

**The Effect of White Mineral Trioxide Aggregate on
Migration, Proliferation, and Odontoblastic
Differentiation of Stem Cells from the Apical Papilla.**

By

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**Dissertation submitted in partial fulfillment
Of the requirements for the degree of
Masters of Science in Endodontics
The University of Michigan
2013**

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Special Thanks

Songtao Shi

Funding

American Association of Endodontics Foundation Grant

CRSE Graduate Master's Funding

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Abstract

Introduction: White Mineral Trioxide Aggregate (WMTA) is a biocompatible dental material with osteoinductive and osteogenic properties recommended for use in regenerative endodontic protocols. There is no evidence on the potential effect of WMTA on the migration, proliferation, and odontoblastic differentiation of Stem Cells from Apical Papilla (SCAP). Our hypothesis is that WMTA will induce migration, proliferation, and odontoblastic differentiation in SCAP cells. **Methods:** SCAP cell migration was assessed by transwell-migration assay at 30 minutes to 72 hours, proliferation by WST-1 assay at 1 to 14 days and odontoblastic differentiation by RNA expression of odontoblastic markers at 7, 14 and 21 days. Calcium enriched media was used to determine the role of calcium during this cellular activation. The cell population was characterized by flow cytometry. **Results:** WMTA significantly increased the migration of SCAP cells at 6 hours and media containing 0.3 and 0.03 mmol calcium ions significantly increased SCAP cell migration after 24 hours. WMTA significantly increased proliferation on days 1 and 5, while calcium enriched media showed a significant increase on day 7. SCAP cell proliferation was influenced strongly by the presence of both 2% and 10% FBS in the media. Odontoblastic markers were seen consistently in all samples, indicating a mixed cell population, which was confirmed by flow cytometry. **Conclusions:** WMTA showed a significant effect on the migration and proliferation of SCAP cells. Calcium enriched media significantly increased SCAP cell migration and proliferation. Media containing FBS significantly increased SCAP cell proliferation, even at the lower 2% concentration.

Chapter 1 –INTRODUCTION

An immature tooth with an open apex and a necrotic pulp poses a challenging clinical problem (1, 2). Dental caries, dens evaginatus or trauma can result in bacterial invasion of the pulp space, leading to necrosis (3). The bacterial infection leads to pulpal necrosis, apical periodontitis and the cessation of root development, which in turn results in fragile and thin root dentin walls (1, 2). This weakened root structure is highly susceptible to fracture (2). Traditional methods to aid in apexification using calcium hydroxide have been shown to weaken the radicular dentin even further, complicating the restorative treatment to a greater degree (4). Similar consequences are found after the traumatic avulsion of immature permanent incisors, most often seen in children between 8 to 11 years of age (5). Avulsion results in severe damage to the tissues involved in the developing root and pulpal structures (6, 7). This extensive damage halts root development and offers a relatively poor prognosis that often leads to the loss of the tooth, with a survival rate of only 57.3 months as reported by Pohl et al (8). In light of the complications seen with traditional apexification methods, several recent studies have begun to look at the possibility of using stem cells to regenerate the pulpo-dentin complex and induce continued root development (1-3, 9, 10).

Adult stem cells are multipotential progenitor cells that have the ability to differentiate into many cell types. They are found throughout the body, including the odontogenic tissues (11). There is tremendous potential for dental stem cells to induce dentinogenesis, apexogenesis, and the regeneration of the dental pulp (9, 12-14). Stem Cells from the Apical Papilla (SCAP) are found in the apical papilla of immature

permanent human teeth during root formation (14, 15). The apical papilla is induced by the Primary Enamel Knot during the early stages of tooth development and plays an important role in the development of the tooth by moving apically to continue forming the radicular dentin as well as forming the dental pulp (13). Huang and collaborators, have speculated that as a precursor to the radicular pulp, SCAP cells may give rise to odontoblasts and Dental Pulp Stem Cells (DPSCs) (16). As a progenitor cell line and the source of primary odontoblasts that are involved in root dentin formation, SCAP may prove to be an excellent source of cells for regeneration of the pulpo-dentin complex (16). Studies by Sonoyama have shown that SCAP cells proliferated 2-3 times faster than other pulp organ cells in culture and that they were as potent in both osteogenic and odontogenic differentiation as Mesenchymal Stem Cells (MSCs) (14, 15). Recent studies have shown that MSCs and human dental pulp cells react favorably to the presence of dental materials such as Mineral Trioxide Aggregate (MTA, Dentsply Endodontics, Tulsa, OK), which has been shown to be biocompatible and to promote cell growth (12, 17-21).

MTA has been used since the early 1990's (22). The main components of unset white MTA (WMTA) are tricalcium silicate, dicalcium silicate, tricalcium aluminate, bismuth oxide, and calcium sulphate dehydrate (22, 23). The composition of gray MTA is similar, except that it contains tetracalcium aluminoferrite (22, 23). The composition of set MTA is mostly calcium silicate hydrate (C-S-H) and calcium hydroxide ($\text{Ca}(\text{OH})_2$). A large release of calcium ions results from the dissociation of $\text{Ca}(\text{OH})_2$ and the decomposition of the C-S-H (22, 24). $\text{Ca}(\text{OH})_2$ formation is greatest at 4 to 24 hours and present in the maximum amount at 7 days, but decreases by day 30, yielding a high calcium ion release that declines over 5 weeks (22, 24, 25). It has excellent

biocompatibility and has been used in root end fillings, perforation repair, pulp capping, and apexification procedures (12, 22, 26). MTA has been shown to stimulate hard tissue formation, pulp cell migration, proliferation, and differentiation into odontoblast-like cells (12, 17, 19). A recent study by Kim et al., investigated the gene expression in human pulp cells exposed to MTA for 72 hours, in which 109 genes were up-regulated more than 2 fold and 69 genes down regulated by more than half, indicating that MTA's effects on cells can be detected within the first hours of contact with the material (18). The high calcium ion release of MTA leads to the hypothesis that it is responsible for the biological response (27).

Calcium ions have been shown to be a potent signaling molecule in many cell types and to play a role in the regulation of most cellular physiologic processes (27-33). Migration of mesenchymal stem cells, bone marrow-derived progenitor cells, and tumor cells are affected by calcium ion concentration (28-30). Human dental pulp cells were shown to have a similar increase in proliferation rates when exposed to media containing MTA and media containing exogenous calcium ions at the same concentration (27). In addition, the mineralization potential of human periodontal ligament cells and adipose-derived stem cells is promoted with increased calcium ion concentrations (31, 32). The differentiation of pulpal cells into odontoblasts may also be influenced by the levels of calcium ions present in the surrounding environment (33). The ability of calcium ions to influence cell migration, proliferation, and differentiation makes it a sensible choice to explore in regenerative endodontic procedures.

Regenerative endodontics has received growing interest in recent years (1, 3, 34). Numerous case studies have shown that following disinfection with triple antibiotic paste,

MTA used in conjunction with a stimulated cell source from induced bleeding at the open apex can result in continued root development in immature teeth that present with open apices and necrotic pulps (1, 3, 34, 35). Lovelace et al., have shown that the induction of bleeding at the apex releases a higher concentration of MSCs as compared to the concentration found in the systemic circulation (34). The authors note that while the exact source of these stem cells is, as yet, unknown, it is suspected to be the apical papilla (34).

The rationale for this present study emerged after a review of the literature reporting the effects of MTA on odontogenic stem cell populations. The literature has shown that MTA promotes healing and induces hard tissue formation *in vivo* when used in clinical situations such as pulp capping, perforation repair, root end filling, and apexification procedures (12, 22, 26). The review has also shown that SCAP cells are present in the area of the developing root and are capable of high proliferation rates as well as odontoblastic differentiation (14-16). There is a limited number of studies evaluating the mechanism of action of White MTA as well as its effect on the migration, proliferation, and odontoblastic differentiation of SCAP cells.

MTA promotes a favorable wound healing environment and increases cell survival (12). Mesenchymal Stem Cells (MSCs) have shown a significant increase in proliferation rates after 7 days as compared to controls. Results of a transwell migration study also showed a significant increase in the migration of MSCs in the presence of MTA. The ability of MTA to promote migration, adhesion, and proliferation in these cells mimics key wound healing phases (17). MTA has been shown to affect pulp cells, but not SCAP cells, within 72 hours and to initiate expression of the putative

odontoblastic markers DSPP, DMP-1, and MEPE in 7-14 days (12, 18, 36). The expression of these markers correlates to the $\text{Ca}(\text{OH})_2$ formation from MTA at 24 hours to 7 days and is concurrent with the large calcium ion release (22, 24, 25). The possible correlation between the expression of odontoblastic markers and MTA's calcium release has not been investigated.

SCAP cells have higher proliferation and doubling rates and increased migration when compared to DPSC (37). They were recently shown to produce odontoblast like cells and dentin like tissue onto existing dentinal walls and MTA plugs in an *in vivo* study by Huang et al (37). This was an observation by the authors, who did not investigate the effect of MTA on SCAP cells. The study was designed to investigate the effect of poly-D,L-lactide/glycolide scaffolds seeded with either SCAP cells or DPSC. The seeded scaffolds were placed in a 6-7mm x 1-2.5mm human root fragment with a MTA plug in one end and exposed to the growth factors sequestered in the dentin of the root fragment. More relevant to our topic, the authors note "*The most interesting and important finding of this study is the formation of a continuous layer of dentin-like tissue with uniform thickness on the existing canal dentin walls and the MTA cement surface, especially with SCAP-seeded samples*" (37). The incidental finding of the uniform dentinal layer on the MTA plug leads us to the speculation that the SCAP cells were recruited into the area by the MTA plug. This has yet to be investigated.

The understanding of the role of Stem Cells from Apical Papilla in the regeneration process of injured immature teeth could provide a valuable treatment option for the retention of compromised immature permanent teeth. This effort

could be enhanced by MTA's ability to promote migration, proliferation, and differentiation of stem cells.

Hypothesis: White mineral trioxide aggregate can induce migration, proliferation and differentiation in stem cells from the apical papilla.

Null Hypothesis: White mineral trioxide aggregate cannot induce migration, proliferation and differentiation in stem cells from the apical papilla.

Our goal is to identify if WMTA plays a role in the migration, proliferation, and differentiation of SCAP cells and to understand the role of the calcium ion release associated to this induction.

The following specific aims will address this hypothesis:

- 1) To assess whether WMTA induces migration of SCAP cells.
- 2) To assess whether WMTA induces proliferation of SCAP cells.
- 3) To assess whether WMTA induces differentiation of SCAP cells into odontoblast-like cells measured by the expression of putative odontoblastic markers: dentin sialophosphoprotein (DSPP), dentin matrix protein (DMP-1), and matrix extracellular phosphoglycoprotein (MEPE).
- 4) To determine if calcium ions released from WMTA are responsible for inducing the migration, proliferation, and odontoblastic differentiation induced in SCAP cells.

To facilitate the reading, the next chapters are organized according to aims. Each chapter contains a description of the methods used and results.

Chapter 2 – SCAP CELL MIGRATION

To determine whether WMTA induces migration in SCAP cells, a trans-well migration assay was used. The SCAP cells were challenged with 1 or 24 hour set WMTA and as control groups plain or 10% FBS medium were used starting from 1/2 hour up to 72 hours exposure. The first portion of this materials and methods section contains a description of the general materials and methods that were used with all the experiments. After this, each chapter will describe in detail the methods used for the experiment related to each aim.

Materials and Methods

Cell Culture

The SCAP cells used in all experiments were provided by Dr. Songtao Shi (University of Southern California, Los Angeles, USA). All cell culture tasks were carried out under a laminar flow hood. A passage Zero SCAP cell vial stored at -80°C was thawed in a 37°C warm water bath and the contents transferred to a T-75 flask (BD Falcon, San Jose, CA) with 10ml of minimal essential medium alpha Eagle (α -MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Hereinafter “10% FBS media”) and incubated at 37°C in 5% CO₂ and 100% humidity. After 24 hours, the media was removed by vacuum pipetting and 10ml of fresh media was added. Cells were allowed to grow until reaching 80% confluence. At that point the media was removed by vacuum pipetting and the cells were passaged by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to

inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 6ml of 10% FBS media. Three 2ml aliquots of re-suspended cells were then placed in 3 T-75 flasks each containing 10ml of 10% FBS media and incubated at 37°C in 5% CO₂ and 100% humidity. Cells were used between passages 3 and 5.

MTA Preparation

White MTA ProRoot (Dentsply Endodontics, Tulsa, OK) was mixed per the manufacturer's recommendations. Specifically, 100mg of WMTA was mixed with 35µl of sterile water under a laminar flow hood in a sterile Dappen Dish (Keystone Industries, Cherry Hill, NJ) for approximately 1 minute. Ten milligram pellets were formed by placing the mixed WMTA into the large groove of a Lee MTA Pellet Forming Block (G. Hartzell & Son, Concord, CA) with the mixing spatula. The pellets were then placed in a 100 mm Cell Culture Dish (BD Falcon, San Jose, CA) and allowed to set for 1 hour or 24 hours at 37°C in 5% CO₂ and 100% humidity.

Transwell Migration Assay

SCAP cell migration was assessed by using a Transwell Migration Assay. SCAP cells were prestained with Cell Tracker Green (CTG) (Invitrogen Corp., Carlsbad, CA) as follows: the media from a T-75 flask containing passage 4 SCAP cells at 80% confluence was removed by vacuum pipetting and 3ml of plain α -MEM media (Hereinafter "plain media") plus 6µl of 10mM CTG stock solution were added to the flask (a 2µM final

concentration of CTG). The flask was incubated at 37°C in 5% CO₂ and 100% humidity for 30 minutes. The plain media with CTG was removed by vacuum pipetting and 10ml of 10% FBS media was added to the flask and incubated for 30 minutes at 37°C in 5% CO₂ and 100% humidity. The plain media was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100µl of re-suspended cells in 900µl of plain media into a 2ml Eppendorf tube. 1×10^5 cells in 250µl of plain media were seeded into the upper chambers of transwell 8-µm-size pore FluoroBlok 24 well multiwell system membrane filters (BD Falcon, San Jose, CA) (Hereinafter “FluoroBlok inserts”). The lower chambers of the 24 well multiwell system contained 500µl of plain media. The FluoroBlok inserts were placed into the lower chambers of the 24 well multiwell system and incubated for 4 hours at 37°C in 5% CO₂ and 100% humidity to allow for attachment of the cells to the membrane filter. After 4 hours, the FluoroBlok inserts were transferred to a new set of lower chambers of the 24 well multiwell system which contained the following test conditions: plain media with 1 hour set WMTA, plain media with 24 hour set WMTA, plain media, α -MEM supplemented with 2% FBS and 1% penicillin/streptomycin (Hereinafter “2% FBS media”) with 1 hour set WMTA, 2% FBS media with 24 hour set WMTA, 3.0mmol Ca

media, 0.3mmol Ca media, 0.03mmol Ca media, 2% FBS media, and 10% FBS media. One pellet of WMTA was used in each well that contained WMTA and 500µl of media was used in each well. The plate was incubated at 37°C in 5% CO₂ and 100% humidity. Cells that migrated to the bottom of the FluoroBlok inserts were read by fluorescence at 485/585 nm using a microplate reader (Genius; Tecan, Grödig, Austria) at 30 minutes, 60 minutes, 3, 6, 12, 24, 48, and 72 hours. This experiment was repeated 3 times in triplicate.

Statistical Analysis

The Statistical Analysis was completed with the support of the University of Michigan's Center for Statistical Consultation and Research (CSCAR). All data was analyzed using one-way ANOVA with a Bonferroni comparison and a *P* value less than or equal to 0.05. The analysis was completed using SigmaStat 2.0 Software (Systat Software, San Jose, California). This process was repeated for the remaining experiments.

Results

WMTA Significantly Increased SCAP Cell Migration at 6 Hours

The results for the transwell migration assay are described in relative fluorescence units (RFU) and are listed in Table 1. There were no significant differences between any groups at the 30 minute reading (Figure 1). During the 60 minute reading, there were no significant differences among the plain media groups; however there was a significant increase in migration for the 2% FBS media group as compared to all groups (*P* = <0.002). Both the 24 hour set WMTA with 2% FBS media and the 10% FBS media groups showed a significant increase in migration as compared to the 1 hour set WMTA

with 2% FBS media ($P = <0.001$, Figure 2). No significant differences were observed in the plain media groups at the 3 hour reading. The 2% FBS group did show a significant increase in migration as compared to the remaining FBS groups in the 3 hour reading ($P = <0.004$, Figure 3). The 6 hour reading showed that both 1 and 24 hour set WMTA in plain media had significantly increased migration as compared to the plain media control ($P = <0.038$). There was no difference observed in the FBS media groups (Figure 4). No significant differences were observed between any of the plain media groups or any of the FBS media groups in the 12 hour reading (Figure 5). The 24 hour reading showed no differences in the plain media groups. A significant difference was observed between the 2% and 10% FBS groups, with the 2% FBS group showing increased migration ($P = 0.008$, Figure 6). During the 48 hour reading, the only significant differences seen were in the FBS containing media groups where the 10% FBS group showed significantly less migration than both the 2% FBS media and 1 hour WMTA with 2% FBS media group ($P = <0.05$, Figure 7). There were no significant differences seen in the plain media groups during the 72 hour reading, however both the 10% FBS media group and the 1 hour WMTA with 2% FBS media group showed significantly increased migration when compared to the 2% FBS media group ($P = <0.047$, Figure 8). Media containing both 2% and 10% FBS induced a significant increase in migration as compared to the plain media groups after 12 hours ($P = <0.05$, Figures 5-8) and media with 2% FBS also showed a significant increase in migration at both 60 minutes and 3 hours ($P = <0.05$, Figures 2 & 3).

In summary, we found that migration of SCAP cells was induced after 6 hours of exposure to 1h or 24 h set WMTA in plain medium. Other times of exposure did not show any significant differences.

**Table 1: SCAP Cell Migration
(in Relative Fluorescence Units)**

		1hr MTA Plain	24hr MTA Plain	Plain	3.0mmol Ca	0.3mmol Ca	0.03 mmol Ca	1hr MTA 2% FBS	24hr MTA 2% FBS	2 % FBS	10% FBS
30 mins	Average	13803	15056	13032	-3254	10669	10677	9040	11744	11177	12108
	Sdev	1208	1719	1152	74	1425	1132	3781	2820	946	1458
60 mins	Average	10606	11506	8241	-3254	7993	7925	7253	11318	15252	11131
	Sdev	2013	2300	2327	74	1425	1132	541	503	946	1045
3 hours	Average	9896	12241	8788	-4015	9675	8410	7788	9018	15287	10305
	Sdev	552	1371	2826	997	2835	1426	1323	1110	1123	1078
6 hours	Average	14908	17234	10390	-3288	9313	8140	13207	11971	8373	12821
	Sdev	1834	1398	1820	852	1793	1476	2966	1641	1005	1588
12 hours	Average	8055	8914	5758	4283	5898	8662	11112	12429	11379	14516
	Sdev	1507	110	1352	1031	3365	850	3014	2005	1767	1077
24 hours	Average	5771	6958	5484	-1301	11312	9805	14776	16121	18466	13094
	Sdev	1312	1223	394	533	1577	1731	1277	1807	1807	215
48hours	Average	3029	3438	2758	-1337	12014	11126	18810	17148	19323	15419
	Sdev	1613	596	1055	615	825	1448	774	1053	1966	1062
72 hours	Average	6739	7793	7103	-1023	11068	10687	17817	17334	14109	18723
	Sdev	1487	218	868	682	502	1298	805	996	2151	1247

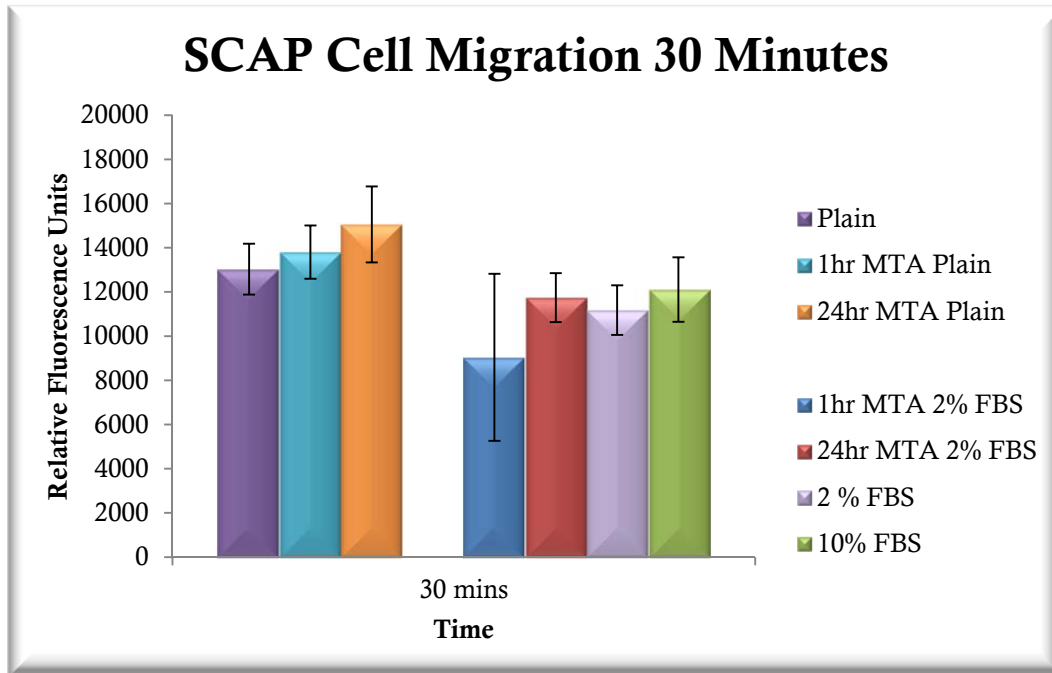


Figure 1. SCAP Cell Migration at 30 Minutes.

