated five-fold).

**Comparison of biomembranes with longer versus shorter PMMA spacer residence.** The second type of gene analysis performed was a comparison of genes expressed in biomembranes in spacers that resided within the host subject for > 12 weeks compared with those from spacers that had a duration of < 12 weeks (Table III). For spacers with longer maturation times, it was noted that there remained a modest upregulation of several genes with recognised relationships to stem cells (BMP7, MYST histone acetyltransferase 3, fibroblast growth factor (FGF) receptors 1 and 2, FGF 4 and sonic hedgehog). With respect to bone- and cartilage-related genes, upregulation was seen in oestrogen receptor 1 (upregulated 3.1-fold), growth and differentiation factor 5 (upregulated 2.4-fold) and a number of matrix-related genes.
Discussion

The ability to reconstruct large bone defects that occur as a result of open fractures and/or infections remains a significant challenge in orthopaedic trauma. The Masquelet technique, which uses an induced biomembrane as a conduit for bone graft, has increasingly been used in treatment of patients with complicated segmental bone loss. The purpose of this study was to investigate the cell biology and stem cell content of the human biomembrane formed during the induced biomembrane technique.

Our results demonstrated that the histological presence of isolated bone islands formed with the biomembrane tissue harvested at the time of spacer removal was consistent with intramembranous ossification. This is an important finding in human biomembrane research and is similar to previous studies from our laboratory that have evaluated the properties of biomembranes formed in a rat segmental defect model.

Stem cell differentiation analyses in the current study used cells isolated from the human biomembrane. These cells showed a greater variability in pluripotency for osteogenic potential (70%) compared with chondrogenic or adipogenic potentials (100% and 90%, respectively). Since the clinical outcome desired with the Masquelet approach is bone formation, we chose an osteoblast cell line rather than fibroblasts for comparison with the biomembrane in our gene expression analyses as osteoblasts are more relevant to expression patterns related to bone formation. Strong expression and high fold changes were found for bone-, cartilage-, stem cell- and angiogenesis-related genes (Table II). The high expression level of asporin (upregulated 208 times) merits comment. As shown in previous osteoarthritis literature, not only can this extracellular matrix protein inhibit TGF-ß and regulate chondrogenic potential with additional evidence for a role in osteoarthritis, but also, some research points to elevated asporin expression in osteoblasts in subchondral bone in osteoarthritic patients. In the second part of our gene analysis studies, we looked for differences in expression patterns in PMMA spacers with longer versus shorter maturation periods. Upregulation of several stem cell-related genes was identified as well as bone-, cartilage- and vascular-related genes (Table III). Downregulation of several genes was also seen. We would postulate that this is because these genes, including type I collagen, were more actively expressed in the early biomembrane stages during formation of the stroma and islands of trabecular bone.

The biomembrane lies within a highly complex biological milieu in which many cytokine systems are active. The biomembrane is used clinically as both a receptive bed for bone formation and a source of mesenchymal stem cells which may be recruited and directed to this bed during the healing process. Although several small animal models have been used to evaluate the biomembrane, few studies have analysed the cellular features and stem cell content of biomembranes isolated from humans. A recent paper by Aho et al. analysed properties of the induced biomembrane from 14 human subjects. They concluded that the biomembrane consisted of mature vascularised fibrous tissue with some osteogenic and chondrogenic time-sensitive potential. However, unlike the current study, their work did not extensively evaluate the difference in gene expression patterns of several bone-, cartilage- and vascular-related genes. The gene expression...
work reported in the present study may serve as a foundation for future studies that focus on specific gene up- or down-regulation in order to optimise the biomembrane osteogenic potential.

This study has several strengths and limitations. One strength is that data presented herein are shown in relation to well characterised clinical findings, including PMMA spacer duration, energy levels of the trauma source, AO/OFTA and Gustilo classifications, fracture fixation descriptions, presence or absence of infection, and measurements of bone defect lengths and volume of the PMMA spacer (Table I). One other important consideration and possible limitation of this study is that the biomembrane analyses presented here are based upon specimens derived from variable sites in the tibia and femur, and the biomembranes formed in response to variable PMMA spacer sizes which resided in the site for different time periods (six to 18 weeks). There was also significant variation in subject ages (18 to 62 years). However, this variability in study population structure reflects the potential age range of trauma patients with severe large bone defects who may be effectively treated with the

### Table II. Major bone-, cartilage- and vascular-related gene expression findings in biomembrane specimens vs osteoblast cells.

