

RESEARCH ARTICLE

Intermediate layer contribution in placental membrane allografts

Annelise Roy  | Sarah Griffiths 

Research and Development Department,
StimLabs, LLC, Roswell, GA, USA

Correspondence

Sarah Griffiths, StimLabs, LLC, 1225
Northmeadow Parkway, Suite 104, Roswell,
GA 30076, USA.
Email: sarah@stimlabs.com

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StimLabs, LLC

Abstract

Placental membrane (PM) allografts are commonly used to treat chronic wounds. Native PM is composed of an amnion, chorion, and intermediate layer (IL) that contain matrix structures and regulatory components beneficial in wound healing. Historically, commercially available allografts were composed of only one or two layers of the PM. To maximize the conserved material in PM allografts, a dehydrated complete human placental membrane (dCHPM) allograft processed using the Clarify™ process was developed. Histological and proteomic characterization comparing dCHPM allografts with native PM demonstrated that the majority of matrix structures and regulatory proteins are retained in dCHPM allografts through processing. To evaluate the importance of maintaining the entire intact PM and the contribution of the IL, the structural and proteomic makeup of the IL was compared with that of dCHPM allografts. This is the first known characterization of regulatory proteins in the IL. Results demonstrate that the IL contains over 900 regulatory and signaling components, including chemokines, growth factors, interleukins, and protease inhibitors. These components are key regulators of angiogenesis, neurogenesis, osteogenesis, inflammation, tissue remodeling, and host defense. The results show that the proteomic composition of the IL is consistent with that of the entire dCHPM allograft. Although further investigation is required to fully understand the contribution of the IL in PM allografts, these results demonstrate that the IL contains structural and regulatory proteins that can enhance the barrier and wound healing properties of PM allografts.

KEYWORDS

allograft, amnion, chorion, extracellular matrix, placental membrane, wound healing

1 | INTRODUCTION

The recent increased use of PM allografts in wound healing applications stems from their proven efficacy, unique bioscaffold, and therapeutic components, as well as the immunological privilege of fetal tissue (Castellanos, Bernabé-García, Moraleda, & Nicolás, 2017; Parolini, Solomon, Evangelista, & Soncini, 2010). Studies performed on composite or single layer allografts confirm the presence of growth factors, cytokines, and tissue inhibitors of metalloproteinases (TIMPs; Koob, Lim, Masee, Zabek, & Denozière, 2014; Mrugala et al., 2016). Additionally, composite and single layer PM allografts

have demonstrated success in wound healing applications. Since the mid-1990s, single layer placental membranes have been popular in ophthalmology as a treatment in corneal surgeries (J. C. Kim & Tseng, 1995; Solomon et al., 2002). Across a range of applications, these grafts have been shown to accelerate wound closure and successfully heal chronic wounds (Castellanos et al., 2017; Mrugala et al., 2016).

Anatomically, the human PM surrounds the fetus and separates it from maternal tissue. The PM is a metabolically active tissue that continually develops, expands, and remodels during gestation. The membrane provides elasticity and strength to hold, cushion, and

protect a developing fetus and acts as a critical barrier, preventing a mixing of maternal and fetal blood and the transfer of pathogens, while allowing key nutrients to pass to the fetus (Gude, Roberts, Kalionis, & King, 2004; Watson & Burton, 1998). Malfunctions of the PM are associated with a host of complications, such as premature rupture of fetal membranes, underlining the importance of PMs (Janzen et al., 2017; Kumar et al., 2016; Watson & Burton, 1998). As donor eligibility for transplantable material would exclude these donors for acceptance in manufacturing, the potential impacts on efficacy in wound repair are not known.

Native PM has eight identifiable layers that each contribute to the overall strength, elasticity, and barrier properties of the tissue, as summarized in Table 1 (Bryant-Greenwood, 1998). Commonly, these are described as two major layers: the amnion and the chorion. On the fetal side of the PM, the amnion is composed of an epithelium, basement membrane, compact layer, and fibroblast layer. The amnion is the innermost membrane to the fetus, providing tensile strength

and acting as a fibrous skeleton (Niknejad et al., 2008). Toward the maternal side is the chorion, which includes the reticular layer, a pseudobasement membrane, and trophoblast layer that contacts the maternal decidua (Niknejad et al., 2008). The chorion contributes to elasticity and stability and provides a scaffold for native cells (Bryant-Greenwood, 1998; Hieber et al., 1997; Mamede et al., 2012; Niknejad et al., 2008). The amnion and chorion attach and interact through a third, dynamic middle layer called the intermediate layer (IL), also referred to as the spongy layer or zona spongiosa.