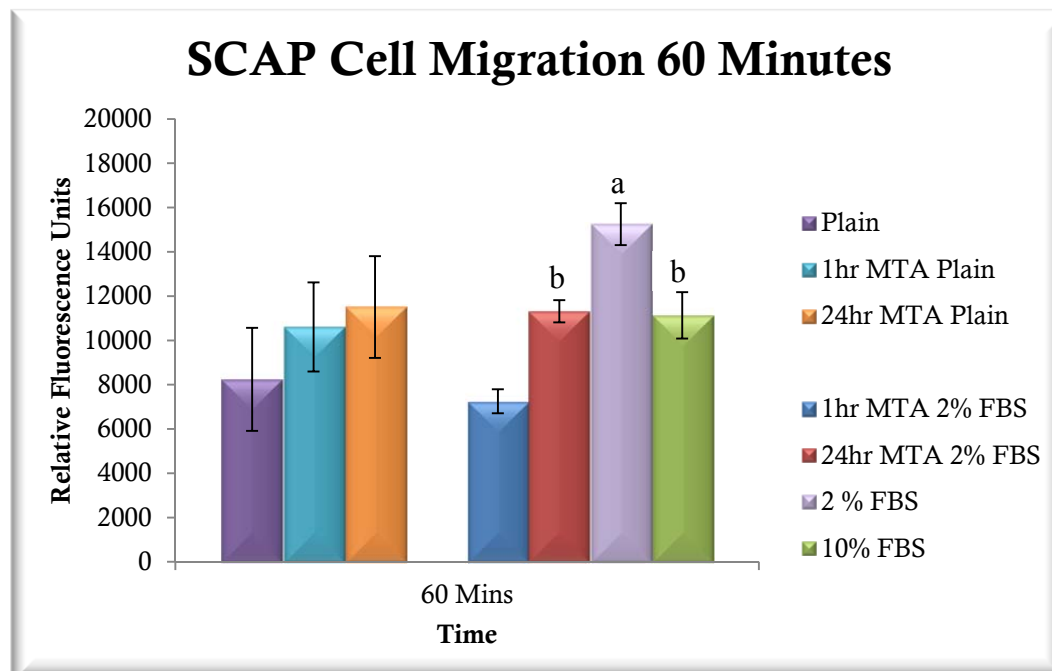


Figure 2. SCAP Cell Migration at 60 Minutes. No SS difference found with cells seeded in plain medium, a $P \leq 0.05$ compared with all groups, b $P \leq 0.05$ compared with 1hr MTA 2% FBS.

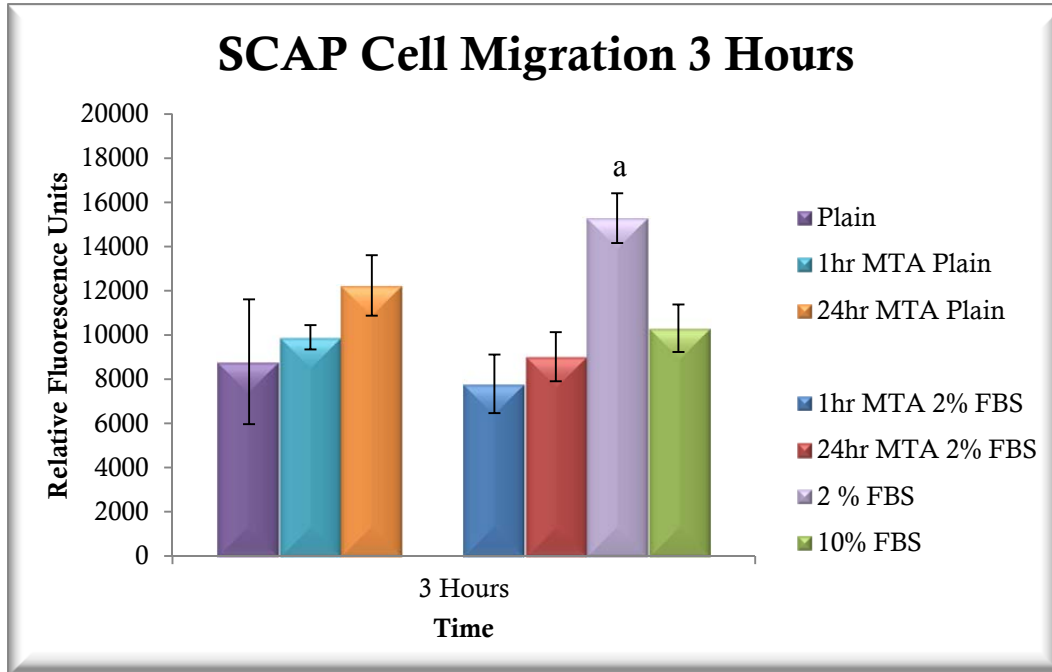


Figure 3. SCAP Cell Migration at 3 Hours. No SS difference found with cells seeded in plain medium, a $P \leq 0.05$ compared with all groups.

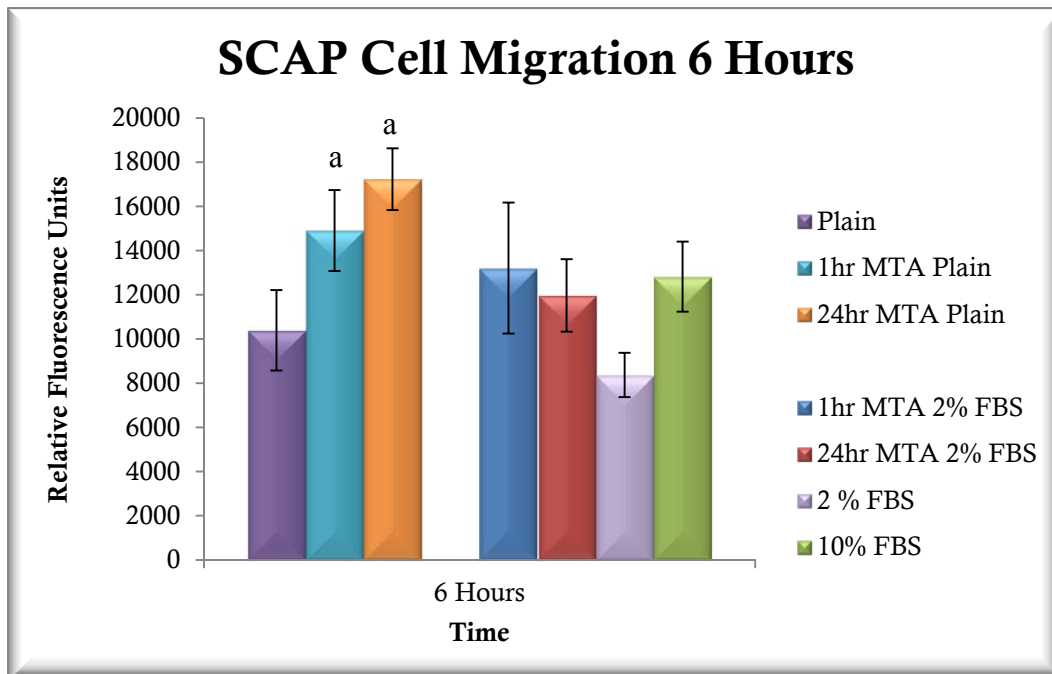


Figure 4. SCAP Cell Migration at 6 Hours. There was a SS difference in migration when the cells were seeded with WMTA 1h or 24h in plain medium. a $P \leq 0.05$ compared with Plain control.

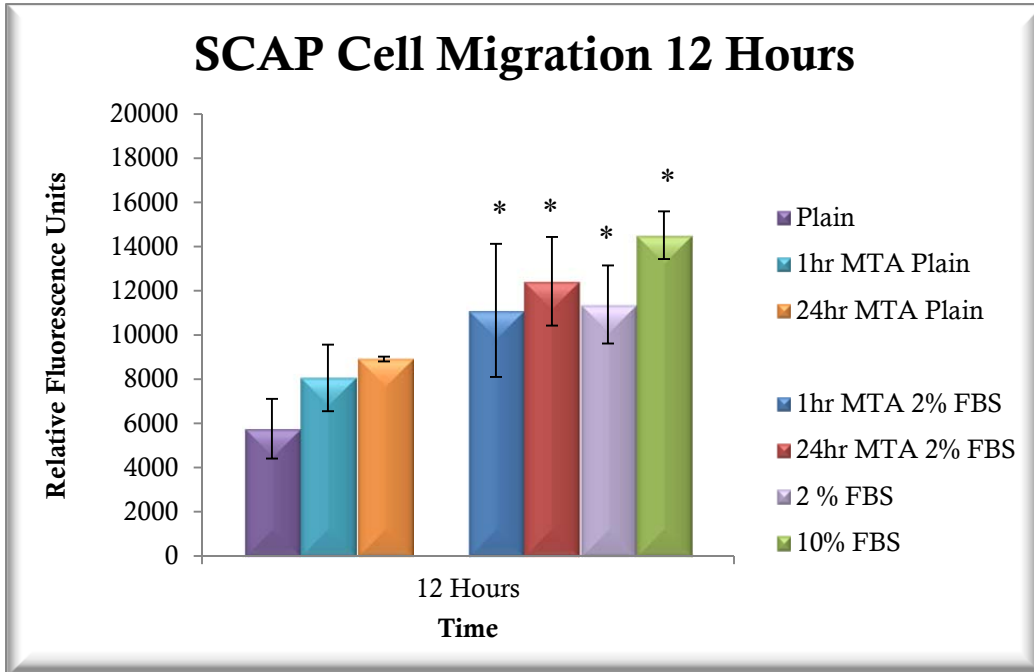


Figure 5. SCAP Cell Migration at 12 Hours. No SS difference found with cells seeded in plain medium,* $P \leq 0.05$ for all FBS groups compared with Plain control.

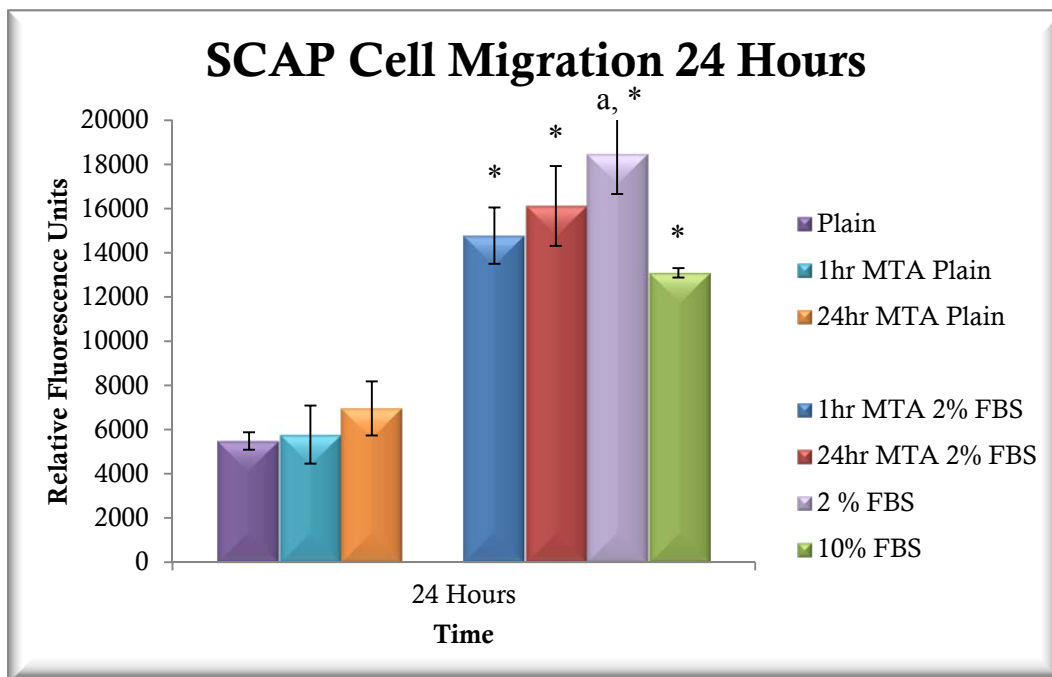


Figure 6. SCAP Cell Migration at 24 Hours. No SS difference found with cells seeded in plain medium, ^a $P \leq 0.05$ compared with 10% FBS, * $P \leq 0.05$ for all FBS groups compared with Plain control.

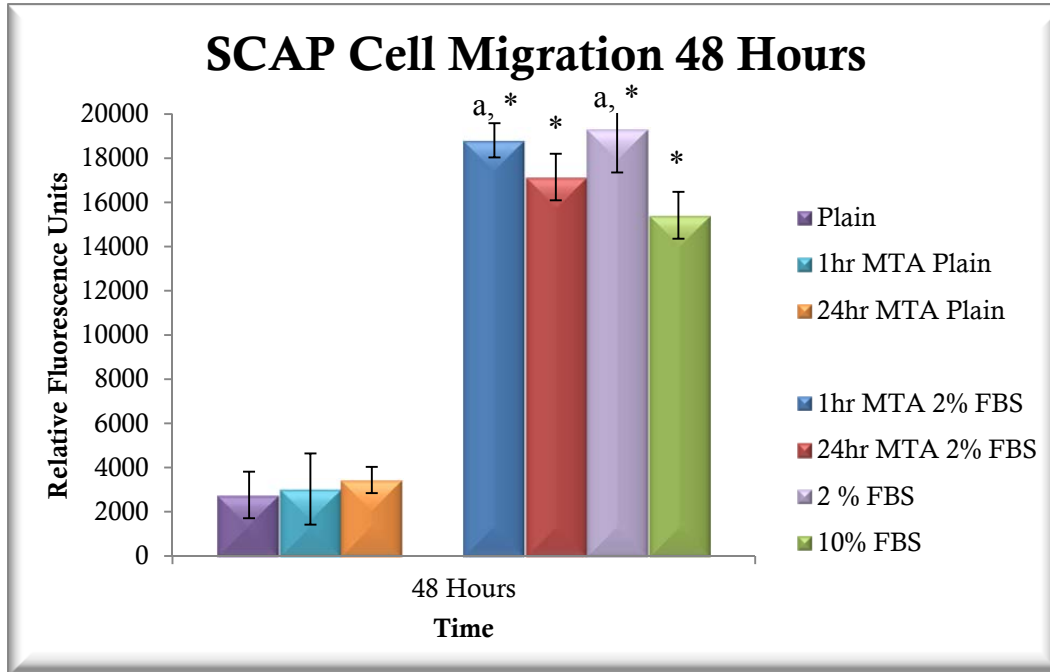


Figure 7. SCAP Cell Migration at 48 Hours. No SS difference found with cells seeded in plain medium, a $P \leq 0.05$ compared with 10% FBS, * $P \leq 0.05$ for all FBS groups compared with Plain control.

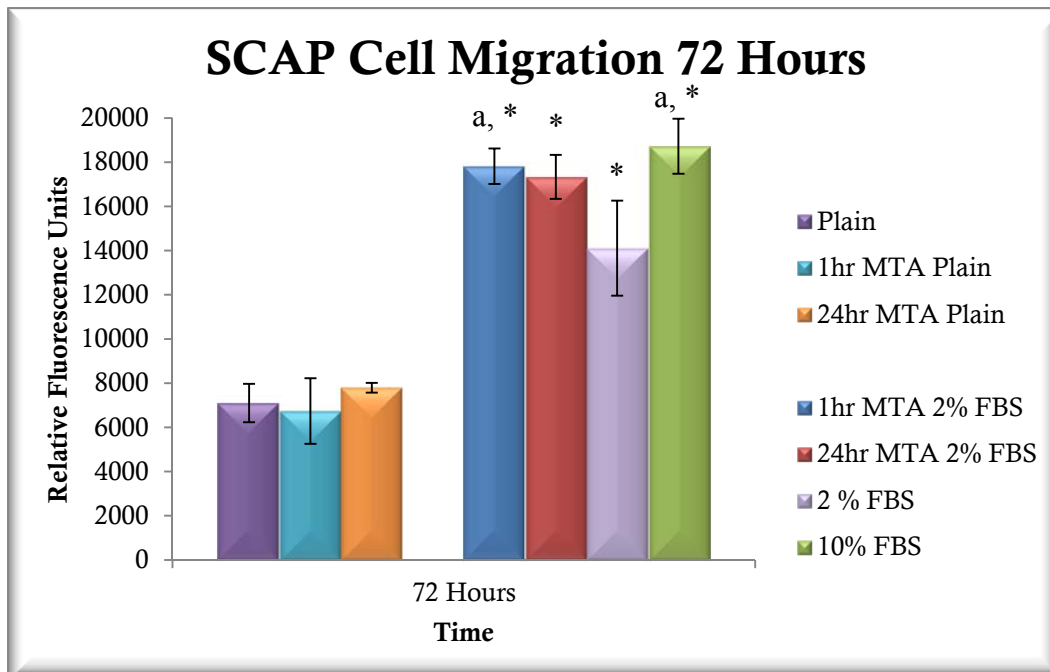


Figure 8. SCAP Cell Migration at 72 Hours. No SS difference found with cells seeded in plain medium, a $P \leq 0.05$ compared with 2% FBS, * $P \leq 0.05$ for all FBS groups compared with Plain control.

Chapter 3 – SCAP CELL PROLIFERATION

To determine whether WMTA induces proliferation of SCAP cells, a WST-1 proliferation assay was used. The SCAP cells were challenged with 1 or 24 hour set WMTA and as control groups plain or 10% FBS medium were used starting from 1 day up to 14 days of exposure.

Materials and Methods

WST-1 Proliferation Assay

SCAP cell proliferation was evaluated after exposure to 1h or 24 hr set WMTA in plain or 2% FBS medium by WST-1 proliferation assay method. The media from a T-75 flask containing passage 4 SCAP cells at 80% confluence was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100µl of re-suspended cells in 900µl of plain media into a 2ml Eppendorf tube. 4×10^3 cells were seeded into 12 well companion plates (BD Falcon, San Jose, CA) with 1.5ml of media in the following test conditions: 3 sets of 3 wells containing plain media, 3 sets of 3 wells containing 2% FBS media, 1 set of 3 wells containing 3.0 mmol Ca media, 1 set of 3 wells containing 0.3 mmol Ca media, 1 set of 3 wells containing 0.03 mmol Ca

media, and 1 set of 3 wells containing 10% FBS media. Cell culture transwell inserts, 3- μ m-size pore (BD Falcon, San Jose, CA), were placed into each well. Two WMTA pellets were placed into the transwell inserts of the test groups as follows: plain media with 1 hour set WMTA, plain media with 24 hour set WMTA, 2% FBS media with 1 hour set WMTA, and 2% FBS media with 24 hour set WMTA. The plates were incubated at 37°C in 5% CO₂ and 100% humidity and the media changed every 3 days. The plates were evaluated at 1, 3, 5, 7, 9, 11 and 14 days via WST-1 Proliferation Assay.

The WST-1 proliferation assay was performed as follows: the transwell inserts were removed and discarded, the media was removed via vacuum pipetting and 300 μ l of new media containing 30 μ l of WST-1 reagent (Roche, Mannheim, Germany) (1:10 dilution) was added to each well. The plates were incubated at 37°C in 5% CO₂ and 100% humidity for 1 hour. One-hundred microliters of media plus WST-1 reagent was transferred from each well in the 12 well plate to a single well in a 96 well plate. Controls of WST-1 reagent in a 1:10 dilution with plain, 2% FBS, and 10% FBS media were also placed in wells of the 96 well plate. The absorbance (450nm-685nm) was determined in a microplate reader (Genius; Tecan, Grödig, Austria). This experiment was repeated 3 times in triplicate.

Results

WMTA Significantly Increased SCAP Cell Proliferation after 1 and 5 Days of exposure.

The results for the WST-1 proliferation assay are described in absorbance (A450nm-A685nm) and are listed in Table 2. After 1 day, both 1 and 24 hour set WMTA in plain

media showed a significant increase SCAP proliferation as compared to the plain media control ($P = 0.023$ and $P = 0.008$, respectively). In the FBS media group, a significant increase in proliferation was seen in both the 1 hour set WMTA with 2% FBS ($P = 0.018$) and the 2% FBS media group ($P = 0.011$, Figure 9). There were no significant differences between any groups on day 3 reading (Figure 10). A significant increase in proliferation was seen in the 24 hour set WMTA with plain media group as compared to the plain media control on day 5 reading ($P = 0.023$, Figure 11). No significant differences were seen between any of the plain media groups at both the 7 and 9 day readings, however the 2% FBS group showed increased proliferation as compared to the 24 hour set WMTA with 2% FBS group ($P = <0.025$). The 10% FBS group had significantly increased SCAP cell proliferation when compared to all other groups ($P = <0.001$, Figures 12 & 13). At the 11 day reading no differences were seen in any of the plain media groups. In the FBS media groups, all groups showed significantly increased proliferation to 24 hour set WMTA with 2% FBS group ($P = <0.008$). The 2% FBS media group showed a significant increase as compared to 24 hour set WMTA with 2% FBS group ($P = <0.001$), but significantly less proliferation as compared to the 10% FBS group ($P = <0.001$, Figure 14). The 2% FBS group showed increased proliferation as compared to the 24 hour set WMTA with 2% FBS group ($P = 0.011$) and the 10% FBS group had significantly increased SCAP cell proliferation when compared to all other groups ($P = <0.002$) at the 14 day reading. There were no differences seen in the plain media groups (Figure 15).

In Summary we found that SCAP cells exposed to 1 h or 24 h set WMTA in Plain medium showed a statistically significant difference in proliferation after 1 and 5 days of

exposure. The 10% FBS group showed a constant increase in proliferation over time after 3 days of exposure.

