Chapter 7

Placental Cells and Tissues: The Transformative Rise in Advanced Wound Care

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Additional information is available at the end of the chapter

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Abstract

The fetal environment has a remarkable capacity for facilitating and guiding tissue development. Placental tissues including the placental disc, umbilical cord, amniotic fluid and amniotic sac are highly specialized tissues responsible for transporting nutrients and coordinating developmental cues during pregnancy and fetal development. Placental tissues are nutrient-rich, structurally complex and immunologically privileged, making them promising allograft therapies for advanced wound care. Amniotic membrane allografts in particular have been shown to be effective therapies for treatment of chronic wounds, including diabetic and venous ulcers, by modulating inflammation, reducing scar tissue formation and enhancing healing. Amniotic membrane has also demonstrated the ability to promote cell proliferation, cell migration and modulate cytokine secretion by a variety of cell types involved in wound healing, including human dermal fibroblasts, microvascular endothelial cells and stem cells. In addition, amniotic membrane allografts have been shown to stimulate stem cell activity, promote angiogenesis and modulate inflammation *in vitro* and *in vivo*. Placental tissues are complex tissues composed of extracellular matrix (ECM), cells and a broad array of cytokines that may collectively enhance wound healing by modulating wound environments and stimulating endogenous cells to progress through the normal healing stages of inflammation, proliferation and remodeling.

Keywords: placenta, umbilical cord, amniotic fluid, amniotic membrane, dHACM

1. Introduction

The fetal environment has a remarkable capacity for facilitating and guiding tissue development. Starting from a single fertilized egg, fetal cells proliferate, migrate, differentiate and

respond to local and external environmental cues to develop into a fully healthy human body, including musculoskeletal, neural and cardiovascular systems. The fetal environment is critical in embryogenesis and fetal development, as the maternal environment provides specific cues for development and fetal cells respond to those maternal cues. This remarkable sequence of signals guides cell cleavage and differentiation throughout gestation in a spatially and temporally coordinated fashion.

In particular, the placenta is a highly specialized organ that develops as a conduit between maternal and fetal tissues with the primary function of transporting nutrients and developmental signals between the mother and the fetus. The placenta is composed of four distinct tissues, as depicted in **Figure 1**, including:

- placental disc,
- umbilical cord,
- amniotic fluid and
- amniotic sac or membrane.

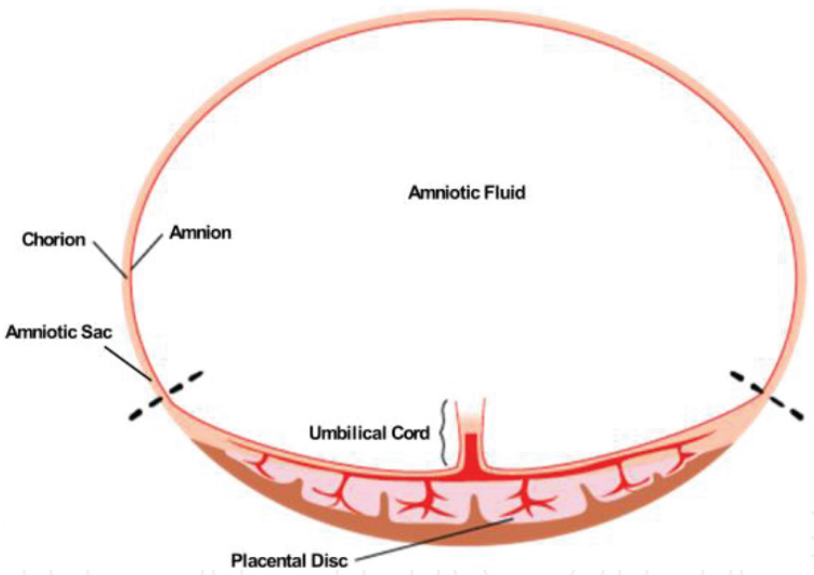

Figure 1. Placental tissues include the placental disc, umbilical cord, amniotic fluid and amniotic sac. The amniotic sac is composed of the amnion and chorion layers.

The placental disc connects the blood supply of the developing fetus with the mother to regulate nutrition, waste removal, hormonal balance and the immune system, while also acting as an immunologically privileged barrier to prevent direct contact between the maternal and fetal blood [1]. Placental tissues including the umbilical cord, amniotic fluid and amniotic sac play significant roles in regulating tissue development by maintaining the fetal environment. The developing fetus receives nutrients from the placenta through the umbilical cord vessels and the fetus is continually bathed in amniotic fluid, which cushions and protects the fetus. The amniotic fluid is physically contained and biologically regulated by the amniotic sac,

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which secretes regulatory proteins and signals into the amniotic fluid and to the fetus. Together these placental tissues, including the placental disc, umbilical cord, amniotic fluid and amniotic sac, provide nutrition, protect the fetus and act as an immunologically privileged barrier to regulate fetal development.

Due to their role in tissue development, placental tissues are nutrient-rich and structurally complex tissues, which have been investigated as advanced wound care therapies. Additionally, fetal tissues are immunologically privileged [2] and the placenta is normally discarded after birth, making placental tissues an available source of donor tissue with low risk of immunological rejection. Placental tissues, including amniotic membrane, umbilical cord, amniotic fluid and placental disc, have rapidly escalated in use as allografts to enhance healing of wounds. These placental allografts are naturally derived tissues composed of cells, extracellular matrix (ECM) and a complex array of regulatory cytokines with the inherent function of supporting tissue growth and modulating inflammation. Amniotic membrane allografts in particular have been shown to be effective therapies for healing of chronic wounds, including diabetic and venous ulcers [3, 4]. Placental tissues have also demonstrated the ability to promote cell proliferation, cell migration and modulate cytokine secretion by a variety of cell types involved in wound healing, including human dermal fibroblasts, microvascular endothelial cells and stem cells [5–8]. This chapter will provide a review of placental tissues and their role in wound healing.

Published literature was reviewed for *in vitro*, *in vivo* and clinical studies on the use of placental tissues for wound care and soft tissue healing. Databases such as PubMed, Google Scholar and Google Books were searched for terms relevant to the structure, function and biomedical application of placental tissues, including the amniotic membrane, umbilical cord, amniotic fluid and placental disc and various review articles and citations were used to identify applicable publications. Where appropriate, discussion was primarily limited to recently published, peer-reviewed studies and completed randomized controlled clinical trials to focus on high quality research.

2. Role of placental cells and tissues in fetal development

2.1. Placental development

The placenta is a remarkable organ with very unique characteristics, including being a nutrient-rich and immunologically privileged tissue. The structure and function of the placenta during pregnancy and fetal development gives placental tissues unique characteristics that can be utilized to enhance healing of wounds. The placental disc is composed of both maternally- and fetally-derived tissues to form a specialized maternal/fetal barrier that facilitates transport between maternal and fetal blood without direct contact. The fetal component of the placenta, called the chorion frondosum, develops from the fetal blastocyst, while the maternal component, called the decidua basalis, develops from the maternal uterine tissue. Placental development is initiated after fertilization and implantation.

Upon fertilization, an egg undergoes cleavage, cavitation and differentiation to form a multicellular structure called a blastocyst. A blastocyst is composed of an inner cell mass, which contains embryonic stem cells that become the embryo and an outer cell layer that is called the trophoblast. As a part of the female menstrual cycle, the maternal uterus undergoes a process called decidualization in response to hormonal changes, including cyclic secretion of 17β-estradiol and progesterone [1]. The maternal endometrium subsequently undergoes remodeling, which includes increased glandular epithelial secretion, influx of specialized uterine natural killer cells and vascular remodeling, to prepare itself for blastocyst implantation [9].

After the blastocyst implants into the maternal endometrium, the trophoblast develops to form the outer layer of the placenta. Trophoblast cells rapidly proliferate and differentiate to form an inner layer of cytotrophoblast cells and the cytotrophoblast cells differentiate and fuse to form an outer multinucleated syncytiotrophoblast cell layer that covers the placenta. The syncytiotrophoblast extends into the endometrial epithelium and invades the connective tissue, as the blastocyst sinks beneath the endometrial surface. Lacunar networks form within the syncytiotrophoblast, allowing maternal blood to flow in and out of the networks and extensions of proliferating cytotrophoblasts evaginate into the syncytiotrophoblast forming the chorionic villi of the placenta [1]. As the placenta develops, the trophoblast layers form a placental barrier, where a layer of cells separate the maternal blood in the intervillous space from the fetal blood in the villi.

In response to blastocyst implantation, the maternal endometrium undergoes a decidual reaction in which the decidual stromal cells accumulate glycogen and nutrients and increase secretory function to support the early embryo [1]. Also, decidualizing stromal cells acquire the unique ability to regulate trophoblast invasion, to resist inflammatory and oxidative insults and to dampen local maternal immune responses. Stromal cells increase expression of various factors including prolactin, insulin-like growth factor binding protein 1 (IGFBP-1), tissue factor, interleukin 15 (IL-15) and vascular endothelial growth factor (VEGF) [9].

