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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 osteogenic features of the cell population, and on ways to direct inherent stem cells towards the osteogenic 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 al[14] examined histological properties of the biomembrane in 14 subjects and concluded that it consisted of mature vascularised fibrous tissue with some time-sensitive osteogenic 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% H_2O_2 (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-ß3 (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% CO_2 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^{-8}M menadione, 5 mM ß-glycerol phosphate, and 10^{-7}M 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] and the Gustilo classification.[21] 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 cm^3) (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

Table I. Demographic and clinical features of study population.*

Age (yrs)/ gender	Fracture location	Cause of trauma	AO/OTA class-ification	Gustilo class-ification	Fracture fixation during cement spacer	Bone infection (Y/N)	Bony defect length (cm)*	Volume of PMMA spacer (cm^3)	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 tibia	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)

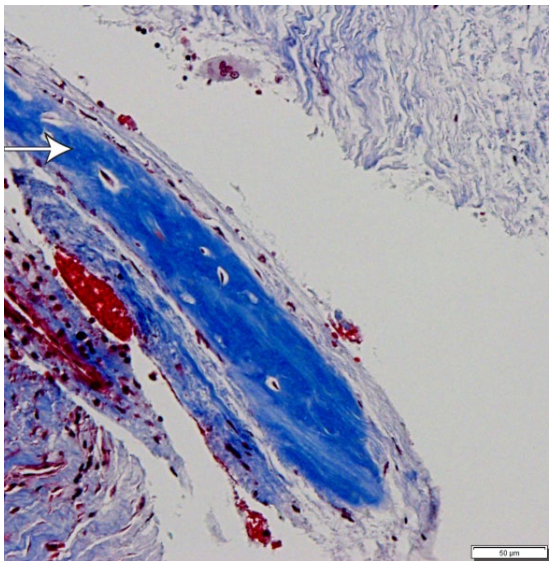

Fig. 1

Light microscopic features of the biomembrane tissue. Trabecular bone (arrow) was present in 4/13 (33.3%) of specimens examined for morphological features. (Masson trichrome stain; bar = 50 µm).

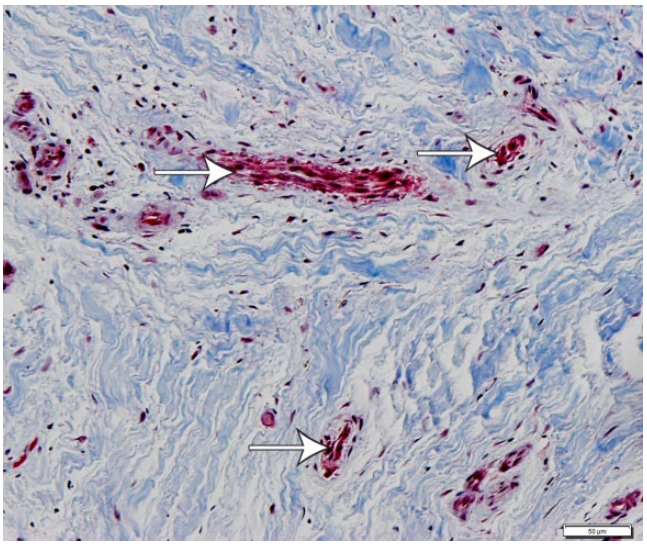

Fig. 2

Light microscopy showing the highly vascular nature of the biomembrane. Arrows mark vasculature. (Masson trichrome stain; bar = 50 µm).

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

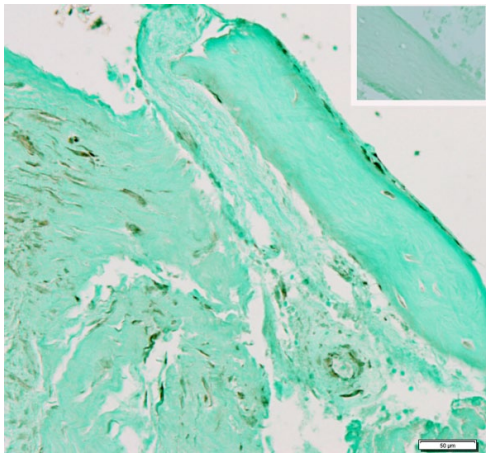

Fig. 3

Immunohistochemical localisation of bone morphogenetic protein 2 in the biomembrane. Insert upper right shows an adjacent section processed as a negative control (Bar = 50 µm).