**Table 2: SCAP Cell Proliferation
(in absorbance (A450nm-A685mm))**

		1hr MTA Plain	24hr MTA Plain	Plain	3.0mmol Ca	0.3mmol Ca	0.03 mmol Ca	1hr MTA 2% FBS	24hr MTA 2% FBS	2 % FBS	10% FBS
Day 1											
	Average	0.1203	0.1275	0.0816	-0.0866	0.1022	0.1080	0.1690	0.1367	0.1740	0.1175
	Sdev	0.0107	0.0182	0.0169	0.0077	0.0050	0.0049	0.0163	0.0134	0.0158	0.0182
Day 3											
	Average	0.1146	0.135033	0.097867	-0.03487	0.135233	0.134533	0.326433	0.300333	0.2895	0.287033
	Sdev	0.0329	0.0077	0.0213	0.0102	0.0216	0.0197	0.0509	0.0242	0.0205	0.0262
Day 5											
	Average	0.1103	0.297033	0.1527	-0.12337	0.2021	0.1553	0.814833	0.6856	0.8292	0.890867
	Sdev	0.0266	0.0145	0.0257	0.0029	0.0070	0.0150	0.0360	0.0928	0.0300	0.1624
Day 7											
	Average	0.142033	0.180867	0.1972	-0.1377	0.277367	0.3297	1.121367	0.911067	1.253067	1.958333
	Sdev	0.0256	0.0634	0.0228	0.0148	0.0145	0.0210	0.1305	0.0582	0.1046	0.1416
Day 9											
	Average	0.0794	0.209567	0.139767	-0.09617	0.1738	0.1197	1.640667	1.204833	1.866067	3.7938
	Sdev	0.0144	0.0662	0.0730	0.0055	0.0283	0.0218	0.2689	0.1083	0.1283	0.2775
Day 11											
	Average	0.023267	0.065333	0.110567	-0.06973	0.146767	0.133633	1.464667	0.833833	2.307267	5.228367
	Sdev	0.0056	0.0562	0.0212	0.0156	0.0597	0.0505	0.0524	0.0269	0.2249	0.2472
Day 14											
	Average	0.035467	1.524	0.083467	-0.0526	0.142533	0.098967	2.365933	1.524	2.853867	4.625367
	Sdev	0.0708	0.0408	0.0267	0.0055	0.0376	0.0418	0.0708	0.0408	0.1781	0.7289

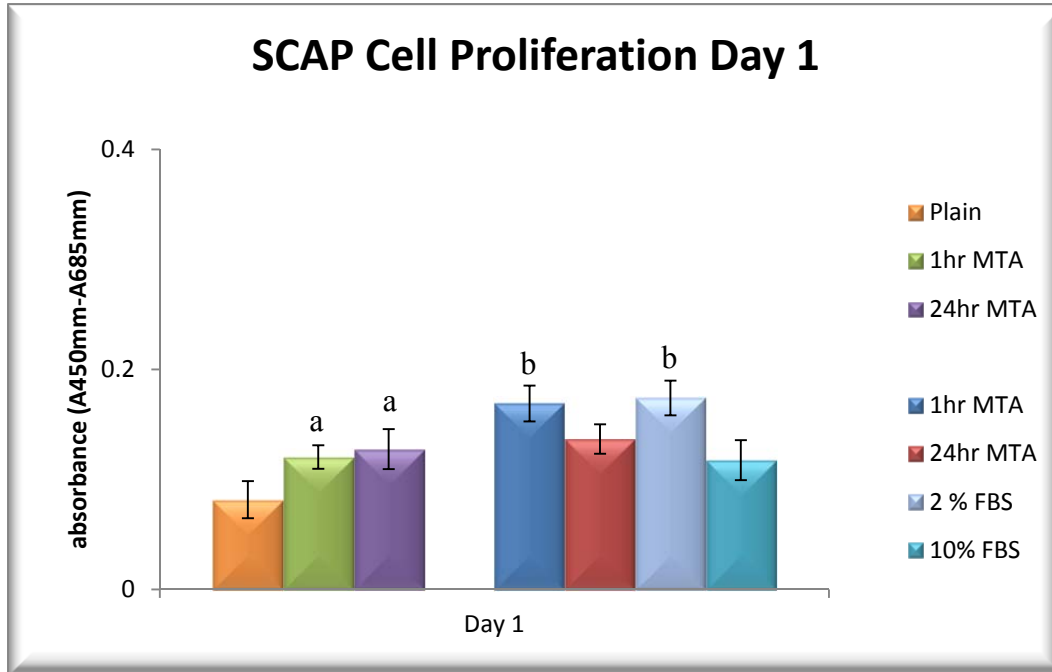


Figure 9. SCAP Cell Proliferation at Day 1. There is SS difference when the cells were exposed to 1 hr and 24 hr WMTA, a $P \leq 0.05$ compared with Plain control, b $P \leq 0.05$ compared with 10% FBS.

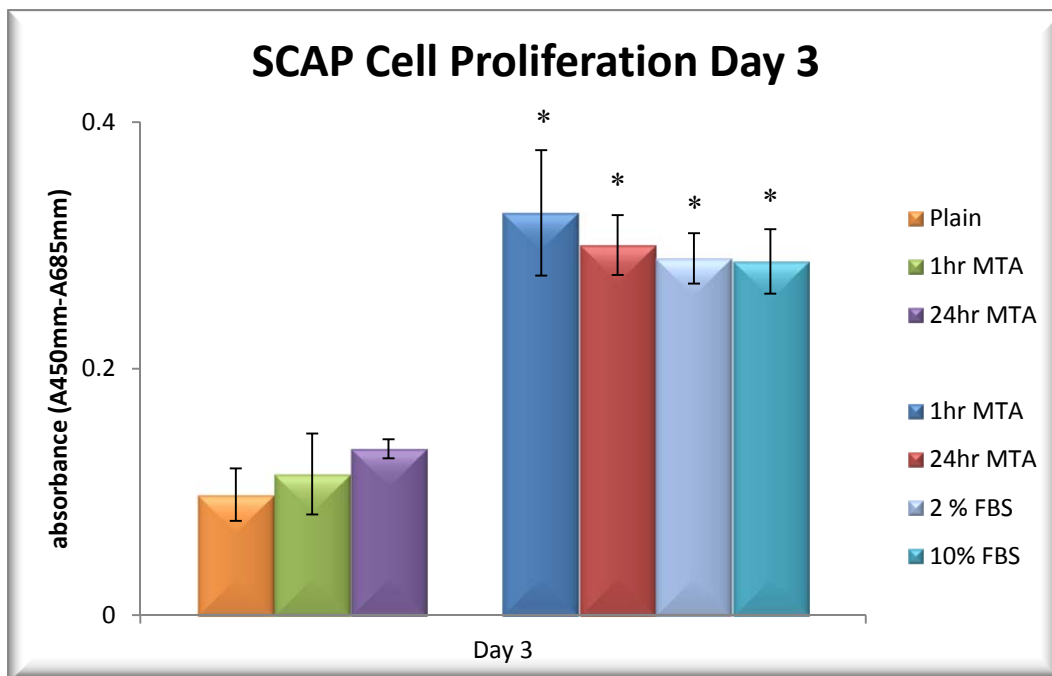


Figure 10. SCAP Cell Proliferation at Day 3. There is SS difference when the cells were exposed to all FBS groups, * $P \leq 0.05$ compared with the Plain control.

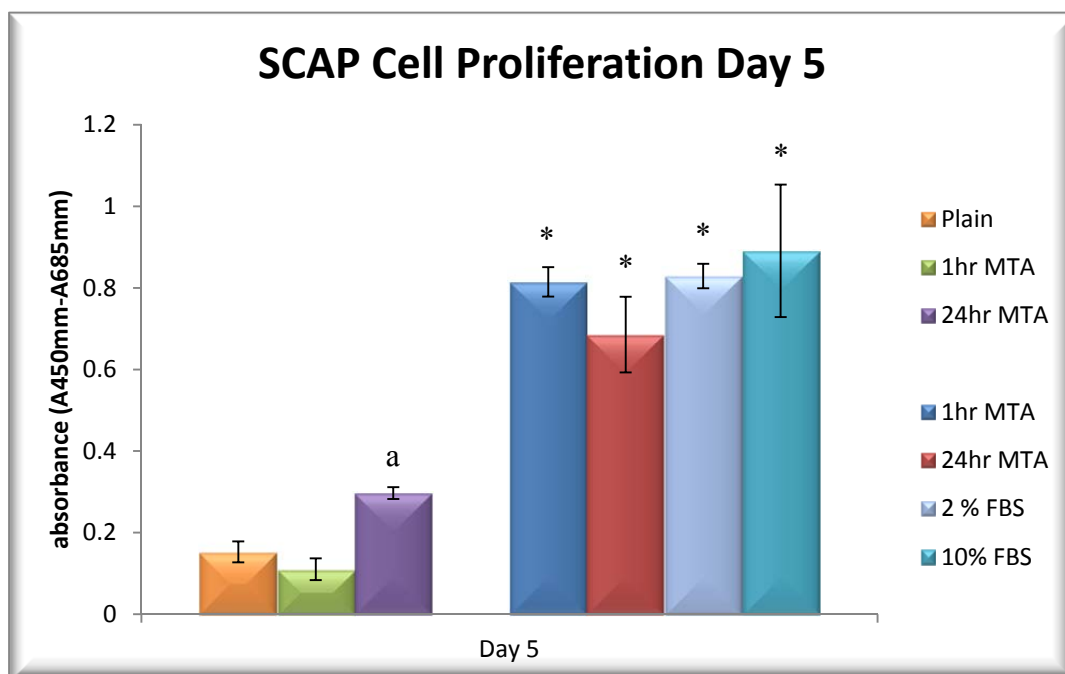


Figure 11. SCAP Cell Proliferation at Day 5. There is SS difference when the cells were exposed to 24 hr WMTA in Plain media, a $P \leq 0.05$ compared with Plain control; * $P \leq 0.05$ for all FBS groups compared with Plain control.

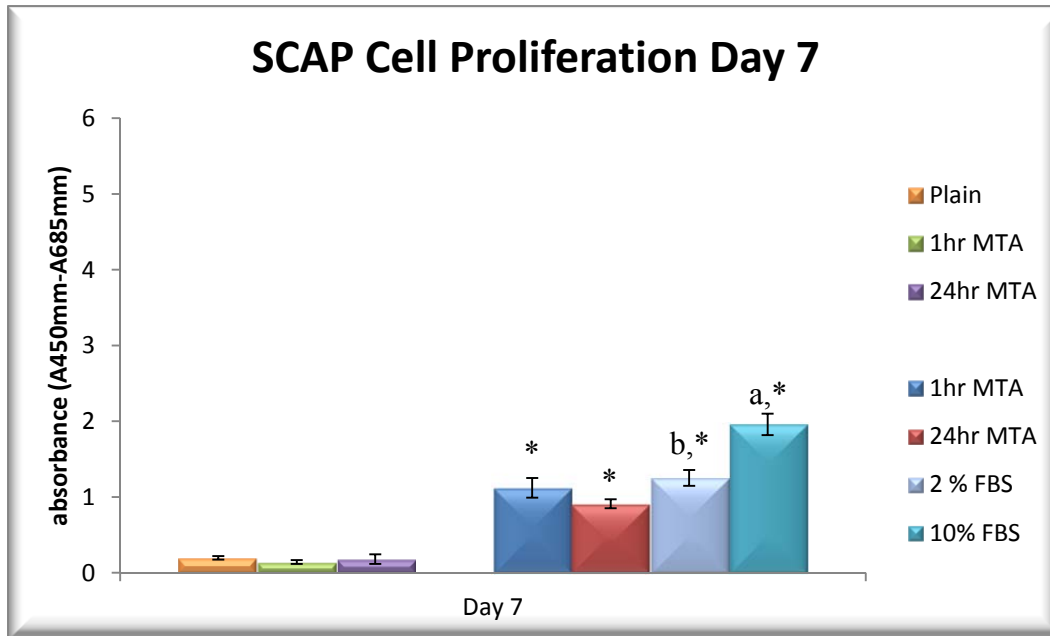


Figure 12. SCAP Cell Proliferation at Day 7. There is SS difference when the cells were exposed to 10% FBS, a $P \leq 0.05$ compared with all groups; b $P \leq 0.05$ compared 2% FBS with 24hr MTA in 2% FBS media; * $P \leq 0.05$ for all FBS groups compared with Plain control.

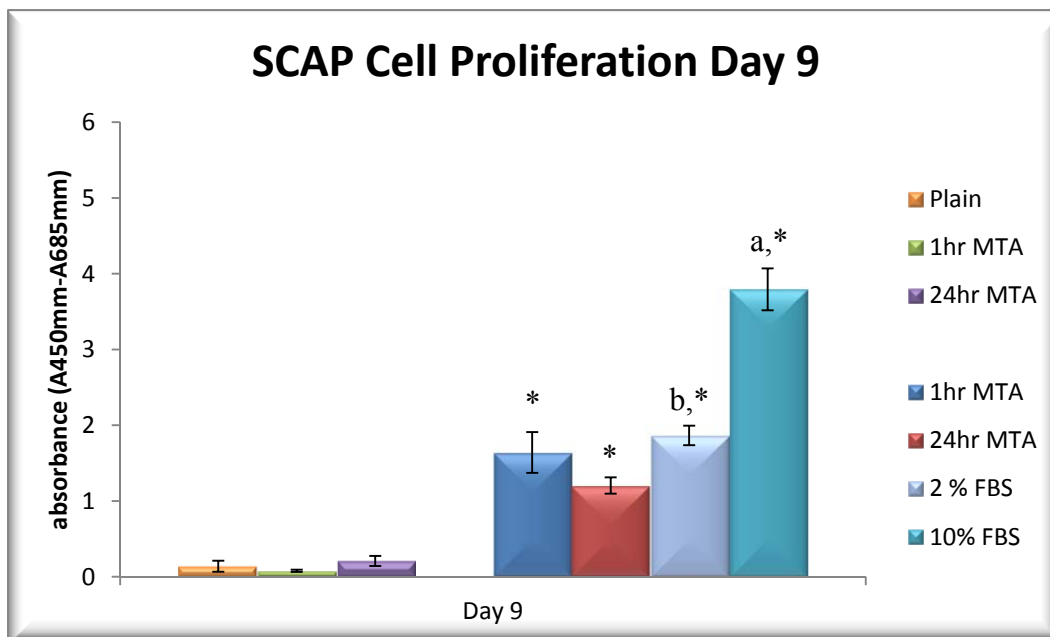


Figure 13. SCAP Cell Proliferation at Day 9. There is SS difference when the cells were exposed to 10% FBS, a $P \leq 0.05$ compared with all groups; b $P \leq 0.05$ compared 2% FBS with 24hr MTA in 2% FBS media; * $P \leq 0.05$ for all FBS groups compared with Plain control.

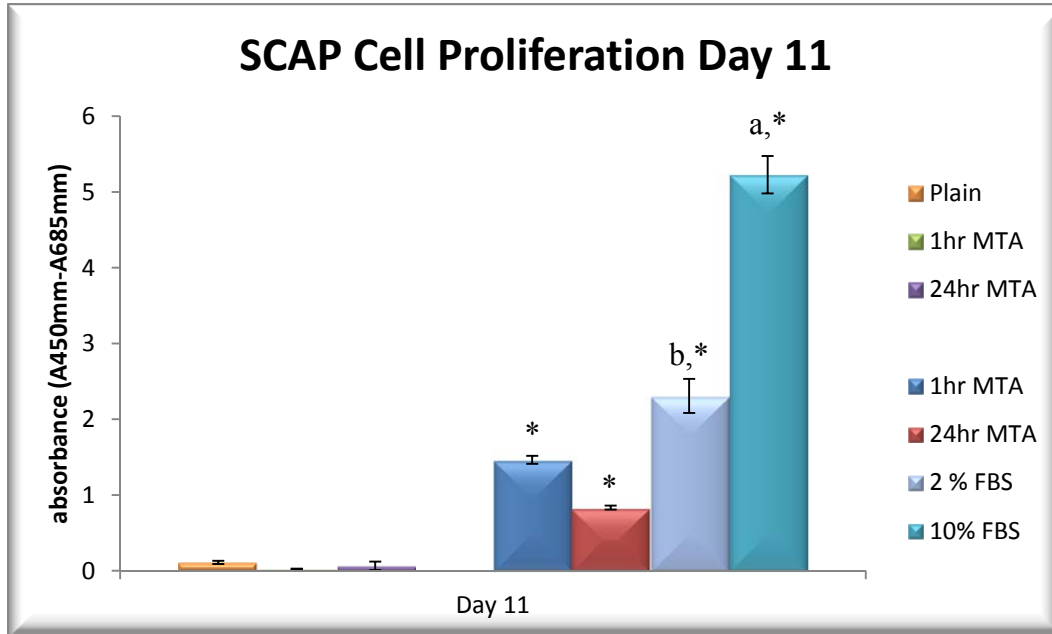


Figure 14. SCAP Cell Proliferation at Day 11. There is SS difference when the cells were exposed to 10% FBS, a $P \leq 0.05$ compared with all groups; b $P \leq 0.05$ compared 2% FBS with 24hr MTA in 2% FBS media; * $P \leq 0.05$ for all FBS groups compared with Plain control.

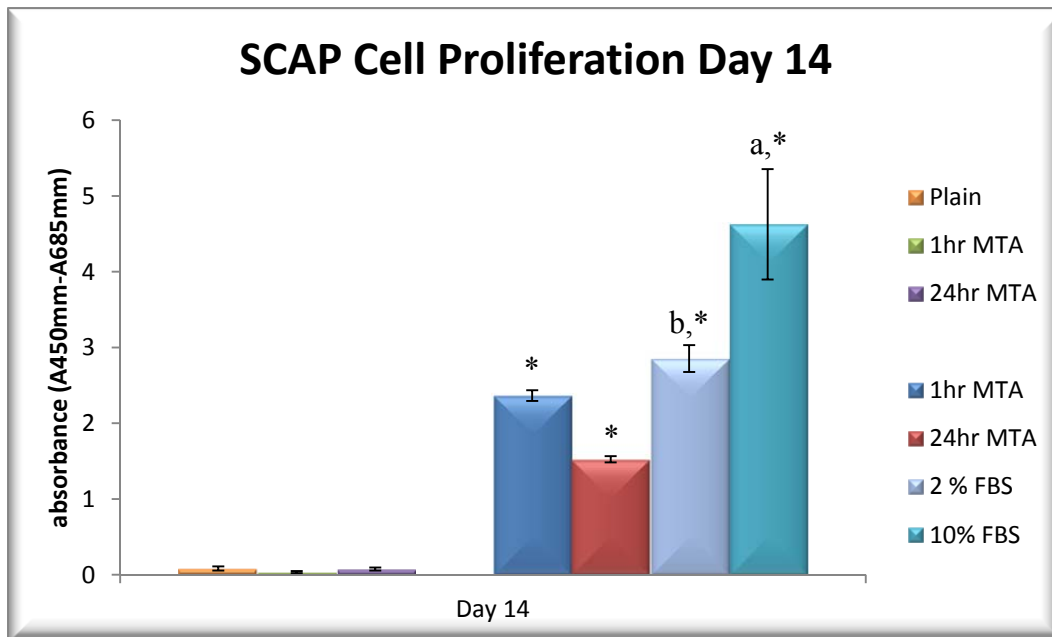


Figure 15. SCAP Cell Proliferation at Day 14. There is SS difference when the cells were exposed to 10% FBS, a $P \leq 0.05$ compared with all groups; b $P \leq 0.05$ compared 2% FBS with 24hr MTA in 2% FBS media; * $P \leq 0.05$ for all FBS groups compared with Plain control.

Chapter 4 – SCAP CELL DIFFERENTIATION

To determine whether WMTA induces differentiation in SCAP cells, an RT-PCR assay was used. The SCAP cells were challenged with 1 or 24 hour set WMTA and control groups of plain and 10% FBS medium were used starting from 1 day up to 21 days of exposure.

Materials and Methods

RT-PCR Analysis

SCAP cell differentiation was evaluated using RT-PCR analysis and gel electrophoresis. The media from a T-75 flask containing passage 4 SCAP cells at 80% confluence was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100µl of re-suspended cells in 900µl of plain media into a 2ml Eppendorf tube. Four-thousand cells were seeded into the wells of 6 well companion plates (BD Falcon, San Jose, CA) with 3ml of media in the following test conditions: 3 sets of 3 wells containing plain media, 3 sets of 3 wells containing 2% FBS media, 1 set of 3 wells containing 3.0mmol Ca media, 1 set of 3 wells containing 0.3mmol Ca media, 1 set of 3 wells containing 0.03mmol Ca media, and 1 set of 3 wells containing 10% FBS

media. Three micrometer-size pore transwell cell culture inserts (BD Falcon, San Jose, CA) were placed into each well. Three WMTA pellets were placed into the transwell inserts of the test groups as follows: plain media with 1 hour set WMTA, plain media with 24 hour set WMTA, 2% FBS media with 1 hour set WMTA, and 2% FBS media with 24 hour set WMTA. The plates were incubated at 37°C in 5% CO₂ and 100% humidity and the media changed every 3 days. The plates were evaluated at 7, 14 and 21 days via RT-PCR Analysis.

The RNA isolation was performed with the use of an RNeasy Mini Kit (Qiagen, Valencia, CA) The media was removed from all wells in the 6 well plate by vacuum pipetting and the cells were harvested by adding 200µl of freshly mixed RLT lysis buffer (15ml RLT lysis buffer + 150µl 2-mercaptoethanol) to each well and vigorously scraping with a cell scraper (Corning Inc., Corning, NY) for 2 cycles of 2 minutes per well. The contents of the wells were then agitated by gentle pipetting and transferred to a collection tube supplied in the kit. All 3 wells for each condition were combined into a single collection tube (600µl total volume). The collected sample was placed in a QIAshredder (Qiagen, Valencia, CA) and centrifuged at 13,000 RPM for 2 minutes at 4°C. The QIAshredder insert was discarded and 600µl of 70% alcohol was added to the tube. Half of the sample was placed in a Mini Spin Column from the RNeasy kit and centrifuged at 13,000 RPM for 2 minutes at 4°C. The remaining sample was passed through the same Mini Spin Column. The solution that passed into the collection tube was discarded and 700µl of Buffer RW1 was adding to the Mini Spin Column and centrifuged at 13,000 RPM for 15 seconds at 4°C. The solution that passed into the collection tube was discarded and 500µl of Buffer RPE was adding to the Mini Spin Column and centrifuged

twice at 13,000 RPM for 15 seconds at 4°C. The collection tube was discarded. The Mini Spin Column placed on a new collection tube centrifuged at 13,000 RPM for 2 minutes at 4°C. The Mini Spin Column was rotated 180° and centrifuged at 13,000 RPM for 1 minute at 4°C. The Mini Spin Column transferred to a new Eppendorf tube and 35µl of RNase-Free Water was added to the middle of the Mini Spin Column, which was then centrifuged at 13,000 RPM for 1 minute at 4°C. The solution that passed into the Eppendorf tube was collected by pipette and placed again in the middle of the Mini Spin Column and centrifuged at 13,000 RPM for 1 minute at 4°C. The Mini Spin Column was discarded and the purified RNA sample was contained in the Eppendorf tube. The purity of the RNA sample was measured on cuvette/spectrophotometer a by placing 1µl of RNA sample into 100µl of RNase-free water and reading the optical density. Working solutions were standardized to 100ng for the RT-PCR reactions.