As the maternal uterine endometrium undergoes decidualization, the spiral arteries in the decidua are remodeled to become less convoluted and larger to increase maternal blood flow to the placenta. Maternal vessels are disrupted to form the intervillous space, where maternal blood comes in direct contact with fetal chorion frondosum, though no fluid is exchanged across the membrane.

2.2. Placental structure and composition

The intervillous space lies in between the fetal chorionic villi and the maternal blood vessels and contains the main functional units of the placenta. In the intervillous space, extensively branched and closely packed villous structures contain fetal blood vessels. The intervillous space is lined with syncytiotrophoblasts and at this border, maternal blood enters via spiral endometrial arteries [1]. To support blood flow and nutrient transport, the relatively high pressure of maternal blood fills the intervillous space of the placenta and bathes the fetal villi in blood. Maternal-fetal exchange of nutrients occurs at the terminal regions of the chorionic

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villi. Then as the maternal blood pressure decreases, the deoxygenated blood drains out of the intervillous space into the maternal bloodstream through the endometrial veins.

In the fetal circulation, the umbilical cord connects the fetal blood to the placental circulation. The umbilical cord connects to the chorionic plate of the placental disc and the vessels branch radially over the surface of the placenta to form a network of villous tree structures [1]. The umbilical vessels branch in the placenta to form chorionic vessels, which then branch again to form cotyledon vessels. These vessels in the chorionic villi form an extensive network, which brings fetal blood extremely close to maternal blood with no intermingling. In the intervillous space, maternal and fetal blood come as close as 2–4 μm of each other to facilitate transport across the placental barrier without direct contact or mixing of blood [1]. As a result, signals and nutrients in the maternal and fetal blood become intertwined throughout pregnancy.

Given the unique function of the placenta, it is no surprise that placental tissue components, including ECM, cells and cytokines, are intricately organized. These bioactive tissue matrices can be used for a variety of medical applications, including treatment of wounds, especially when the biological components of the tissues are preserved.

2.2.1. Placental disc

The placental disc is composed of a highly vascularized extracellular matrix. Collagens I, III, IV and VI have been identified in the placental disc, with collagen type I being the predominant structural component [10]. Additionally, the placenta disc contains a vast distribution of noncollagenous glycoproteins and proteoglycans, including fibronectin, fibrillin I, laminin, thrombospondin I, tenascin C, decorin, heparan sulfate proteoglycans and elastin [11]. This distribution of diverse ECM components can influence cellular differentiation, hormone and protein production, proteolytic activity, as well as various repair mechanisms. Of note, collagen IV, laminin and heparan sulfate, which are normally associated with basement membranes in most adult organs, are expressed weakly throughout the villous stroma and this distribution may facilitate remodeling of basement membranes and increase morphogenetic and functional flexibility of various villous cell populations [10].

The various cell types in the placental disc include trophoblasts, connective tissue fibroblasts, vascular cells, as well as a population of placental mesenchymal stem cells (MSCs) [12]. Due to the role of the placenta in nutrient transport, the placenta is also rich in a number of nutrients and cytokines, including water, electrolytes, vitamins, glucose, proteins, amino acids, lipids and triglycerides. However, because the placental disc contains maternally derived tissue, placental disc tissue typically requires complete decellularization to remove immunological components and use for transplantation is limited to the extracellular matrix.

2.2.2. Umbilical cord

In addition to the placental disc, other placental tissues include the umbilical cord, amniotic sac and amniotic fluid, which are derived from fetal tissues and also have unique structures and functions to support pregnancy and development.

The umbilical cord is composed of Wharton's jelly, which surrounds the umbilical vein and umbilical arteries, contained by an epithelium. The umbilical vein carries oxygenated, nutrient-rich blood from the placenta to the fetus and two umbilical arteries return deoxygenated blood and waste products away from the fetus to the placenta. The umbilical cord connects to the fetal blood supply through the abdomen which will become the navel after birth and within the fetus, the umbilical vein carries oxygenated blood to the hepatic portal vein which carries blood to the liver and the inferior vena cava which carries blood to the heart. The fetal internal iliac arteries then connect to the two umbilical arteries to return deoxygenated blood back into the umbilical cord toward the placenta.

Wharton's jelly is a gelatinous substance composed largely of a diffuse ECM that is rich in collagen and hyaluronic acid, as well as low cellularity of fibroblasts. Collagens I, III, V and VI form an insoluble collagen fibril network, along with an interpenetrating glycoprotein network of fibrillin-rich microfibrils [13, 14]. Hyaluronic acid, the predominant glycosaminoglycan in Wharton's jelly, is immobilized within the insoluble network, forming a hydrated gel that maintains the tissue architecture of the cord and protects the umbilical vessels from extension and compression [15, 16]. The umbilical cord also contains lower amounts chondroitin sulfate, dermatan sulfate, keratin sulfate and heparan sulfate proteoglycans [13].

The Wharton's jelly contains a population of stromal fibroblast-like and myofibroblast-like cells, along with a population of MSCs [15]. Though cell density in the umbilical cord is relatively sparse, these cells are encased in a high volume of ECM suggesting that umbilical cord cells are responsible for secreting large amounts of ECM in order to maintain the tissue matrix [16]. Wharton's jelly also acts as a reservoir of growth factors, which are bound to high molecular weight ECM components. The Wharton's jelly contains acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-I), IGFBPs, platelet-derived growth factor (PDGF), transforming growth factor α (TGF-α) and TGF-β [15, 16] and these growth factors and cytokines control cell proliferation and differentiation, protein synthesis and remodeling of the ECM.

2.2.3. Amniotic sac

The human amniotic sac is a thin membrane that contains the amniotic fluid and holds the developing fetus. The amniotic sac comprises two distinct but conjoined membranes—the amnion and chorion. The amnion comprises the inner surface nearest the fetus and contacts the amniotic fluid, while the chorion is nearest the uterus. The membranes consist of an organized collagen-rich ECM, various cells and an abundance of regulatory proteins and signaling molecules. The amnion is composed of an epithelium, followed by a basement membrane, compact layer and fibroblast layer. The epithelium, which faces the developing fetus, consists of a single layer of epithelial cells uniformly arranged on the basement membrane. The basement membrane is a thin layer composed of collagens III and IV and noncollagenous glycoproteins laminin, nidogen and fibronectin [17]. The compact layer is a dense layer almost totally devoid of cells and forms the main fibrous structure of the amnion. Interstitial collagens I and III form bundles in the compact layer that maintain the mechanical integrity of the membrane, while collagens V and VI form filamentous connections to the

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basement membrane [17]. The fibroblast layer consists of fibroblasts embedded in a loose collagen network with islands of noncollageneous glycoproteins [18].

An intermediate, or spongy, layer separates the amnion and chorion membranes. A nonfibrillar meshwork of collagen III and an abundance of proteoglycans and glycoproteins form a loosely connected jelly-like structure that is high in water content [17, 19]. The intermediate layer acts as an interface that allows the amnion and chorion to glide over one another.

The chorion layer is three to four times thicker than the amnion. Chorion is composed of a reticular layer, pseudobasement membrane and trophoblast layer [17]. The reticular layer contacts the intermediate layer and is composed of collagens I, III, IV, V and VI [17, 20]. The pseudobasement membrane anchors the trophoblasts to the reticular layer with collagen IV, fibronectin and laminin [17, 20]. The trophoblast layer faces the maternal tissue and consists of 2–10 layers of trophoblasts [20]. The trophoblast layer of the chorion frondosum is adhered to the maternal decidua on the surface of the placental disc; however, in the amniotic sac, the chorion does not integrate with decidual tissue.

The amnion and chorion contain no blood vessels and have no direct blood supply; thus, required nutrients are supplied to the amniotic membranes directly through diffusion out of the amniotic fluid or from the underlying decidua [19]. Likewise, the membranes also secrete substances both into the amniotic fluid and toward the uterus, influencing both amniotic fluid homeostasis and maternal cellular physiology, respectively [1]. To date, 226 growth factors, cytokines, chemokines and regulatory proteins have been identified in amniotic membrane-derived tissues [21]. These molecules include growth factors, immunomodulatory cytokines and chemokines and tissue inhibitors of metalloproteinases (TIMPs), such as PDGF-AA, PDGF-BB, TGF-α, TGF-β, bFGF, EGF, VEGF, IL-10, IL-4, placental growth factor (PlGF), TIMP-1, TIMP-2 and TIMP-4, which possess important regulatory roles in regulating fetal development and pregnancy [5].