Each layer has a characteristic extracellular matrix (ECM) makeup that is instrumental to the structural integrity and barrier properties of the PM. The epithelial layer is made up of a thin layer of tightly packed epithelial cells that sit directly on a basement membrane. The amniotic basement membrane is composed of densely packed collagens, laminin, nidogen, fibronectin, and proteoglycans like heparan sulfate (Keene, Sakai, Lunstrum, Morris, & Burgeson, 1987; Malak et al., 1993; Mamede et al., 2012; Niknejad et al., 2008;

TABLE 1 Extracellular matrix components and functions of the native placental membrane layers

Layer		ECM components	Function	Sources
Amnion	Epithelium	•Epithelial cells	•Contains growth factors that promote epithelialization	Parolini et al. (2010); Mamede et al. (2012); Malak et al. (1993); Keene et al. (1987); Rousselle et al. (1997); Smith et al. (1994); Niknejad et al. (2008)
	Basement membrane	•Collagens •Laminin •Nidogen •Fibronectin •Heparan sulfate	•Acts as permeable barrier, allowing transport of nutrients and building blocks •Hemostatic properties prevent hematoma and reduce microbial accumulation •Increases barrier integrity, stabilizing membrane and cells •Provides scaffolding for other extracellular components	
	Compact layer	•Collagen types I, III, V, and VI •Fibronectin	•Main fibrous skeleton of native scaffold •Increases tensile strength	
	Fibroblast layer	•Collagen types I, III, and IV •Proteoglycans	•Anchors cells to scaffolding •Increases tensile strength	
Intermediate layer	Intermediate layer	•Collagen types I, III, and IV •Heparan sulfate •Hyaluronic acid	•Creates an acellular barrier between amnion and chorion •Nonfibrillar meshwork structure that provides scaffold and cushioning •Tissue hydration and lubrication •Mechanical support to membranes •Provides elasticity and tractional resistance to membrane	Meinert et al. (2001); Mamede et al. (2012); Bryant-Greenwood (1998)
Chorion	Reticular layer	•Collagen types I–VI •Elastin •Proteoglycans	•Contributes to membrane integrity •Provides a scaffold for other layers of membrane and cell growth •Contributes to elasticity of the membrane	Malak et al. (1993); Hieber et al. (1997); Malak et al. (1993); Niknejad et al. (2008); Keene et al. (1987); Rousselle et al. (1997); Smith & Ockleford (1994); Bryant-Greenwood (1998)
	Basement membrane	•Collagen type IV •Fibronectin •Laminin	•Increases integrity of membrane barrier •Provides scaffolding for other extracellular components •Stabilizes membranes and helps stabilize cells	
	Trophoblasts	•Trophoblasts •Collagen	•Chemical barrier to maternal hormones and local maternal signals	

Abbreviation: ECM, extracellular matrix.

Rousselle et al., 1997; Smith & Ockleford, 1994). The compact layer and fibroblast layer are also made up of collagen type I, type III, type V, and type VI, along with laminins, nidogens, and fibronectins, creating the fibrous structure of the amnion (Keene et al., 1987; Mamede et al., 2012; Niknejad et al., 2008; Rousselle et al., 1997; Smith & Ockleford, 1994).

The chorion layers provide structural support and anchoring for cells across the PM (Bryant-Greenwood, 1998; Malak et al., 1993). The reticular layer is composed of collagen types I–VI and an elastin network contributing added elasticity (Hieber et al., 1997; Malak et al., 1993; Niknejad et al., 2008). The trophoblasts and collagen in the trophoblast layer facilitates attachment of the PMs to the maternal decidua (Bryant-Greenwood, 1998).

The IL acts as an interface between the maternal-facing and fetal-facing side of the PM and varies in thickness (Baergen, 2011). It is composed of collagen type I, type III, and type IV and a high density of proteoglycans and glycoproteins, including hyaluronic acid and heparan sulfate (Bryant-Greenwood, 1998; Mamede et al., 2012; Meinert et al., 2001; Niknejad et al., 2008). These ECM proteins exist in a loose matrix that forms a spongy, acellular network (Niknejad et al., 2008). It acts as a barrier between the amnion and chorion layers and allows the amnion layer to glide along the chorion (Bryant-Greenwood, 1998). Although the mechanical characteristics are imperative to PM integrity, little else has been studied about the IL.

Composite PM allografts have been shown to be efficacious in wound healing, especially in chronic wounds, corneal wounds, and burns (Castellanos et al., 2017; Mermet et al., 2007; Solomon et al., 2002). Typically, following debridement, PM allografts are applied to the wound surface to stimulate healthy wound closure, followed by applying a secondary dressing (Ganatra, 2003). The allografts are reapplied until resolution of the wound, similar to methods described by Ganatra (2003) and Mermet et al. (2007). Animal and clinical research has shown that PM is anti-inflammatory, antimicrobial, analgesic, pro-angiogenic, immunologically privileged, and has properties that reduce scarring and enhance cellular proliferation and migration, many of which can be attributed to the reciprocity of the ECM structure and the regulatory proteins present in PM (Castellanos et al., 2017; Malhotra & Jain, 2014; Mermet et al., 2007; Schultz & Wysocki, 2009). Since the commercial success of these allografts, a large body of research has focused on identifying the specific cytokines in the amnion or chorion layers of PMs that help to regulate tissue repair (Castellanos et al., 2017; Fetterolf & Snyder, 2012; Tseng et al., 2004). Components that regulate fibroblast migration and proliferation, including basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF- β 1), and platelet-derived growth factors (PDGF-BB and PDGF-AA), and those that regulate re-epithelialization, like epithelial growth factor (EGF), have been identified in PM (Barrientos, Stojadinovic, Golinko, Brem, & Tomic-Canic, 2008; Koob, Lim, Zabek, & Masee, 2015; Werner & Grose, 2003). TIMPs are regulators of ECM turnover that are critical to mediating fibrosis and healthy ECM deposition and are found in PMs (Arpino, Brock, & Gill, 2015; Visse & Nagase, 2003). Stimulators