| Gene name                        | Direction | Fold change | p-value  | Gene identifier | Gen ID |
|----------------------------------|-----------|-------------|----------|-----------------|-------|
| **Stem cell-related genes**      |           |             |          |                 |       |
| Fibroblast-like growth factor 2  | Down      | 45.8        | < 0.0001 | NM_002006       | FGF2  |
| Notch homolog 2                  | Down      | 2.6         | 0.001    | AF308601        | Notch2|
| Transforming growth factor, beta 2| Down      | 27.2        | < 0.0001 | M19154          | TGF-β2|
| Wingless-type MMTV integration site family, member 3| Down      | 7.2         | < 0.0001 | AAA63626       | WNT3  |
| Wingless-type MMTV integration site family, member 5A | Down      | 40.9        | 0.0002   | NM_003392       | WNT5A |
| Insulin-like growth factor 1 (somatomedin C) | Up        | 30.7        | 0.0001   | AU44912         | IGF1  |
| Secreted frizzled-related protein 2 | Up        | 77.7        | < 0.0001 | AF11912         | SFRP2N|
| **Bone or cartilage-related genes** |           |             |          |                 |       |
| Bone gamma-carboxyglutamate (gla) protein | Down      | 29.1        | < 0.0001 | NM_000711       | BGLAP |
| Cyclin-dependent kinase 6         | Down      | 4.3         | 0.04     | AW274756        | CDK6  |
| Fibronectin 2                     | Down      | 21.3        | 0.0009   | NM_001999       | FN2   |
| Interleukin 6                     | Down      | 22.5        | 0.0002   | NM_001600       | IL-6  |
| SATB homeobox 2                   | Down      | 3.5         | 0.013    | AB028957        | SATB2 |
| Tumour necrosis factor, alpha-induced protein 3 | Down      | 2.6         | 0.04     | AI738969        | TNFαP3|
| Acid phosphatase 5, tartrate resistant | Up        | 5.0         | 0.01     | NM_001611       | ACPS  |
| Asporin                           | Up        | 208.6       | < 0.0001 | NM_017680       | ASPN  |
| Biglycan                          | Up        | 16.9        | 0.03     | AAB45258        | BGN   |
| Bone morphogenetic protein 2      | Up        | 3.6         | 0.003    | NM_001200       | BMP2  |
| Bone morphogenetic protein 6      | Up        | 2.1         | 0.009    | NM_001277       | BMP6  |
| Chemokine (C-C motif) ligand 5    | Up        | 4.2         | 0.006    | NM_002985       | CCL5  |
| Collagen, type I, alpha 2         | Up        | 3.7         | 0.006    | AA628535        | COL1A2|
| Collagen, type III, alpha 1       | Up        | 4.5         | 0.02     | AIU146808       | COL3A1|
| Collagen, type V, alpha 3         | Up        | 9.9         | 0.001    | A1994221        | COL5A3|
| Collagen, type VI, alpha 2        | Up        | 2.8         | 0.04     | AL531750        | COL6A2|
| Collagen, type VIII, alpha 1      | Up        | 7.0         | 0.008    | AI806793        | COL8A2|
| Collagen, type XI, alpha 1        | Up        | 11.1        | 0.003    | J04177          | COL11A1|
| Collagen, type XII, alpha 1       | Up        | 11.0        | 0.006    | AIU146651       | COL12A1|
| Collagen, type XIV, alpha 1       | Up        | 27.8        | 0.001    | BFG49063        | COL14A1|
| Decorin                           | Up        | 7.7         | 0.003    | AI281593        | DCN   |
| Growth hormone receptor           | Up        | 4.9         | 0.04     | NM_000163       | CHR   |
| Interleukin 23, alpha subunit p19 | Up        | 4.6         | 0.005    | AL599222        | IL23A |
| Matrix Gla protein                | Up        | 158.4       | < 0.0001 | NM_00090000     | MGP   |
| Matrix metalloproteinase 14       | Up        | 2.5         | 0.004    | Z48481          | MMP14 |
| Matrix metalloproteinase 9 (type IV collagenase) | Up        | 12.0        | 0.01     | NM_004994       | MMP9  |
| Runt-related transcription factor 2| Up        | 6.2         | 0.0005   | AW465946        | RUNX2 |
| **Vascular-related genes**        |           |             |          |                 |       |
| Angiopoietin 1                    | Down      | 2.6         | 0.003    | U83508          | ANGPT1|
| BMP binding endothelial regulator  | Down      | 5.0         | < 0.0001 | AH423201        | BMPER |
| Bradykinin receptor B2            | Down      | 5.5         | 0.0003   | NM_000623       | BDKRB2|
| Endothelin 1                      | Down      | 2.4         | 0.03     | J05008          | EDN1  |
| Vascular endothelial growth factor A| Down      | 2.1         | 0.004    | M272781         | VEGFA |
| Vascular endothelial growth factor C| Down      | 8.8         | 0.005    | US8111          | VEGFC |
| Vasodilator 2                     | Down      | 15.0        | < 0.0001 | AI961235        | VASH2 |
| Angiogenin, RNase A family, 5     | Up        | 5.6         | 0.01     | AI761728        | ANG   |
| Angiopoietin 2                    | Up        | 12.7        | 0.001    | AA0835514       | ANGPT2|
| Aquaporin 1                       | Up        | 34.2        | 0.003    | AL518391        | AQP1  |
| Endothelial cell-specific chemotaxis regulator | Up        | 5.6         | 0.01     | AI422211        | ECSCR |
| Endothelin receptor B             | Up        | 3.1         | 0.002    | NM_003991       | EDNRB |
Table III. Major bone-, cartilage- and vascular-related gene expression findings in human biomembrane specimens with longer spacer duration (> 12 weeks) versus those with shorter duration.

