RICHGEN: A safe and effective combination cryopreserved allograft enhances regeneration

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ABSTRACT

RICHGEN, a cryopreserved flowable allograft product (For Homologous Use Only), is prepared using a proprietary patented process from a healthy young woman's post-birth donated amniotic fluid, amnion membrane, placenta, and Wharton's jelly and extracellular matrix. It is minimally manipulated according to current FDA and Public Health Services Act regulations. The cutting edge manufacturing technique uses only single donor tissues and maintains the functions of the combined human tissue including immunomodulatory, anti-inflammatory, and tissue regenerative properties that are highly effective and safe, providing significant value in pain control, orthopedic pathologies, and organ system pathologies. The bioassay results presented in this paper demonstrate that the RICHGEN combination allograft presents a significant advancement in off the shelf, ready to use, injectable providing high levels of cytokines, growth factors, cellular components and extracellular matrix for tissue regeneration in patients.

1. Introduction

This white paper describes the scientific background and significant advancement in the therapeutic uses of the cryopreserved allograft composition RICHGEN (For Homologous Use Only) that is prepared from four materials: amniotic fluid, amnion membrane, placenta, and Wharton's jelly, and amniotic extracellular matrix (ECM). Advanced biologics technology is used to process RICHGEN that are well within minimal manipulation guidelines set forth by the FDA in November 2017, such that the final product has high concentrations of cellular components, full spectrum of growth factor proteins, chemokines, cytokines, associated exosomes, collagen substrates (I, II, IV, V and VI), hyaluronic acid, alpha-2 macroglobulin, secretomes, and miRNA that are highly useful in regenerative therapeutics.

The purpose of this white paper is to describe the properties of RICHGEN (For Homologous Use Only), the power of the combined component derived allograft for off the shelf use by physicians and aestheticians. A detailed description of the therapeutic applications of RICHGEN in regenerative processes such as wound repair (skin, eyes, deep tissues, and bone and cartilage), pain, anti-inflammatory, and immunomodulatory processes is given. In addition, the results of a bioassay analysis of RICHGEN components are presented.

2. RICHGEN Composition

RICHGEN (For Homologous Use Only) is a cryopreserved flowable allograft injectable product that is prepared using a proprietary patented processing of a healthy young woman's post birth amniotic fluid, amnion membrane, placenta, and Wharton's jelly and ECM. The combination of tissue is processed appropriately and safely from one single donor each time the product is made. It is available as multiple size cryopreserved aliquots called RICHGEN (0.5 ml), RICHGEN I (1 ml), and RICHGEN II (2 ml). RICHGEN is also available as amnio membrane in sizes 1 X 1 cm to 8 X 12 cm sizes; and amnio plus chorion membrane with Wharton's jelly sandwiched in sizes: 1 X 1 cm to 4 X 8 cm; and an optic disc membrane available in 8 mm to 12 mm in diameter size.

The components of RICHGEN are as follows: there are 1500 signaling proteins in the full spectrum of growth factors, cytokines, chemokines, and associated exosomes; extracellular matrix proteins such as collagen (collagen substrates I, II, IV, V and VII), fibronectin, laminin and hyaluronic acid; mesenchymal stromal cells (MSCs; expressing markers for CD73, CD95, CD105 and CD45), pluripotent MSCs, epithelial cells and endothelial cells; fibroblasts and keratinocytes, and subcellular components such as secretomes, and ribonuclear complex proteins including miRNA (listed in **Table 2**).