RT-PCR was performed using SuperScript III Platinum kit (Invitrogen Corp., Carlsbad, CA). The primer sequences used were as follows: GAPDH (sense 5' gacccttcattgacctcaact 3'; antisense 5' caccacattcttgatgcatc 3'; 683-bp amplicon); DSPP (sense 5' gacccttcattgacctcaact 3', antisense 5' tgccatttgcgtgatggtt 3'; 181-bp amplicon); DMP-1 (sense 5' caggagcacaggaaaaggag 3', antisense 5' ctggtggtatcttgggcact 3'; 213-bp amplicon); and MEPE (sense 5' gcaaaagcaccatcgtatt 3', antisense 5' ctgcctctacaaggetgac 3'; 385-bp amplicon). For each sample, 5µl of 100ng working solution was placed in a 0.2ml PCR tube with 25µl of 2X Reaction Mix, 2µl sense primer, 2µl anti-sense primer, 1µl Taq enzyme, and 15µl of RNase-free water. The PCR tube was tightly capped and placed in a Mastercycler gradient machine (Eppendorf North America, Hauppauge, NY). Polymerase chain reaction (PCR) was performed with 35

cycles of denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec, and extension at 72°C for 60 sec.

The RT-PCR products were evaluated by electrophoresis in 2% agarose gels stained with ethidiumbromide. The 2% agarose gels were made by combining 2g UltraPure agarose (Invitrogen Corp., Carlsbad, CA) with 100ml TAE (1X) buffer in a 125ml Erlenmeyer flask and microwaving for 1-1.5 minutes until the solution was clear, then adding 5 μ l of ethidiumbromide to the flask and swirling to mix thoroughly. The gel mixture was then poured into a 15cm x 10 cm Gel Caster (Bio-Rad, Hercules, CA) with a 20 well comb and allowed to set for 45 minutes. Well #1 in each gel was loaded with 5 μ l of loading dye and 10 μ l of 100kb Plus DNA Ladder (Life Technologies, Grand Island, NY), 15 μ l of sample and 5 μ l of dye were loaded into the sample wells. The gels were connected to a Bio-Rad Power Pak 300 (Bio-Rad, Hercules, CA) and run at 80V for 45 minutes. Gel photographs were taken with a Kodak EDAS 290. A densitometric analysis was performed using ImageJ software (NIH). This experiment was repeated 3 times in triplicate.

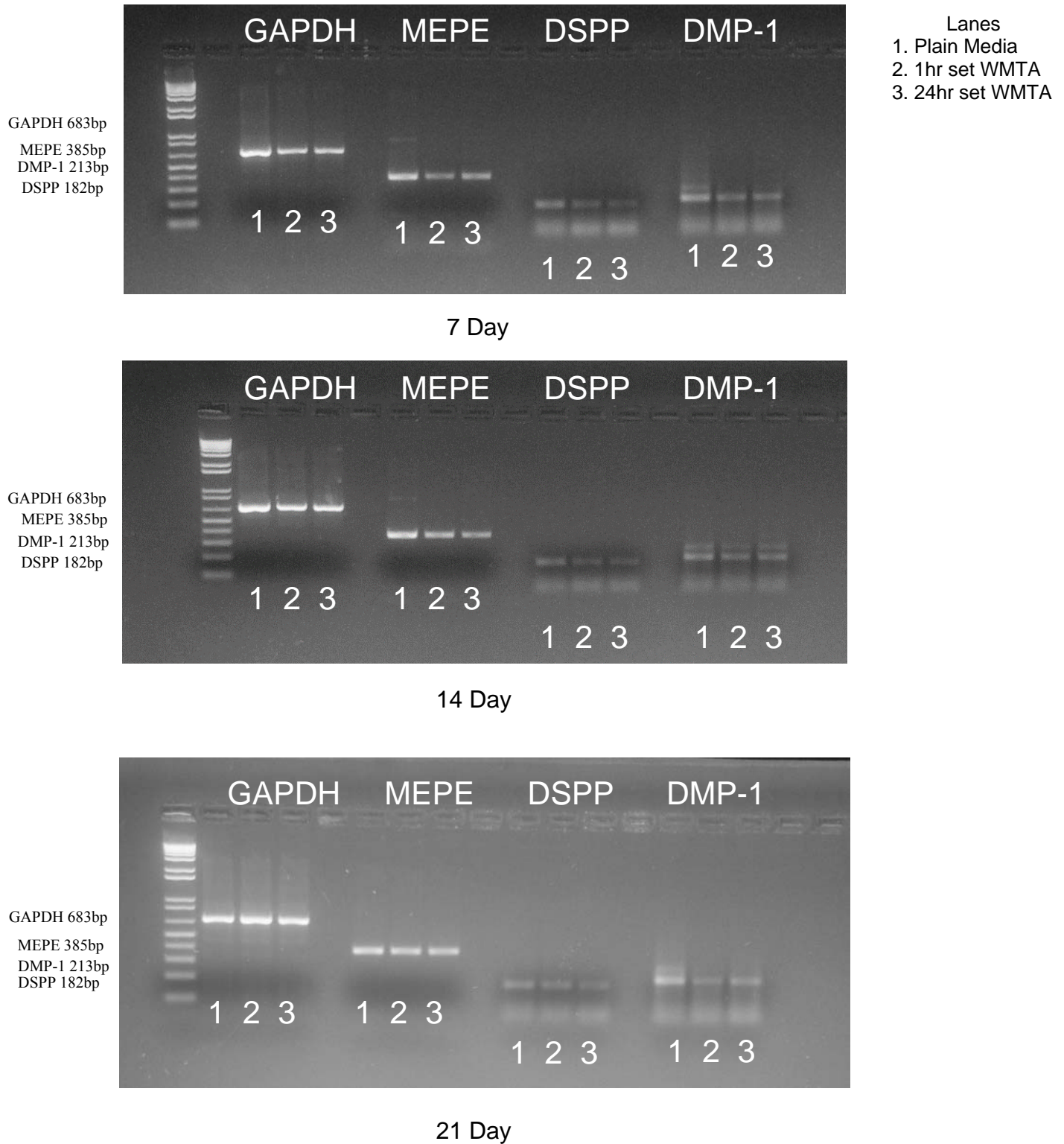
Results

WMTA had No Effect on SCAP Cell Differentiation

The results for the RT-PCR Analysis are relatively homogenous. The Odontoblastic markers dentin sialophosphoprotein (DSPP), dentin matrix protein (DMP-1), and matrix extracellular phosphoglycoprotein (MEPE) are present in all samples and at all time points. The plain media groups show a higher expression of MEPE, followed by DMP-1, then DSPP at all time points (Figure 16). The FBS groups show a similar pattern of expression, however there is an increased density of the bands as compared to the plain

media groups (Figure 17). There was no increase in expression for any marker from the WMTA groups with either Plain media or FBS media as shown by densitometric analysis (Figure 18-26).

SCAP Cell Differentiation in Plain Media



SCAP Cell Differentiation in 2% and 10 FBS Media

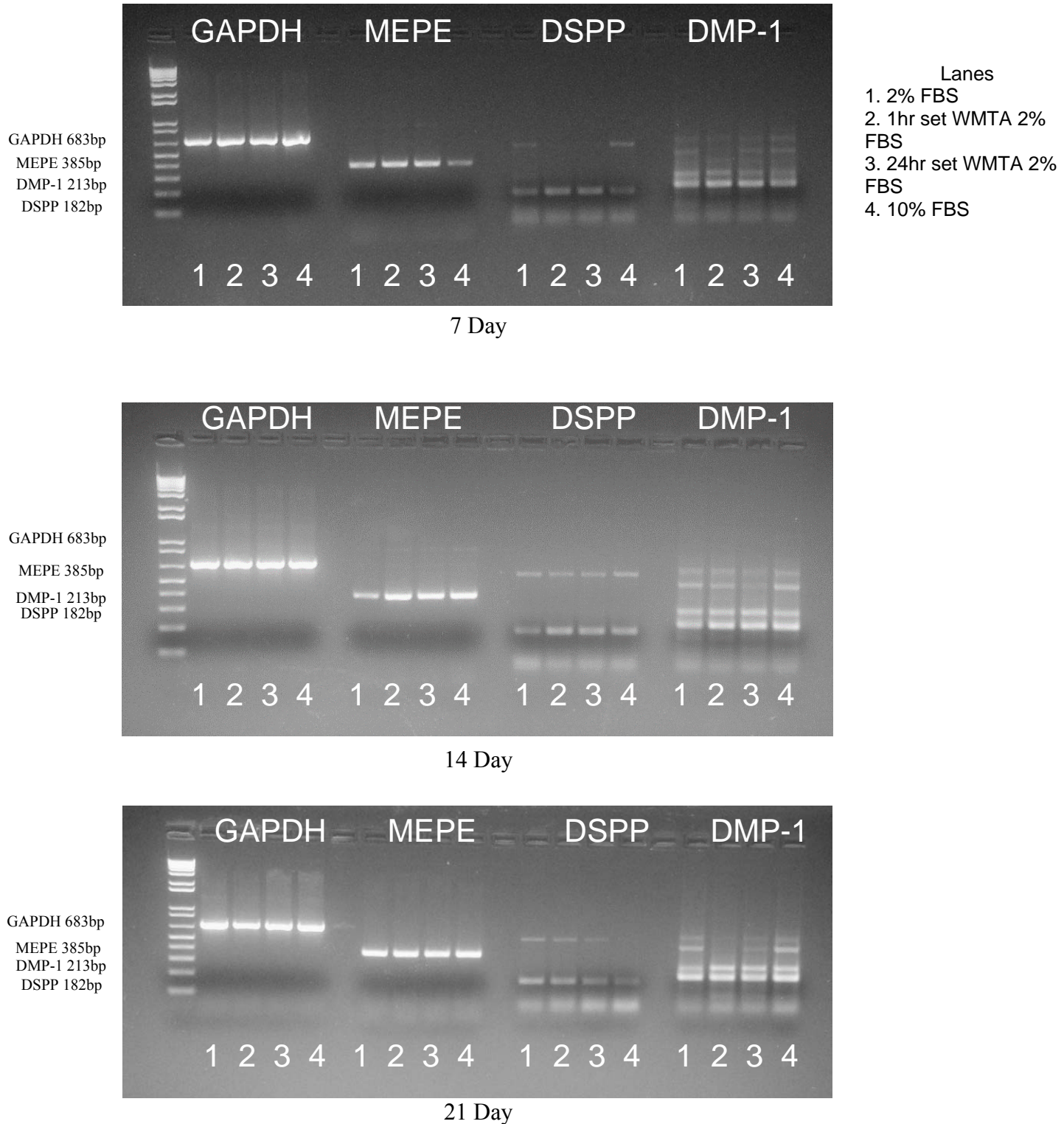


Figure 17. SCAP Cell Differentiation in FBS Media at 7, 14, and 21 Days. Lane 1: 2% FBS media; Lane 2: 1hr set WMTA in 2% FBS media; Lane 3: 24hr set WMTA in 2% FBS media; Lane 4: 10% FBS media.

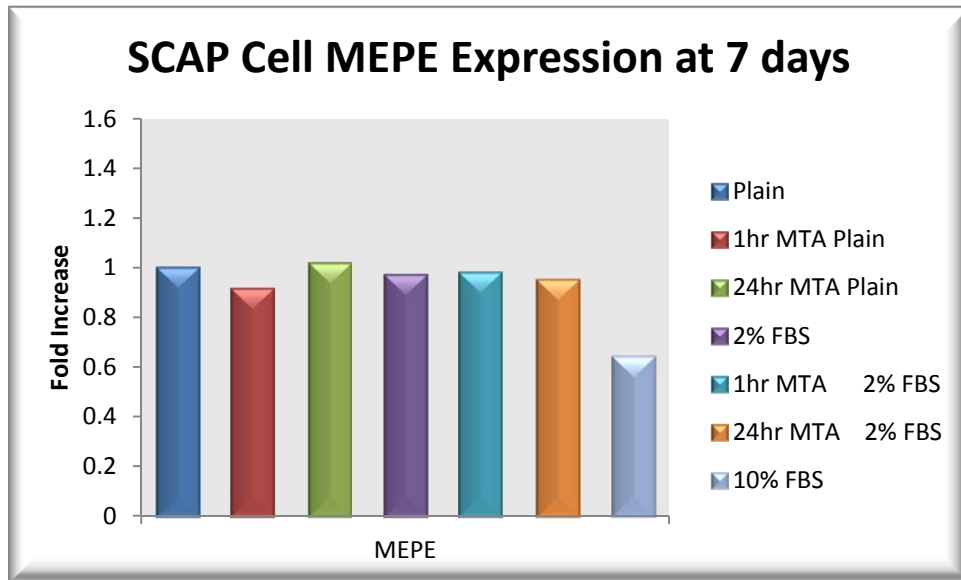


Figure 18. SCAP Cell MEPE Expression at 7 days as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

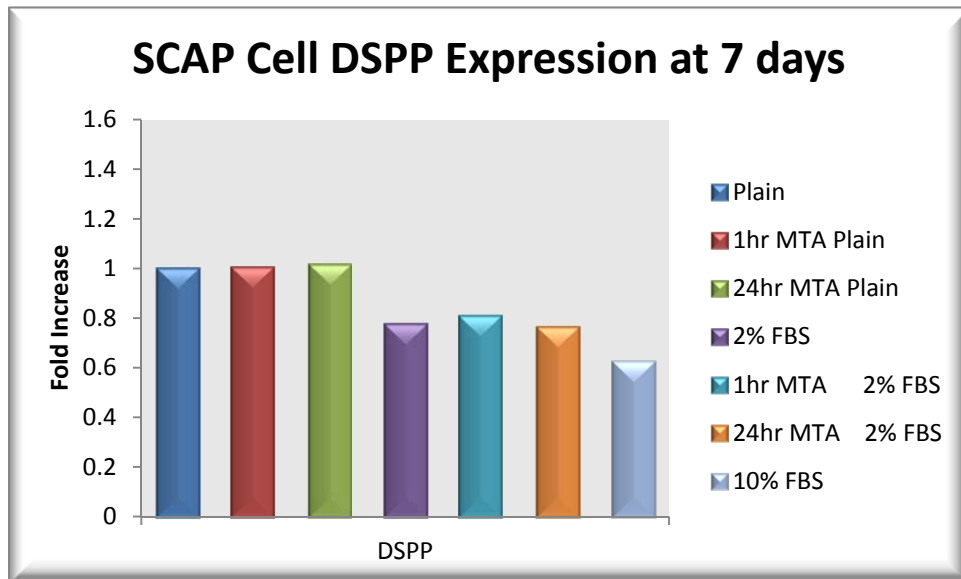


Figure 19. SCAP Cell DSPP Expression at 7 days as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

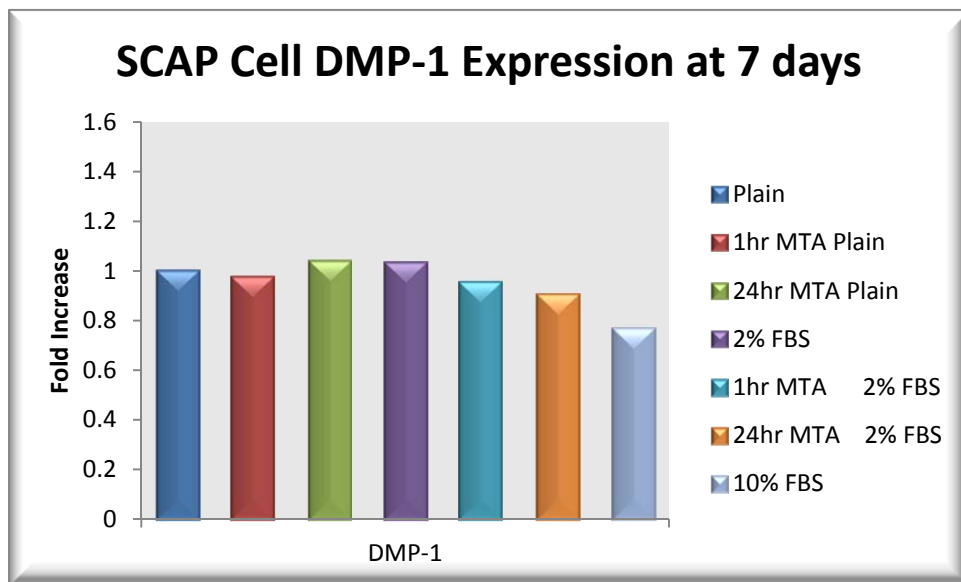


Figure 20. SCAP Cell DMP-1 Expression at 7 days as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

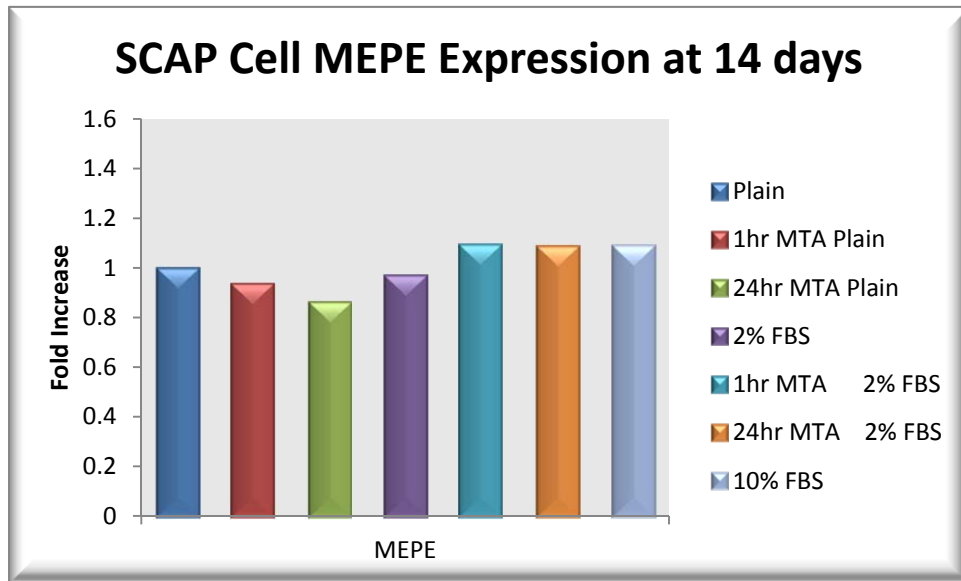


Figure 21. SCAP Cell MEPE Expression at 14 days as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

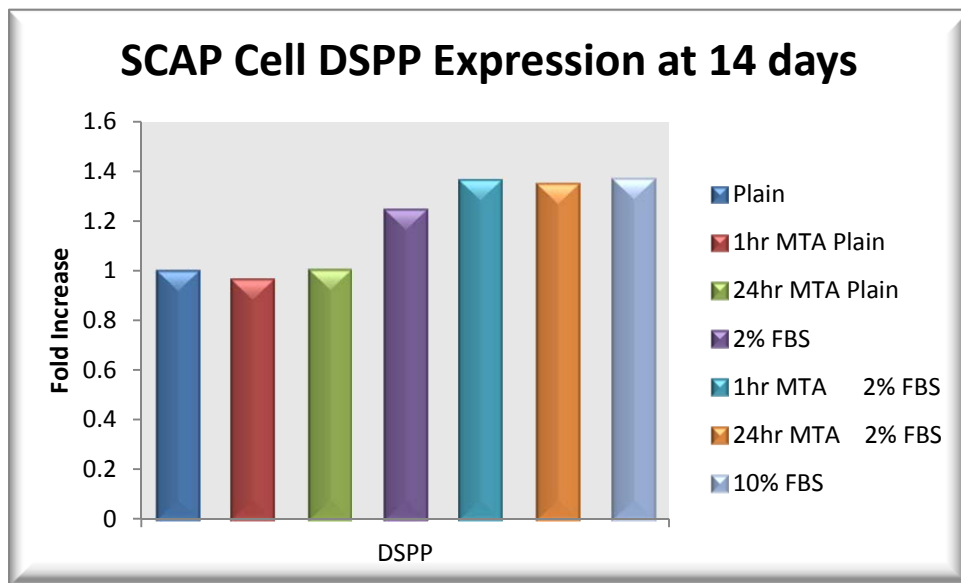


Figure 22. SCAP Cell DSPP Expression at 14 days as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

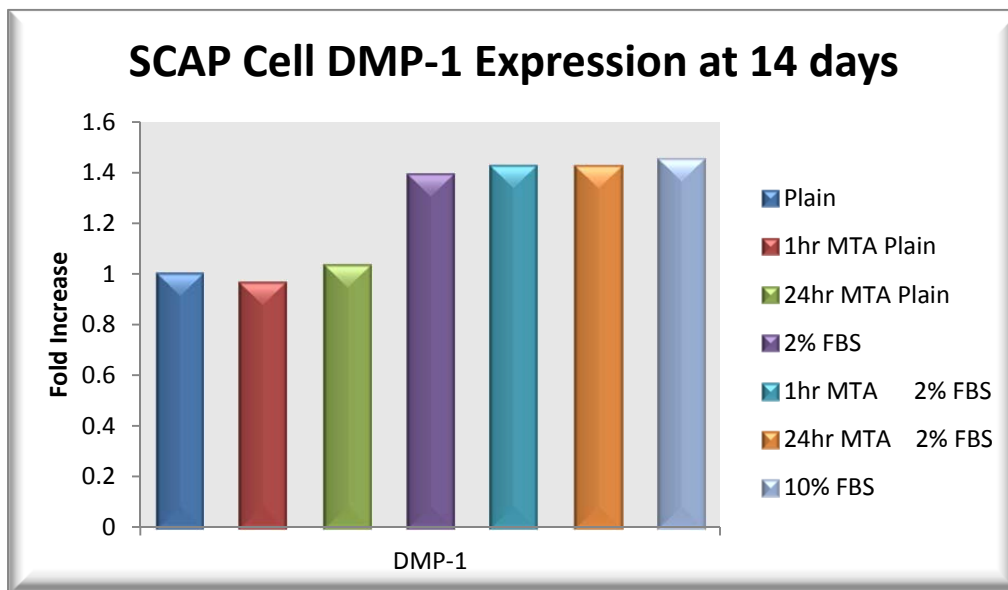


Figure 23. SCAP Cell DMP-1 Expression at 14 days as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