2.2.4. Amniotic fluid

Amniotic fluid surrounds and bathes the fetus during development. Amniotic fluid is generated from the maternal plasma, which passes through the amniotic membranes through osmotic and hydrostatic forces. Early in development, amniotic fluid has a similar composition to fetal plasma and is absorbed through the fetal skin, amniotic membrane, placental surface and umbilical cord [22]. After keratinization of the fetal skin, the fluid is primarily absorbed by the fetus through breathing and swallowing of the amniotic fluid. The amniotic fluid is also exchanged through fetal urination and secretion of oral, nasal, tracheal and pulmonary fluids. Amniotic fluid is predominantly composed of water and contains carbohydrates, proteins, lipids, electrolytes, fetal waste products such as urea and meconium and low numbers of a heterogeneous population of fetal-derived cells.

The volume of amniotic fluid increases during pregnancy up to a peak volume of 800 mL by 28 weeks as the fetus grows, in order to cushion and protect the developing fetus. Near term, the volume declines to approximately 400 mL [22]. Despite a continual flow of fluid between the fetus and placenta, the volume and composition of the amniotic fluid are highly controlled

by various mechanisms. Hyaluronic acid is the primary extracellular matrix component that is suspended with the amniotic fluid. Hyaluronic acid increases the viscosity of amniotic fluid, supporting lubrication and movement within the amniotic sac. Hyaluronic acid may also play an important role in fetal healing, which is known to lack fibrous scarring, since hyaluronic acid is deposited early in the healing process in adult tissues and is also known to interact with a variety of growth factors and signaling molecules during the wound healing process [23].

The amniotic fluid is rich in a number of key signaling molecules that play critical roles in fetal development. Amniotic fluid contains a variety of growth factors, carbohydrates, proteins, lipids, electrolytes and other nutrients, including high levels of EGF, TGF-α, TGF-β, IGF-I, erythropoietin (EPO), granulocyte colony-stimulating factor (GCSF) and macrophage colony-stimulating factor (MCSF) [22]. As a key regulator of the fetal innate immune system, amniotic fluid also contains a variety of enzymes, antimicrobial peptides and immunomodulatory mediators that protect the fetus from infection [22, 24]. Amniotic fluid contains a low cellularity of heterogeneous fetal-derived cell types, including epithelial cells from the fetal skin and amnion membrane, cells from the digestive, respiratory and urogenital tracts, as well as a small population of multipotent stem cells [25].

2.3. Stem cells derived from placental tissues

Placental tissues, including the amniotic fluid, amniotic membrane and umbilical cord, are a rich source of multipotent stem cells with the ability to differentiate down multiple lineages and possessing potent immunomodulatory properties. Placental stem cells include hematopoietic and mesenchymal stem cells. Because placental stem cells can be derived from a readily available source of tissue that involves minimal ethical concerns, placental stem cells present significant promise as a cell-based therapy to treat disease [25].

The amniotic membrane contains several populations of multipotent cells, which include amniotic epithelial cells (AECs), amnion-derived MSCs and chorion-derived MSCs [26]. AECs express surface markers that are characteristic of embryonic stem cells, such as stage-specific embryonic antigen 3 (SSEA-3), SSEA-4, TRA-1-60 and TRA-1-81, as well as transcription factors that are commonly associated with pluripotent stem cells, including Oct-4 and Nanog [27]. AECs can give rise to cells in all three germ layers. AECs express nonclassical human leukocyte antigen G (HLA-G), but low levels of HLA-A and -B antigens suggesting that they are immunologically inert [19, 26]. The amnion and chorion are also a source of amniotic membrane stem cells that closely resemble MSCs. Amniotic membrane MSCs (AM-MSCs) express surface markers and differentiation potential consistent with bone marrow MSCs and also express low levels of HLA-A and -B [28].

The amniotic fluid also contains a population of multipotent stem cells. Similar to AM-MSCs, amniotic fluid stem cells (AFSCs) are phenotypically similar to bone marrow MSCs and possess similar multilineage potential [25, 29]. AFSCs may be derived from fetal tissue or multipotent cells from the amniotic membrane. AFSCs also retain some characteristics of embryonic stem cells like expression of SSEA-4 and Oct-4 and differentiation potential down all the three germ lineages [25, 29], suggesting that AFSCs are an intermediate cell type between embryonic stem cells and adult mesenchymal stem cells [30].

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Umbilical cord blood is known to be a rich source of hematopoietic stem cells (HSCs) and MSCs [31]; however, Wharton's jelly also acts as a niche, which contains umbilical cord stem cells (UCSCs). UCSCs express similar phenotypic surface markers and differentiation potential to bone marrow MSCs [15, 32].

Placental-derived MSCs possess potent immunomodulatory and reparative properties [26, 33]. Therefore, placental-derived MSCs have been investigated clinically and preclinically for regeneration of tissues and treatment of a variety of diseases and disorders, including treatment of dermal wounds [25, 26, 34, 35]; however, to date live cell therapies have demonstrated limited clinical efficacy due to limited viability and engraftment, as well as phenotypic changes involved with expansion and cryopreservation of live cells [36–39].

2.4. Placental function

By supplying nutrients to the fetus without direct mixing of maternal and fetal blood that could lead to immunological rejection, the placenta plays a key role in supporting fetal development. Through the unique structure of the placental tissues, the placenta connects the developing fetus to the maternal blood supply to provide thermoregulation, nutrient exchange, waste elimination, as well as immunological and physical protection.

2.4.1. Nutrient and waste transport

Perfusion of blood through the placental disc allows transfer of critical nutrients and oxygen from the maternal blood to the fetal circulation. The umbilical cord and vessels connect the fetal blood supply to the placenta and facilitate delivery of nutrients to the developing fetus. Transport occurs through passive diffusion of soluble components across the placental membrane, as well as active transport, which requires expenditure of energy. The placenta transports a full array of nutrients from maternal to fetal blood to support fetal development. The placenta controls transport of water, electrolytes, vitamins, glucose, proteins, amino acids, lipids and triglycerides through both active and passive mechanisms [1]. Many nutrients are present in similar concentrations in fetal and maternal blood and can travel across the placental membranes by simple diffusion down a concentration gradient. However, some nutrients are required in higher concentrations in fetal blood than in the maternal plasma to support fetal development. Therefore, these nutrients require active transport across the placental membrane to concentrate these molecules in fetal blood against a concentration gradient.

The placental membrane is highly permeable to respiratory gases, including rapid diffusion of oxygen from the maternal to fetal blood and of carbon dioxide from fetal to maternal blood. In fact, fetal hemoglobin has a higher affinity for oxygen and lower affinity for carbon dioxide than maternal hemoglobin, which supports favorable gas exchange between the fetal and maternal blood [1]. Transport of water across the placental membrane occurs readily by hydrostatic forces and osmotic pressure and electrolyte balance within the fetal plasma is critical to the function of cellular environments. Ions such as sodium and chloride are present in similar levels in fetal and maternal blood and transport occurs largely by passive diffusion. However, some ion levels including potassium, magnesium, calcium and phosphate are

generally higher in fetal blood than maternal plasma, indicating that they undergo active transport across the placental membrane via a number of ion pumps and transporters [1].

Glucose is a critical carbohydrate transported across the placenta and is the primary source of energy for the fetus. Glucose is transported across the placental membrane by facilitated diffusion through protein channels and glucose transporters [1]. Amino acids, which are the building blocks that make up proteins, are generally more abundant in fetal plasma than in maternal plasma, indicating that amino acids undergo active transport across the placental membrane [1]. Lipid transport across the placenta includes free fatty acids, triglycerides, phospholipids, glycolipids, sphingolipids, cholesterol, cholesterol esters, fat-soluble vitamins and other compounds. These lipids remain bound to water-soluble lipoproteins in plasma and are transported by active and passive mechanisms.

Along with the transport of nutrients, the placenta also supports removal of waste products from the fetal blood including urea, uric acid, creatinine and carbon dioxide back to the maternal blood [1]. Due to the immature fetal liver, the placenta is also responsible for breaking down various waste products through endogenous enzymes and transport proteins involved in the handling of bile acids, biliary pigments and xenobiotics and for transporting waste products to the maternal blood where they are processed and removed from the maternal circulation by the maternal liver and kidney [40].

2.4.2. Endocrine secretion

Beyond transport between the mother and child, the placenta also acts as an endocrine organ by producing hormones and steroids. The mother's hormone levels change throughout pregnancy and the placenta secretes various hormones essential to support pregnancy and fetal development, including estrogen, progesterone, chorionic gonadotrophin, placental lactogen and placenta growth hormone [1]. These hormones control different aspects of the maternal reproductive organs, uterine contraction, placental development, metabolism, cell differentiation and fetal development. The placenta also produces a large number of growth factors including EGF, IGF and PDGF, as well as various cytokines, chemokines, eicosanoids, vasoactive autacoids and others to support pregnancy and development.

The amniotic membrane also sends signals to the fetus through the amniotic fluid and to the mother through the uterus. In addition to physically encasing the amniotic fluid and developing fetus, the amniotic membrane is a bioactive tissue that plays an integral biological role in fetal development and progression of pregnancy through secretion of growth factors, cytokines, chemokines and related regulatory factors produced by endogenous cells. Therefore, the amniotic membrane and amniotic fluid harbor significant biological activity, including a significant number of developmental cytokines that play important roles in tissue formation.