3. Background: Applications of combination allograft

The structure of the placenta along with a historical overview of its clinical applications was recently reviewed by Pogozhykh et al (1). The main function of the placenta is to support fetal growth and remove metabolic waste with low immunogenic profile and this combined with an abundant availability has led to clinicians using the

placental tissue for several therapeutic applications. The mature placenta consists of chorionic plate, amnion and umbilical cord. The human amnion is a single layer of epithelial cells separating the amniotic cavity from the vascularized chorion. The Amniotic Membrane (AM) is the innermost layer of the placenta and consists of a thin epithelial layer, a thick basement membrane, and an avascular stroma. It contains collagen (IV, and VII), fibronectin, and laminin. It also contains fibroblasts and growth factors, cytokines, exosomes, alpha-2-macroglobulin, miRNA and a wide spectrum of growth factors at high concentrations. Chorionic membrane consists of fibroblasts and trophoblasts. Postpartum placental cells are composed mainly of fetal tissue and some maternal cells that include natural killer (NK) cells, macrophages, and other immune cells (1, 2).

Cells derived from human placenta have been used in surgical applications, spinal injuries, lung diseases, ischemic diseases, autoimmune diseases, hematologic malignancies, Peyronie's disease and erectile dysfunction (2). Placental tissue has been applied to a wide variety of pathologies due to its wound-healing, antimicrobial, anti-inflammatory, and low immunogenic properties, and each of these properties will be discussed in detail in the below subsections (2).

3.1 Tissue regenerative properties: The placenta has demonstrated versatility as a biological dressing in hundreds of cases over the past hundred years. The earliest record of placental tissue to enhance wound healing was in the early 1900s by Davies J in burns and ocular wounds (3). In 1980, the amnion membrane (AM) was used to successfully treat chronic leg ulceration (4). The major types of placental tissue used as biological dressing and tissue replacement therapy include amnion and chorion membranes, amniotic fluids, umbilical cord, cord blood, total placental extracts, and stem cells (5-9). Placental tissue replacement therapies have been used in subcutaneous, intramuscular, intravenous, intraoperative, orthopedic pain, skin substitution, and ophthalmic use (10-13). In one study 79 patients undergoing orthopedic surgery (open fracture reduction, spine surgery and wound debridement) had a topical application of placental extract and showed normal wound healing with minimal side effects (10). In another study 27 patients treated with placental extract to cover non-healing wound showed >50% epithelialization compared to only 7 patients in the control group (12). Another study showed that subcutaneous injection of placental extract was effective in alleviating the symptoms of chronic fatigue syndrome (11). A review on applications of AM in orthopedics in humans describes the beneficial effects of amniotic injections in alleviating pain in symptomatic knee osteoarthritis and in refractory plantar fasciitis (13). Two additional reviews describe the role of MSCs derived from multiple sources (one being umbilical cord) in the repair of fractures (14,15). At present the use of MSCs in bone tissue engineering is in preclinical stages in humans with MSCs being used as seed cells to build tissue engineered bone grafts (14, 15).

Wounds unresponsive to standard therapeutic measures such as ulcers, orthopedic and ophthalmologic pathologies have responded to AM treatment (16-19). In a study of patients with chronic venous leg ulcers using AM, 100% adherence was seen within 7 days for all patients, 80% showed significant response and 20% had complete healing within 3 months (16). All patients showed significant decrease in wound area and pain (16). In an experimental rabbit model with severely damaged cornea, transplantation with AM showed improved ocular surface reconstruction (17).

Wharton's jelly is rich in mesenchymal stromal cells and has been used for transplantation of hematopoietic stem cells in a variety of hematologic and non-hematologic disorders (20). Wharton's jelly stem cells have also been studied as transplantable cells in various conditions including cancer, chronic liver disease, cardiovascular diseases, nerve, cartilage, and tendon injury (reviewed in 21). Stem cells derived from Wharton's jelly can differentiate into hepatocyte-like cells, and express markers similar to liver cells than can be used in liver disease (22). In a large animal model (sheep), stem cells from Wharton's jelly engineered to form cardiovascular tissue were successfully implanted for 20 weeks (23). In addition, the use of Wharton's jelly stem cells differentiated into chondrocytes was proposed for cartilage regeneration in the repair of high impact injury (during sports and exercise), osteoarthritis, and rheumatoid arthritis (24).