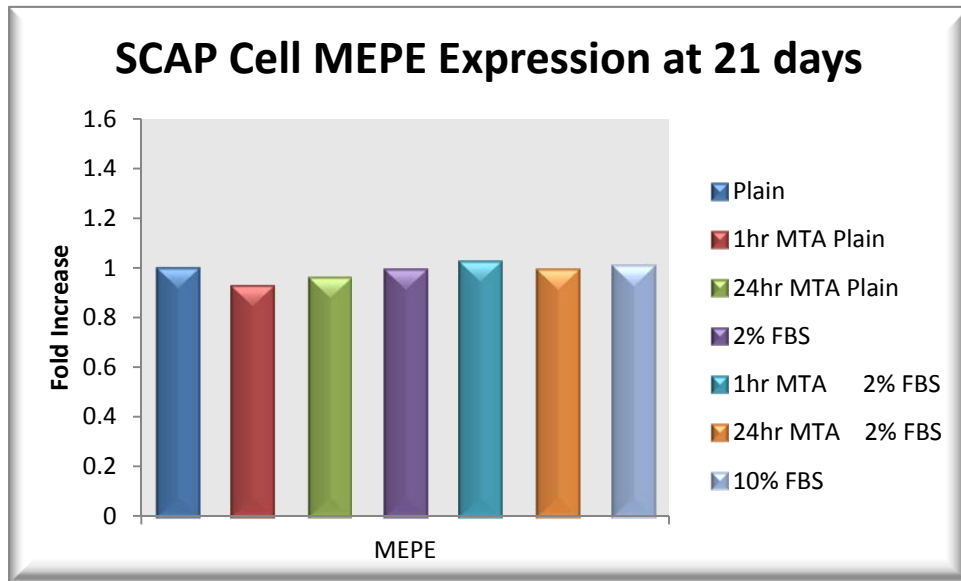


Figure 24. SCAP Cell MEPE Expression at 21 days as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

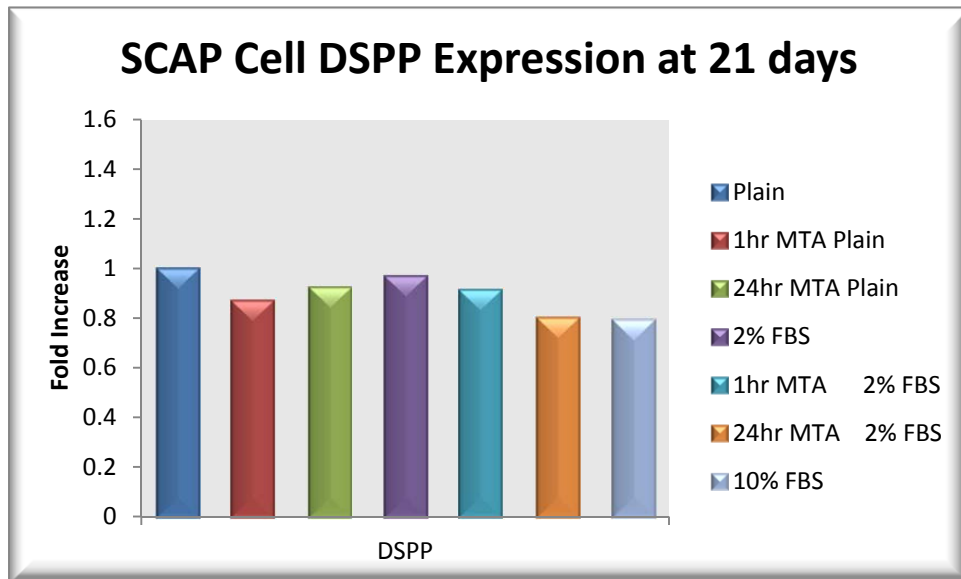


Figure 25. SCAP Cell DSPP Expression at 21 days as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

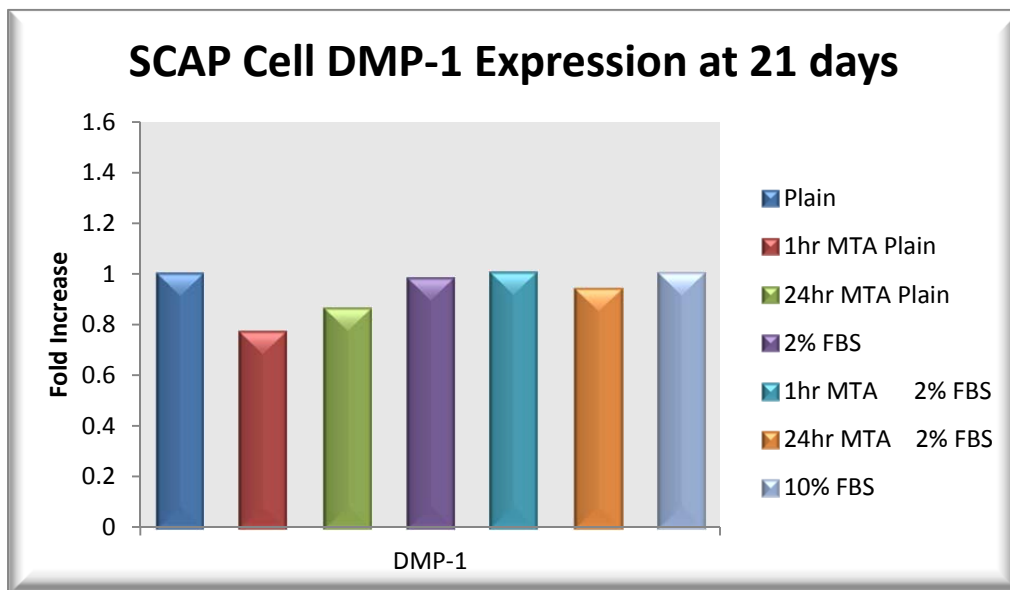


Figure 26. SCAP Cell DMP-1 Expression at 21 days as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

Chapter 5 – CALCIUM ENRICHED MEDIA

To determine whether calcium enriched medium induces migration, proliferation or differentiation in SCAP cells, all the assays previously described were used. The SCAP cells were challenged with 3.0mmol, 0.3mmol, and 0.03mmol free calcium ions and control groups of plain, 2% FBS, and 10% FBS medium were used.

Materials and Methods

Calcium Enriched Media Preparation

The calcium enriched media was prepared by making a 1M CaCl₂ stock solution by dissolving 5.549g of anhydrous CaCl₂ in 50ml of plain α -MEM media. The desired serial dilutions of 3.0mmol, 0.3mmol, and 0.03mmol free calcium ions were then made by taking 5 μ l of 1M stock solution into 5ml of plain α -MEM media to form the 3.0mmol Ca media. Then 500 μ l of the 3.0mmol Ca media was added to 4.5ml of plain α -MEM to form the 0.3mmol Ca media. Finally, 500 μ l of the 0.3mmol Ca media was added to 4.5ml of plain α -MEM to form the 0.03mmol Ca media.

Calcium Enriched Media Experiments

The three previous experiments were all conducted using calcium enriched media as previously described in each chapter's Materials and Methods section. The calcium enriched media was used as an additional experimental condition in each experimental trial.

Results

Calcium Enriched Media Significantly Increased SCAP Cell Migration

The results for the Calcium Enriched Media transwell migration assay are described in relative fluorescence units (RFU) and are listed in Table 1. The 3.0 mmol calcium enriched media group was cytotoxic and therefore showed no significant migration at any time point. No significant increase in SCAP cell migration was seen in either the 0.3mmol or 0.03mmol calcium enriched media groups as compared to the plain control for the time points from 30 minutes to 12 hours (Figures 27-31). The 0.3mmol calcium enriched media group showed a significant increase in migration starting at 24 hours ($P = 0.001$, Figure 32), 48 hours ($P = <0.001$, Figure 33), and 72 hours ($P = 0.003$, Figure 34). The 0.03mmol calcium enriched media group also showed a significant increase in SCAP cell migration starting at 24 hours ($P = 0.011$, Figure 32), 48 hours ($P = <0.001$, Figure 33), and 72 hours ($P = 0.006$, Figure 34).

Calcium Enriched Media Significantly Increased SCAP Cell Proliferation

The results for the Calcium Enriched Media WST-1 proliferation assay are described as absorbance at (A450nm-A685nm) and are listed in Table 2. The 3.0 mmol calcium enriched media group was cytotoxic and therefore showed no SCAP cell proliferation at any time point. Only the 0.03mmol calcium enriched media group showed a significant increase in SCAP cell proliferation at the 7 day time point ($P = 0.006$, Figure 38). All other readings showed no significant effect on proliferation for either calcium enriched media group (Figures 35-41).

Calcium Enriched Media had No Effect on SCAP Cell Differentiation

The results for the Calcium Enriched RT-PCR Analysis are displayed in Figure 42. The 3.0 mmol calcium enriched media group was cytotoxic and no bands were seen in any gels for all three time points. The Odontoblastic markers DSPP, DMP-1, MEPE are present in all samples and at all time points. Both groups show a higher expression of MEPE, followed by DMP-1, then DSPP at all-time points. No difference in expression was seen in any marker for either calcium enriched media group and the controls (Figures 43-51). A sample of whole SCAP cells, which were taken directly from a flask as a control is shown on the 14 day gel. They show the same levels of expression as the calcium enriched media group and both the plain media and FBS media groups (Figures 42 and 46-48).

In Summary we found that SCAP cells exposed to 0.3mmol or 0.03mmol calcium enriched media showed a statistically significant difference in migration after 24 hours and that SCAP cells exposed to 0.03mmol calcium enriched media showed a statistically significant difference in proliferation at 7 days, but no induction was seen in terms of dentinogenesis differentiation.

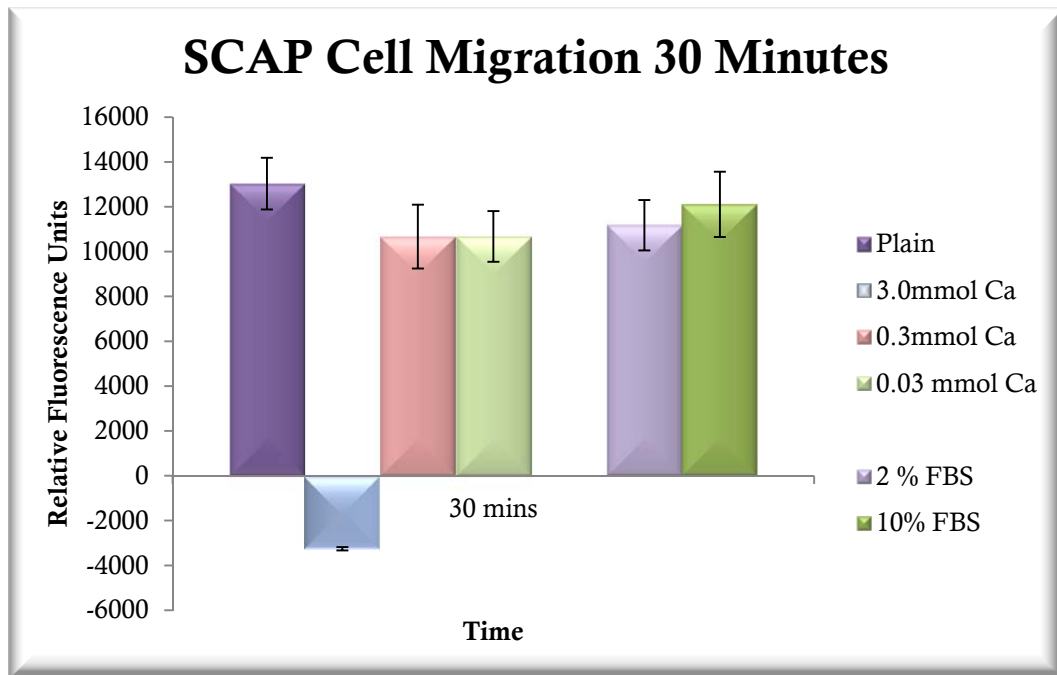


Figure 27. SCAP Cell Migration at 30 Minutes.

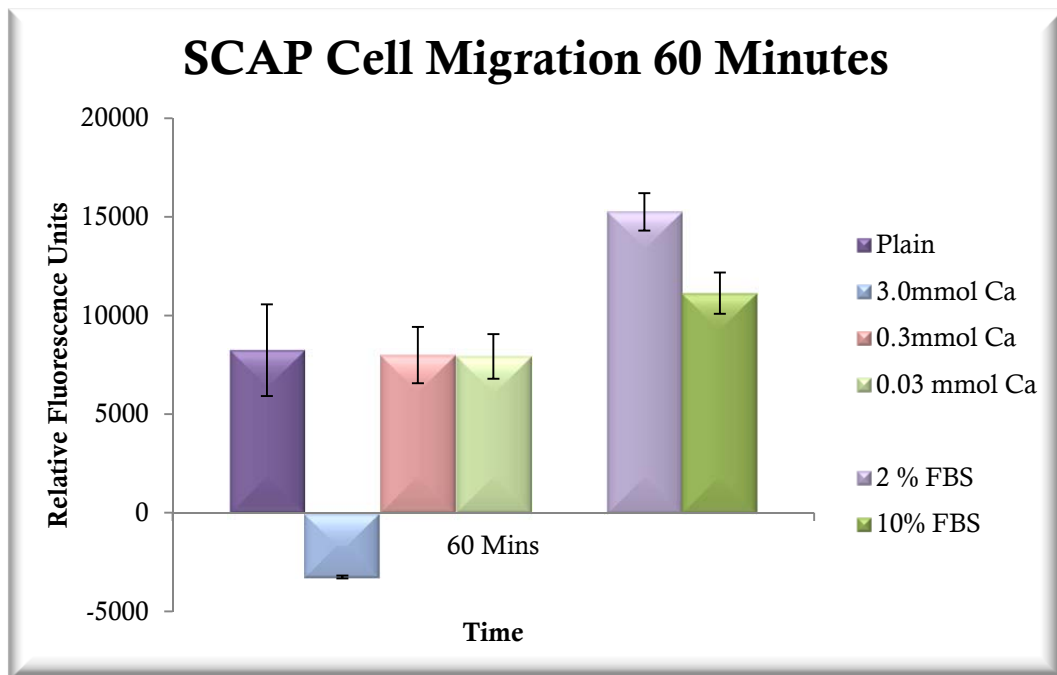


Figure 28. SCAP Cell Migration at 60 Minutes.

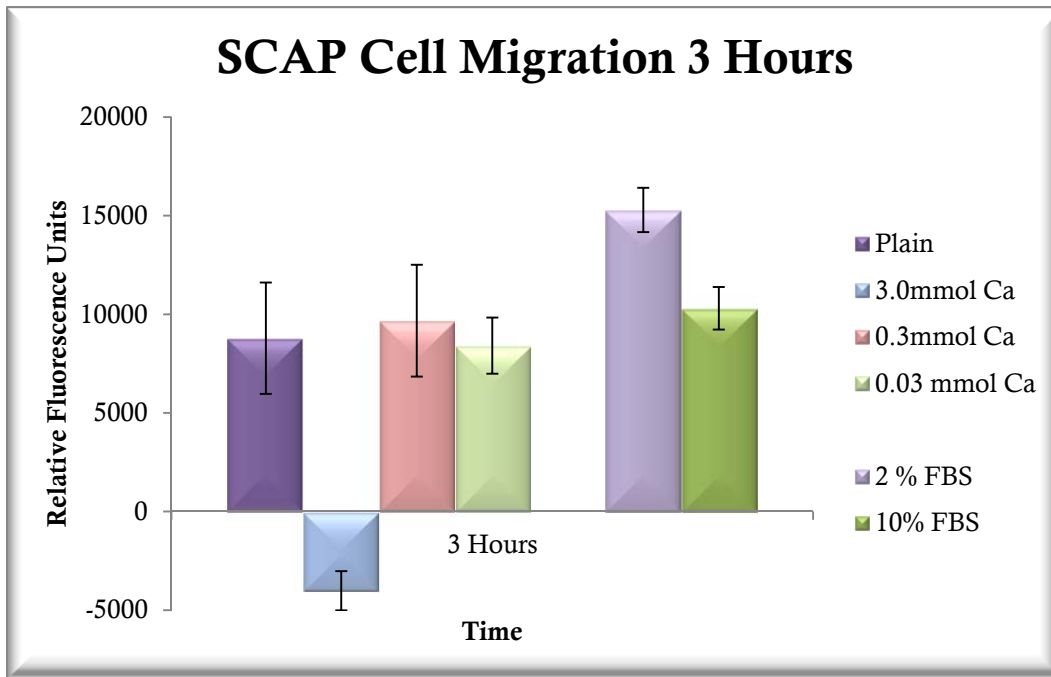


Figure 29. SCAP Cell Migration at 3 Hours.

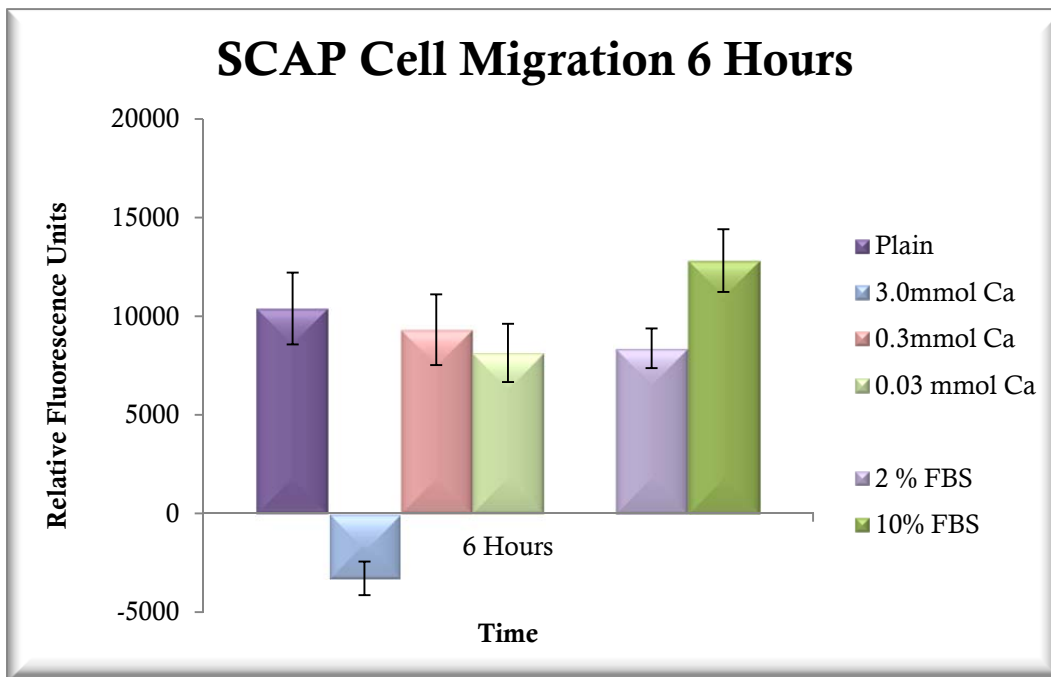


Figure 30. SCAP Cell Migration at 6 Hours.

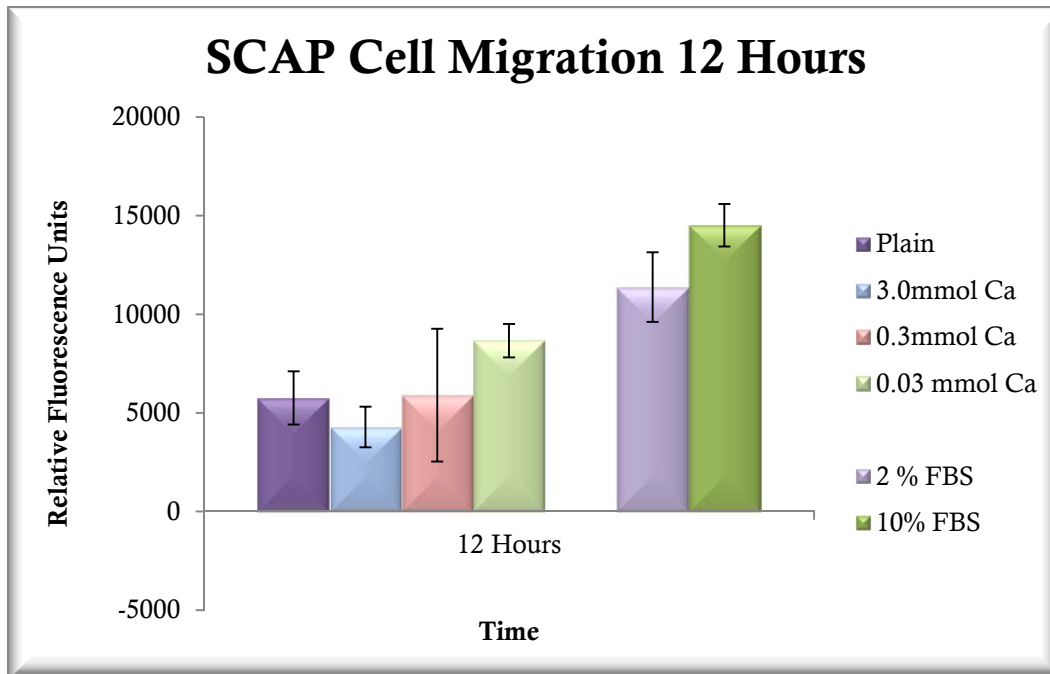


Figure 31. SCAP Cell Migration at 12 Hours.

