The Effect of Acellular Dermal Matrix and PRGF on Gene Expression in Human Gingival Fibroblasts In Vitro.

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The effect of two preparations of acellular dermal matrix and PRGF on gene expression in human gingival fibroblasts *in vitro*.

When used with freeze-dried acellular dermal matrix (FD-ADM), PRGF led to a reversal in gene expression reduction compared to FD-ADM alone.

Keywords: Acellular dermal matrix, plasma rich in growth factors, gingival recession, periodontal plastic surgery.

Abstract:

Background: Gingival recession is a condition affecting the periodontium that is characterized by the displacement of the gingival margin apically from the cementoenamel junction (CEJ) and leads to compromised support of the tooth. While the gold standard of treatment often consists of harvesting autogenous tissue from a separate surgical site, other options involving cadaver tissue have been utilized. Acellular dermal matrices (ADMs) have been investigated in many studies. The purpose of this study was to investigate the effect of adding additional growth factors during the rehydration of two preparations of ADM on gene expression in gingival fibroblasts. Methods: Gingival fibroblasts were grown on ADMs with and without plasma rich in growth factors (PRGF). PCR analysis was used to analyze the effect on gene expression. Results: The presence of ADMs led to a reduction in gene expression compared to control groups. The reduction in gene expression was reversed in the freeze-dried preparation of ADM when combined with PRGF, though this effect was not seen in the solvent-dehydrated preparation. PRGF alone did not show any significant changes in gene expression. Conclusion: When combined with PRGF, reductions in gene expression seen with ADMs alone were reversed, though this reversal was only seen in the freeze-dried preparation of the ADMs.

1 | INTRODUCTION

Periodontal tissues (gingiva, bone, cementum and periodontal ligament) are supporting structures surrounding teeth. Gingival recession is a condition affecting the periodontium that is characterized by the displacement of the gingival margin apically from the cementoenamel junction (CEJ) and leads to compromised support of the tooth. Recession can have various etiologies, such as variations in normal anatomy of the teeth or gums[1], aggressive tooth brushing[2], parafunctional habits[3], orthodontics[4], or periodontal disease[5]. Gingival recession in the adult population over age 29 has been estimated to be as high as 22.5% and has also been shown to increase with age [6]. Gingival recession is frequently associated with problems such as compromised esthetics, pain due to root sensitivity or a lack of an attached tissue seal at the gingival margin. Patients with advanced recession are also at a higher risk of developing root caries.

Various treatment modalities have been utilized for the treatment of gingival recession, in order to augment the lost soft tissue. Commonly, a coronally positioned gingival flap with the addition of an autogenous connective tissue graft harvested from the patient's palate has been used for root coverage. The outcomes of this procedure have been proven to be predictable and stable over time [7]. However, the addition of a second surgical site for graft harvesting has many drawbacks, such as significant patient discomfort and complications at the harvest site[8]. Anatomical limitations may also limit the extent of harvesting autogenous connective tissue[9]. Allogenic graft materials such as acellular dermal matrices (ADM) have been developed, eliminating the need for a second surgical site. Processing these materials includes removal of the epidermis and the cellular elements from the donor tissue, leaving behind a matrix of mostly elastin and a fibrillar collagen mesh [10]. Two of the most common commercially available ADMs differ greatly in their preparation:

1. Freeze-dried ADM (FD-ADM)* is prepared beginning with removal of the epidermis using a buffered salt solution. A non-denaturing detergent is then used to dissolve and wash away additional cells. Cryopreservant is then added and the material is freeze-dried.
2. Solvent-dehydrated ADM (SD-ADM)† undergoes a 5-stage *Tutoplast*® sterilization process used to remove unwanted materials (cells, antigens, viruses).

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Clinical human studies comparing these two types of allogenic graft materials with a coronally positioned flap found no significant difference in the amount of root coverage achieved with up to a year post-operative observation[10],[11]. Plasma rich in growth factors (PRGF) is an autologous product that can potentially be used to accelerate soft tissue healing and stimulate regeneration [12]. Numerous studies have demonstrated that PRGF stimulates increased cellular proliferation, migration, and chemotaxis of cell types including fibroblasts [13] and osteoblasts [14]. Additionally, studies have also demonstrated an antibacterial effect of PRGF on some *Staphylococcus* species[15], and due to the absence of leukocytes in PRGF, the release of pro-inflammatory cytokines is not seen with its use[16]. PRGF contains many growth factors found in would healing, notably transforming growth factor β1 (TGF-β1), insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF)[17].