3.2 Antimicrobial properties: The placenta tissue serves as a barrier against microorganisms and plays a role in protecting the damaged tissue from infections during the wound-healing process. The placenta expresses several proteins that have bactericidal, antiviral and antifungal properties. These include the defensins (human neutrophil defensin, lactoferrin, lysozyme, bactericidal /permeability-increasing protein) calprotectin, secretory leukocyte protease inhibitor, RIS-1/psoriasin, and a cathelicidin (25, 26). The defensins are found in the AM and are active against both gram-positive and gram-negative bacteria, enveloped viruses and fungi (27). Lactoferrin shows its bactericidal action by chelating iron, rendering it inaccessible to microbes, and by binding the outer membranes of

bacteria to release the lipopolysaccharide lactoferricin. In addition, lactoferrin has antimicrobial activity against viruses, protozoa, and fungi (28). Thus, antimicrobial properties of placenta play a key role in tissue regenerative therapies.

3.3 Anti-inflammatory properties: Trelford et al in 1979 (5) found that AM transplantation promoted epithelial healing, reduced inflammation, increased comfort, and decreased the severity of insufficient vascularization. The epithelial cells of AM express several anti-inflammatory proteins such as interleukin-1 receptor antagonist, tissue inhibitors of metalloproteinase, collagen XVIII, and interleukin-10 (IL-10) that can inhibit the proinflammatory cytokine interleukin-6 (IL-6), that are responsible for its tissue protective properties (29-31). Several growth factors produced by AM are also involved in these processes, including transforming growth factor beta (TGF-β) and fibroblast growth factor (FGF) (32). In addition to AM epithelial cells, the mesenchymal stem cells (MSCs) play a role in inflammation through secretion of cytokines and growth factors with antiapoptotic, proangiogenic and immune-regulatory properties. Both the AM epithelial cells and the MSCs mediate their anti-inflammatory action by decreasing pro-inflammatory signals (cytokines), inhibiting T and B cell proliferation, suppressing inflammatory properties of monocytes, macrophages, dendritic cells, neutrophils, and natural killer cells (33). Both AM and MSCs increase anti-inflammatory immune components such as regulatory T cells (Tregs) and M2 macrophages (33). Studies have shown that transplantation with AM promotes re-epithelialization, decreases inflammation and fibrosis, and modulates angiogenesis (34).

3.4 Low immunogenicity: The structure of the placenta has several features to reduce activation of the maternal immune system, and therefore protect the fetus. The trophoblast cells in the placenta express very low amounts of major histocompatibility complex (MHC) proteins (human leukocyte antigen: HLA-A, HLA-B, and HLA-C) thereby making it difficult for the immune system to recognize these cells (35-37). Immune rejection does not occur after allotransplantation with human amniotic epithelial cells (since no antibodies to HLA are generated). Reports on the immunogenicity of human amniotic epithelial cells after transplantation into human volunteers have also been published (38, 39). None of the volunteers showed clinical signs of rejection (graft versus host reaction), and amniotic epithelial cells were demonstrated by biopsy up to 7 weeks after implantation. HLA antibodies were not detected in serum samples, and no in vitro lymphocyte reaction to the amniotic cells was found in 2 out of 7 volunteers (39). Thus, using AM cells for transplantation does not cause acute immune rejection.

3.5 Evidence in tissue regeneration: Human amniotic fluid stem cells (hAFS) have shown a distinct secretory profile and significant regenerative potential in several preclinical models of disease. hAFS actively release extracellular vesicles (EV) that have significant paracrine potential and regenerative effect (40-42). EV are membrane-bound cellular components enriched with soluble, bioactive factors (proteins, lipids, etc.) and RNA (mainly regulatory microRNA—miRNA). They elicit a wide range of effects while mediating intercellular transfer of information on the responder cell, consequently modulating its function. These include the activation of antiapoptotic and pro-survival pathways eliciting angiogenic, anti-inflammatory, and antifibriotic responses and the stimulation of resident endogenous progenitors, overall enhancing organ function (43). In particular, many studies have reported the potential efficacy of EV from adult mesenchymal stem cells (MSC) in providing cardioprotection against acute myocardial infarction (MI) (44, 45), in enhancing wound healing (46), counteracting graft-versus-host-disease (47), reducing renal injury (48), mediating liver regeneration (49), and stimulating neural plasticity following stroke (50). Thus, hAFS containing EV offer several benefits over bone marrow or conventional therapy in tissue regeneration.