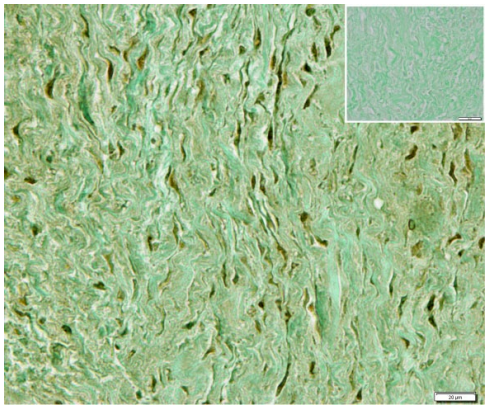

Fig. 4

Immunohistochemical localisation of runt-related transcription factor 2 within the biomembrane. Insert upper right shows an adjacent section processed as a negative control (Bar = 20 µm).

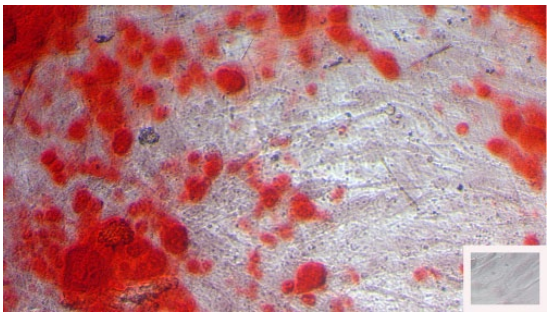

Fig. 5

Biomembrane cells cultured in osteogenic differentiation media showed development of calcified nodules as verified here with alizarin red staining. Insert lower right shows no development of calcified nodules in cells grown in control medium. Original magnification ×200.)

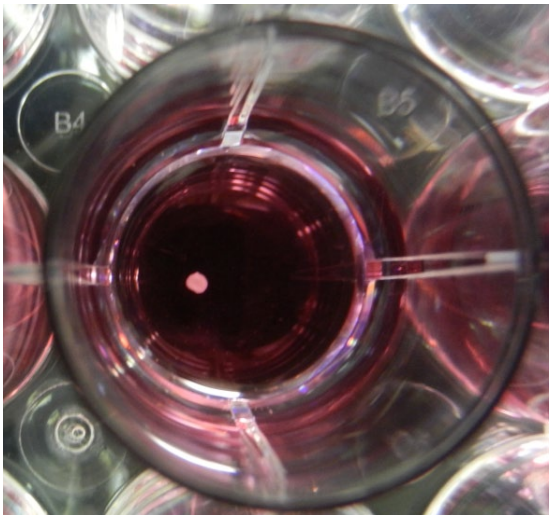

Fig. 6

Biomembrane cells cultured in chondrogenesis media showed the formation of compact micromasses *in vitro* (diameter of the culture plate well shown is 16 mm).

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.

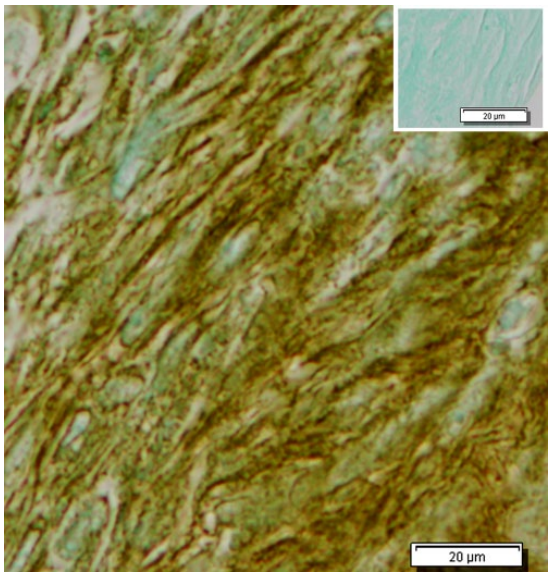

Fig. 7

Micromass specimen embedded in paraffin and processed for immunohistochemical localisation of chondroitin sulfate. Note the abundant presence of this matrix component. Insert upper right shows an adjacent section processed as a negative control (Bar = 20 µm).