2.4.3. Fetal immunity

The placenta also plays critical roles in supporting fetal immunity. The placenta and amniotic membrane fight against infection by acting as a selective barrier to inhibit transmission of certain microbes, including bacteria, viruses and xenobiotics. The placenta, amniotic fluid and

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amniotic membrane also contain a number of enzymes that metabolize drugs and xenobiotics, as well as antimicrobial effectors and immunomodulatory mediators that protect the fetus from infection [22, 41]. Additionally, the placenta permits transport of IgG antibodies from the maternal plasma to support passive immunity in the fetus by providing a copy of maternal humoral immunity [1].

The placental barrier is also critical to preventing immunological rejection of the fetal tissues by the maternal immune system, as the trophoblast layers come in direct contact with maternal decidua and blood, including maternal natural killer (NK) cells and macrophages, but are not rejected. Though the various mechanisms for immune tolerance remain under investigation, the syncytiotrophoblast and cytotrophoblast lack HLA-A and -B tissue antigens, while expressing HLA-C, -E, -F and -G antigens. Absence of classical HLA-A and -B antigens likely prevent recognition by cytotoxic T cells, while the presence of nonclassical HLA-G antigens is necessary to prevent destruction by maternal NK cells. HLA-C is the only classical MHC Class I antigen expressed and HLA-G in particular does not distinguish between individuals but may support antiviral, immunosuppressive and nonimmunological functions [42]. Similarly, as a biological barrier between the mother and child, amniotic membrane tissues naturally contain low levels of HLA-A and -B antigens and β2-microglobulin and are therefore considered immunologically privileged tissues.

The placental trophoblasts also secrete an array of signals that inhibit cytotoxic T cells in the maternal decidua, including Fas ligand, indoleamine-2,3-dioxygenase (IDO), vasoactive intestinal peptide (VIP), phosphocholine, programmed death ligand 1 (PDL1) and progesterone [2, 43]. These signals interact with NK cells, helper T cells and regulatory T cells to suppress immune responses [2, 43]. Therefore, placental tissues have immunologically privileged properties, which make them a promising source of allograft tissue for treatment of wounds.

2.4.4. Protection and cushioning

Amniotic fluid physically cushions the fetus within the mother's abdomen, while allowing fetal movement and promoting musculoskeletal development [22]. The fetus and amniotic fluid are enclosed within the amniotic membrane, which acts as a mechanically robust barrier between the mother and the child. This thin membrane must possess the structural integrity to support the pregnancy through term. Therefore, the amniotic membrane is a metabolically active tissue, which continually remodels and grows to accommodate the increasing volume of the conceptus and amniotic fluid without premature rupture.

3. History of placental tissues for wound healing

Placental tissues including umbilical cord, amniotic sac and amniotic fluid are nutrient-rich tissues that support fetal development and are immunologically privileged to prevent rejection by the maternal immune system. These characteristics make them promising tissues to support wound healing. Though the complex cascade of signals involved in tissue development is not fully understood to date, placental tissues are able to control and regulate the proper balance

of many factors to facilitate growth of the embryo. Therefore, the placenta may contain specific regulatory signals that may be critical for tissue growth and these signals may also provide a unique stimulus to promote healing of complex wounds in adults.

At birth, the placenta separates from the wall of the uterus and is expelled from the body. The mother and child no longer require the function of the placenta to facilitate nutrient transport and pregnancy after birth. The umbilical cord is cut from the child and placental tissues are typically discarded as medical waste. Therefore, placental tissues are a plentiful source of donor tissue with significant potential for use as allografts. These tissues are rich in nutrients and the fetal components of the placenta possess significant immunological properties, which make them ideal tissues to promote wound healing.

3.1. Early evidence of efficacy

Placental tissue has been used as allografts since the early twentieth century. In particular, amniotic membrane tissue has been shown to promote healing in a variety of applications including wounds, ophthalmology and surgery [44]. Amniotic membrane allografts have a number of naturally inherent properties that make them beneficial tissues to promote healing. The amniotic membrane tissue provides a natural barrier and ECM scaffold for wound healing and the amniotic membrane also contains an abundance of various growth factors and biological macromolecules important in regulating the physiological healing response [5]. The natural composition of amniotic membrane gives the tissue the biological activity to enhance healing, modulate inflammation and reduce scar formation.

The first reported use of amniotic membrane for skin transplantation was in 1910 [45]. A variety of cases followed including reconstructive OB/GYN surgery, dentistry, neurosurgery and general surgical applications with reports of decreased pain, low rates of infection and improved healing [44]. In the 1940s, promising outcomes were reported for use in healing of the ocular surface [46] and beginning in the 1960s, use of amniotic membrane as wound coverings for treatment of burns and chronic wounds increased [47].

Despite promising results indicating that amniotic membrane was a valuable allograft tissue to promote healing and repair, clinical use of amniotic membrane diminished and failed to achieve widespread use. At the time, amniotic membrane tissue was difficult to reliably source, cleanse, preserve and handle. Additionally, fresh allografts carried a risk of infectious disease transmission such as human immunodeficiency virus (HIV) from the donor tissue. Limited processing and preservation methods also made transportation and storage of the tissue difficult [47].

However, with improvements in processing techniques and quality control of infectious disease testing, amniotic membrane tissues were reintroduced for ophthalmic applications in the 1990s. Use increased rapidly with ophthalmology becoming one of the most popular applications of the material in the late twentieth century [46]. Amniotic membrane is currently used for conjunctival reconstruction, burn treatment, pterygium repair and a number of other similar applications. Following the success of amniotic tissue in ophthalmology, adaption of preserved amniotic tissues for wound care soon followed.

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3.2. Recent advances in wound care

3.2.1. Improvements in placental tissue processing

As processing techniques continued to improve, use of amniotic membrane allografts in wound care increased, as did use in dental and surgical applications. Various methods have been developed to cleanse, prepare and preserve the tissue for surgical use. Additionally, improved controls are now in place to appropriately preserve the tissue and reduce the risk of infectious disease transmission.

In the United States, human placentas are donated under informed consent, in compliance with the Food and Drug Administration's (FDA) Good Tissue Practices (GTP) and the American Association of Tissue Banks' (AATB) standards. Mothers can choose to donate their placentas following full-term, live births that result in both a healthy mother and child. Placentas are typically donated following scheduled Caesarean sections, which allow the tissue to be maintained in the aseptic field without passing through the birth canal. All donors are tested and confirmed free of infectious diseases, including HIV, human T-lymphotropic virus (HTLV), hepatitis B and C and syphilis, in accordance with the AATB standards.

Allograft tissues may be processed using a variety of techniques. For example, many allografts (harvested from another human tissue donor) and xenografts (harvested from animal tissues) are fully decellularized in order to remove immunogenic cellular components and prevent immune rejection by the recipient. The process of decellularization intentionally washes out immunoreactive cellular components including bioactive regulatory factors, leaving a structurally intact but biologically inert extracellular matrix scaffold. While decellularization is necessary to prevent host rejection in xenograft tissues (such as porcine small intestinal submucosa or urinary bladder) and nonimmunologically privileged human allograft tissues (such as human dermis), fetal-derived placental tissue allografts are immunologically privileged tissues. Fetal placental tissues contain low levels of HLA antigens and do not elicit immune rejection. Therefore, placental tissues may be gently cleansed to remove blood and hazardous materials, while preserving the natural biological activity of the tissue for transplantation without complete decellularization.

Human amniotic membranes have increased in popularity as barrier membranes to promote healing of dermal, ophthalmic and surgical wounds, partly due to their immunologically privileged properties [47]. To provide a product for patient use, allograft tissues require preservation techniques to allow for transportation, storage and off-the-shelf usage. The most common method to preserve tissue grafts and prevent degradation is through cryopreservation or freezing. Freezing tissue can prevent degradation by reducing enzymatic and chemical activity in the tissues and inhibiting the growth of microorganisms. However, cryopreserved grafts are often cumbersome to transport and store, requiring temperature-controlled conditions such as liquid nitrogen, dry ice, or large freezers, often at -80°C or below. Cryopreserved grafts also are commonly stored in cryoprotectants, such as dimethylsulfoxide (DMSO) and glycerol, added to mitigate the effects of ice crystal formation within the tissues, which can destroy cellular membranes and disrupt tissue matrix. These cryoprotectants, however, can be

cytotoxic at high concentrations or extended exposure times and must be thoroughly rinsed from the tissues prior to application on patients.

An increasingly popular alternative to cryopreservation is tissue dehydration. Tissue can be dehydrated under heat, open air, or freeze drying (lyophilization). By removing residual moisture, tissue can be preserved by reducing activity of soluble chemical reactions and water-dependent enzymatic activity and inhibiting the viability of microorganisms in a low moisture environment. Dehydration preserves tissue without the need for freezers, dry ice, or liquid nitrogen and certain methods of dehydration have been shown to retain equivalent or superior biological activity compared to cryopreservation, with the benefits of being shipped and stored at ambient conditions. Dehydrated tissues are also typically stronger and easier to handle than wet tissues. Though dehydration may alter the tissue's microstructure by causing the matrix to become more compact in the absence of water, by preserving the native tissue matrix proteins the dehydrated tissue can be rehydrated in the wound environment to return the tissue to its original state.