3.6 MicroRNA profiling of hFAS-EV: Balbi et al provide an excellent review of the miRNA profile in amniotic fluid cellular components (51). hAFS-EV from normal and hypoxic cells contain small non-coding RNAs and miRNAs (20–40 nucleotides). The authors showed that hypoxic preconditioning lead to significant enrichment of a few miRNAs (miR-210, miR-199a-3p, miR-146b, miR-126, miR-21 and miR-let7c). These miRNA are implicated in key biological effects such as fibroblast proliferation, angiogenesis, inhibition of fibrosis, cardioprotection, prosurvival and reducing inflammatory protein levels (51).

4. Methods

4.1 Franciscan cell viability assay: RICHGEN samples were shipped in dry ice and maintained at -85 °C until ready for testing; two samples were thawed in a 37 °C bead bath. The samples were mixed by pipetting up and down, and a 0.1 ml aliquot was transferred to a 1.5 ml microcentrifuge tube, a timer was started, and the sample incubated at room temperature. A 0.02 ml aliquot of the sample was stained at time zero using acridine orange (AO) and propidium iodide (PI)

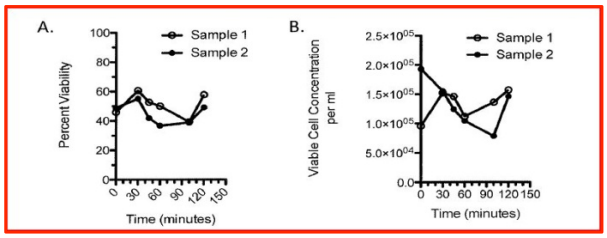
(Nexcellcom, Lawrence, MA) followed by quantification and viability determination using a Nexcellcom Cellometer. This analysis of cell viability was repeated at 30, 45, 60, 100, and 120 minutes after thawing from the same aliquot.

4.2 Franciscan antibody staining and flow cytometric analysis: About 1.9 ml of RICHGEN sample was washed with 5 ml of phosphate buffered saline (PBS, pH 7.4), concentrated by centrifugation and the cells were suspended in fluorescence activated cell-sorting (FACS) buffer (PBS, 1% FBS). Cells were stained with antibodies from BioLegend (San Diego, CA) against the human CD45 (FITC anti-human CD45). Cells were also stained with isotype control antibodies from BioLegend (FITC mouse IgG1 k). Following 20 minute incubation on ice in the dark, cells were washed in FACS buffer and suspended in FACS buffer supplemented with viability stain (7-AAD, BD Pharmingen, [San Jose, CA]). Flow cytometry was performed using an Acuri C6 flow cytometer (BD Pharmingen) followed by analysis with FlowJo (Ashland, OR).

4,3 Laboratory analysis of bioactive proteins: Samples of RICHGEN were analyzed using enzyme linked immunoabsorbent assay (ELISA) for bioactive proteins and known cell surface markers including vascular endothelial growth factor (VEGF) that is involved in angiogenesis, interleukin-1 receptor antagonist (IL-1ra) that regulates pro-inflammatory response, beta fibroblast growth factor that is a strong mitogen and plays a role in cartilage repair, and platelet derived growth factor beta (PDGF-b) that plays a role in cell growth and division and angiogenesis. Quantification analysis was done on 41 proteins, and cellular components of RICHGEN.