of angiogenesis, including vascular endothelial growth factor (VEGF), have also been studied in PM (Werner & Grose, 2003). Focus has been given to identifying components that reduce inflammation, like specific interleukins and hyaluronic acid (Necas, Bartosikova, Brauner, & Kolar, 2008; Werner & Grose, 2003). Hyaluronic acid is a component of interest in PM because of its role in anti-inflammatory, analgesic, and antifibrotic signaling pathways (Gupta, Lall, Srivastava, & Sinha, 2019; Mohan, Bajaj, & Gundappa, 2017; Mohseni, Saem, Sekhavati, Molaszem, & Tabrizi, 2018; Necas et al., 2008). These biochemical properties are complemented by the inherent physical, mechanical, and vapor barrier provided by the layers of PM allografts (Ganatra, 2003).

The manufacturing of PM allografts necessitates washing the tissue to remove residual maternal blood. Popular processing techniques prescribe the separation of the amnion and chorion to facilitate complete washing of the membranes before relamination (Daniel, Tofe, Spencer, & Russo, 2012; Koob et al., 2015). These techniques expose the center of the PM to agitation and manipulation resulting in the loss of substantial ECM content, including the majority of the hydrophilic IL. Commonly, the tissue is then either cryopreserved, dehydrated, or lyophilized (freeze-dried) to preserve the tissue. Commercially available shelf-stable PM allografts are sold as amnion and amnion/chorion composite grafts (Koob et al., 2015). These processes substantially remove the IL, removing key nutrients and structures from the allograft. Furthermore, the separation and oven dehydration disrupts the natural architecture of the grafts (Johnson, Gyurdieva, Dhall, Danilkovitch, & Duan-Arnold, 2017).

A proprietary processing technique (Clarify process) was developed by StimLabs, LLC (Roswell, GA), to produce a dehydrated complete human placental membrane (dCHPM) allograft (Revita™), designed to conserve the intact architecture and optimize the retention of key components through processing. The dCHPM retains the fully intact structure of the amnion, IL, and chorion structures through processing.

The purpose of this study was to compare dCHPM allografts with native PM to demonstrate that the complete native barrier structures and cytokine composition are conserved through processing. Additionally, data are presented on the regulatory component content of the IL and its potential contribution to the overall barrier and wound healing properties of dCHPM allografts.

2 | MATERIALS AND METHODS

2.1 | Tissue processing

dCHPM allografts were processed from donated human placentas (Figure S1). Placentas were recovered from full-term, healthy births under full consent of the mothers. The donation process followed the regulations of the Food and Drug Administration (FDA) and the American Association of Tissue Banks (AATB). Patient screening was performed to test for human immunodeficiency virus type 1 and type 2 antibody, human T-lymphotropic virus type 1 and type 2 antibody,

hepatitis C antibody, hepatitis B surface antigen, hepatitis B core total antibody, rapid plasma regain (RPR) for syphilis or serologic test for syphilis (SRS), human immunodeficiency virus type 1 nucleic acid, hepatitis C virus nucleic acid, hepatitis B virus nucleic acid, cytomegalovirus antibody, and West Nile virus nucleic acid.

Intact PMs were removed from the placental disk by blunt dissection. Samples of fresh, unprocessed tissue were cut and digested for analysis. Membranes were then further processed through the Clarify process. After washing, small sections of membrane were cut to collect IL samples. Using gentle massage, the gelatinous IL was pushed out from between the amnion and chorion layers. Processed membranes and isolated IL were then lyophilized before analysis.

2.2 | Histology

Histological analysis of fresh, unprocessed PM and the dCHPM samples was performed by Premier Laboratory, LLC (Longmont, CO), according to their standard procedures. Stains were selected to highlight the morphology and principle structural proteins in the PM: cell nuclei (hematoxylin and eosin [H&E]), collagen (Masson's trichrome and Verhoeff's stain with van Gieson counterstain), elastic fibers (Verhoeff's stain with van Gieson counterstain; EVG), and glycosaminoglycans (GAGs) and proteoglycans (Alcian blue).

2.3 | PM layer thickness analysis

Scanned histological images of fresh, unprocessed PM samples ($n = 3$ donors) were analyzed in ImageScope v 12.2.2.5015. Multiple ($n > 10$) measurements of the amnion, IL, and chorion thickness were taken at consistent intervals across each sample. The thickness measurements were averaged to obtain a mean layer thickness for each layer in each given sample. The mean layer thickness is reported as a mean across donors.