Following dehydration, human amniotic membrane tissue is easy to handle and can be stored at ambient conditions with a shelf-life of up to 5 years, while preserving the structural integrity and biochemical activity of native amniotic membrane. Even though dehydration renders amniotic cells nonviable, these cells remain structurally intact, including the cellular and pericellular components that play essential roles in regulating biological activity. Retention of bioactive factors is thought to be critical to the clinical efficacy of amniotic tissue allografts in wound repair and tissue regeneration. Therefore, harsh cleansing processes that wash bioactive material out of the grafts may greatly reduce the cytokine content and diminish the clinical efficacy of the naturally derived tissues.

An additional benefit of tissue dehydration is that the allografts can be terminally sterilized to reduce the risk of infectious disease from the donor tissue. While all allograft tissues are aseptically processed to reduce the risk of bacterial or viral contamination, dehydrated tissues can be terminally sterilized using techniques such as gamma ray or electron beam irradiation to further reduce the risk of disease transmission. Though high levels of radiation may potentially crosslink or denature proteins within tissues, terminally sterilized amniotic membranes allografts have been proven to retain biological activity both clinically and through *in vitro* experiments [3, 5]. These data suggest that sterilization does not significantly diminish the bioactivity of amniotic membrane allografts and is worthwhile to ensure maximal safety to patients.

Each tissue processing technique has differing advantages and disadvantages based on the clinical goal of the resulting allograft tissue. However, with amniotic membrane tissue, the goal of tissue processing is to cleanse the tissue of hazardous materials in order to ensure a safe allograft product for the patient while preserving the natural properties and biological activity of the native amniotic membrane tissue to maximize efficacy and promote tissue healing. With its rapid growth, usage of amniotic membrane has now expanded to include many other promising applications in addition to wound care. It is emerging as a reparative membrane in orthopedics, neurosurgery, periodontology, gynecological surgery, general and reconstructive surgery and a number of other medical fields [47].

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3.2.2. Amniotic membrane allograft composition

Due to remarkable clinical success, use of placental tissue allografts has largely focused on amniotic membrane to date. Amniotic membrane allografts can comprise single-layer amnion tissue, or the amnion can be combined with the chorion layer to form a laminated graft. Beginning with success in ophthalmological applications in which a thin, unobtrusive membrane is often desired, single-layer amnion grafts have increased in popularity to promote healing of dermal wounds. More recently, laminated membranes of amnion and chorion have also been developed to provide thicker, more substantial grafts. In particular, MiMedx Group, Inc. (Marietta, GA) uses a proprietary, patent-protected PURION® Process to manufacture dehydrated human amnion/chorion membrane (dHACM) allografts (EpiFix®). Hematoxylin and eosin (H&E) staining of dHACM, which stains cell nuclei dark blue and stains connective tissue and cytoplasm pink, is shown in **Figure 2**. Because the chorion is dissected from the amniotic sac and not from the placental disc or from the chorionic plate to produce these dHACM grafts, the chorion tissue in PURION® Processed dHACM is nonmaternally derived and is immunologically privileged with a low risk of eliciting an immune response.

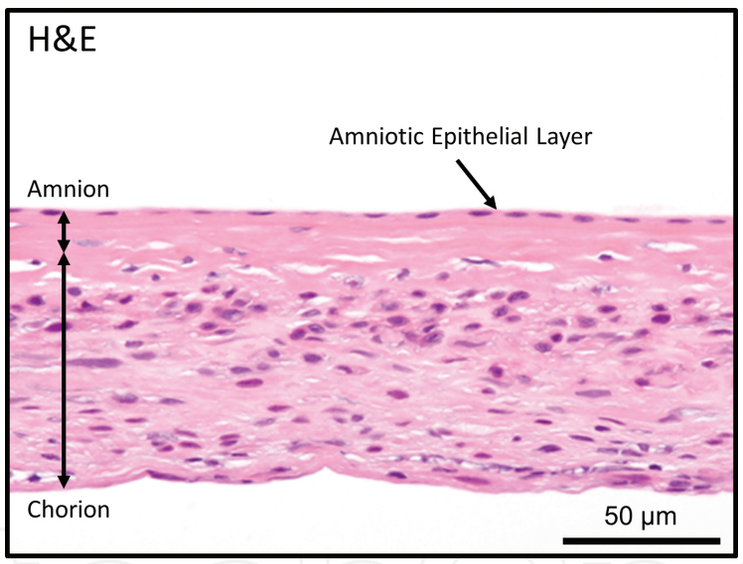

Figure 2. Hematoxylin and eosin (H&E) staining of dehydrated human amnion/chorion membrane (dHACM).

A significant advantage of including chorion tissue in an amniotic membrane allograft is that the chorion is approximately three to four times thicker than the amnion layer alone while containing a similar distribution of bioactive growth factors [48]. Therefore, lamination of the amnion and chorion layers results in a thicker graft with easier handling characteristics and significantly greater total content of growth factors and cytokines than single layer, amnion-only grafts. By preserving the content of amniotic membrane tissue and utilizing the thicker chorion layer, PURION® Processed dHACM grafts have been shown to contain as much as 20-fold greater levels of growth factors and cytokines than other amnion-only allografts [48].

Various amniotic membrane allografts have also been micronized into particulate forms or suspended in fluid to allow injection of the grafts for sports medicine and wound applications.

These injectable tissues have been used in capsular joints to relieve pain and promote soft tissue healing, as well as to modulate inflammation and promote healing of microtears, such as in plantar fasciitis [49].

3.2.3. Regulation of placental tissue allografts

In the United States, placental tissues, including amniotic membrane allografts, are commonly regulated by the FDA as human cells, tissues and cellular and tissue-based products (HCT/Ps) under Section 361 of the Public Health Service (PHS) Act. Tissues regulated as Section 361 HCT/Ps do not require FDA clearance or approval; however, the HCT/P allografts are required to be in compliance with the FDA's current Good Tissue Practices (cGTP) regulations and 21 Code of Federal Regulations (CFR) Part 1270 and 21 CFR Part 1271. These standards are important to prevent introduction, transmission, or spread of communicable diseases. Regulations require that manufactured products are registered with the FDA and that stringent donor eligibility requirements are in place for donor screening and testing of relevant communicable diseases. cGTP establishes guidelines for manufacturing methods, facilities and controls to ensure that HCT/Ps do not contain communicable disease agents, are not contaminated and do not become contaminated during processing. Tissue processing sites must also be registered as tissue banks with the AATB.

Tissues regulated as Section 361 HCT/Ps are required to be "minimally manipulated" human tissues, meaning that processing cannot alter the original relevant characteristics of the tissue and that the tissue cannot be combined with another article. These HCT/P tissues are intended for homologous use, meaning that they perform the same basic functions in the recipient as the donor and they do not have a systemic effect. The 361 HCT/P regulatory pathway allows naturally derived tissues to be transplanted for use, as long as they are safe and used in an appropriate manner. In accordance with these regulations, amniotic membrane allografts act as barriers to modulate inflammation, reduce scar tissue formation and enhance healing.

3.3. Recent clinical data in wound care

To date, clinical data on placental tissues has focused largely on amniotic membrane allografts. Amniotic membrane has proven to be an effective therapy to promote epithelialization, modulate inflammation, inhibit protease activity and enhance wound healing [19, 46]. Another promising characteristic of placental tissue is the ability to reduce fibrous scar tissue formation, as fetal tissue and the fetal environment are known to support scarless healing, though the molecular mechanisms are not yet fully understood [50].

Though many case studies exist documenting the use of amniotic membrane allografts in wound care, the number of prospective, randomized controlled clinical trials (RCTs) on placental tissues are currently limited. However, several RCTs have demonstrated the efficacy of amniotic membrane allografts in healing of chronic wounds. In particular, PURION® Processed dehydrated human amnion/chorion membrane (dHACM) allografts (EpiFix®, MiMedx Group, Inc.) have demonstrated promising clinical results in a number of randomized clinical trials, including studies in diabetic foot ulcers (DFUs) and venous leg ulcers

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(VLU) [3, 4]. A number of additional studies from various sponsors are registered on ClinicalTrials.gov and are currently ongoing [51].