When used for root coverage with a coronally positioned flap and connective tissue graft, less recession is noted upon healing when PRGF was used [18]. PRGF has also been shown to produce favorable changes in gingival fibroblast viability and proliferation [19]. Currently, to our knowledge, no studies have investigated if the addition of PRGF when using ADM would be beneficial. While clinical studies have been done to determine the efficacy of using freeze-dried versus solvent-dried ADM, no studies have been carried out to determine if the two different materials would produce any notable changes on gene expression in gingival fibroblasts. The addition of growth factors has been shown to increase mRNA expression of pro-wound healing molecules in gingival fibroblasts [20]; however, this effect has not been investigated in the presence of ADM.

The objective of the current study was to determine if freeze-dried and solvent-dried ADM preparations would produce any changes on gingival fibroblasts with regard to gene expression. We also aimed to determine if the rehydration of the two types of ADM in PRGF alters gene expression relevant to periodontal tissues, particularly in regard to wound healing. Favorable changes in gene expression patterns would suggest that the addition of PRGF during procedures using ADM could potentially result in improved healing and more favorable clinical outcomes.

2 | MATERIALS AND METHODS

2.1 Cells:

Gingival fibroblasts (GF) were established from explants of healthy gingiva obtained from a 13-year-old boy free of systemic diseases undergoing periodontal surgery for hereditary gingival hyperplasia at the Louisiana State University School of Dentistry[21]. While these cells display reduced regulation in proliferation, they exhibit similar gene expression profiles to other fibroblasts. The tissues were washed several times with medium containing 10% penicillin-streptomycin to reduce subsequent bacterial contamination. They were then transferred to 60 mm Petri dishes containing 2 ml of McCoy's 5A medium supplemented with 20% fetal calf serum (FCS), 200 units/ml of penicillin, and 200 µg/ml streptomycin (Gibco®). The tissue was minced into 1 mm pieces with a sterile scalpel and then pipetted into 25 cm^3 tissue culture flasks. To enhance cell outgrowth, the pieces of tissue were made to adhere to the flask surface by carefully draining the medium and incubating the flasks on end for 20 minutes at 37°C. The cells were maintained in a humidified incubator with 5% CO_2 and 95% air and growth medium was changed twice weekly. After 2 to 3 weeks, the fibroblasts were dissociated with 0.05% trypsin–EDTA for 5 minutes, collected with centrifugation at 300× g and resuspended in culture medium. The resuspended fibroblasts were pipetted into 25 cm^3 tissue culture flasks [21].

2.2 *ADMs*

One x 2 cm ADMs ranging in thickness of 0.8 mm-1.8mm were trimmed using a sterile blade to 1x1 cm. ADMs were rehydrated according to the manufacturer's protocol with sterile saline prior to adding gingival fibroblasts.

2.3 *PRGF*

Four 9 mL vials of venous blood were drawn from a healthy individual and prepared according to the manufacturer's protocol [17]. After collection, blood was centrifuged for 8 minutes, separating the cellular components of blood (red blood cells, etc.) from the overlying plasma. The fraction of the plasma rich in growth factors was then isolated (8 mL).

2.5 Addition of PRGF:

PRGF was also examined as the rehydration liquid for the ADMs. Membranes were initially hydrated with saline for 5 minutes to allow removal of the paper backing from the FD-ADM. Both membranes were then hydrated for 20 minutes with PRGF. The growth factor activator, calcium chloride, was already present in the MEMα (Gibco®) media.

2.4 Addition of cells to ADMs:

FD-ADM and SD- ADM were rehydrated with MEMα medium according to their manufacturers' instructions. Cells in the 11th-16th passage were used in this study. After GF reached confluence, they were removed from tissue culture bottles via trypsinization. Prepared GF were then placed on membranes in agarose-coated wells, in order to prevent sticking of cells to culture well bottoms. GF attached to ADMs were allowed to incubate for 5 days. Membranes were then washed with MEMα and frozen at -80 C to facilitate cell lysis.

2.5 Transcript Analysis

In order to isolate mRNA for transcript analysis, membranes were then vortexed vigorously for 15 minutes in an RNA extraction buffer. RT-PCR will be used to assess transcript expression. cDNAs were made using MMLV-RT and the Retroscript Kit (Invitrogen®) as per the manufacturer's instructions. Briefly, 1 g of total RNA will be reverse-transcribed using 100 units of MMLV-RT in 20 μl reactions. 2 μl of each reaction was used as template in 25 μl polymerase chain reactions (PCR), using 50 pmoles of primers, 250 μM of dNTPs, and 1 mM Mg^{2+}. PCR reactions were performed using the following protocol: 93°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 25 cycles. The number of cycles was chosen by creating a standard curve using serial dilutions of DNA template known to include the genes in question and determining the PCR conditions that revealed 2-fold differences in gene expression over three (3) orders of magnitude. Twenty-five (25) cycles of PCR were chosen to be within the linear range of detection for all the genes examined. Genes examined included S15, BMP2, BMP4, TGFβ1, GDF5

FGF2, FGF4, CTGF, and Collagens 1-14. Fibroblasts grown on plastic well surface were used as controls. These particular genes were selected due to their key roles in the regulation of periodontal tissue healing[22]. Quantification of transcript expression was carried out using ImageJ. Statistical analysis included ANOVA and multiple paired t-tests.