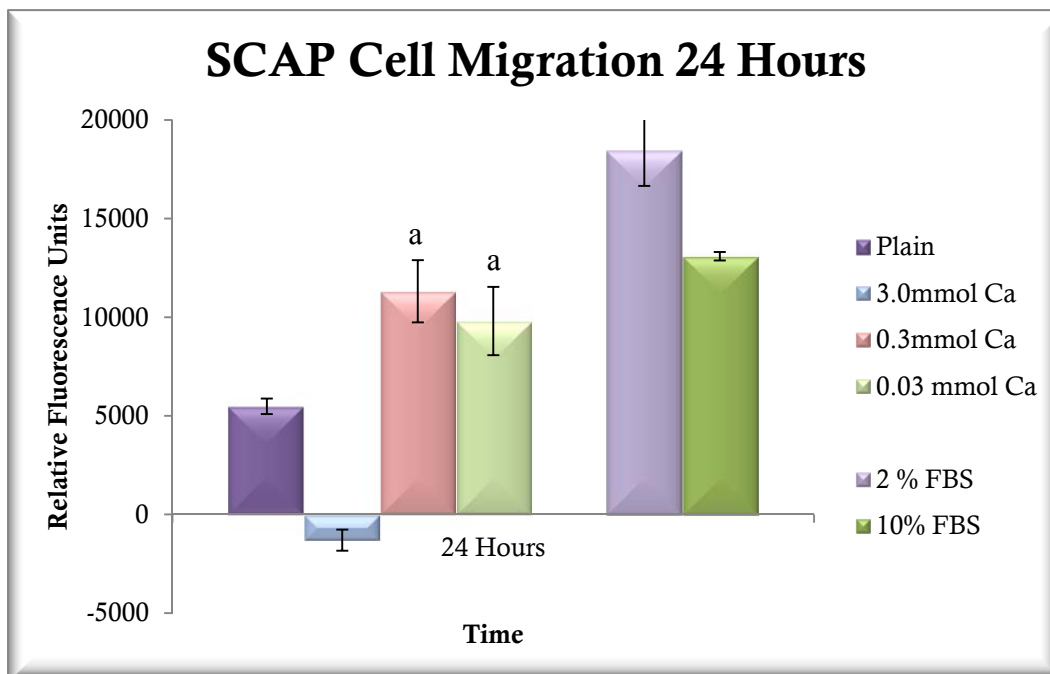


Figure 32. SCAP Cell Migration at 24 Hours. There was a SS difference in migration when the cells were seeded with 0.3 and 0.03mmol Ca enriched media, a $P \leq 0.05$ compared with Plain control

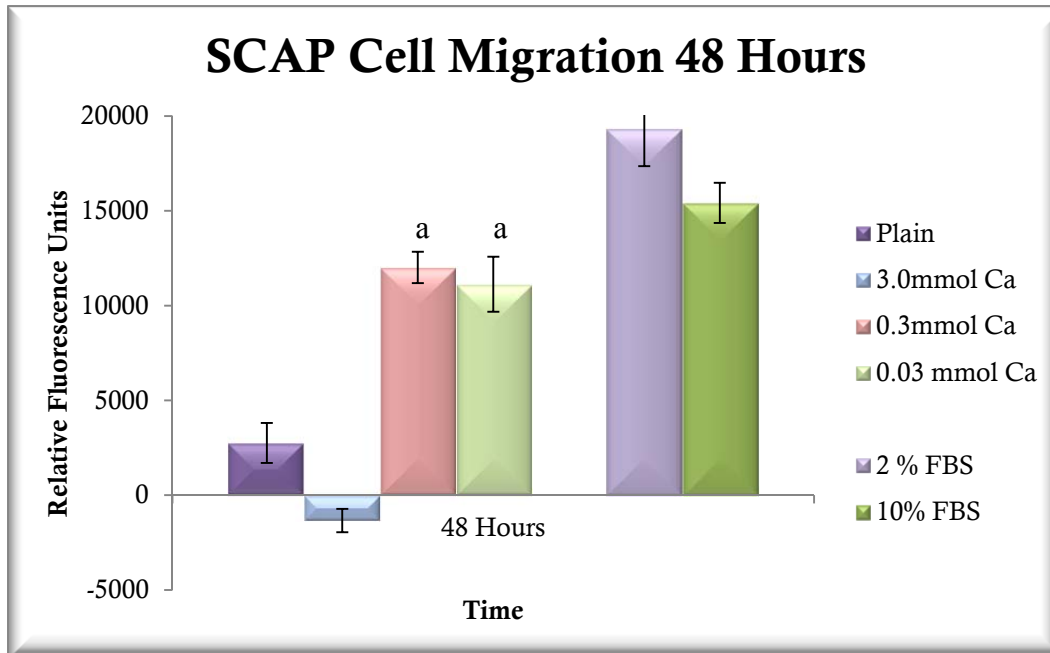


Figure 33. SCAP Cell Migration at 48 Hours. There was a SS difference in migration when the cells were seeded with 0.3 and 0.03mmol Ca enriched media, a $P \leq 0.05$ compared with Plain control

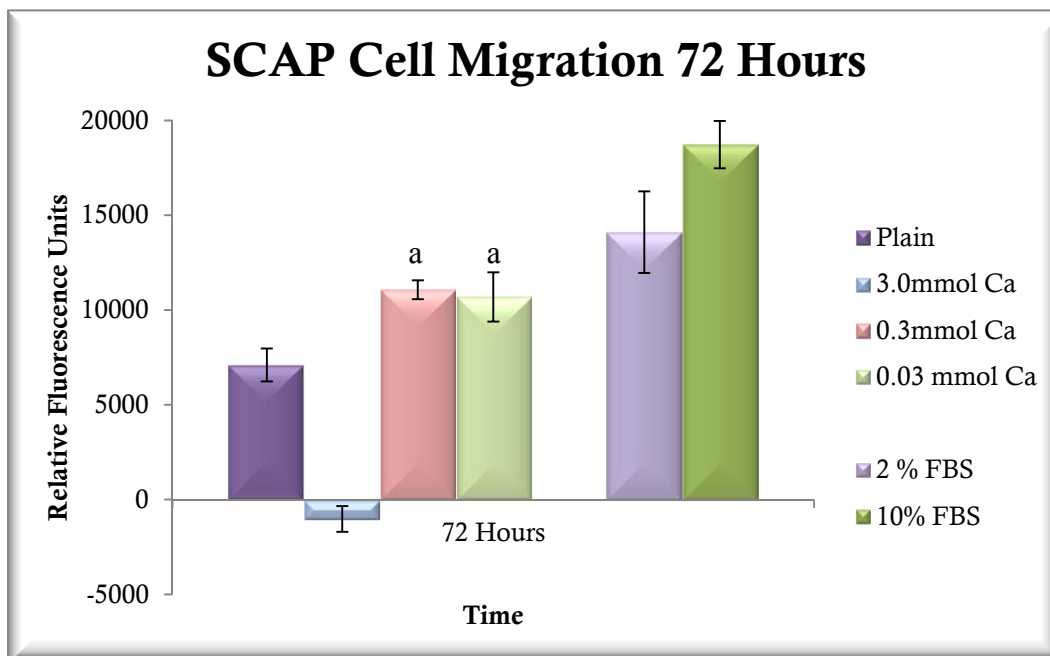


Figure 34. SCAP Cell Migration at 72 Hours. There was a SS difference in migration when the cells were seeded with 0.3 and 0.03mmol Ca enriched media, a $P \leq 0.05$ compared with Plain control

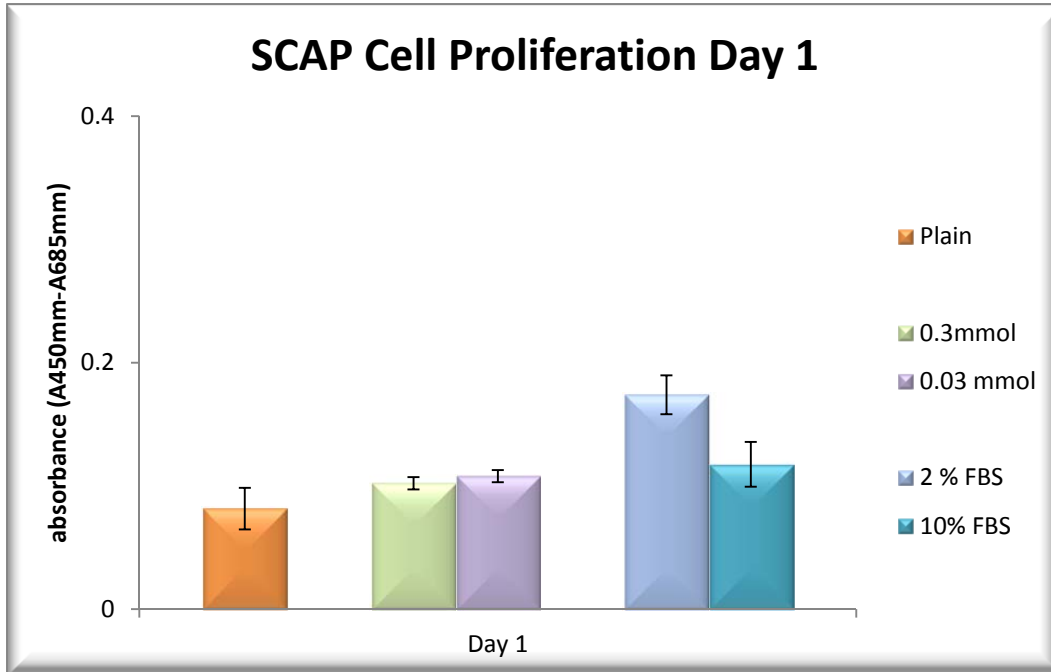


Figure 35. SCAP Cell Proliferation at Day 1

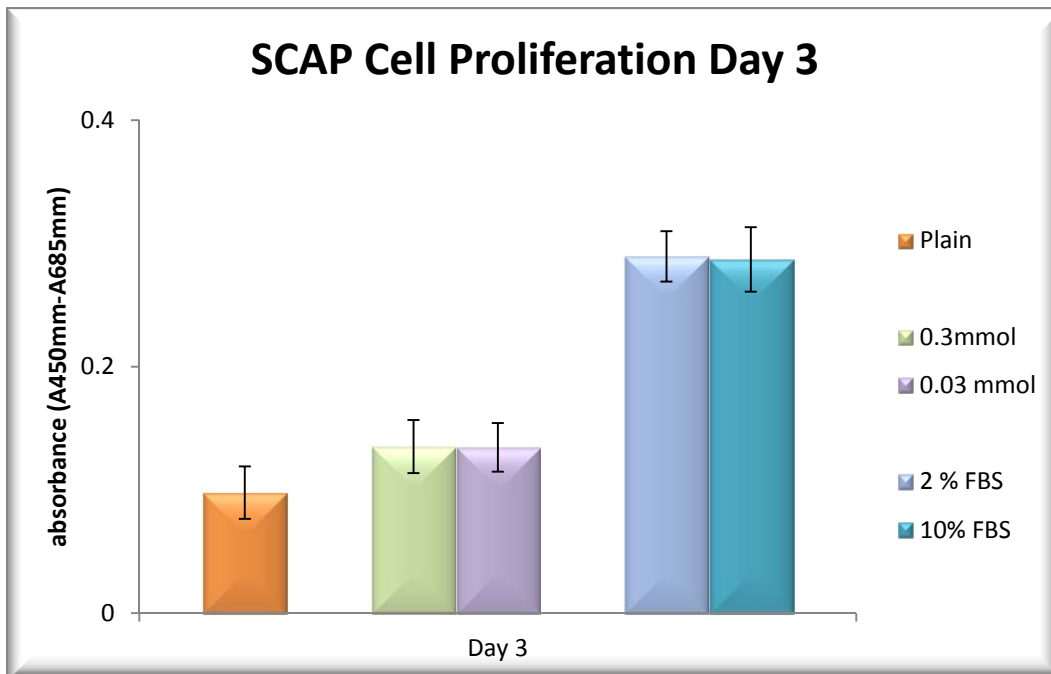


Figure 36. SCAP Cell Proliferation at Day 3

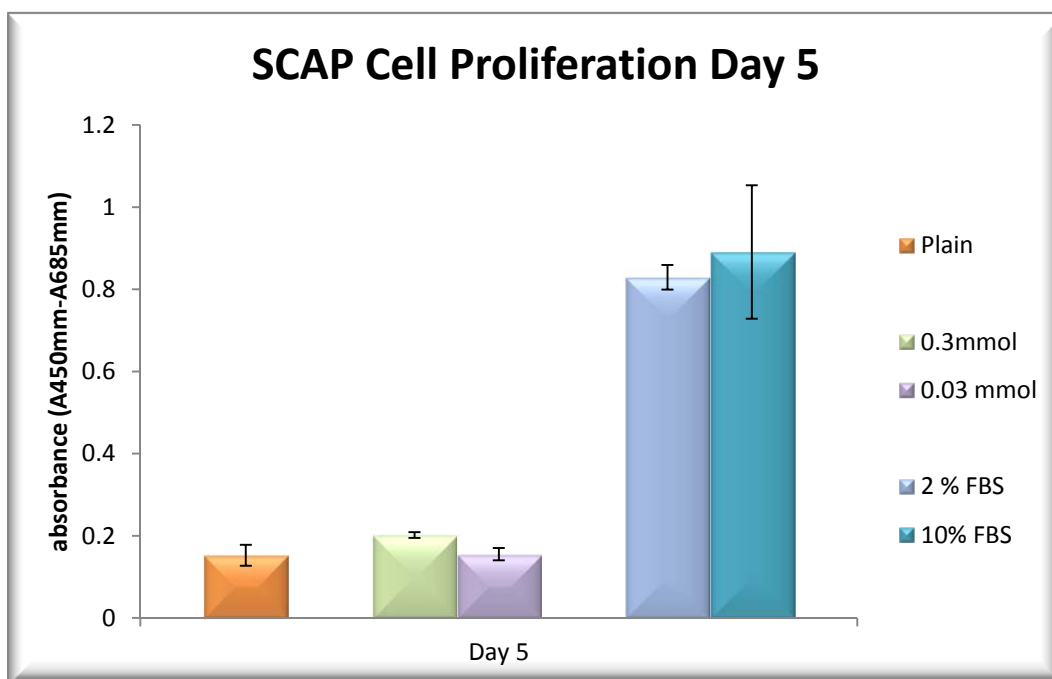


Figure 37. SCAP Cell Proliferation at Day 5

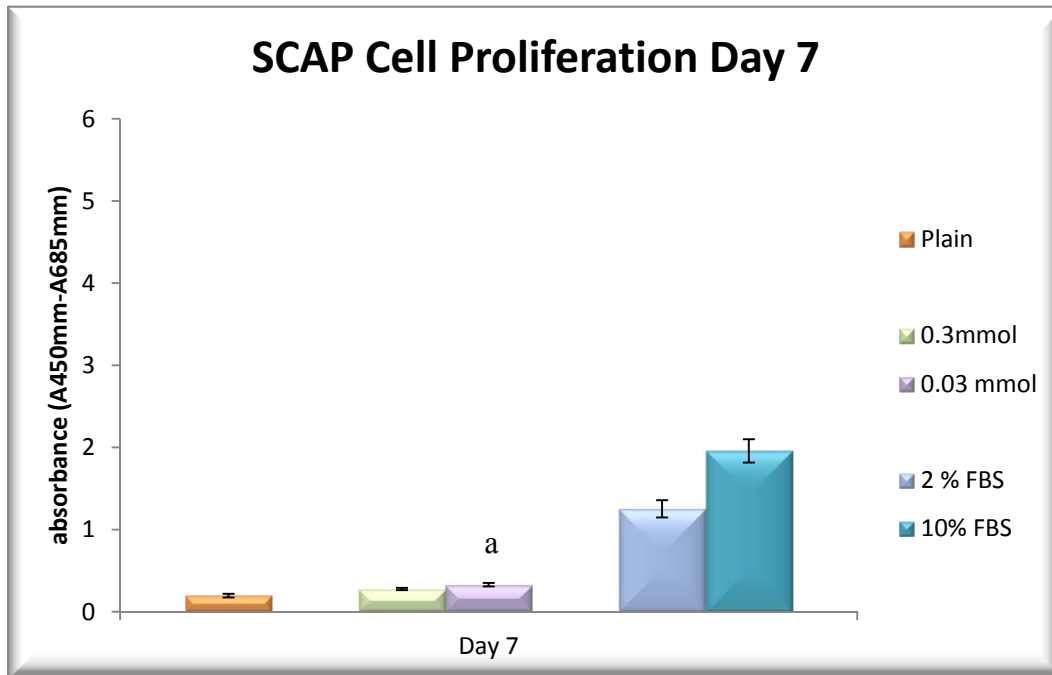


Figure 38. SCAP Cell Proliferation at Day 7. There was a SS difference in proliferation when the cells were seeded with 0.3 and 0.03mmol Ca enriched media, a $P \leq 0.05$ compared with Plain control.