3.3.1. Amnion/chorion allografts in healing of diabetic foot ulcers

A prospective RCT examined healing rates of diabetic foot ulcers (DFUs) treated with a biweekly application of PURION® Processed dHACM allografts (EpiFix®, MiMedx Group, Inc.; $n = 13$), compared to DFUs treated with a standard of care regimen of moist wound therapy alone ($n = 12$). Results demonstrated a statistically significant improvement in healing with 77% and 92% of wounds treated with dHACM completely healed at 4 and 6 weeks, respectively, compared to only 0% and 8% of wounds healed in standard of care controls [3]. Wounds were also reduced in size by an average of 97.1 ± 7.0% and 98.4 ± 5.8% after 4 and 6 weeks, respectively, with dHACM treatment, compared to 32.0 ± 47.3% and -1.8 ± 0.3% reduction in standard of care patients. Despite the relatively small number of patients included in this study, statistically significant differences were observed between treatment groups due to the drastic effect of dHACM on healing rates and the trial was terminated early at 25 patients since the investigator felt that further treatment of patients with standard of care alone would be potentially unethical.

To further support these promising results, patients that failed to heal with standard of care treatment were subsequently treated with a biweekly application of dHACM allografts ($n = 11$). In this crossover study of patients, 55% patients demonstrated complete healing by 4 weeks, along with 64% by 6 weeks and 91% by 12 weeks with application of PURION® Processed dHACM [52]. Additionally, wounds that healed after dHACM treatment in the original and crossover populations were examined for long-term durability, 9–12 months after primary healing. Of the patients that healed in response to dHACM and returned for follow-up ($n = 18$), 94.4% remained fully healed without wound recurrence at the same location [53]. These results reinforce that a significant healing response was observed in chronic DFUs in response to dHACM treatment.

In a separate study to determine the optimal dosing frequency for application of PURION® Processed dHACM allografts, a weekly application ($n = 20$) was compared with biweekly applications ($n = 20$) of dHACM (EpiFix®, MiMedx Group, Inc.) in a prospective, randomized clinical study. The weekly application of dHACM healed diabetic foot ulcers in a mean time to complete healing of 2.4 ± 1.8 weeks, compared to 4.1 ± 2.9 weeks with biweekly applications [54]. Complete healing occurred in 90% of wounds by 4 weeks in the weekly group, while 50% of wounds completely healed by 4 weeks with biweekly treatment. Therefore, these results indicate that with weekly applications wounds healed in approximately half of the time and using a similar number of grafts applied as the biweekly application, even though overall 92.5% of ulcers completely healed during the 12-week study period with dHACM treatment. These results further demonstrated that dHACM allografts are an effective treatment to promote healing in diabetic foot ulcers and suggest that wounds treated with a weekly application of dHACM heal more rapidly than with biweekly application.

3.3.2. Single-layer amnion allografts in healing of diabetic foot ulcers

Only two other amniotic membrane products of note have been used in published prospective RCTs in wounds to date. Using a human viable wound matrix (hVWM; Grafix®, Osiris Therapeutics, Inc., Columbia, MD) composed of cryopreserved amnion, DFUs were treated weekly with hVWM ($n = 50$), compared to standard of care treatment ($n = 47$), in a prospective, randomized multicenter trial. In this study, 62.0% of patients treated with hVWM experienced complete wound closure after 12 weeks, compared to 21.3% of standard of care patients [55]. Among the study participants that healed, ulcers remained closed in 82.1% of patients in the hVWM group and 70% in the control group after an additional 12 weeks.

Dehydrated amniotic membrane allograft (DAMA; AMNIOEXCEL®, Derma Sciences, Inc., Princeton, NJ) composed of dehydrated amnion was also examined in a prospective, randomized multicenter trial for treatment of DFUs. DFUs were treated weekly with DAMA ($n = 15$), compared to standard of care ($n = 14$). In this study, 33% of the subjects treated with DAMA achieved complete wound closure after 6 weeks, compared with 0% of the patients in the standard of care cohort [56].

These studies using single-layer amnion allografts did not achieve healing rates as high or speed of healing as rapid as laminated PURION® Processed amnion/chorion grafts; however, it is difficult to compare healing rates across multiple studies due to the differing patient populations and treatment regimens involved. To compare the effectiveness of therapies, a comparative effectiveness trial is required to compare allograft efficacy in a controlled manner. Nevertheless, the results of these clinical trials indicate that amniotic membrane grafts are safe to use and accelerate healing of chronic DFUs.

3.3.3. Comparative effectiveness of amnion/chorion allografts with bioengineered skin substitute in diabetic ulcers

In a prospective, randomized multicenter comparative effectiveness study, weekly applications of PURION® Processed dHACM (EpiFix®, MiMedx Group, Inc.; $n = 20$) was compared with bioengineered skin substitute (Apligraf®, Organogenesis, Inc., Canton, MA; $n = 20$) and standard of care (collagen-alginate dressing; $n = 20$) for treatment of chronic lower extremity diabetic ulcers. After 4 and 6 weeks, 85 and 95% of ulcers treated with dHACM, respectively, achieved complete wound closure, which was significantly higher than for patients receiving the bioengineered skin substitute which healed 35 and 45% of wounds, respectively, or standard of care treatment with 30 and 35%, respectively [57]. Median time to healing with dHACM was 13 days, compared to 49 days with bioengineered skin substitute and the mean number of dHACM grafts used was 2.5 applications at an average cost of $1669 per patient, compared to 6.2 bioengineered skin substitute grafts used at a cost of $9216, indicating that costs were significantly less for dHACM than the bioengineered skin substitute by a factor of five. These results reaffirm both the efficacy and cost effectiveness of dHACM amniotic membrane allografts to promote healing in chronic diabetic ulcers in comparison with a leading skin substitute.

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3.3.4. Amnion/chorion allografts in healing of venous leg ulcers

To examine the effectiveness of amniotic membrane allografts in difficult to heal venous leg ulcers (VLUs), a prospective, randomized multicenter trial evaluated the safety and efficacy of PURION® Processed dHACM (EpiFix®, MiMedx Group, Inc.) in the treatment of VLUs. Patients with VLUs were treated with either one or two applications of dHACM (n = 53) with multilayer compression therapy (MLCT) versus a standard of care (n = 31) of MLCT alone and patients were examined for an outcome of ≥40% reduction of wound size at 4 weeks, which is a surrogate endpoint found throughout the literature as a strong indicator of healing. After 4 weeks, 62% of patients receiving dHACM and 32% of those receiving MLCT alone demonstrated ≥40% wound closure [4]. Wounds treated with dHACM allograft were reduced in size by a mean of 48.1% after 4 weeks, compared to 19.0% for standard of care controls. This 40% wound closure endpoint was later validated with data demonstrating that 80% of all patients demonstrating ≥40% closure of VLUs after 4 weeks progressed to complete closure within 24 weeks, compared to only 33% of patients who demonstrated <40% healing after 4 weeks [58]. Additionally, 79.5% of patients treated with dHACM reported a reduction in pain using a visual analogue scale (VAS), compared to 52.4% of patients receiving MLCT alone. The results of this trial showed that VLUs treated with only one or two applications of dHACM experienced an accelerated rate of healing which encouraged long-term wound closure and that dHACM allografts significantly improve healing of venous leg ulcers. Together with the DFU data presented above, these clinical trials clearly demonstrate that amniotic membrane allografts promote a more rapid rate of healing in treatment of a variety of dermal wounds.

4. Scientific mechanisms to promote healing using placental tissues

Placental tissue allografts have rapidly escalated as promising advanced wound care therapies; therefore, several scientific studies have sought to improve understanding of the molecular mechanisms by which placental tissue grafts improve healing. While the cellular and molecular mechanisms by which placental tissue allografts enhance healing are still under investigation, scientific and clinical research suggest that placental tissues, including amniotic membrane, umbilical cord and amniotic fluid, possess significant promise as advanced therapies to promote healing of wounds through bioactive modulation of cellular responses and wound environments.

In particular, research has focused on the ability of amniotic membrane tissues as immunologically privileged barriers to modulate inflammation, reduce scarring and enhance healing. PURION® Processed dehydrated human amnion/chorion amniotic membrane (dHACM) allografts (EpiFix®, MiMedx Group, Inc.) have been shown to promote proliferation, migration and modulate cytokine secretion by a variety of cells involved in wound healing.