3 | RESULTS

3.1 Effects of PRGF on GFs

When compared to controls, PRGF showed increased transcript expression for TGFβ1 and collagen-10 (Figure 1). This was demonstrated to be significant after quantification analysis *(*Figure 2).

Compared to control groups, the addition of PRGF alone altered transcript expression in the growth factor TGFβ1 as well as collagen-10 (Figure).

Exposure to 10% PRGF significantly (* = P < 0.05) increased expression of TGFβ1 (Figure 2A) and collagen – 10 (Figure 2B).

3.2 Effects of ADMs on GFs

A general reduction in transcription expression was seen when fibroblasts were added with ADMs, specifically in BMP4, GDF5, FGF2, CTGF, collagen-5 and collagen-8 (Figure 3). For CTGF, collagen-5 and collagen-8. This effect was more pronounced in the FD-ADMs compared to the SD-ADMs. Significance was also demonstrated after quantification analysis (Figure 4).

Compared to control groups, reduced expression of BMP4, GDF5, FGF2, and CTGF were seen, along with collagen-5 and collagen-8 in gingival fibroblasts grown on either ADM (Figure 3). Note the differences in regard to FD-ADM and SD-ADM in the CTGF (Figure 4A), collagen-5 and collagen-8 groups (Figure 4B). Cells grown on ADMS tended to display significant (* = P < 0.05) reduction in the expression of many transcripts compared to controls. Additionally, there was a significant († = P < 0.05) difference in transcript expression between the two ADMs TGFβ1, FGF2, collagen 6 and collagen 8.

3.3 Effects of PRGF and ADMs on GFs

With the addition of PRGF to ADMs, previously noted decreases in transcript expression seen with FD-ADM alone were reversed, particularly in TGF-β1, FGF2, FGF5, CTGF (Figure 5). This effect was not observed with the SD-ADM. Quantification results reflected this observation as well (Figure 6).

TGF-β1, FGF5, and CTGF saw increased transcript expression with the addition of PRGF to FD-ADM (Figure 5). Additionally, this effect was also seen in collagen-3, collagen-5, collagen-6, collagen-8, and collagen-10. This effect was not observed in the SD-ADMs.

Cells grown on ADMS tended to display significant (* = $P < 0.05$) reduction in the expression of many transcripts compared to controls (Figure 6). There was a significant († = $P < 0.05$) difference in transcript expression between the two ADMs for TGFβ1, FGF2 (Figure 6A), collagen 6 and collagen 8 (Figure 6B). Additionally, exposure to to 10% PRGF and grown on FD-ADM displayed a significant (‡ = $P < 0.05$) increase in the expression of GDF5, CTGF, and collagne-3, collagen-5, collagen-6, and collagne-8 and collagen-10. In contrast, cells grown on SD-ADM only displayed an significant (‡ = $P < 0.05$) increase in the expression of GDF5, CTGF, and collagne-3, collagen-5, collagen-6, and collagne-8 and collagen-10.

4 | DISCUSSION

Wound healing is a complex process that is not fully understood. Though their exact role is unknown, the growth factors investigated in this study are known to play a significant role[24]. TGFβ1 is known to play a role in fibroblast and macrophage chemotaxis, extracellular matrix synthesis, and secretion of protease inhibitors[23]. FGF2 and FGF4 also play a role in fibroblast proliferation and angiogenesis[23]. TGFβ1 has been shown to produce an increase in proliferation of both gingival and PDL fibroblasts[25]. In animal studies, BMPs have also been shown to enhance regeneration in periodontal defects[26]. CTGF has demonstrated a pro-reparative role in early wound healing in mice studies[27]. These genes, along with others and collagens thought to contribute to wound healing and periodontal support, were selected for examination.

Results from this *in vitro* study showed that PRGF alone had little effect on gene expression. This study demonstrated slight differences between FD-ADM and SD-ADM in regard to gene expression, which was observed in CTGF, collagen-5, and collagen-8. This could be due to the different preparations of the materials, with the SD-ADM process removing more cellular elements and thus leading to less reduction in transcription expression. The addition of PRGF reversed this effect in the FD-ADM group, though no effect was seen in the SD-ADM.