2.4 | Sample preparation for proteomic analysis

Tissue samples were weighed, and surface area measurements were taken, where applicable. Samples were then minced and placed in cell lysis buffer with 1% v/v protease inhibitor. Tissues were digested overnight at 4°C and then homogenized. The homogenate was centrifuged and the supernatant collected for analysis.

3 | ENZYME-LINKED IMMUNOSORBENT ASSAY

Specific components relevant to wound healing were identified and concentrations of these components were measured using enzyme-linked immunosorbent assay (ELISA) in each sample type. PDGF-BB, PDGF-AA, TIMP-1, TIMP-2, TIMP-4, TGF- β 1, TGF- α ,

bFGF, and EGF ELISA kits were obtained from RayBiotech, Inc (Norcross, GA). Hyaluronan (Hyaluronic acid [HA]) and VEGF ELISA kits were obtained from R&D Systems (Bio-Techne Corporation, Minneapolis, MN). Lactoferrin ELISA kits were obtained from AssayPro, LLC (St. Charles, MO). Assays were performed according to each kit manufacturer's instructions. Component concentrations in the IL are reported. To compare component concentrations, dCHPM component concentrations were normalized to the component concentrations of fresh, unprocessed PM.

3.1 | Kiloplex ELISA array

Concentrations of 1,000 cytokines, growth factors, and regulatory proteins in dCHPM and IL samples were measured with kiloplex ELISA array (Raybiotech, Inc). Testing was performed by RayBiotech, Inc (Norcross, GA). Concentrations were normalized to dry weight for comparison.

3.2 | Statistical analyses

All statistical comparisons were performed using unpaired Student *t* tests with a 95% confidence interval. Concentrations of components were compared between dCHPM and fresh, native PM tissue. *p* values <0.05 were considered to be statistically significant.

4 | RESULTS

4.1 | Histology of dCHPM and native PM

Histological analysis of dCHPM and fresh PM (Figure 1) shows that dCHPM retains the eight individual layers and membrane structure of fresh PM through the Clarify process. This is evident in the cell nuclei of the epithelial layer and trophoblast layer clearly in dark purple, and the discernable, dense pink of the basement membranes, compact layer, and reticular layer that are visible in both tissue types. Importantly, the lightly stained, wavy, and substantially acellular IL is identifiable in both samples. EVG staining of dCHPM shows the defined collagen content of the fibroblast and IL and basement membranes in red, as well as the elastin content of the reticular layer in black (Figure 2a). The collagen content of dCHPM (stained blue, Figure 2b) appears to be most heavily stained in the amnion and IL. The dense Alcian blue staining of dCHPM shows the rich GAG content of the IL, the fibroblast layer, and the reticular layer (Figure 2c).

4.2 | Native PM layer thickness

The thicknesses were measured for each of the three major membrane layers (amnion, IL, and chorion) in fresh PM samples. The mean amnion layer thickness was $69.43 \mu\text{m} \pm 22.34 \mu\text{m}$ ($n = 3$ donors,

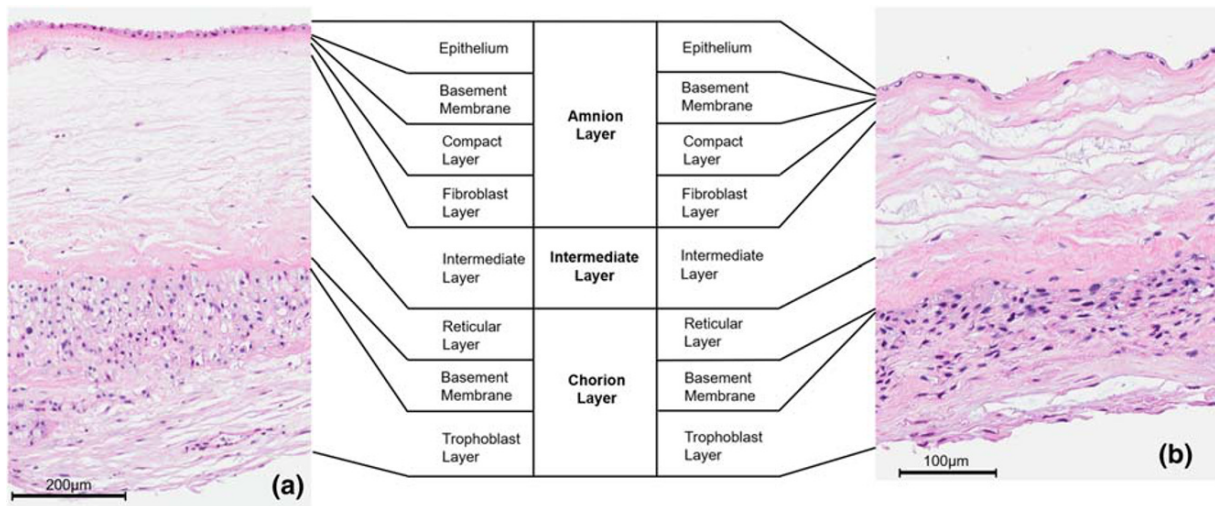
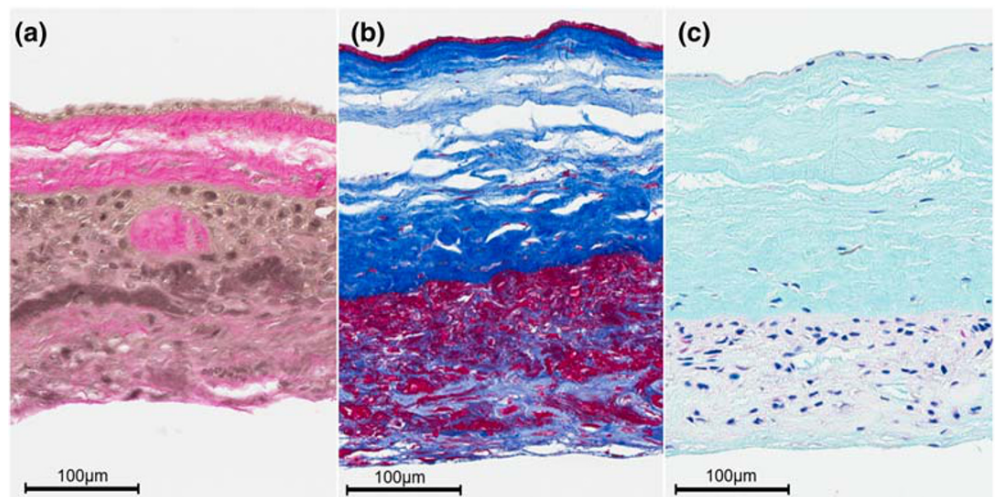