4.1. Growth factor content

To date, over 226 growth factors, cytokines, chemokines and regulatory proteins have been identified in PURION® Processed dHACM allografts, as shown in **Figure 3** [21]. These

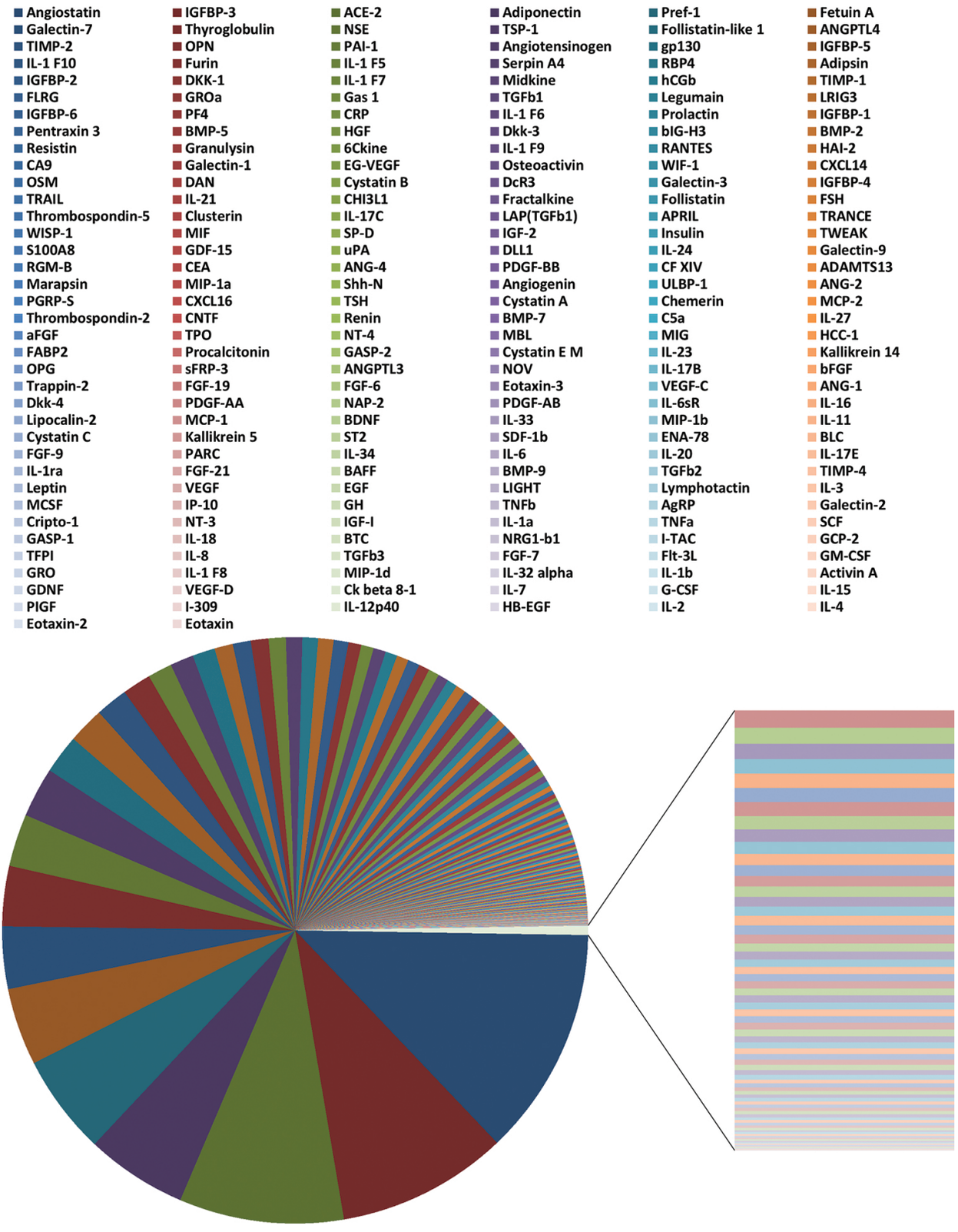

Figure 3. Relative content of 226 various growth factors, cytokines and regulatory molecules identified in dehydrated human amnion/chorion membrane (dHACM) allografts. Factors are listed in order of decreasing abundance, reading from left to right.

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molecules, which include a wide array of growth factors, immunomodulatory cytokines and chemokines and TIMPs, possess important roles in regulating fetal development and pregnancy and therefore may modulate various stages of tissue healing and regeneration. dHACM allografts deliver these bioactive molecules into the wound environment, as an initial fraction of these critical factors are freely soluble and elute out from the grafts, while the remaining fraction remains bound within the tissue extracellular matrix [5]. As the remaining tissue is resorbed over time by matrix metalloproteinases in the wound, the growth factors bound to the extracellular matrix can be released into the surrounding tissue, providing a sustained release of growth factors during the tissue regeneration process.

These results suggest that dHACM grafts deliver active growth factors, cytokines, chemokines and regulatory proteins including an abundance of protease inhibitors that are essential for soft tissue healing [72]. In particular, modulation of inflammation is critical during the early stages of wound repair and dHACM contains an array of immunomodulatory cytokines and chemokines that regulate the activity of immune cells, suggesting dHACM allografts deliver a balance of inflammatory regulators which may modulate the inflammation response within healing wounds.

4.2. Cell proliferation

PURION® Processed dHACM has also been shown to promote cellular proliferation of a variety of cell types involved in wound healing, including human dermal fibroblasts, microvascular endothelial cells and adult stem cells such as bone marrow mesenchymal stem cells (BM-MSCs), adipose-derived stem cells (ADSCs) and hematopoietic stem cells (HSCs) *in vitro*. When cultured in the presence of soluble extracts of dHACM tissue containing a cocktail of naturally derived growth factors and cytokines from amniotic tissue, dHACM was shown to stimulate proliferation *in vitro* in all of these cells types relevant to healing and repair [5–7]. These results demonstrate that dHACM directly causes human dermal fibroblasts, microvascular endothelial cells, mesenchymal stem cells, adipose-derived stem cells and hematopoietic stem cells to proliferate *in vitro* by releasing growth factors that activate the proliferative response and therefore may act by amplifying the respective populations of these cells in wound environments.

4.3. Stem cell migration

In addition to promoting cell proliferation, PURION® Processed dHACM was shown to recruit migration of adult stem cells, including mesenchymal stem cells, adipose-derived stem cells and hematopoietic stem cells *in vitro* and *in vivo*. Using *in vitro* assays, dHACM promoted chemotactic migration of MSCs across porous membranes toward dHACM tissue and accelerated migration of MSCs and ADSCs in closure of cell-free zones [5, 7]. The ability to promote chemotactic stem cell migration was confirmed *in vivo* using a murine ischemic wound model. Increased numbers of MSCs and HSCs were measured at the site of subcutaneous dHACM implantation using flow cytometry, compared to sham wounds without dHACM [5, 8]. Additionally, a parabiosis model was used in which the circulation of a green fluorescent protein (GFP^+) mouse was linked with a wild-type mouse as shown in **Figure 4**.

Flow cytometry and immunohistochemistry identified GFP⁺ stem cells at sites of neovascularization within implanted dHACM grafts in wild-type mice (**Figure 4**, blue), compared to sham and acellular dermal matrix (ADM) controls, indicating that stem cells were recruited through the blood circulation toward the dHACM grafts [8]. Cellular expression of stromal derived factor 1α (SDF-1α), a known stem cell recruiting factor, was also upregulated after dHACM implantation, which may attract additional cells to the site and further promote repair. Stem cells are pivotal cells that are normally recruited to sites of injury, where they mount a multifaceted cascade regulating inflammatory mechanisms, angiogenesis and tissue regeneration. Therefore, these data indicate that dHACM may stimulate healing by recruiting the patient's own reservoir of reparative stem cells from the circulation toward sites of implantation within healing wounds, thereby acting as a "stem cell magnet" to amplify stem cell populations within healing wounds.

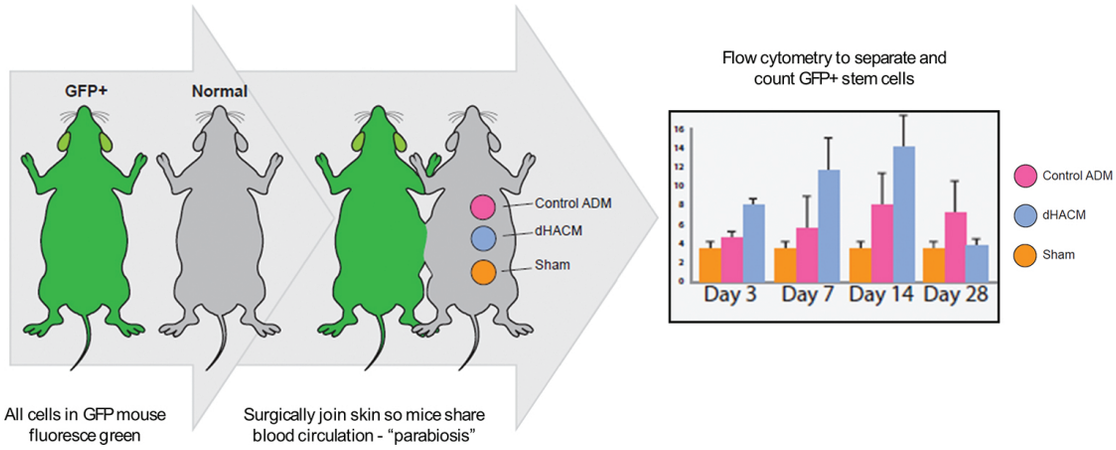

Figure 4. Parabiosis of a GFP⁺ mouse with a normal mouse demonstrated the ability of dehydrated human amnion/chorion membrane (dHACM) to recruit circulating stem cells from the bloodstream. Greater numbers of GFP⁺ stem cells were identified in sites of subcutaneous dHACM implantation (blue) by flow cytometry, compared to sham (orange) and acellular dermal matrix (ADM; pink) controls.