PRGF has been shown to significantly increase proliferation of GF[19]. Platelet preparations have also been shown to favorably affect GF in adhesion and migration in previous studies[28].
ADMs have been shown to effectively treat gingival recession[29]. It has also been demonstrated previously that gingival fibroblasts remain viable on ADMs at 14 and 21 days[30]. The reduction in gene expression seen in this study could be due to a difference in the environment the incubated cells were grown in; cells grown on the tissue culture plastic are flattened and have one side exposed, placing them in a constant semi-wounded state. In this state, increased expression of growth factors would be expected. In the ADM groups, cells are able to exist within the matrix in a rounded, “healthier” state, which would not elicit the additional expression of growth factors required during wound healing. Reduction in collagen transcript expression is thought to be due to the presence of collagen in ADMs; cells would not be required to produce additional collagen if it is already present in the matrices. The slight differences in between the

ADMs could possibly be explained by their different processing methods. Solvent-dehydration of the ADMs may lead to a more complete removal of agents that would hinder wound healing.

Growth factors in combination with ADMs have been shown to accelerate healing when used for surgery in diabetics[31]. Animal studies have also shown improved healing results when growth factors are used in surgery[32]. When ADMs, particularly FD-ADM, were combined with PRGF, a reversal in transcription expression reduction was seen. Different preparations of ADMs could lead to different interactions with PRGF. More studies are needed to determine if differences in gene expression would be encountered. Clinical studies would also be needed to determine if any differences may occur in treatment outcomes, such as recession reduction and post-operative discomfort, when ADMs are combined with PRGF.

The combination of PRGF and ADMs may lead to increased gene expression with FD-ADM, wich could produce more favorable clinical results. PRGF has been shown to accelerate wound healing and may be beneficial in early wound healing when used with PRGF. Reintroducing growth factors into PRGF may lead to more favorable wound healing and less patient post-operative pain.

5 | CONCLUSION

Based on the results of this study, while there are some small differences in gene expression when ADMs are introduced to gingival fibroblasts, the clinical implications of these findings likely have little significance. The small differences noted between the two different types of ADMs also did not demonstrate a clearly more advantageous product. As seen in the past, the addition of growth factors, in this case PRGF, showed increases in growth factor expression that may be beneficial in early healing, particularly in regard to the FD-ADM.

The use of PRGF in procedures using ADMs is not contraindicated, as clinical results have shown that the addition of PRGF in surgical procedures can be beneficial to patient healing outcomes. Findings of this study do suggest that if FD-ADM is going to be used, the addition of PFGF may lead to increases in transcript production not seen with FD-ADM alone.

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Table 1. Functions of Genes Examined

Gene group:	Functions:
S15	Ribosomal gene, responsible for protein production (control)
BMP2/BMP4	Bone morphogenic protein; induce formation of bone and cartilage
TGFβ1	Transforming growth factor; controls proliferation, differentiation and other functions in many cell types
GDF5	Growth differentiation factor; regulators of cell growth and differentiation
FGF2/FGF4	Fibroblast growth factor; have regulatory, morphological, and endocrine effects
CTGF	Connective tissue growth factor; plays a role in cell adhesion, migration, proliferation, angiogenesis, skeletal development, and tissue wound repair
Collagen-1-14	Variants for producing different types of collagen, with the most important being types 1 and 3

Figure 1: Significant differences were detected in transcript expression in the presence of PFGF alone for TGFβ1 and collagen-10