FIGURE 1 Histological sections of a complete, fresh placental membrane (a) and dehydrated complete human placental membrane (dCHPM) (b) stained with hematoxylin and eosin (H&E). The individual layers and the layer groupings making up the complete placental membrane are listed [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2 Histological sections of dehydrated complete human placental membrane (dCHPM) allografts stained with (a) Verhoeff's stain with Van Gieson counterstain showing collagen in red and elastic fibers in black, (b) Masson's trichrome showing collagen in blue, and (c) Alcian blue showing glycosaminoglycans in light blue [Colour figure can be viewed at wileyonlinelibrary.com]



$p = 0.05$). The mean IL thickness was $257.97 \mu\text{m} \pm 83.45 \mu\text{m}$ ($n = 3$ donors), which is 3.72 times thicker than the mean amnion layer thickness. The mean chorion thickness ($291.04 \mu\text{m} \pm 62.79 \mu\text{m}$, $n = 3$ donors) was 1.1-fold thicker than the IL ($p = 0.61$) and was significantly thicker than the mean amnion thickness ($p = 0.02$). The mean IL thickness accounts for over 40% of the mean overall thickness of the native PM.

4.3 | Proteomic analysis of dCHPM, native PM, and IL

The concentrations of a panel of components that are known to be relevant to wound healing were measured in the IL (Table 2).

The percent of fresh PM protein content retained following processing and dehydration was calculated for dCHPM samples and

TABLE 2 Mean component concentrations in intermediate layer

Component	Intermediate layer		n
	Mean (pg/mg)	SD	
PDGF-BB	5.30	4.77	5
PDGF-AA	255.47	322.81	5
TIMP-1	1,051.26	500.30	3
TIMP-2	877.69	413.65	3
TIMP-4	0.54	0.47	5
TGF- β 1	22.79	8.05	3
bFGF	177.91	55.19	4
EGF	0.05	0.09	8
Hyaluronic acid	5,283,723.82	1,830,219.42	6
Lactoferrin	39,906.38	22,609.99	3
VEGF	0.39	0.36	3

represented in Figure 3. There was no significant difference in concentrations of PDGF-BB, PDGF-AA, TIMP-1, TIMP-2, TIMP-4, bFGF, EGF, HBD-1, lactoferrin, or TGF- α between fresh PM and dCHPM. There was a significantly greater concentration of HA

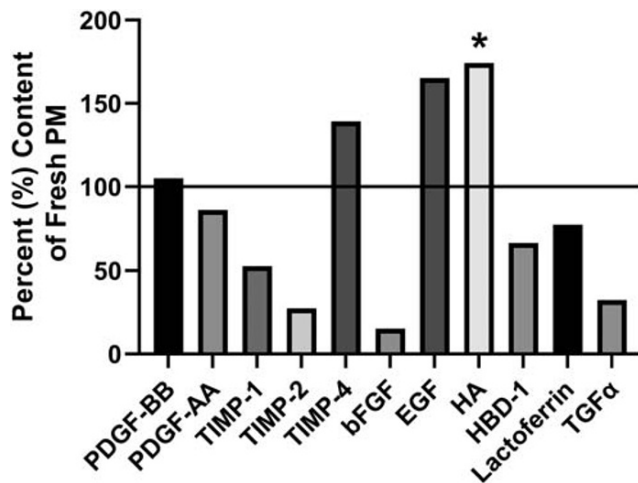


FIGURE 3 Mean target concentrations in dehydrated complete human placental membrane (dCHPM) allografts normalized to target content in fresh PM. Protein analysis performed using enzyme-linked immunosorbent assay. Horizontal line indicates concentrations of fresh placental membrane (PM). *Results of a *t* test with significant differences between the dCHPM concentrations and the fresh PM concentrations ($p < 0.05$)

measured in dCHPM allografts ($43.56 \mu\text{g}/\text{cm}^2 \pm 14.89 \mu\text{g}/\text{cm}^2$, $n = 6$) compared with fresh PM ($25.00 \mu\text{g}/\text{cm}^2 \pm 6.76 \mu\text{g}/\text{cm}^2$, $n = 3$, $p = 0.04$).