| Gene name | Direction | Fold change | p-value | Gene identifier | Gen ID |
|-----------|-----------|-------------|---------|----------------|-------|
| **Stem cell-related genes** | | | | | |
| Frizzled homolog 1 (Drosophila) | Down | 4.54 | 0.02 | NM_003505 | FZD1 |
| Frizzled homolog 7 (Drosophila) | Down | 2.89 | 0.039 | A1333851 | FZD7 |
| Insulin-like growth factor 1 (somatomedin C) | Down | 2.27 | 0.037 | M29644 | IGF1 |
| MYST histone acetyltransferase 3 | Down | 5.46 | 0.009 | A107830 | MYST3 |
| notch homologue 2 | Down | 3.36 | 0.03 | A1158495 | Notch2 |
| Secreted frizzled-related protein 2 | Down | 6.32 | 0.026 | AW003584 | SFRP2 |
| Wingless-type MMTV integration site family, member 3 | Down | 7.2 | <0.0001 | AA463626 | WNT3 |
| Bone morphogenetic protein 7 | Up | 2.03 | 0.009 | M60316 | BMP7 |
| Fibroblast growth factor receptor 1 | Up | 2.14 | 0.009 | NM_023110 | FGFR1 |
| Fibroblast growth factor receptor 2 | Up | 3.23 | 0.009 | AB030073 | FGFR2 |
| Fibroblast growth factor receptor 4 | Up | 3.04 | 0.01 | NM_002007 | FGFR4 |
| Sonic hedgehog homolog (drosophila) | Down | 6.55 | 0.009 | NM_000596 | SHH |
| **Bone or cartilage-related genes** | | | | | |
| ADAM metallopeptidase domain 9 (meltrin gamma) | Down | 6.65 | 0.009 | NM_003816 | ADAM9 |
| ADAM metallopeptidase with thrombospondin type 1 motif, 3 | Down | 2.53 | 0.015 | AB002364 | ADAMTS3 |
| Bone morphogenetic protein receptor, type Ia | Down | 4.26 | 0.013 | A167867 | BMPR1A |
| Bone morphogenetic protein receptor, type II | Down | 6.4 | 0.009 | A0466096 | BMPR2 |
| Cartilage-associated protein | Down | 2.49 | 0.023 | AW024741 | CRTAP |
| Chondroitin sulfate N-acetyl-galactosaminyltransferase 1 | Down | 10.28 | 0.009 | NM_018371 | CSGALNACT1 |
| Chondroitin sulfate N-acetyl galactosaminyltransferase 2 | Down | 3.36 | 0.016 | NM_018590 | CSGALNACT2 |
| Chondroitin sulfate synthase 1 | Down | 4.21 | 0.011 | NM_014918 | CHSY1 |
| Collagen type I, alpha 1 | Down | 3.33 | 0.026 | AA473621 | COL1A1 |
| Collagen type I, alpha 2 | Down | 2.38 | 0.011 | AA62835 | COL1A2 |
| Collagen, type III, alpha 1 | Down | 3.69 | 0.010 | A1444167 | COL1A3 |
| Collagen, type V, alpha 1 | Down | 5.34 | 0.020 | A1130969 | COL5A1 |