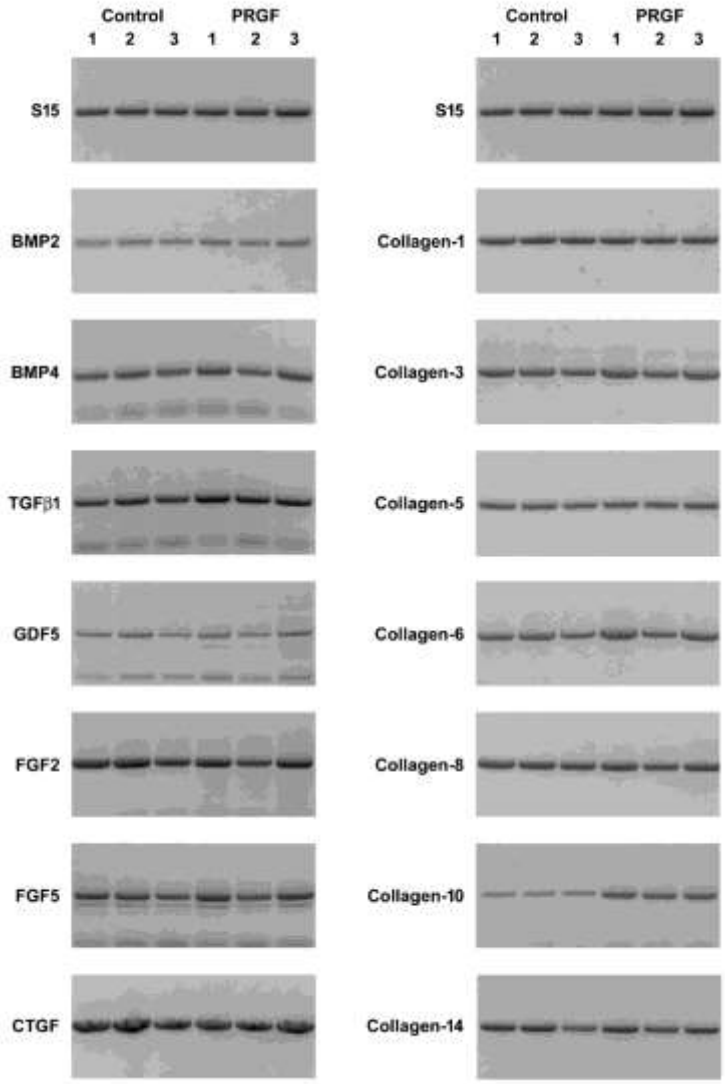

Figure 1: RT-PCR Transcript analysis of gingival fibroblasts treated with 10% PRGF for 5 days, compared to untreated sibling cells grown in MEMα containing 10% FBS. Three samples of each condition are presented. Compared to control groups, the addition of PRGF alone altered transcript expression in the growth factor TGFβ1 as well as collagen-10.

Figure 2: Quantification of Transcription Expression following PRGF Exposure

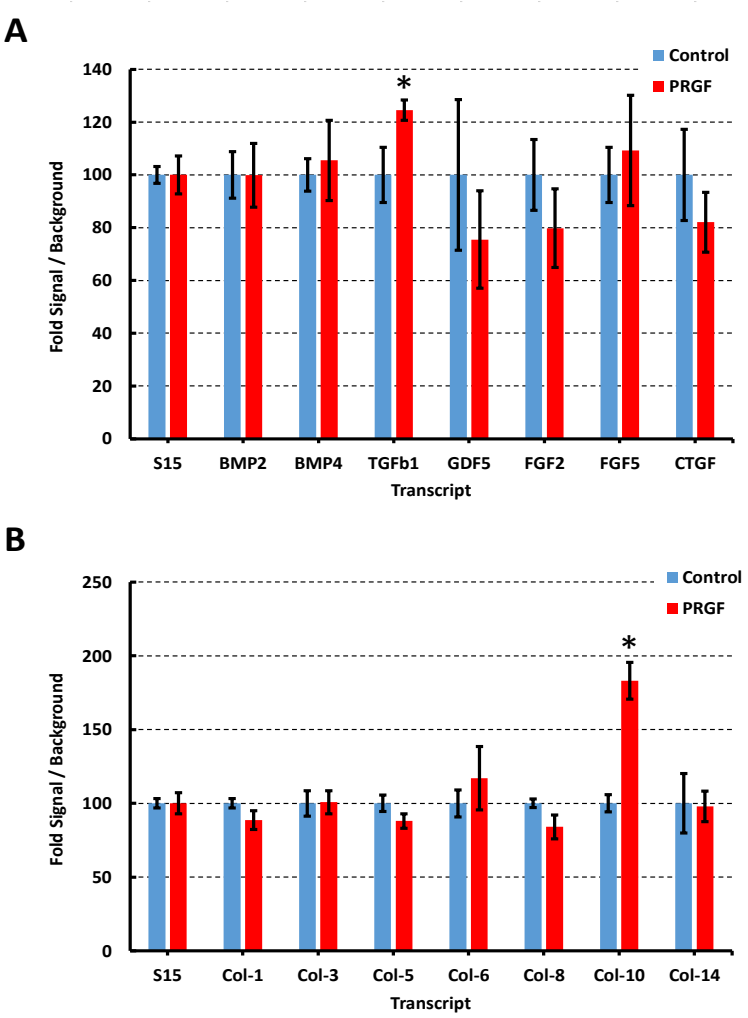

Figure 2: Bars represent the means and standard deviation of RT-PCR transcript three gingival fibroblast samples exposed to 10% PRGF compared to sibling cells exposed to normal growth media containing MEMα and 10% FBS (control). Transcript expression of select growth factors (A) and collagens (B) were compared. Exposure to 10% PRGF significantly (* = $P < 0.05$) increased expression of TGFβ1 (A) and collagen – 10 (B)..

Figure 3: Reduced gene expression for growth factors and collagens in cells associated with ADMs without PRGF.