5. Results

5. 1 Cell viability: As shown in **Figure 1A, B, and C,** immediately after thawing the viability of both samples were between 48 to 60.6% with a cell concentration of ~1.0 X 10^5 cells/ml resulting in a cell yield of ~2.0 X 10^5 cells per vial. Although the viability and cell concentration fluctuated slightly at subsequent time points, the average cell concentration and average viability remained stable or increased over the entire two-hour incubation period (1.33 X 10^5 cells/ml) for both samples (**Table 1**).



C.

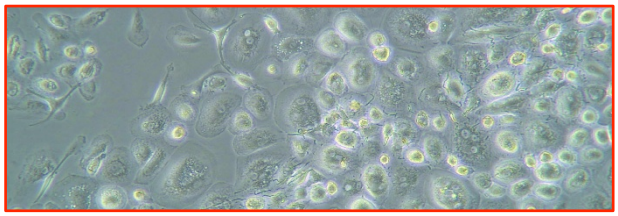


Figure 1: **(A)** Cell viability, **(B)** Cell concentrations for 2 hours after thawing **(C)** Viable cells picture

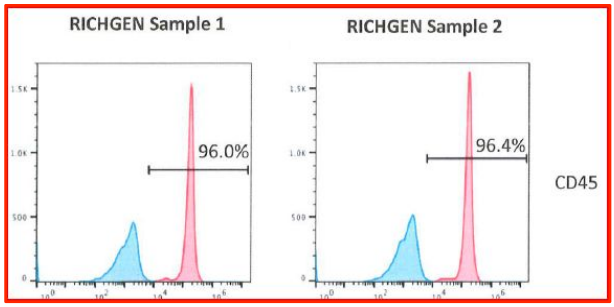
Table 1: Cell concentrations for 2 hours after thawing

Time (min)	Sample 1 Cell conc/ml	Sample 1 % live cells	Sample 2 Cell conc/ml	Sample 2 % live cells
0	9.58 X 10^5	45.9	1.93 X 10^5	48.8
30	1.52 X 10^5	60.6	1.55 X 10^5	55.1
45	1.46 X 10^5	52.6	1.24 X 10^5	41.9
60	1.12 X 10^5	50.0	1.04 X 10^5	36.6
100	1.36 X 10^5	39.3	7.85 X 10^5	38.8
120	1.57 X 10^5	57.7	1.46 X 10^5	49.0

Conc = concentration; min = minutes

5. 2 Antibody staining: For the two RICHGEN samples tested, a majority of cells were positive for CD45 (**Figure 2**). Sample 1 had 96.03% (96.9% - 0.87%) positivity and sample 2 had 96.4% (97.4% - 0.99%) positivity for CD45 antibody. Additional markers for CD73, CD90 and CD105 were present.

Figure 2: Flow cytometry analysis for CD45



Note: Percentages are normalized by subtracting the percentage of positive cells stained with matched isotope control antibodies.

5.3 Analysis of bioactive proteins: We assessed bioactive proteins and cell markers indicative of fibroblast, MSCs and keratinocytes, S100-A4, CD90 and Cytokeratin 7 respectively (**Table 2**). There were 82% of the cells positive for fibroblast cell marker S100-A4, 23.9% positive for MSCs marker CD90 and 0.3% of

the cells positive for keratinocyte marker cytokeratin 7. In many instances there could be overlap of cell markers (i.e. cells positive for both S100-A4 and CD90).