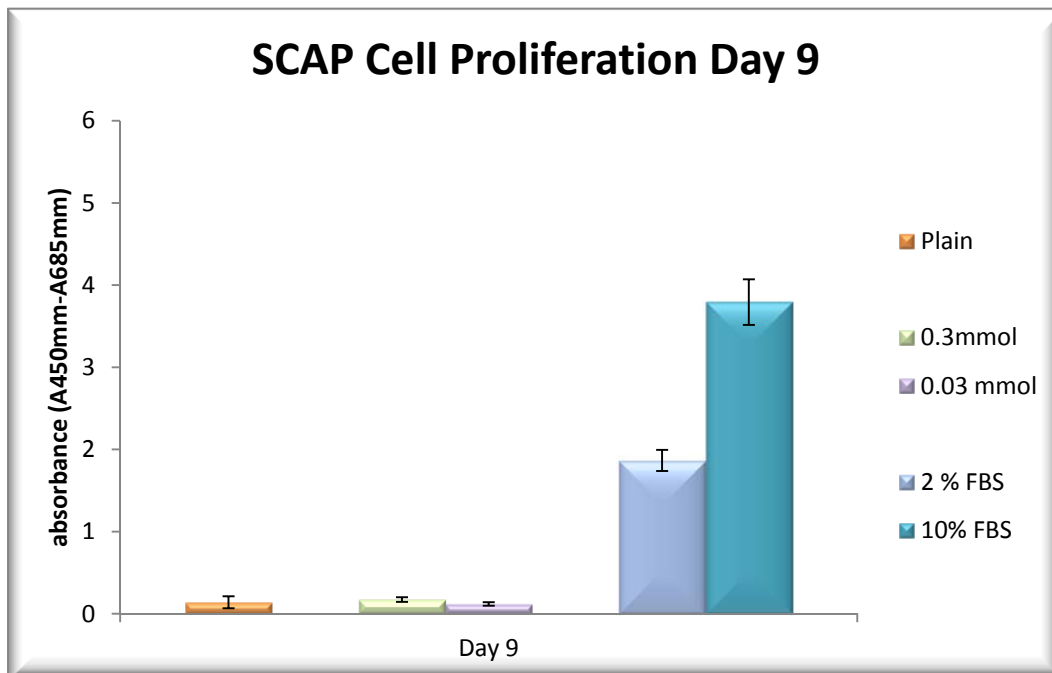


Figure 39. SCAP Cell Proliferation at Day 9

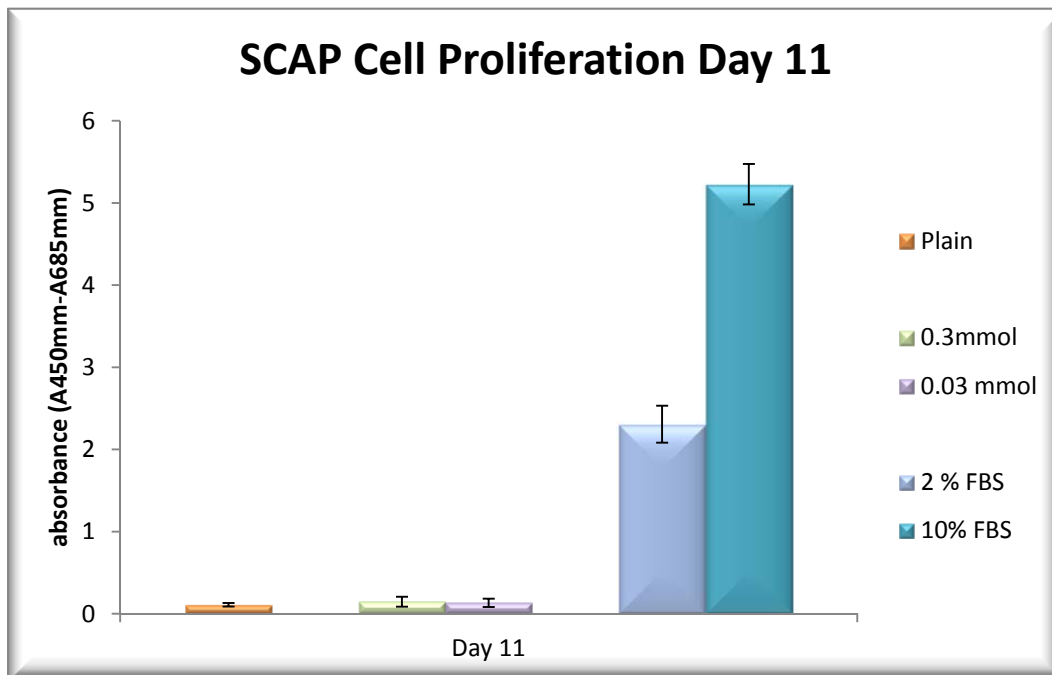


Figure 40. SCAP Cell Proliferation at Day 11

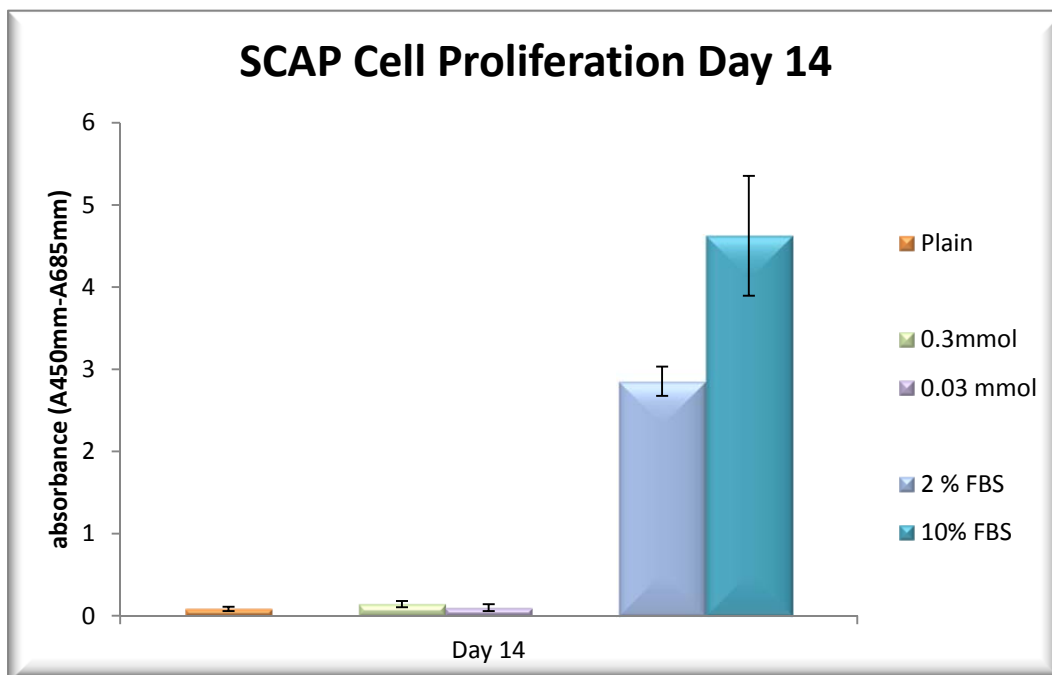
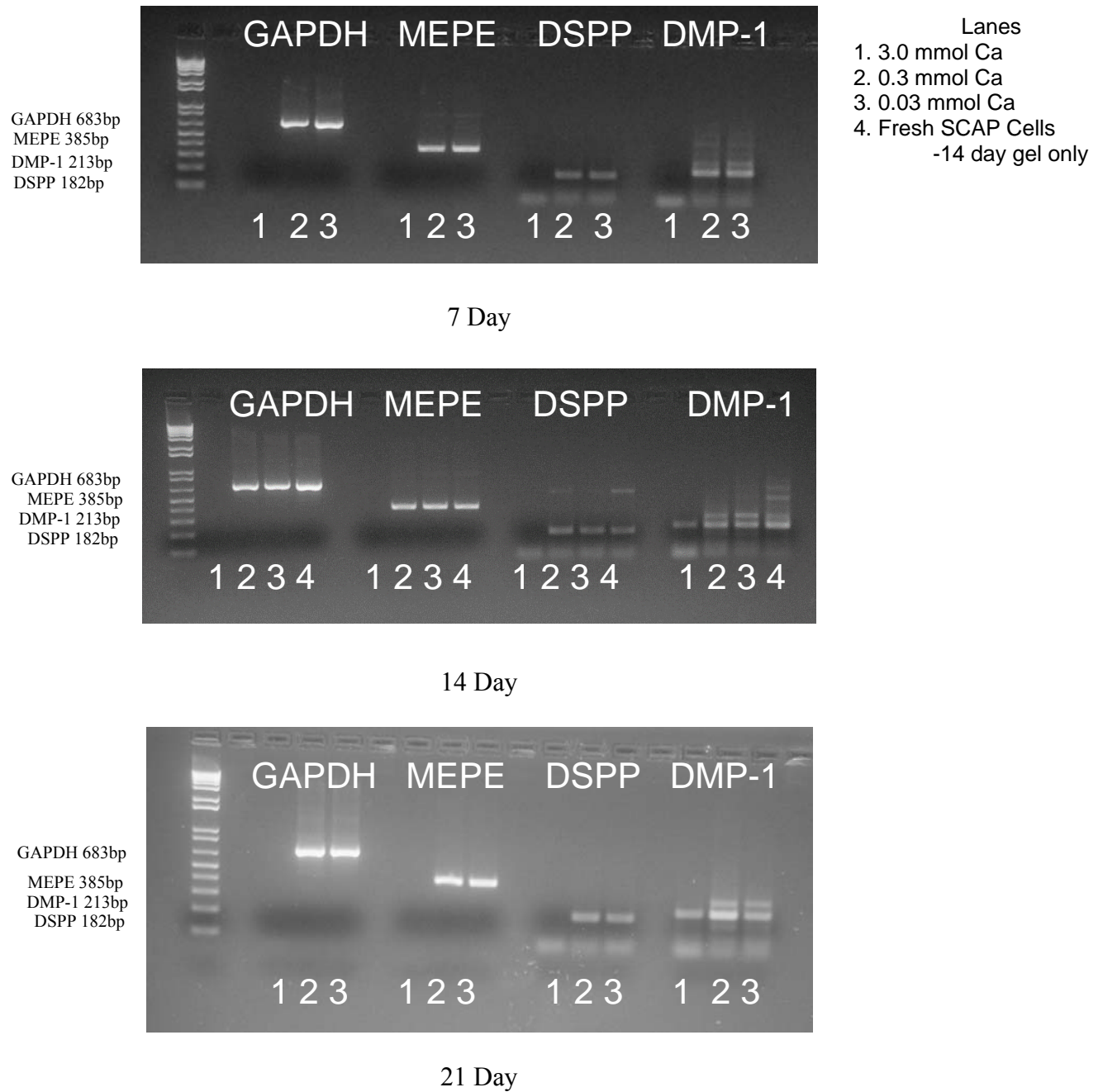


Figure 41. SCAP Cell Proliferation at Day 14

SCAP Cell Differentiation in Ca Media



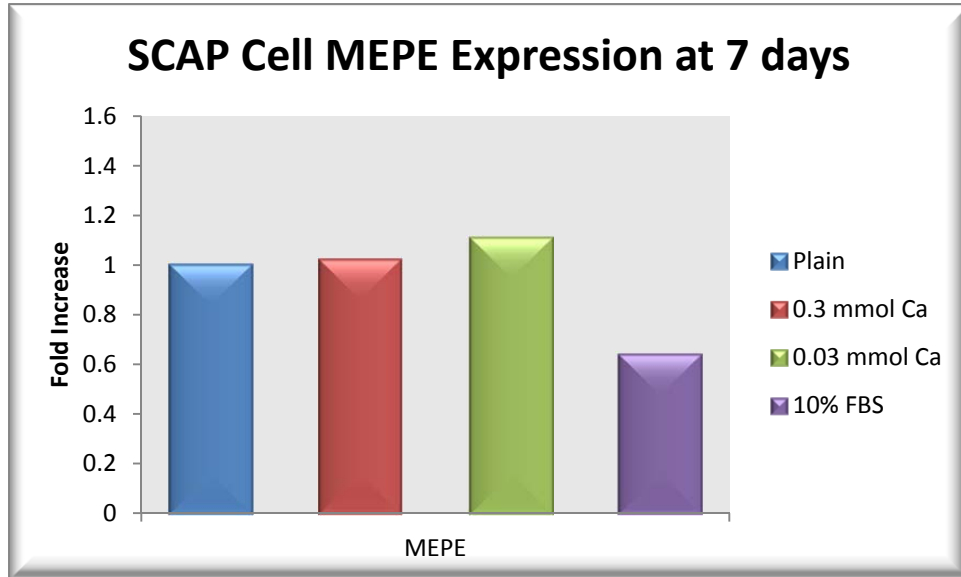


Figure 43. SCAP Cell MEPE Expression after 7 days of exposure to Calcium Enriched Media as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

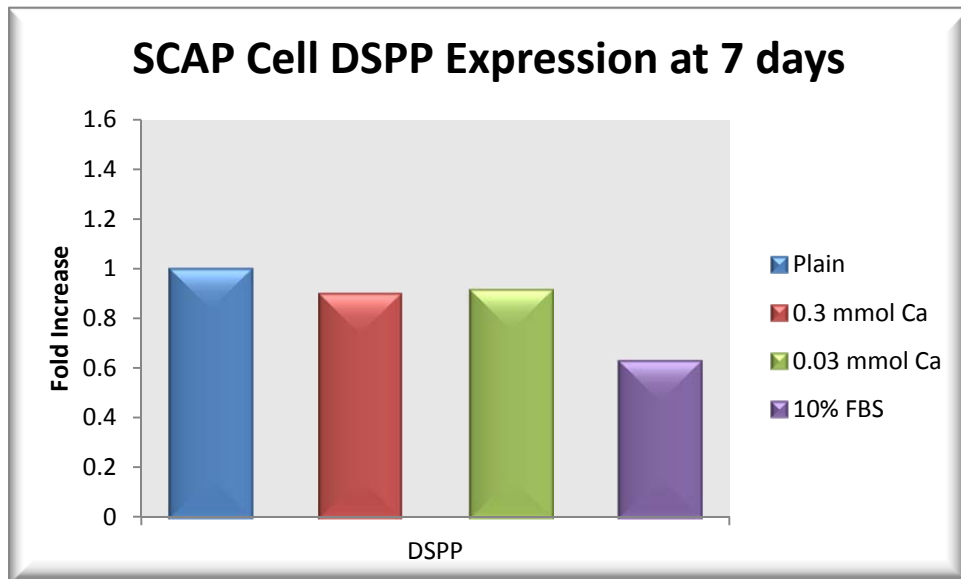


Figure 44. SCAP Cell DSPP Expression after 7 days of exposure to Calcium Enriched Media as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

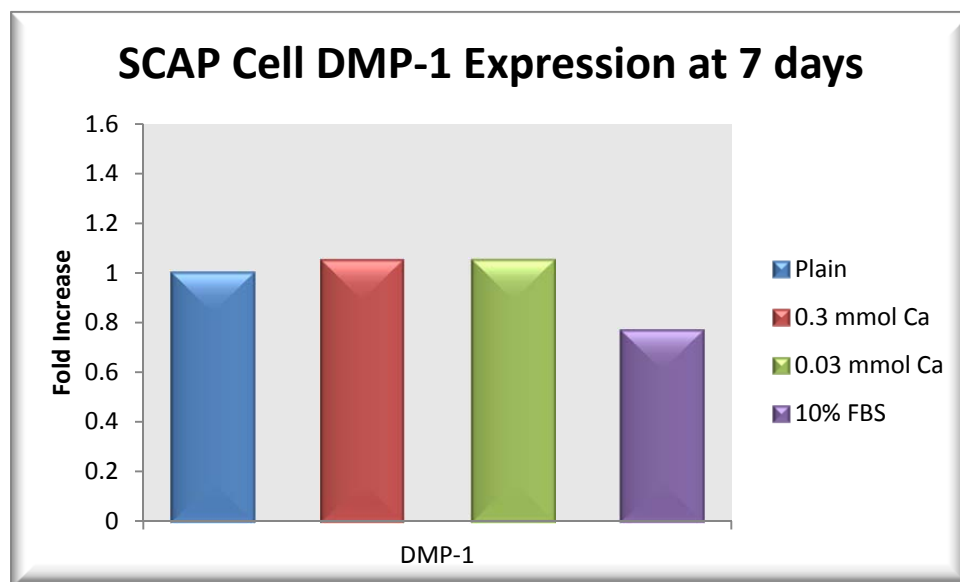


Figure 45. SCAP Cell DMP-1 Expression after 7 days of exposure to Calcium Enriched Media as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

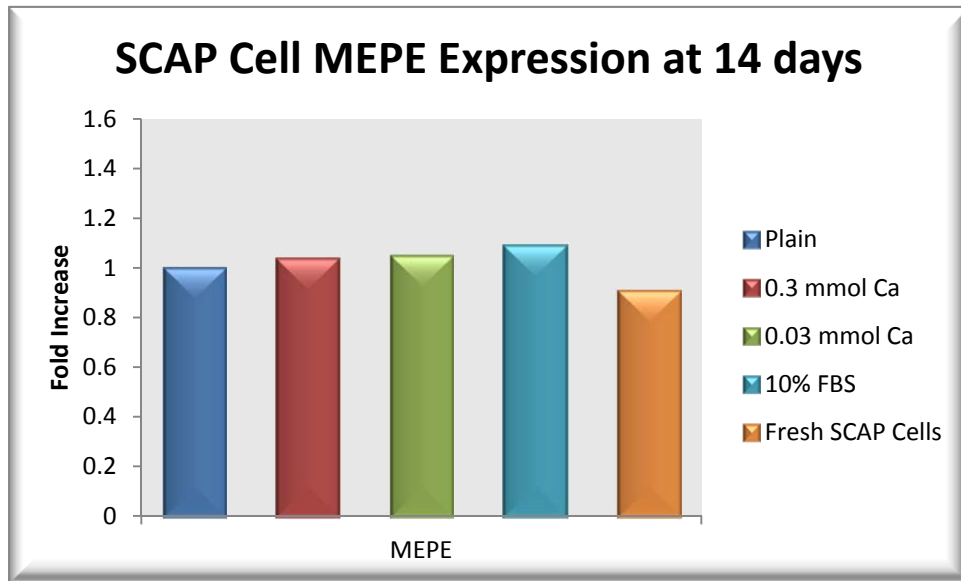


Figure 46. SCAP Cell MEPE Expression after 14 days of exposure to Calcium Enriched Media as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

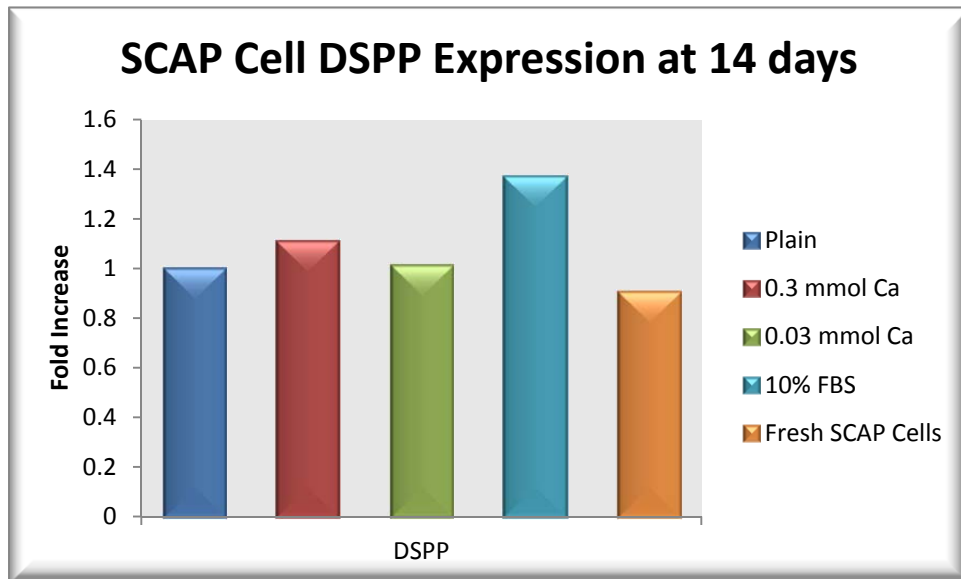


Figure 47. SCAP Cell DSPP Expression after 14 days of exposure to Calcium Enriched Media as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

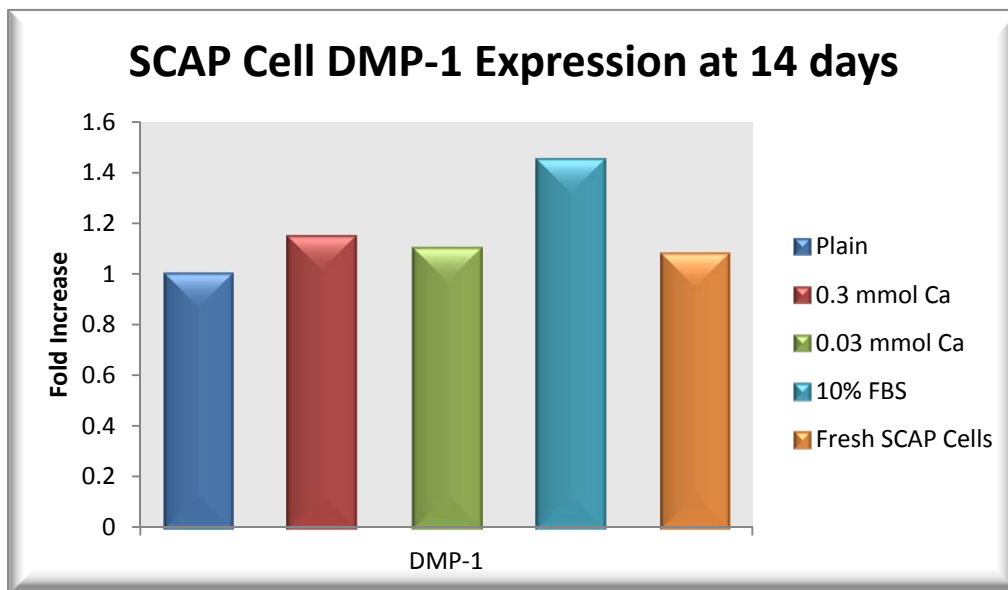


Figure 48. SCAP Cell DMP-1 Expression after 14 days of exposure to Calcium Enriched Media as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

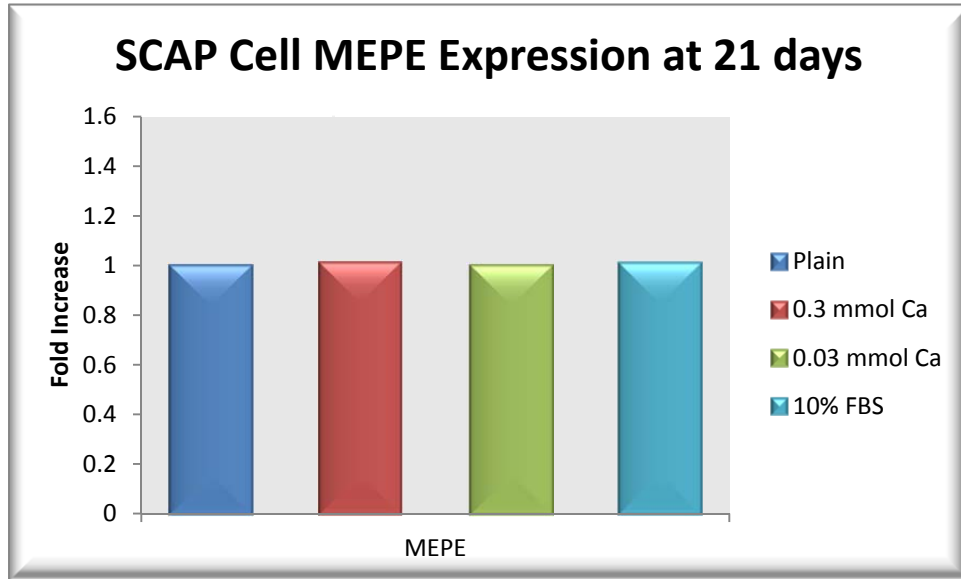


Figure 49. SCAP Cell MEPE Expression after 21 days of exposure to Calcium Enriched Media as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

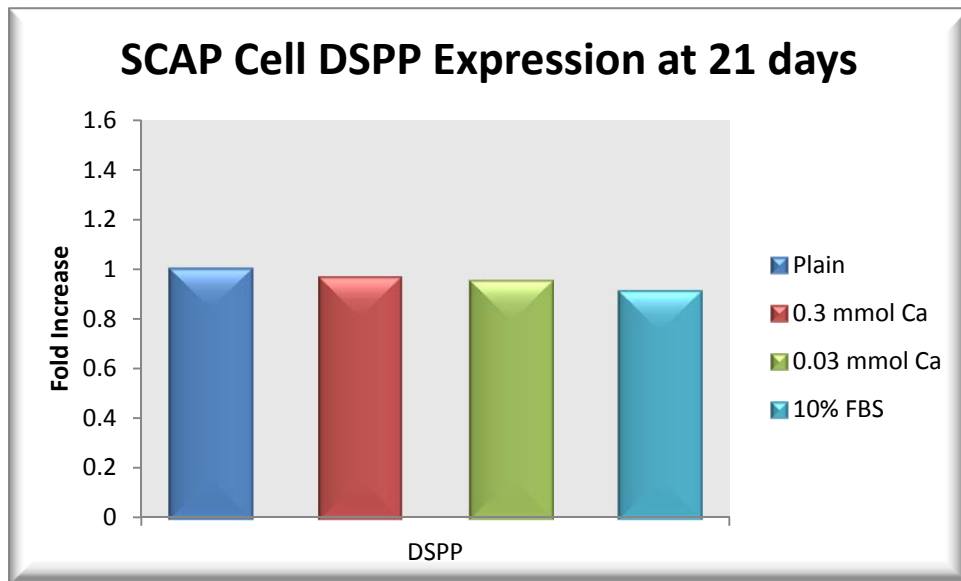


Figure 50. SCAP Cell DSPP Expression after 21 days of exposure to Calcium Enriched Media as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

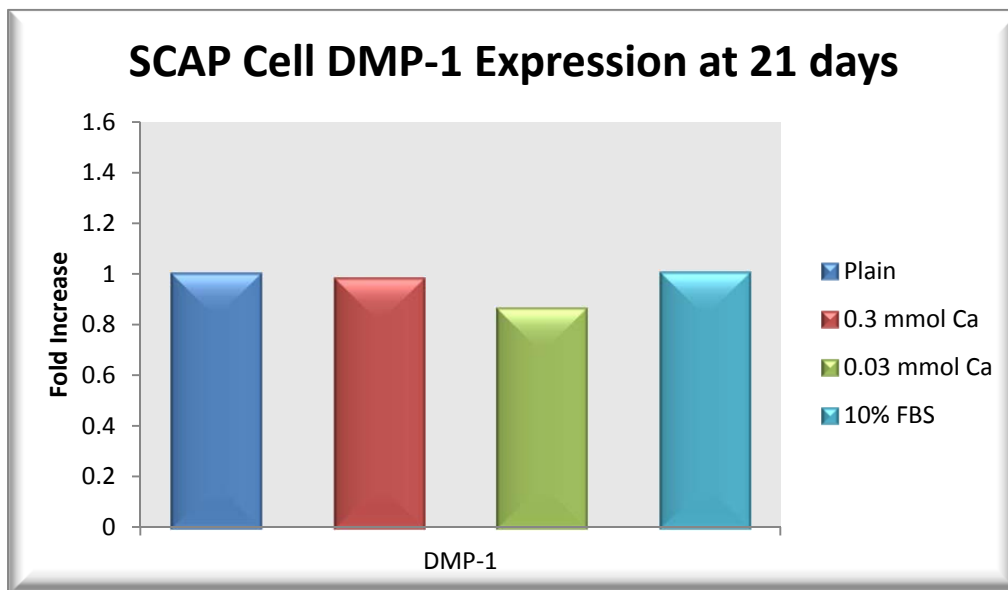


Figure 51. SCAP Cell DMP-1 Expression after 21 days of exposure to Calcium Enriched Media as shown by densitometric analysis of RT-PCR data as compare to the untreated plain group and normalized to GAPDH.

Chapter 6 –QUESTIONS RAISED DURING EXPERIMENTATION

During the experimental stages of this research, several questions arose regarding the results we found and those in previous reports. The expression of odontoblastic markers found in untreated SCAP cells by RT-PCR led us to question whether our cell population had already undergone differentiation or was a mixed population with a lower number of stem cells. We decided to characterize the cell population by using flow cytometry and to evaluate cells at different passages.