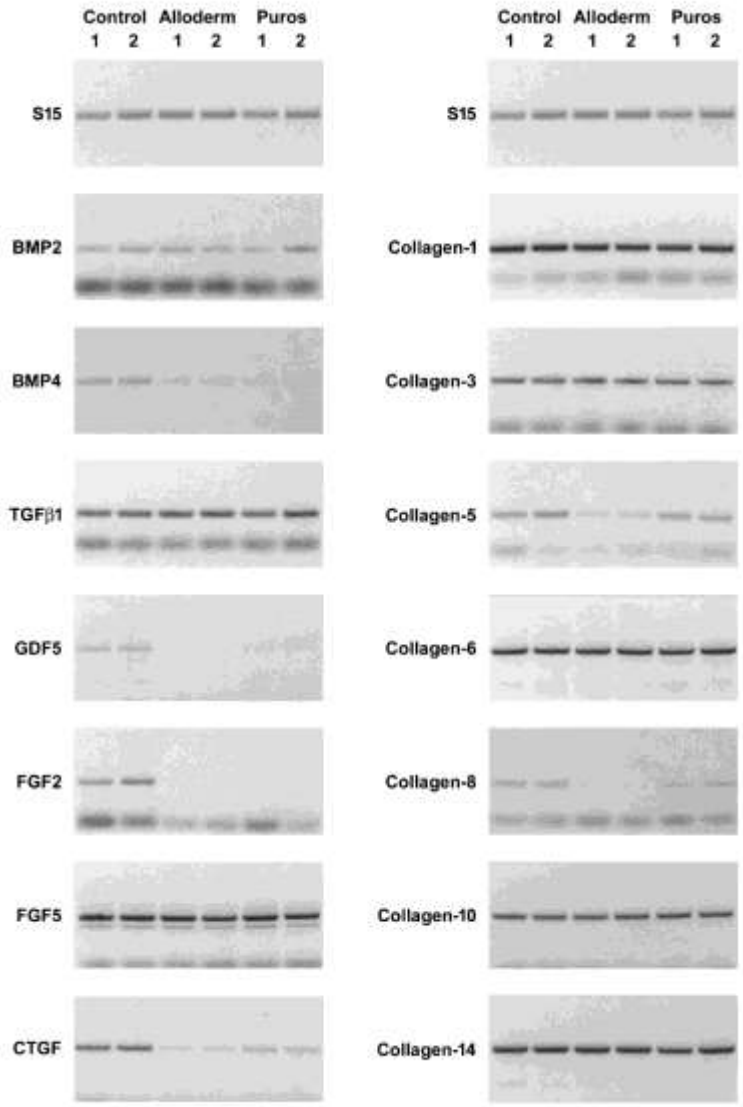

Figure 3: RT-PCR Transcript analysis of gingival fibroblasts exposed to 2 ADMs for 5 days, compared to untreated sibling cells grown in MEMα containing 10% FBS on tissue culture treated polystyrene. Two samples of each condition are presented. Compared to control groups, reduced expression of BMP4, GDF5, FGF2, and CTGF were seen, along with collagen-5 and collagen-8 in gingival fibroblasts grown on either ADM. Note the differences in regard to FD-ADM and SD-ADM in the CTGF, collagen-5 and collagen-8 groups.