| Collagen, type V, alpha 3 | Down | 3.09 | 0.009 | A0948221 | COL5A2 |
| Collagen, type VI, alpha 1 | Down | 2.98 | 0.047 | AA293737 | COL6A1 |
| Collagen, type VI, alpha 2 | Down | 2.54 | 0.009 | AL531750 | COL6A2 |
| Decorin | Down | 4.35 | 0.011 | NM_001920 | DCN |
| Dystroglycan | Down | 4.12 | 0.012 | NM_003933 | DAG1 |
| Fibroblast growth factor receptor 1 | Down | 3.19 | 0.012 | M061985 | FGRF1 |
| Growth hormone receptor | Down | 4.21 | 0.011 | NM_00163 | GHR |
| Heparan sulfate 2-O-sulfotransferase 1 | Down | 4.31 | 0.011 | NM_012262 | HS2ST1 |
| Hypoxia inducible factor 1, alpha subunit | Down | 3.72 | 0.012 | NM_001330 | HIF1A |
| Insulin-like growth factor 1 (somatomedin C) | Down | 2.27 | 0.039 | M29644 | IGF1 |
| Insulin-like growth factor binding protein 3 | Down | 5.5 | 0.015 | BF140228 | IGFBP3 |
| Insulin-like growth factor binding protein 5 | Down | 2.83 | 0.047 | AW007321 | IGFBP5 |
| Interleukin 6 signal transducer | Down | 7.07 | 0.009 | AW242916 | IL6ST |
| Matrilin 2 | Down | 4.71 | 0.049 | NM_002380 | MATN2 |
| Matrix GlA protein | Down | 3.55 | 0.013 | AW512787 | MGP |
| Matrix metalloproteinase 2 | Down | 2.66 | 0.039 | NM_004530 | MMP2 |
| Matrix metalloproteinase 9 | Down | 4.37 | 0.030 | NM_004994 | MMP9 |
| Matrix metalloproteinase 13 (collagenase 3) | Down | 4.13 | 0.024 | NM_002427 | MMP13 |
| Osteoglycin | Down | 4.07 | 0.042 | NM_014057 | OCN |
| Osteoclast stimulating factor 1 | Down | 4.46 | 0.009 | NM_012383 | OSTE1 |
| Periostin, osteoblast specific factor | Down | 7.45 | 0.054 | AW171748 | POSTN |
| Runt-related transcription factor 2 | Down | 4.47 | 0.012 | AL135944 | RUNX2 |
| SMAD family member 1 | Down | 7.26 | 0.009 | US4826 | SMAD1 |
| Sulfatase 2 | Down | 3.69 | 0.012 | AL133001 | SULF2 |
| Tenascin C | Down | 7.85 | 0.016 | NM_002160 | TNC |
| Thyroid hormone receptor, beta | Down | 3.22 | 0.019 | BG494007 | THRB |
| TIMP metalloproteinase inhibitor 2 | Down | 3.4 | 0.01 | NM_003255 | TIM2 |
| Transforming growth factor, beta 3 | Down | 2.17 | 0.011 | J03241 | TGF-B3 |
| Tumour necrosis factor, alpha-induced protein 3 | Down | 3.22 | 0.009 | NM_006290 | TNFAIP3 |