4.4. Secretion of immunomodulatory, angiogenic and tissue promoting cytokines

PURION® Processed dHACM has also been shown to modulate cellular activity by stimulating the secretion of cytokines by fibroblasts, vascular endothelial cells and adult stem cells, including secretion of immunomodulatory, angiogenic and tissue growth promoting cytokines [6, 7, 59]. In particular, the role of stem cells in healing has recently focused on the paracrine signaling properties of these cells, including their influence on the inflammatory status of injured tissues. ADSCs, BM-MSCs and HSCs were shown to modulate secretion of a number of cytokines involved in immunoregulation and mitogenesis in response to dHACM extracts, including chemokines and proteins related to leukocyte migration, immunomodulatory cytokines and mitogenic growth factors and proteins related to tissue growth [7]. These results indicate that in addition to the growth factors and cytokines released from dHACM tissue into the wound, dHACM continues to amplify these paracrine signals by inducing

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resident cells to produce additional regenerative growth factors and this balance of regulatory cues may modulate the wound environment to promote healing.

Although stem cells in diabetic patients are believed to be impaired and less responsive due to hyperglycemia and reduced cytokine bioavailability resulting from the disease state, PURION® Processed dHACM was capable of stimulating ADSCs from type I and type II diabetic donors to proliferate, migrate and modulate gene expression and secretion of immunomodulatory cytokines *in vitro*, similar to levels observed by ADSCs from a healthy donor [60]. These results demonstrate that while stem cells from diabetic donors may have decreased capacities for healing, contributing to the development of chronic wounds, stem cells derived from diabetic patients were capable of responding to treatment with dHACM, suggesting that dHACM treatment may stimulate stem cell activity to promote healing in diabetic ulcers.

4.5. Angiogenesis

An array of angiogenic cytokines have been identified in PURION® Processed dHACM and dHACM was shown to stimulate human dermal microvascular endothelial cells *in vitro*. dHACM caused endothelial cells to proliferate, migrate and induce production of over 30 angiogenic factors *in vitro* [6]. Additionally, following subcutaneous implantation in a murine ischemic wound model, a steady increase in microvessels in dHACM implants was observed *in vivo* over a 4-week period. These levels were equivalent to healthy and healed skin indicating a dynamic intra-dHACM implant neovascular process. Angiogenesis is paramount during the late inflammatory and proliferative phases of wound healing since chronic wounds are commonly associated with poor circulation and vascularization. Therefore, these results demonstrate that dHACM grafts: (1) contain angiogenic growth factors retaining biological activity; (2) promote amplification of angiogenic cues by inducing endothelial cell proliferation and migration and by upregulating production of endogenous angiogenic growth factors by endothelial cells; and (3) support the formation of blood vessels *in vivo*.

Together, these *in vitro* and *in vivo* scientific results strongly suggest that PURION® Processed dHACM is intimately involved with modulation of the cellular environments in wounds to elicit an improved healing response and the reparative attributes of dHACM allografts are further supported by the numerous published clinical trials on their use to promote healing of diabetic foot ulcers and venous leg ulcers. Through the delivery of a diverse cocktail of biologically active signals into the wound environment, PURION® Processed dHACM tissues directly promote cell proliferation and migration to amplify cell populations in the wound and stimulate cytokine secretion of important growth factors and immunomodulatory regulators by these cells. Collectively these cellular cues work together to stimulate stem cell activity, angiogenesis and modulation of inflammation and may reset the wound environment from one of a stalled, chronic state to an acute wound that can progress through the normal healing stages of inflammation, proliferation and remodeling. Though these mechanisms may be characteristic of other placental tissues and amniotic membrane allografts, it should be noted that these cellular responses were demonstrated using PURION® Processed dehydrate human amnion/chorion membrane (dHACM) allografts (EpiFix®, MiMedx Group, Inc.).

4.6. Scientific data from single-layer amnion allografts

Though limited in number, to date, two single-layer amniotic membrane products have been examined for their ability to modulate cellular activity during wound healing and the results have been published in peer-reviewed scientific journals. A study using devitalized, cryopreserved amnion (TissueTech, Inc., Miami, FL) demonstrated the ability of amniotic membrane allografts to suppress macrophage viability and proliferation and to inhibit TGF-β1 signaling, supporting the immunomodulatory properties of amniotic membrane tissue [61]. Additionally, a series of studies on viable, cryopreserved amnion (Grafix®, Osiris Therapeutics, Inc.) demonstrated that amniotic membrane allografts possess angiogenic, antiinflammatory and antioxidant capacity, as indicated by *in vitro* experiments of endothelial cell migration and tube formation [62]; peripheral blood mononuclear cell (PBMC) secretion of tumor necrosis factor α (TNF-α), IL-1α and IL-10 and inhibition collagenase [63]; and reduced oxidant-induced damage in dermal fibroblasts and migration of fibroblasts and keratinocytes [64].

5. Promise of placental tissues for wound healing

Though scientific and clinical data have focused largely on amniotic membrane tissues thus far, other placental tissues are generating significant interest as tissue allografts to support healing of wounds. Due to the structural and functional differences of the placental tissues, these tissues may provide alternative treatment regimens. For example, the structural and biological composition of umbilical cord and amniotic fluid suggests that they may facilitate additional applications beyond amniotic membrane grafts including thicker grafts to better facilitate suturing or liquid grafts for delivery through injection.

Umbilical cord allografts have begun to increase usage as wound therapies (e.g., EpiCord™, MiMedx Group, Inc.). Umbilical cord is composed of Wharton's jelly with a high content of hyaluronic acid, as well as a number of regulatory growth factors and cytokines. Due to the increased thickness of the umbilical cord matrix relative to amniotic membrane, umbilical cord may be easier to handle and suture into place when a thicker graft is desired for deeper wounds, while retaining a similar array of bioactive proteins to promote healing.

As a liquid allograft, amniotic fluid can be delivered in a unique form including injection into wounds or joint spaces (e.g., OrthoFlo, MiMedx Group, Inc.). Amniotic fluid is composed of a complex solution of growth factors, cytokines, proteins, carbohydrates, lipids, hormones, electrolytes, hyaluronic acid, as well as other nutrients, which function to protect and cushion, modulate inflammation and enhance mobility *in utero* [22, 24, 65]. Though clinical data on amniotic fluid are limited, a number of cases have demonstrated that injection of amniotic fluid is safe and anecdotal results suggest that amniotic fluid reduces pain and promotes healing [66, 67]. Additional *in vivo* preclinical models have demonstrated that amniotic fluid promotes healing in a variety of applications including healing of wounds, burns, bone, cartilage, tendon and nerves [68–71].

The placental disc is a rich source of fetal nutrients; however, because it contains both maternal and fetal components, the placental disc tissue requires decellularization to prevent immuno-

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logical rejection following implantation and wound healing applications are generally limited to the use of the decellularized placental disc matrix. Decellularization removes biologically active components; however, with appropriate processing methods, the extracellular matrix can be preserved. The placental disc is a rich source of collagen, particularly collagen type I; therefore, placental collagen is currently under investigation to develop collagen-based scaffolds in the form of sponges or void fillers (e.g., AmnioFill™, MiMedx Group, Inc.). Additionally in an unrelated application, purified placental collagen has also been used to manufacture cross-linked collagen fibers for use as sutures and tendon repair devices (e.g., CollaFix™, MiMedx Group, Inc.).

Overall, the fetal environment has tremendous capacity to support and guide tissue development and enable scarless healing and placental tissue including the umbilical cord, amniotic fluid and amniotic membrane actively regulate and maintain the composition and structure of the fetal environment. These tissues are also immunologically privileged as barrier tissues that separate the mother from the fetus and support immunological tolerance. These complex, nutrient-rich tissues are created biologically to support growth during pregnancy and clinical results indicate that they possess significant promise to enhance wound healing by delivering cytokines, which alter the wound environment and stimulate endogenous cells to reset the natural wound healing process.

The unique characteristics of placental tissue allografts make them promising therapies for wound care and soft tissue healing, including for a variety of chronic and acute wounds, burns, plastic and reconstructive surgery, as well as various surgical and sports medicine applications. Placental tissue allografts provide a bioactive therapy for treatment of complex wounds where standard of care treatment is not sufficient, with the ability to modulate inflammation and reduce scar tissue formation. Placental tissue also has specific advantages over many other available bioactive therapies, including reduced cost, an abundance of donor tissue, ease of handling and being immunologically privileged. While the exact mechanisms by which placental tissue allografts promote healing remain under investigation, *in vitro* and *in vivo* research suggests that they alter cellular activity within the wound environment by modulating inflammation, promoting cellular migration and proliferation and stimulating stem cell activity. These cellular responses may then reset the healing trajectory and encourage progression through the natural stages of inflammation, proliferation and remodeling to enhance wound healing.

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