Figure 4: Quantitative analysis comparing ADMs

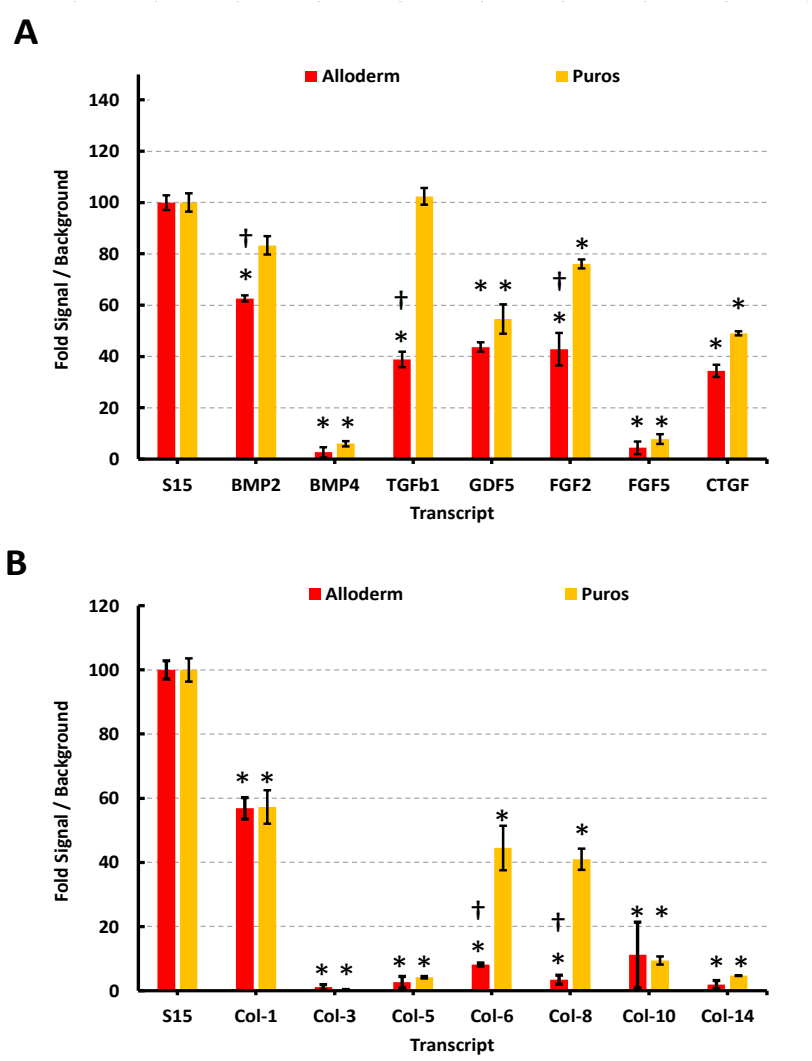

Figure 4A:

Bars represent the means and standard deviation of RT-PCR transcript two gingival fibroblast samples grown on ADMs compared to sibling cells exposed to normal growth media containing MEMα and 10% FBS grown on tissue culture treated polystyrene (control). Transcript expression of select growth factors (A) and collagens (B) were compared. Cells grown on ADMS tended to display significant (* = $P < 0.05$) reduction in the expression of many transcripts compared to controls. Additionally, there was a significant († = $P < 0.05$) difereence in transcript expression between the two ADMs TGFβ1, FGF2, collagen 6 and collagen 8.

Figure 4B: Significant differences were seen in collagen transcript expression compared to controls (*). Differences were also noted between the two AMDs (†).

Figure 5: Increased gene expression in growth factors in cells when ADMs exposed to PRGF

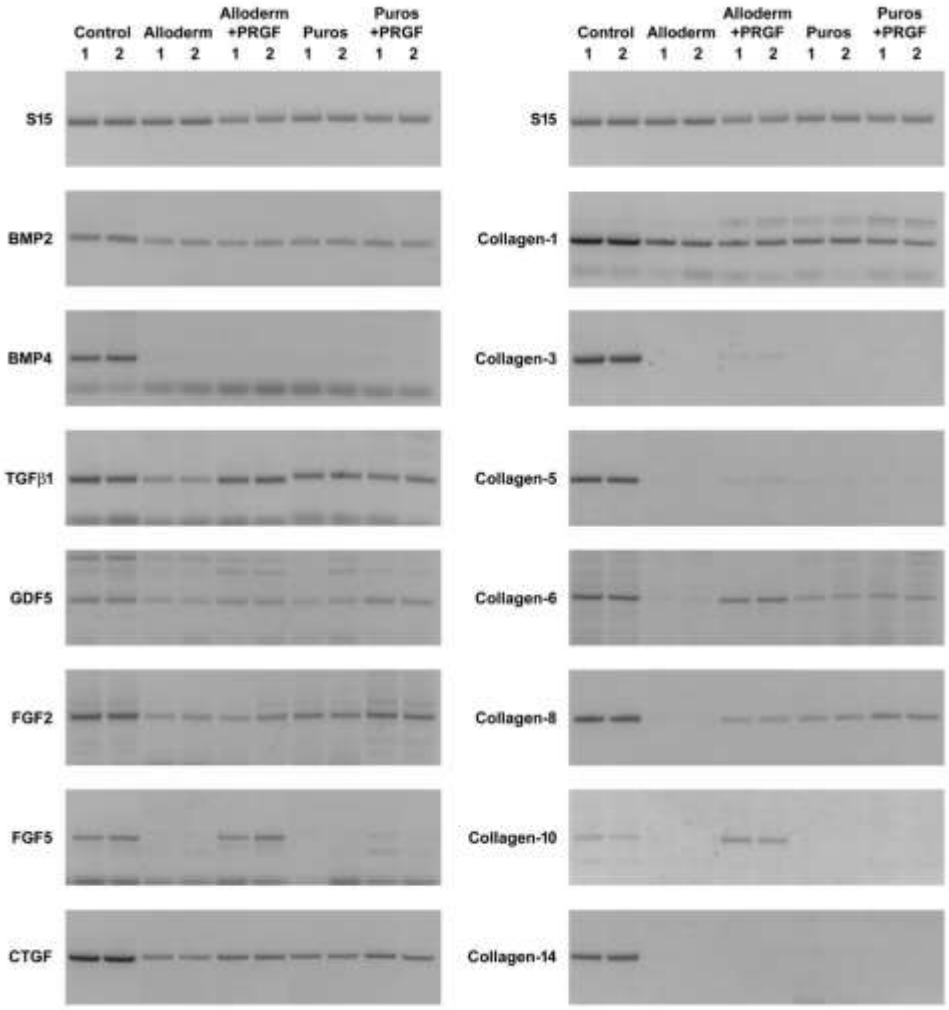

Figure 5: RT-PCR Transcript analysis of gingival fibroblasts exposed to 2 ADMs for 5 days (with and without 10% PRGF), compared to untreated sibling cells grown in MEMα containing 10% FBS on tissue culture treated polystyrene. Two samples of each condition are presented. TGF-β1, FGF5, and CTGF saw increased transcript expression with the addition of PRGF to FD-ADM. Additionally, this effect was also seen in collagen-3, collagen-5, collagen-6, collagen-8, and collagen-10. This effect was not observed in the SD-ADMs.