Masquelet technique. Results from age- and site-controlled small animal studies were previously reported by our group.\textsuperscript{24,25} Another limitation of this study is that it has a relatively small sample size for the gene expression analyses. Although many of the differences in gene expression reached statistical significance, future studies with larger sample sizes are needed.

Our findings add additional clinical cases to the literature, and advance this research by quantifying the osteogenic, chondrogenic and adipogenic differentiation potential of cells within the biomembrane. This novel work establishes baseline data for the process during which biomembrane cells differentiate into important stem cells. We look forward to such

(continued)
Table III. (continued)

| Gene name                        | Direction | Fold change | p-value | Gene identifier | Gen ID |
|----------------------------------|-----------|-------------|---------|-----------------|-------|
| Versican                         | Down      | 9.07        | 0.011   | D32029          | VCAN  |
| Vitamin D receptor               | Down      | 3.12        | 0.009   | NM_000376       | VDR   |
| ADAM metalloprotease with thrombospondin type 1 motif, 9 | Up        | 3.3         | 0.009   | AB037733        | ADAMTS9 |
| Aggrecan                         | Up        | 3.9         | 0.009   | BC036445        | ACAN  |
| Bone morphogenetic protein 7     | Up        | 2.03        | 0.019   | NM_014482       | BMP7  |
| Bone morphogenetic protein 10    | Up        | 2.04        | 0.019   | NM_014482       | BMP10 |
| Epidermal growth factor receptor | Up        | 4.69        | 0.01    | AF277897        | EGFR  |
| Estrogen receptor 1              | Up        | 3.13        | 0.009   | AF258450        | ESR1  |
| Fibrillin 2                      | Up        | 2.63        | 0.009   | AF193046        | FB2   |
| Fibroblast growth factor 4       | Up        | 3.04        | 0.01    | NM_002007       | FGFR2 |
| Fibroblast growth factor receptor 2 | Up      | 3.23        | 0.009   | AB030073        | FGFR2 |
| Growth differentiation factor 5   | Up        | 2.4         | 0.009   | NM_000557       | GDF5  |
| G protein-coupled receptor S5     | Up        | 2.48        | 0.027   | NM_005683       | GPR5S |
| Heparan sulfate (glucosamine) 3-O-sulfotransferase S | Up        | 2.43        | 0.011   | AW449310        | HSST5 |
| Hyaluronan and proteoglycan link protein 4 | Up    | 2.59        | 0.009   | W63783          | HAPLN4|
| Interleukin 21                    | Up        | 2.26        | 0.012   | NM_021803       | IL21  |
| Interleukin 23, alpha subunit p19 | Up        | 2.86        | 0.031   | AJ296370        | IL23A |
| Natriuretic peptide receptor C/guanylate cyclase C | Up      | 2.35        | 0.009   | AK029660        | ANGPT1|
| Retinoic acid receptor, gamma     | Up        | 2.32        | 0.011   | MS7707          | RARG  |
| Sonic hedgehog homolog (Drosophila) | Up    | 2.42        | 0.01    | AI192528        | SHH   |
| Tenasin R                        | Up        | 2.23        | 0.009   | Y13359          | TNR   |
| Thrombospondin 1                 | Up        | 2.65        | 0.013   | BF084105        | THBS1 |
| **Vascular-related genes**        |           |             |         |                 |       |
| Angiopoietin 1                   | Down      | 3.36        | 0.012   | NM_001146       | ANGPT1|
| Angiopoietin 2                   | Up        | 6.28        | 0.001   | NM_001147       | ANGPT2|
| Aquaporin 1                      | Down      | 3.7         | 0.011   | AL518391        | AQP1  |
| Lysyl oxidase                    | Down      | 5.01        | 0.013   | NM_002317       | Lox   |
| Endothelin 1                     | Down      | 2.76        | 0.01    | NM_0001955      | EDN1  |
| Endothelial cell-specific chemotaxis regulator | Down | 5.13        | 0.009   | AH222111        | ECCR1 |
| Endothelin receptor type A       | Down      | 6.09        | 0.009   | AU118882        | EDNRA |
| Vascular endothelial growth factor A | Down   | 7.33        | 0.017   | AF022375        | VEGFA |
| Angiopoietin-like 3              | Up        | 2.21        | 0.009   | AV659209        | ANGPT3|
| Angiopoietin 4                   | Up        | 2.33        | 0.012   | NM_015985       | ANGPT4|
| Endoglin                         | Up        | 2.08        | 0.009   | AA906156        | ENG   |
| Epir Colin                      | Up        | 2.45        | 0.009   | BC03506         | EREG  |
| Vasohibin 2                      | Up        | 2.7         | 0.01    | BC028194        | –     |

information being used in work designed to optimise the osteogenic or chondrogenic potential of the biomembrane, potentially expediting the timeline for healing segmental bone defects in patients with complex fractures.

In conclusion, the Masquelet technique (PMMA-induced biomembrane) is successfully employed in current bone defect reconstruction treatment. Limited data exist on detailed characterisation of the cell biology of the human biomembrane. We suggest that future research directed towards optimising the biological features of the biomembrane should focus on optimisation of the osteogenic features of the cell population and on ways to direct the stem cells present in the biomembrane into the osteogenic lineage (25% of our biomembrane cell specimens did not differentiate into osteoblasts). This is vital since timely formation of high-quality bone is of paramount importance in the clinical patient population with segmental bone defects. The work presented herein represents an important step forward in the advancement of our understanding of the cellular features and stem cell properties of the human biomembrane and highlights the importance for future research in which biomembranes may be modified to create an optimal bone healing microenvironment.

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ICMJE conflict of interest
None declared.

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