Table 2: List of bioactive proteins in RICHGEN

Growth Factor Cytokines	Function
AR	Amphiregulin (AR) (Colorectum cell-derived growth factor) (CRDGF)
BDNF	Brain-derived neurotrophic factor (BDNF) (Abrineurin)
bFGF	Fibroblast growth factor 2 (FGF-2) (Basic fibroblast growth factor) (bFGF) (Heparin-binding growth factor 2) (HBGF-2)
BMP-4	Bone morphogenetic protein 4 (BMP-4) (Bone morphogenetic protein 2B) (BMP-2B)
BMP-5	Bone morphogenetic protein 5 (BMP-5)
BMP-7	Bone morphogenetic protein 7 (BMP-7)
OP-1	(Osteogenic protein 1) (OP-1) (Eptotermin alfa)
b-NGF	Beta-nerve growth factor (Beta-NGF)
EGF	Pro-epidermal growth factor (EGF) [Cleaved into: Epidermal growth factor (Urogastrone)]
EGF R	Epidermal growth factor receptor (EC 2.7.10.1) (Proto-oncogene c-ErbB-1) (Receptor tyrosine-protein kinase erbB-1)
EG-VEGF	Prokineticin-1 (Endocrine-gland-derived vascular endothelial growth factor) (EG-VEGF) (Mambakine)
FGF-4	Fibroblast growth factor 4 (FGF-4) (Heparin secretory-transforming protein 1) (HST) (HST-1) (HSTF-1) (Heparin-binding growth factor 4) (HBGF-4) (Transforming protein KS3)
FGF-7	Fibroblast growth factor 7 (FGF-7) (Heparin-binding growth factor 7) (HBGF-7) (Keratinocyte growth factor)
GDF-15	Growth/differentiation factor 15 (GDF-15) (Macrophage inhibitory cytokine 1) (MIC-1) (NSAID-activated gene 1 protein) (NAG-1) (NSAID-regulated gene 1 protein) (NRG-1) (Placental TGF-beta) (Placental bone morphogenetic protein) (Prostate differentiation factor)
GDNF	Glial cell line-derived neurotrophic factor (hGDNF) (Astrocyte-derived trophic factor) (ATF)
GH	Somatotropin (Growth hormone) (GH) (GH-N) (Growth hormone 1) (Pituitary growth hormone)
HB-EGF	Proheparin-binding EGF-like growth factor [Cleaved into: Heparin-binding EGF-like growth factor (HB-EGF) (HBEGF) (Diphtheria toxin receptor) (DT-R)]
HGF	Hepatocyte growth factor (Hepatopoietin-A) (Scatter factor) (SF) [Cleaved into: Hepatocyte growth factor alpha chain; Hepatocyte growth factor beta chain]
IGFBP-1	Insulin-like growth factor-binding protein 1 (IBP-1) (IGF-binding protein 1) (IGFBP-1) (Placental protein 12) (PP12)
IGFBP-2	Insulin-like growth factor-binding protein 2 (IBP-2) (IGF-binding protein 2) (IGFBP-2)
IGFBP-3	Insulin-like growth factor-binding protein 3 (IBP-3) (IGF-binding protein 3) (IGFBP-3)
IGFBP-4	Insulin-like growth factor-binding protein 4 (IBP-4) (IGF-binding protein 4) (IGFBP-4)
IGFBP-6	Insulin-like growth factor-binding protein 6 (IBP-6) (IGF-binding protein 6) (IGFBP-6)
IGF-1	Insulin-like growth factor I (IGF-I) (Mechano growth factor) (MGF) (Somatomedin-C)
IL-1ra	Interleukin 1 receptor antagonist
Insulin	Insulin [Cleaved into: Insulin B chain; Insulin A chain]
MCSF R	Macrophage colony-stimulating factor 1 receptor (CSF-1 receptor) (CSF-1-R) (CSF-1R) (M-CSF-R) (EC 2.7.10.1) (Proto-oncogene c-Fms) (CD antigen CD115)
NGF R	Tumor necrosis factor receptor superfamily member 16 (Gp80-LNGFR) (Low-affinity nerve growth factor receptor) (NGF receptor) (p75 ICD) (CD antigen CD271)
NT-3	Neurotrophin-3 (NT-3) (HDNF) (Nerve growth factor 2) (NGF-2) (Neurotrophic factor)
NT-4	Neurotrophin-4 (NT-4) (Neurotrophin-5) (NT-5) (Neutrophic factor 4)
OPG	Tumor necrosis factor receptor superfamily member 11B (Osteoclastogenesis inhibitory factor) (Osteoprotegerin)
PDGF-AA	Platelet-derived growth factor subunit A (PDGF subunit A) (PDGF-1) (Platelet-derived growth factor A chain) (Platelet-derived growth factor alpha polypeptide)
PDGF-BB	Platelet-derived growth factor subunit B homodimer
PIGF	Placenta growth factor (PIGF)
SCF	Kit ligand (Mast cell growth factor) (MGF) (Stem cell factor) (SCF) (c-Kit ligand) [Cleaved into: Soluble KIT ligand (sKITLG)]
SCF R	Mast/stem cell growth factor receptor Kit (SCFR)