Figure 6: Quantification analysis of data combining ADMs with PRGF

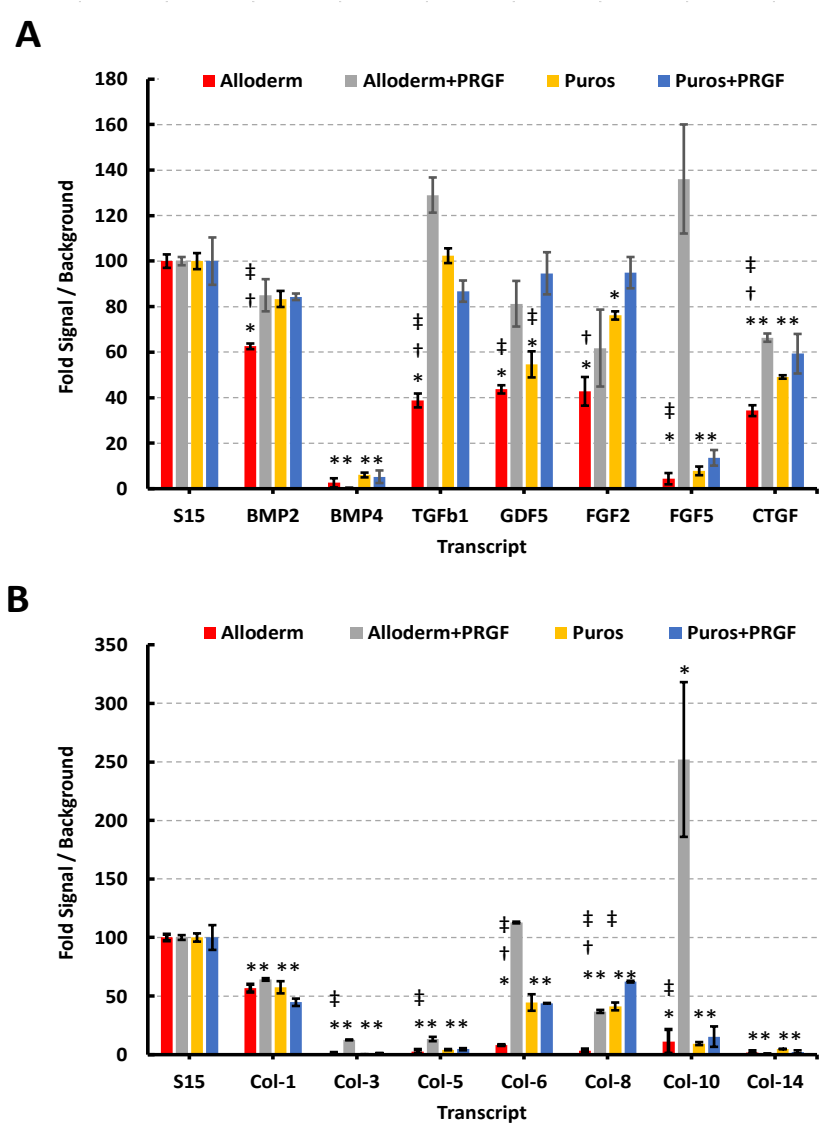

Figure 6A: Bars represent the means and standard deviation of RT-PCR transcript two gingival fibroblast samples grown on ADMs compared to sibling cells exposed to normal growth media containing MEMα and 10% FBS grown on tissue culture treated polystyrene (control). Cells were grown in the presence or absence of 10% PRGF for 5 days. Transcript expression of select growth factors (A) and collagens (B) were compared. Cells grown on ADMS tended to display significant (* = $P < 0.05$) reduction in the expression of many transcripts compared to controls. There was a significant († = $P < 0.05$) difference in transcript expression between the two ADMs for TGFβ1, FGF2, collagen 6 and collagen 8. Additionally, exposure to to 10% PRGF and grown on FD-ADM displayed a significant (‡ = $P < 0.05$) increase in the

expression of GDF5, CTGF, and collagne-3, collagen-5, collagen-6, and collagne-8 and collagen-10. In contrast, cells grown on SD-ADM only displayed an significant (‡ = $P < 0.05$) increase in the expression of GDF5, CTGF, and collagne-3, collagen-5, collagen-6, and collagne-8 and collagen-10