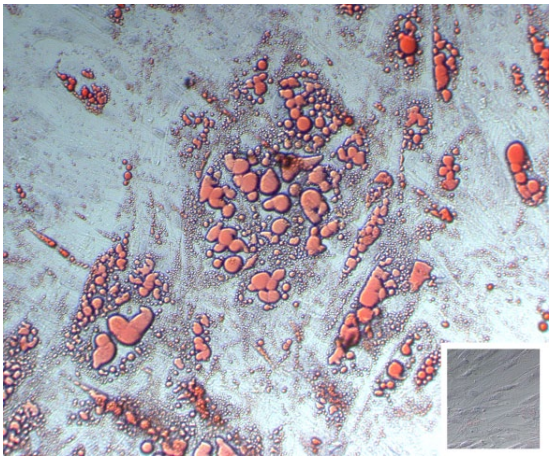

Fig. 8

Biomembrane cells cultured for adipogenesis show the presence of fat droplets (stained red in the micrograph). Insert lower right shows that biomembrane cells cultured in control media showed no presence of fat droplets (original magnification ×200).

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.[24,25]

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).[22,23] 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,[26,27] but also, some research points to elevated asporin expression in osteoblasts in subchondral bone in osteoarthritic patients.[28] 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.[29-31] 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[14] 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

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 (basic)	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	AA463626	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
Fibrillin 2	Down	21.3	0.0009	NM-001999	FBN2
Interleukin 6	Down	22.5	0.0002	NM_000600	IL-6
SATB homeobox 2	Down	3.5	0.013	AB028957	SATB2
Tumour necrosis factor, alpha-induced protein 3	Down	2.6	0.04	AI738896	TNFAIP3
Acid phosphatase 5, tartrate resistant	Up	5.0	0.01	NM_001611	ACP5
Asporin	Up	208.6	< 0.0001	NM_017680	ASPN
Biglycan	Up	16.9	0.03	AA845258	BGN
Bone morphogenetic protein 2	Up	3.6	0.003	NM_001200	BMP2
Bone morphogenetic protein 6	Up	2.1	0.009	NM_0017_PM_at	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	AU146808	COL3A1
Collagen, type V, alpha 3	Up	9.9	0.001	AI984221	COL5A3
Collagen, type VI, alpha 2	Up	2.8	0.04	AL531750	COL6A2
Collagen, type VIII, alpha 1	Up	7.0	0.008	AI806793	CO:8A2
Collagen, type XI, alpha 1	Up	11.1	0.003	J04177	COL11A1
Collagen, type XII, alpha 1	Up	11.0	0.006	AU146651	COL12A1
Collagen, type XIV, alpha 1	Up	27.8	0.001	BF449063	COL14A1
Decorin	Up	7.7	0.0003	AI281593	DCN
Growth hormone receptor	Up	4.9	0.04	NM_000163	GHR
Interleukin 23, alpha subunit p19	Up	4.6	0.005	AL559122	IL23A
Matrix Gla protein	Up	158.4	< 0.0001	NM_0009000	MGP
Matrix metallopeptidase 14	Up	2.5	0.004	Z48481	MMP14
Matrix metallopeptidase 9 (type IV collagenase)	Up	12.0	0.01	NM_004994	MMP9
Runt-related transcription factor 2	Up	6.2	0.0005	AW469546	RUNX2
Vascular-related genes					
Angiopoietin 1	Down	2.6	0.003	U83508	ANGPT1
BMP binding endothelial regulator	Down	5.0	< 0.0001	AI423201	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	U58111	VEGFC
Vasohibin 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	0.0003	AL518391	AQP1
Endothelial cell-specific chemotaxis regulator	Up	5.6	0.01	AI422211	ECSCR
Endothelin receptor type B	Up	3.1	0.002	NM_003991	EDNRB