An array of 1,000 protein targets was performed on IL and dCHPM samples to compare the protein categories detectable in the tissues. Detected proteins were categorized into groups by the type of protein and the functional role they perform. A comparison of the number of proteins in each category detected in IL and dCHPM samples, as well as specific signaling factors measured in each sample type, is detailed in Figure 4. Functional roles were assigned based upon activities relevant to tissue repair and remodeling, as reported by the <http://www.uniprot.org> database. Proteins that were not associated with these functional roles were not included in this analysis.

5 | DISCUSSION

Histological analysis shows that the Clearify processed dCHPM preserves the native architecture of the PM, including the IL (Figure 1). The layers are never separated during processing and so retain their natural borders, connections, and substance. Further, the freeze-drying method preserves the natural structure of the tissue, unlike heat dehydrated alternatives that can cause the tissue layers to compact, which has been shown to alter biomatrix function (Johnson et al., 2017; Koob et al., 2014). The dCHPM still retains the open, spongy tissue of the IL. This open architecture and the intact processing technique make dCHPM more analogous to the native

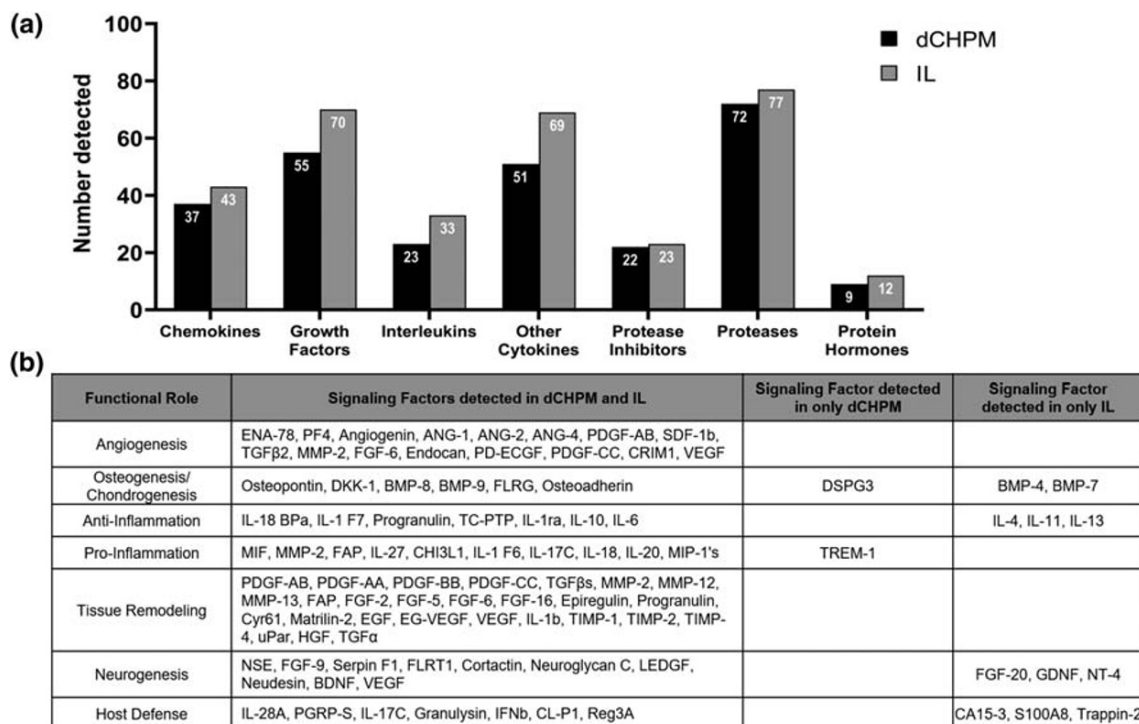


FIGURE 4 Protein categories and functional groups of proteins detected in dehydrated complete human placental membrane (dCHPM) and intermediate layer. (a) Comparison of the number of proteins detected in each category between dCHPM and intermediate layer. (b) Table of signaling factors detected in dCHPM and/or intermediate layer grouped by functional roles. The components included in this figure are a selection of relevant components tested in the kiloplex assay and are not a complete list of detected components

tissue compared with single layer or composite PM allografts. Native tissue matrix structure is a key component of advanced wound healing, acting as a scaffold for more facile cell infiltration (Schultz, Davidson, Kirsner, Bornstein, & Herman, 2011). The conserved elastin content of the reticular and trophoblast layers is highlighted in the EVG staining of dCHPM (Figure 2a). This composition adds to the integrity of the membrane, giving elasticity and scaffolding to support connections to other layers (Hieber et al., 1997; Malak et al., 1993). Preserving these ECM structures within dCHPM allografts allows them to act as natural physical barriers capable of protecting both surface and surgical wounds during the healing process.