Growth Factor Cytokines	Function
	(EC 2.7.10.1) (Piebald trait protein) (PBT) (CD antigen CD117)
TGFa	Protransforming growth factor alpha [Cleaved into: Transforming growth factor alpha (TGF-alpha) (EGF-like TGF) (ETGF) (TGF type 1)]
VEGF	Vascular endothelial growth factor

6. Discussion

This review indicates that the RICHGEN cryopreserved amniotic fluid, amnion membrane, placenta and Wharton's jelly, ECM combination allograft process is successful. With minimal manipulation of the allograft components, RICHGEN retains functional stem cells, bioactive proteins (cytokines, chemokines, growth factors, and robust cellular components). The RICHGEN allograft combined with the presence of an extracellular matrix, lamin, and fibronectin, present a functional allograft that is effective in a wide array of applications. The applications range from topical wound healing to rapid response for use in orthopedic pathologies, organ system pathologies, pain/anti-inflammatory control, and scar management. This is achieved by regulating inflammatory and chemotactic pathways and providing scaffolding for healthy tissue generation and repair of injured tissue.

The anti-inflammatory action appears to require contact with the various growth factors/ cytokines in suspension. The mechanism by which RICHGEN exerts its anti-inflammatory action is by decreasing pro-inflammatory signals (cytokines), inhibiting T and B cell proliferation, and increasing anti-inflammatory immune components such as Tregs and M2 macrophages (33). RICHGEN contains stem cells and many growth factor proteins such as VEGF, B-FGF, IL-1ra and PDGF-BB. These growth factors play a role in growth, cartilage repair, reducing the inflammatory response and creating new vasculature which increases blood flow.

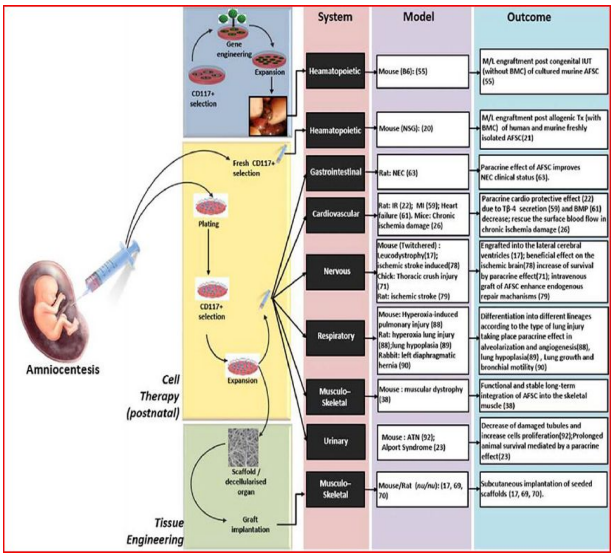
RICHGEN has cell surface markers of MSCs (CD73, CD90 and CD105) and Figure 2 above shows the presence of the ubiquitous stem cell marker CD45 in flow cytometric assays. CD45 plays an important role in signal transduction and lymphocyte development. Several different isoforms of CD45 exist and the expression of a specific isoforms depends on cell type, developmental state, and activation state of the cell, for example CD45 on T cells play an important role in T-cell activation and immune response (52, 53).