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/OTA 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). These factors undoubtedly influenced important features such as vascularisation since the vascularity and healing potential in a younger subject would presumably be greater than that of a middle-aged subject. 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 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	AI333651	FZD7
Insulin-like growth factor 1 (somatomedin C)	Down	2.27	0.037	M29644	IGF1
MYST histone acetyltransferase 3	Down	5.46	0.009	AI817830	MYST3
Notch homolog 2	Down	3.36	0.03	AU158495	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 4	Up	3.04	0.01	NM_002007	FGF4
Sonic hedgehog homolog (drosophila)	Up	2.42	0.01	AI92528	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	AI678679	BMPR1A
Bone morphogenetic protein receptor, type II	Down	6.4	0.009	AL046696	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	5.21	0.011	NM_014918	CHSY1
Collagen type I, alpha 1	Down	3.33	0.026	AI743621	COL1A1
Collagen, type I, alpha 2	Down	2.38	0.011	AA62835	COL1A2
Collagen, type III, alpha 1	Down	3.69	0.010	AU144167	COL3A1
Collagen, type V, alpha 1	Down	5.34	0.020	AI130969	COL5A1
Collagen, type V, alpha 3	Down	3.09	0.009	AI984221	COL5A3
Collagen, type VI, alpha 1	Down	2.98	0.047	AA292373	COL6A1
Collagen, type VI, alpha 2	Down	2.54	0.009	AL531750	COL6A2
Decorin	Down	4.35	0.011	NM_001920	DCN
Dystroglycan 1	Down	4.12	0.012	NM_004393	DAG1
Fibroblast growth factor receptor 1	Down	3.19	0.012	M60485	FGFR1
Growth hormone receptor	Down	4.21	0.011	NM_000163	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_001530	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	BF340228	IGFBP3
Insulin-like growth factor binding protein 5	Down	2.83	0.047	AW007532	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 metallopeptidase 2	Down	2.66	0.039	NM_004530	MMP2
Matrix metallopeptidase 9	Down	4.37	0.030	NM_004994	MMP9
Matrix metallopeptidase 13 (collagenase 3)	Down	4.13	0.024	NM_002427	MMP13
Osteoglycin	Down	4.07	0.042	NM_014057	OGN
Osteoclast stimulating factor 1	Down	4.46	0.009	NM_012383	OSTF1
Periostin, osteoblast specific factor	Down	4.65	0.034	AW137148	POSTN
Runt-related transcription factor 2	Down	4.47	0.012	AL353944	RUNX2
SMAD family member 1	Down	7.26	0.009	U54826	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 metallopeptidase inhibitor 2	Down	3.4	0.01	NM_003255	TIMP2
Transforming growth factor, beta 3	Down	2.17	0.011	J03241	TGF-β3
Tumour necrosis factor, alpha-induced protein 3	Down	3.22	0.009	NM_006290	TNFAIP3

(continued)

Masquelet technique. Results from age- and site-controlled small animal studies were previously reported by our group.[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

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 metallopeptidase 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	0.009	M60316	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	FBN2
Fibroblast growth factor 4	Up	3.04	0.01	NM_002007	FGF4
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 55	Up	2.48	0.027	NM_005683	GPR55
Heparan sulfate (glucosamine) 3-O-sulfotransferase 5	Up	2.43	0.011	AW449310	HS3ST5
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	0.013	AJ296370	IL23A
Natriuretic peptide receptor C/guanylate cyclase C	Up	2.35	0.009	AI628360	NPR3
Retinoic acid receptor, gamma	Up	2.32	0.011	M57707	RARG
Sonic hedgehog homolog (Drosophila)	Up	2.42	0.01	AI192528	SHH
Tenascin 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	Down	6.28	0.01	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_001955	EDN1
Endothelial cell-specific chemotaxis regulator	Down	5.13	0.009	AI422211	ECSCR
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	ANGPTL3
Angiopoietin 4	Up	2.33	0.012	NM_015985	ANGPT4
Endoglin	Up	2.08	0.009	AA906156	ENG
Epiregulin	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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Author Contribution

- H. E. Gruber, Conceived of idea, wrote manuscript, obtained research funding, selected gene expression data, prepared illustrations, reviewed histology/immunohistochemistry, did revisions 1 and 2.
- G. Ode, Conceived of idea, assisted with manuscript preparation, reviewed patient charts, observed surgeries, reviewed literature.
- G. Hoelscher, Performed ontology searches for gene expression data.
- J. Ingram, Performed histology and immunohistochemistry on harvested biomembranes, assisted with methods text.
- S. Bethea, Performed cell cultures and mRNA harvesting.
- M. J. Bosse, Conceived of idea, performed surgeries on patients, helped with obtaining funding, reviewed and revised manuscript, senior MD on project.

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ICMJE conflict of interest

- None declared.