The majority of the PM ECM is interstitial collagen, which mechanically strengthens the tissue, and makes it more resistant to proteolytic enzymes (Bryant-Greenwood, 1998; Mamede et al., 2012). This is advantageous in chronic wound applications that, by definition, do not properly regulate matrix degrading enzymes, such as matrix metalloproteinases (MMPs; Caley, Martins, & O'Toole, 2015). Based on Masson's trichrome staining in this study, a large quantity of collagen was found to be present in the IL. In addition, it was demonstrated that the most heavily stained regions of mucopolysaccharides and glycoproteins, including GAGs and proteoglycans, are found in the IL and reticular layer. The hydrophilic nature of the GAG content of the IL, specifically the hyaluronic acid, gives it lubricating, elastic, and cushioning properties, which make it an almost incompressible fluid or jelly (Goa & Benfield, 1994; Meinert et al., 2001). Our results support studies that demonstrate that the IL is predominantly composed of collagen, proteoglycans, and GAGs that exist in a loose matrix that forms a spongy, acellular network (Bryant-Greenwood, 1998; Meinert et al., 2001; Niknejad et al., 2008).

Although the IL is crucial to the mechanical and barrier properties of the PM, it is commonly grouped together as part of the amnion. Because of this, there is little research available characterizing the IL specifically, even though the layer is visibly distinct in histological staining and can account for over 40% of the full thickness of native PM. This designation overlooks its unique contribution to the barrier, mechanical, and metabolic properties of the PM (Bryant-Greenwood, 1998; Niknejad et al., 2008). Our data demonstrate that the majority of the thickness of native PM comes from the chorion and IL. Although the thickness of the IL is variable between donors, these data demonstrated that on average, it is 3.72 times as thick as the amnion layer (Baergen, 2011).

Optimal PM allografts intended for use in wound healing should maximize the conservation of placental ECM proteins. Structural ECM, such as collagen and elastin, provides tensile strength. GAGs and proteoglycans, being extremely hydrophilic proteins, promote tissue hydration, flow resistance, and molecular exclusion. Adhesive glycoproteins such as fibronectin and laminin provide structural integrity, a matrix for cell growth, and facilitate interactions between cells (Schultz & Wysocki, 2009). Wound healing depends on the interaction of ECM, cytokines, and cells, in a process known as dynamic reciprocity (Schultz et al., 2011). Although ECM helps manage cytokine activity, cellular attachments to ECM structures are generally required for a cellular response to cytokines signaling

(Schultz et al., 2011). The presence of enough intact ECM components and scaffolding is crucial to the healing process and important to maintain in PM allografts intended to aid in tissue regeneration. The results described above provide evidence that dCHPM allografts conserve a majority of the ECM of native PM, including collagens, elastins, and proteoglycans. These are important for tissue remodeling and further assist in the function of cytokines known to improve the progression of tissue remodeling (Schultz et al., 2011).

The PM is also a selective immunological barrier (H. S. Kim et al., 2002). Extensive studies have been done on the antimicrobial properties of PM, its role in modulating inflammation, and the interplay of innate and adaptive immunity in the tissue (Frew & Stock, 2011). The PM contains a wealth of antimicrobial peptides (AMPs), including α - and β -defensins and lactoferrin, which is an iron-binding peptide that inhibits bacterial growth and is known to play a key role in immunological defense during gestation (Frew & Stock, 2011; Niemelä, Kulomaa, Vija, Tuohimaa, & Saarikoski, 1989; Underwood, Gilbert, & Sherman, 2005). *in vitro* assays have shown PM to be bactericidal to a host of bacteria, including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, which is a major concern in the treatment of burns, and indicate that PMs have an independent mechanism of antimicrobial decontamination (Kjaergaard et al., 2001; Zare-Bidaki, Sadrinia, Erfani, Afkar, & Ghanbarzade, 2017). Previously, no study has described the antimicrobial protein content of the IL. Kiloplex array data offer the first insight into the additional bacteriostatic barrier properties of the IL. Whereas 17 host defense proteins were detected in both dCHPM and IL samples, including IL-28A, PGRP-S, IL-17C, granulysin, IFN β , CL-P1, and Reg3A, seven specific factors were only detected in the IL, specifically including CA15-3, S100A8, and Trappin-2 (Figure 4). Our results also show that lactoferrin is present in the IL. This further suggests an immunological barrier role of the IL and underlines the therapeutic importance of conserving this layer in PM allografts. Our data indicate that the IL can enhance the host defense content of PM allografts and potentially their antimicrobial properties.