The high efficiency of Wharton's jelly and MSC material recovery, the ethical procurement of material, the low immunogenicity, and the fact that they are from healthy, young donors (only single donor processing for each batch) makes RICHGEN an ideal product for transplantation use in regenerative medicine. The amniotic fluid has been identified as an untapped source of embryonic stem cells which possess immunomodulatory properties. CD117(c-Kit) cells selected from amniotic fluid have been shown to differentiate into cell lineages representing all three embryonic germ layers without generating tumors, making them ideal candidates for regenerative medicine applications (51). Moreover, their ability to engraft in injured organs and modulate immune and repair responses of host tissues suggest that transplantation of such cells may be useful for the treatment of various degenerative and inflammatory disease (51). The amniotic fluid provides the signaling required for these cells to maintain their undifferentiated status, which would be consistent with a stem cell niche role for the amniotic fluid.

Additional components of the RICHGEN such as the ECM are also useful in tissue regeneration and cartilage repair. A study in rats show that an amnion derived ECM scaffolding was effective in repairing osteochondral defect in rat knees due to the presence of collagen, fibronectin, hyaluronic acid, and chondroitin sulfates (54).

In **Figure 3** below based on work in animal models, multiple centers have demonstrated successful use of amniotic fluid stem cells. The therapeutic effect appears to be related to the paracrine and immunomodulating effects (55).

Figure.3: Summary of key supporting evidence from animal models for potential clinical applications of freshly isolated AFSC figure from Loukogeorgakis et al (55)



The anti-fibrosis anti-scarring capacity of the ECM present in RICHGEN is due to the presence of the anti-inflammatory cytokine, IL-10, which can inhibit the production of IL-6 (30). Diminished IL-6 production contributes to fetal wound repair without scarring (56). The phenomenon of fetal wounds healing without scar was confirmed in a study by Liechty et al (57). The three different TGF-beta (types 1, 2, 3) are the most potent cytokines, promoting myofibroblast differentiation by upregulating expression of alpha- smooth muscle actin, integrin alpha5, and EDA containing fibronectin Fn- 46 in a number of cell types, including fibroblasts (58). These factors contribute to healthy tissue healing to form fully functional, flexible tissue, with strong anatomic layering. TGF-beta also upregulates the expression of matrix components such as, collagens and proteoglycans, and downregulates proteinase and matrix metalloproteinase, while upregulating their inhibitors (58).

It is the combination of each of these powerful cytokines, chemokines and ECM present in RICHGEN amniotic fluid, amnion membrane, placenta, and Wharton's jelly flowable allografts that amplify the healing potential of the recipients.

7. Conclusions

In this review we have demonstrated that regenerative therapeutics requires a combination of components to drive the healing regenerative process using healthy processed multiple tissue materials. The proprietary processing techniques provide enhanced levels of the MSCs, full spectrum of growth factors, miRNA, exosomes, and cellular components in the allografts. The strong viability demonstrated in Figure 1 and Table 1 show an increase in cell division and proliferation after thawing. The MSCs, growth factors, cytokines, and ECM combine together to provide a safe and efficacious pathway to healing injured tissue, bone and cartilage.

The data above represents the characterization of RICHGEN allograft product done by an independent lab. It characterizes the product by assessing what the end user will get in terms of cells (viability, quantity and cell type) and proteins/growth factors. The product does contain high quality signaling stem cells and the full spectrum of 1500 growth factors of which many have been assayed. The full spectrum of growth factors play a role in growth, cartilage repair, reducing the inflammatory response and creating new vasculature.

RichSource Stem Cells Inc. is educating practitioners of the understanding and awareness of the plethora of proteins and extra-cellular matrix signaling interactions in the RICHGEN combination tissue allografts to optimize regeneration.

RICHGEN brings the entire symphony orchestra of proteins to maximize signaling for cutting edge regenerative healing of injured, traumatized and/ or diseased tissue, or deteriorated bone and cartilage.

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Figure 3 Expanded from Loukogeorgakis et al (53)