Along with the properties mentioned above, PM allografts have been shown to have anti-inflammatory, analgesic, and pro-angiogenic properties, to reduce scarring and enhance cellular proliferation and migration due to the signaling protein and matrix content (Castellanos et al., 2017; Malhotra & Jain, 2014; Mermet et al., 2007; Schultz & Wysocki, 2009). Here, we compared fresh, unprocessed PM to processed dCHPM to investigate the conservation of components relevant to applications in wound healing. The Clarify process did not significantly decrease the amount of PDGF-AA or PDGF-BB, bFGF, EGF, TGF- α , TIMP-1, TIMP-2, TIMP-4, hyaluronic acid, or lactoferrin found in PM tissue. These growth factors are important for re-epithelialization and matrix remodeling, whereas TIMPs are crucial to the regulation of ECM proteolysis and remodeling (Arpino et al., 2015); specifically, TIMPs can provide localized control of ECM turnover and remodeling (Riley et al., 1999). These data demonstrate that the Clarify process is able to conserve these critical components at the physiologically relevant concentrations found in fresh PM. It should be noted that several protein targets were measured at higher

concentrations in dCHPM than in fresh PM when normalized to surface area, including HA. It is possible that there exists greater donor variability in the concentration of these target proteins, which would account for these apparent increases. Hyaluronic acid content is reported as significantly higher in dCHPM than in fresh PM; however, HA in native PM may not be as readily digested due to the gelatinous nature of fresh PM samples when compared with the dehydrated HA content in dCHPM. These results do confirm that HA is highly conserved in dCHPM allografts relative to unprocessed PM, as is also suggested by the histological staining. HA is a beneficial ECM component for wound care products as it contributes to cell proliferation, cellular migration, tissue hydration, and lubrication, as well as signaling anti-inflammatory pathways (Goa & Benfield, 1994; Pienimäki et al., 2001).

Importantly, this study demonstrates that the same components found in dCHPM allografts are also found in the IL. Notably, there are additional proteins relevant to wound healing that were detected in the IL that were not at detectable levels in dCHPM samples as a whole (Figure 4). A comparison of protein categories and functional groups detected in dCHPM and IL alone further highlights the compositional similarity between the IL and the entire dCHPM. Proteomic comparison of the two notably shows that a greater number of proteins in each category were at detectable levels in IL, including growth factors, interleukins, and cytokines. Although the detection limits of the kiloplex assay performed resulted in the detection of more components in the IL samples, which may have been more dilute in dCHPM, the results highlight the rich proteomic array that exists in the IL. These similarities in detectable components show that the developed processing method for dCHPM allografts conserves a majority of the unique proteinaceous content found in the IL. The IL contributes additional concentrations of almost all components tested in the PM. Of 1,000 tested proteins, 834 were detected in dCHPM and 927 were detected in IL. The detection overlap demonstrates that over 98% of the components tested in the whole dCHPM allografts are also found in IL. These similarities hold throughout the functional roles of the detected proteins, including the number of angiogenic factors detected, as well as proteins involved in tissue remodeling (Figure 4b). Notably, a number of additional proteins relating to anti-inflammatory and host defense roles were detected in IL that were not detected in dCHPM as a whole, including IL-4, IL-11, IL-13, CA15-3, S100A8, and Trappin-2. This extensive array shows, for the first time, the complex proteomic composition of the IL and the signaling content that it contributes to the overall native PM. The angiogenic, anti-inflammatory, host defense, and tissue remodeling components, specifically, suggest a role for the IL in the use of PM allografts in wound healing. These findings warrant further study and clinical evaluations to determine the potential therapeutic benefit of the inclusion of the IL in PM allografts. At the time of this study, an ongoing randomized clinical trial is evaluating the efficacy of dCHPM in diabetic foot ulcers when compared with standard of care (clinicaltrials.gov, NCT 03708029).

Previously reported placental characterization focuses on composite or single layer PM allografts that lack an IL and/or a chorion, as no

previously available products preserved all three major layers of the PM (Koob et al., 2014; Koob et al., 2015; McQuilling, Vines, Kimmerling, & Mowry, 2017). Historically, it has been assumed that the majority of components relevant to wound healing are found in the amnion or chorion of PM allografts. This is supported by the lack of literature available on the proteomic makeup of the IL. However, although these components do exist in the amnion and chorion, these results show that there is also a dense concentration of these wound healing components in the IL, some of which may account for the majority of the component concentration in the complete membrane. Inclusion of the IL into PM allografts can increase the concentration of proteins shown to modulate wound healing. Furthermore, the IL may contribute other physical, mechanical, and antimicrobial barrier characteristics to PM allografts used to treat wounds. These results are the first to describe the processing of native PMs to preserve the core matrix layer. Characterization of the IL distinguishes it as a functionally distinct and vital layer of the PM, rather than a subcomponent of the amnion stroma.

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CONFLICT OF INTEREST

Authors disclose that they are employees of and hold equity interest in StimLabs, LLC. SG is an inventor on patent applications encompassing the intellectual property of the Clearify process.

ORCID

Annelise Roy  <https://orcid.org/0000-0001-8649-0941>

Sarah Griffiths  <https://orcid.org/0000-0001-8444-8344>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Figure S1. Macroscopic images of placental membrane. (A) Image of fresh, unprocessed placental membrane. (B) Image of processed placental membrane before dehydration. (C) Image of lyophilized dCHPM.

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