Materials and Methods

Flow Cytometric Analysis

SCAP cells from passages 2 and 10 were evaluated by Flow Cytometry to characterize the cell population of each passage. A buffer solution of 2% FBS in PBS (hereinafter “buffer”) was made for use in the dilution of the antibodies. The media from a T-75 flask containing passage 2 and 10 SCAP cells at 80% confluence was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100µl of re-suspended cells in 900µl of plain media into a 2ml Eppendorf tube. 5×10^5 cells were seeded into 5ml polystyrene round-bottom tubes (BD

Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The supernatant was discarded via gentle vacuum pipetting and the pellet of cells was re-suspended in 100µl of 5% bovine serum albumin (BSA) and incubated on ice for 30 minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting. The pellet of cells was re-suspended in 1ml of PBS, centrifuged for 5 minutes at 1000 RPM at 4°C, the supernatant was discarded via gentle vacuum pipetting, and this step was repeated with an additional 1ml of PBS (Hereinafter “wash cycle”). A control sample from each passage was then re-suspended with 100µl of buffer and incubated in the dark on ice until read by flow cytometry. The supernatant from the remaining tubes was discarded via gentle vacuum pipetting and the samples divided into 3 groups based on the antibody staining received. In the CD146 group, the pellet of cells was re-suspended in 100µl of 1:11 (1µl antibodies/11µl buffer) human CD146-APC antibodies (Milenyi Biotec, Auburn, CA) and incubated in the dark on ice for 30 minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting and a wash cycle performed. The supernatant was discarded via gentle vacuum pipetting and samples from each passage were then re-suspended with 100µl of buffer and incubated in the dark on ice until read by flow cytometry. Next, in the CD24 group the pellet of cells was re-suspended in 100µl of 2:10 (2µl antibodies/10µl buffer) PE mouse anti-human CD24 antibodies (BD Pharmingen, San Jose, CA) and incubated in the dark on ice for 30 minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting and a wash cycle performed. The supernatant was discarded via gentle vacuum pipetting and samples

from each passage were then re-suspended with 100 μ l of buffer and incubated in the dark on ice until read by flow cytometry. Then, in the STRO-1-FITC group the pellet of cells was re-suspended in 100 μ l of 1:25 (1 μ l antibodies/25 μ l buffer) mouse anti-STRO-1 primary antibodies (Invitrogen Corp., Carlsbad, CA) and incubated in the dark on ice for 60 minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting and a wash cycle performed. The supernatant was discarded via gentle vacuum pipetting and a control sample from each passage was then re-suspended with 100 μ l of buffer and incubated in the dark on ice until read by flow cytometry. The pellet of cells in the remaining tubes were re-suspended in 100 μ l of 1:100 (1 μ l antibodies/100 μ l buffer) FITC rabbit anti-mouse IgG conjugate antibodies (Invitrogen Corp., Carlsbad, CA) and incubated in the dark on ice for 30 minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting and a wash cycle performed. The supernatant was discarded via gentle vacuum pipetting and samples from each passage were then re-suspended with 100 μ l of buffer and incubated in the dark on ice until read by flow cytometry. A control tube of cells stained with only the FITC rabbit anti-mouse IgG conjugate antibodies for each passage was also made. All samples were re-suspended with a pipette prior to flow cytometry. The samples were processed at the University of Michigan Life Science Center Flow Cytometry Core on a BD FACSAria3 Flow Cytometry machine using the BD FACSDiva software and 10,000 events. This experiment was repeated 4 times in triplicate.

Results

The Number of CD24+ Cells Decreased to Zero by Passage 10

The results for the Flow Cytometric Analysis are displayed as the percentage of cells in total sample population and shown in Figure 52. The percentage of cells that were CD24+ in passage 2 was 3.9%, which decreased to 0% in the passage 10 cells. Passage 2 cells were CD146+ in 73.2% of the population decreasing to 60% of the cell population in the passage 10 cells. Only 5.1% of the cell population was STRO-1+ in the passage 2 cells. This number decreased to 0.8% in the passage 10 cells.

In Summary we can conclude that the cells used for most of the experiments were between passages 4 and 6 and that they were of a mixed population where the percentage of stem cells, analyzed by CD24, CD146 and STRO-1 markers, was reduced while the experiments were conducted.

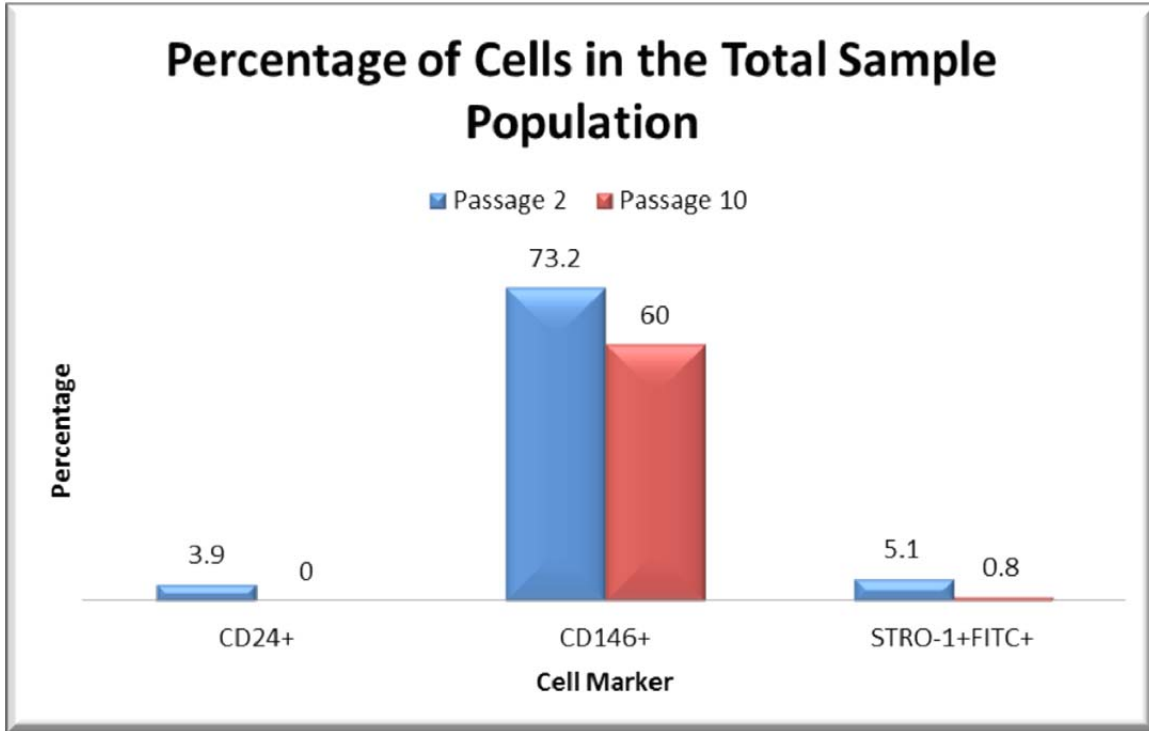


Figure 52. Percentage of positive CD24, CD146, and STRO-1 cells in the Total Sample Population.

While many studies reported significant increases in both migration and proliferation of cells with MTA, our results were more subtle. We noted the extensive use of Gray MTA (12, 17, 19, 27) instead of WMTA as well as the fact that in some studies the cells were in direct contact with the MTA (12, 17, 38). Also, these studies used a much larger amount of MTA. So we began to question whether the GMTA has a different effect on cells than WMTA, whether the direct contact enhances this effect, and whether the amount of MTA used has any bearing on the effects seen. Here we considered it important to evaluate our WMTA results to GMTA as pilot studies to further explore these questions.

Materials and Methods

Gray MTA Pilot Studies

SCAP cell proliferation in the presence of gray MTA (GMTA) (Gray MTA ProRoot Dentsply Endodontics, Tulsa, OK) was evaluated using the WST-1 proliferation assay method. The media from a T-75 flask containing passage 4 SCAP cells at 80% confluence was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100µl of re-suspended cells in 900µl

of plain media into a 2ml Eppendorf tube. 4×10^3 cells were seeded into the wells of 12 well companion plates (BD Falcon, San Jose, CA) with 1.5ml of media in the following test conditions: 7 sets of 3 wells containing 2% FBS media, 1 set of 3 wells containing plain media, and 1 set of 3 wells containing 10% FBS media. 3- μ m-size pore transwell cell culture inserts (BD Falcon, San Jose, CA) were placed into each well. Twenty-four hour set GMTA and WMTA pellets were placed into the transwell inserts of 6 sets of the wells containing 2% FBS media as follows: 3 pellets of GMTA, 6 pellets of GMTA, 9 pellets of GMTA; and 3 pellets of WMTA, 6 pellets of WMTA, 9 pellets of WMTA. Additionally, 4×10^3 cells were seeded into the wells of 12 well companion plates (BD Falcon, San Jose, CA) with 1.5ml of media in the following test conditions: 7 sets of 3 wells containing 2% FBS media, 1 set of 3 wells containing plain media, and 1 set of 3 wells containing 10% FBS media. Prior to cell seeding, GMTA and WMTA pellets were freshly mixed and placed wet into the 6 sets of the wells that would contain 2% FBS media as follows: 3 pellets of GMTA, 6 pellets of GMTA, 9 pellets of GMTA; and 3 pellets of WMTA, 6 pellets of WMTA, 9 pellets of WMTA. The wet GMTA and WMTA pellets were spread over the bottom surface of the well to provide a uniform thickness covering the bottom of the well. The plates were then placed in an incubator and allowed to set for 24 hours at 37°C in 5% CO₂ and 100% humidity. The plates were incubated at 37°C in 5% CO₂ and 100% humidity and the media changed every 3 days. The plates were evaluated 7 days via WST-1 proliferation assay.

The WST-1 proliferation assay was performed as previously described in Chapter 3. These experiments were repeated twice in triplicate.

Results

Indirect GMTA Significantly Increased SCAP Cell Proliferation

The results for the Gray MTA Pilot Studies are described as absorbance at (A450nm-A685nm). In the study where the MTA was placed in transwell-inserts, all 3 groups with GMTA showed significantly increased SCAP cell proliferation as compared to the WMTA groups ($P = <0.001$, Figure 53). All 3 WMTA groups showed significantly decreased proliferation as compared to the 2% FBS control ($P = <0.002$). No difference was seen between either the GMTA or WMTA groups when the MTA was placed directly in the well, however both GMTA and WMTA significantly decreased SCAP cell proliferation as compared to the 2% and 10% FBS controls ($P = <0.05$, Figure 54).

In Summary we found that all GMTA groups showed significantly increased SCAP cell proliferation as compared to the WMTA groups after 7 days of exposure.

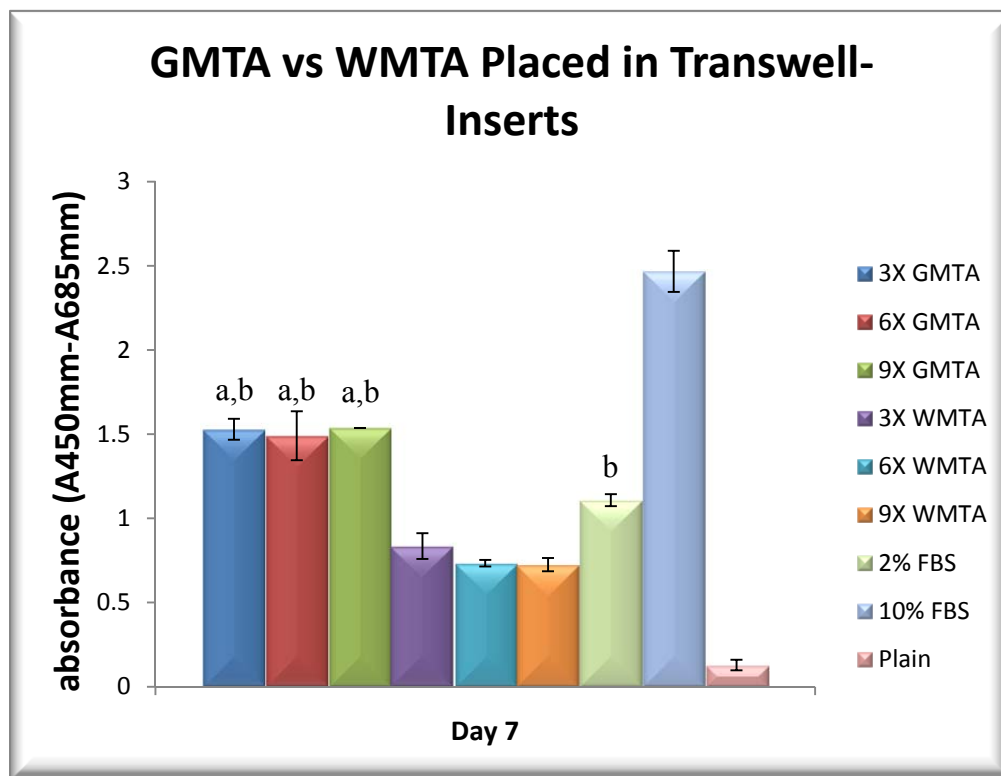


Figure 53. GMTA vs WMTA proliferation when in Indirect (Transwell Inserts) Contact with SCAP Cells. There was a SS increase in proliferation seen with all GMTA groups, a $P \leq 0.05$ compared with 2% FBS control, b $P \leq 0.05$ compared with all WMTA groups

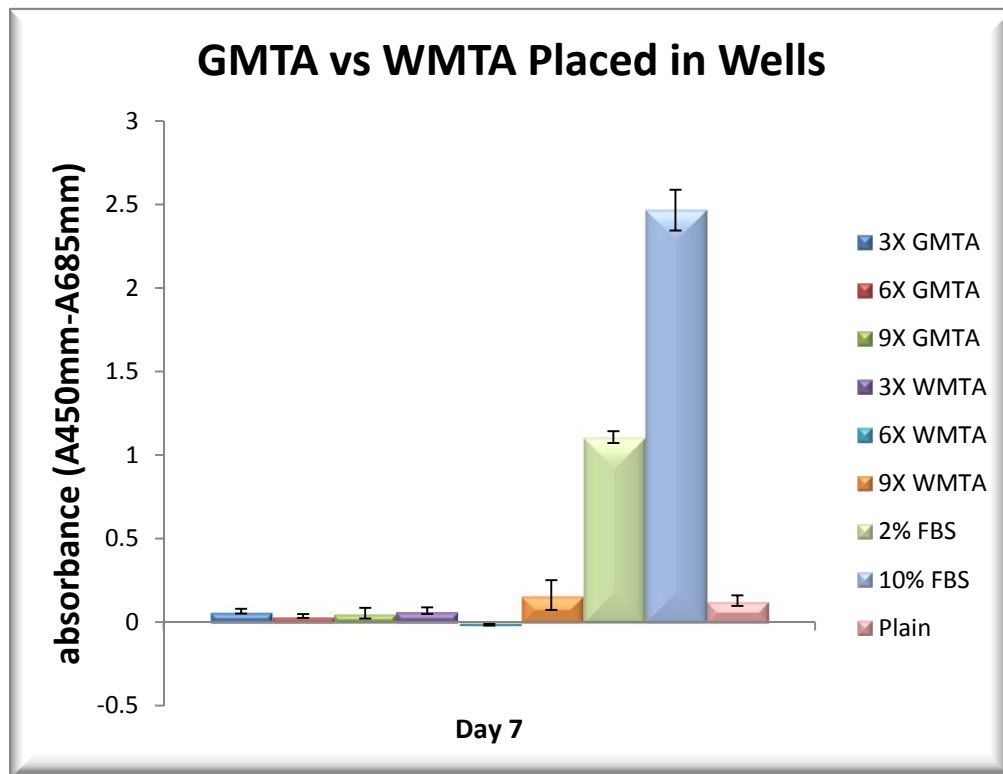


Figure 54. GMTA vs WMTA proliferation when in Direct Contact with SCAP Cells.

Chapter 7 – DISCUSSION

Pulpal regeneration in the immature tooth aims to ensure the continued development of the root, gaining width of the dentinal walls and length of the root. The increased width of dentin will help in strengthening the weak and thin root structure that is often seen in these cases (35, 39). The source of the stem cells responsible for the continued development of the root structure has yet to be identified, however SCAP cells have been postulated as a likely source (34). The properties of SCAP cells regarding their robust growth and multi-potentiality make them an attractive target for tissue regeneration and other efforts to enhance the regeneration of the pulp-dentin complex (14, 15, 40). Efforts to increase the attraction and differentiation of SCAP cells are being investigated more commonly today, including growth factors such as basic fibroblast growth factor (bFGF) and materials such as MTA (17, 40). MTA has been shown to promote cell growth and odontoblastic differentiation, two very important processes for pulp regeneration (17, 41, 42). The possible synergistic interaction between the potent SCAP cells and MTA to enhance the regeneration of the pulp-dentin complex is what inspired this study.

In this study, we were able to show a significant increase in SCAP cell migration after 6 hours and proliferation after 24 hours of exposure to WMTA. We also demonstrated that calcium enriched media significantly increased SCAP cell migration after 24 hours. Under the conditions used here with a mixed population of SCAP cells, we did not find any odontoblastic differentiation as analyzed by RT-PCR gene expression. Through the use of Flow Cytometry, we were able to characterize our population of mixed cells and show the decrease of stem cell markers commonly used to identify SCAP

cells. We also demonstrated that GMTA had a stronger effect than WMTA on SCAP cell proliferation.

After 6 hours, we found an increase in SCAP cell migration in contact with media containing 1 or 24 hour set WMTA. At 1/2, 1, 3, and 12 hour periods, we saw an increased trend in cell migration, but it was not statistically significant. Our results are consistent with previous studies that have shown an increase in migration with the use of WMTA (17), although we did not see as strong of an increase. This could be due to the fact that in that study hMSCs were used, which could have inherently different migration ability or stem cell proportions; or the difference in laboratory technique as the MTT Assay was used to measure cell migration. Our migration results comparing media containing FBS with plain media are consistent with the literature.

Our study showed a significant increase in SCAP cell proliferation for either 1 or 24 hour set WMTA in plain media and for the 24 hour set WMTA in plain media at 5 days. Previous reports in the literature have shown little effect of WMTA on cell proliferation until 21 days and the MTA used was not cytotoxic to cells during the earlier time points tested (12, 17, 27, 43). With the exception of the day 1 and day 5 readings, we are in agreement with these results. It should also be noted that the previous studies used FBS containing media and our findings using 10% FBS are in agreement with these results. It is our plain media results that differ from these studies which used FBS containing media and no other studies have investigated the effect of WMTA using plain media. One of the most interesting findings in our study is the dramatic impact that FBS has on SCAP cell proliferation, even at a lower concentration of 2%. Other reports in the literature have used GMTA and shown a significant increase in the proliferation of the

cell types studied (12, 27). It should also be noted that no one has studied the effect of MTA on SCAP cells directly and only hDPCs and hMSCs have been used for those studies (12, 17, 18). While the cell types are very similar if not related in lineage, there may be untold differences.

It was the disparity in the use of GMTA that led us to complete 2 different pilot studies using GMTA. After reviewing the literature, a number of issues became clear. Many of the studies used much larger amounts of MTA than we did; the MTA was in direct contact or indirect contact with the cells; and the GMTA seemed to more dramatically influence the cells than the WMTA did. Our results with GMTA indirectly contacting SCAP cells show the same significant increase as the other studies have shown (27). Our results do agree with the literature regarding direct contact with MTA (12, 17) as no significant increase was seen after 7 days.

The results of our RT-PCR Analysis were unexpected. We saw expression of the Odontoblastic markers DSPP, DMP-1, MEPE in all samples and at all time points, even on the untreated cells. These cells showed the same expression pattern and this pattern even held true for our calcium enriched media groups. These results were not consistent with the odontoblastic differentiation that other studies have found (18, 19, 41, 44). This pattern seemed to suggest that we had a mixed cell population with a low percentage of undifferentiated cells and the SCAP cells had begun to differentiate during the culturing period prior to starting the experiments.

To evaluate the stage of differentiation of the SCAP cells, we evaluated the presence of the cell markers by Flow Cytometry. Our Flow Cytometry results showed lower CD24+ and SRTO-1+ percentages than originally reported by Sonoyama in 2006,

even for the cells at passage 2. Only the percentage of CD146+ cells was in agreement (14). We saw that the CD24+ cells dropped to zero by passage 10. This is crucial as CD24 is considered the identifying marker for SCAP cells (14, 15). It appears that the cells were already differentiated towards an odontoblastic lineage by the expression of odontoblastic markers DSPP, MEPE and DMP-1 or that the percentage of cells expressing these markers was so low that it was difficult to identify their differentiation process.

Although in our hypothesis and aims we were looking for the role of the calcium released in the medium from WMTA, we could not measure this release with the protocols tested. Instead, the use of enriched calcium media helped in part to mimic this effect. There is little in the current literature regarding the effect of calcium ions on the dental pulp stem cells. Only one study done on dental pulp cells has correlated the effect of the calcium released from GMTA with proliferation (27). However, our proliferation study only partially agreed with this study after 7 days of exposure, whereas that study showed a concentration dependent increase in proliferation after 12 days. This is likely explained by the fact that our calcium enriched media was plain media without FBS or other growth factors. In our study both concentrations of the calcium enriched media showed a significant increase in SCAP cell migration after 24 hours. Calcium is well known as an intracellular signaling molecule and has been shown to increase the chemotaxis and migration of hMSCs (28, 29). In this light, our results should be expected, but to the best of our knowledge they have not been reported before.

The results of our experiments point us in several new directions. The use of Flow Cytometry to select the stem cells from the mixed SCAP population would help us

to understand the effects of WMTA on these cells. We have also shown in a pilot study that a clear difference exists between both GMTA and WMTA in terms of cell proliferation. We have also shown that a minimal 2% FBS concentration can influence cells as much as the 10% FBS concentration that is so often used. It is also possible that the effects of the FBS are overpowering the true effects of the agent we are studying. A future study should be done to titrate the FBS and see if there are any changes in the results of our experiments. Finally, the effect of calcium ions on the cells needs to be investigated more thoroughly. The large release of calcium ions by set MTA results from the dissociation of calcium hydroxide and the decomposition of calcium silicate hydrate, the two main components of set MTA. The greatest rate of Ca(OH)_2 formation during the setting of MTA occurs between 4 to 24 hours (22, 24, 25). Future studies into the amount of calcium ions released by MTA at 1 and 24 hours and its availability to nearby cells needs to be investigated.

In conclusion, we have shown that WMTA can induce SCAP cell migration and proliferation while using a mixed population of SCAP cells; SCAP cells in our population are at different stages of differentiation, and that calcium enriched media increased SCAP cell migration and proliferation. The use of calcium releasing materials, such as MTA, could have biologic impact on the recruitment of cells into a tooth root undergoing dentinogenesis. Future investigations are needed to determine if a more undifferentiated population of SCAP cells has a different reaction regarding migration, proliferation, and differentiation to the released compounds or final products of set MTA.

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Appendix

Lab Protocols

Cell Culture

The SCAP cells used in all experiments were provided by Dr. Songtao Shi (University of Southern California, Los Angeles, USA). All cell culture tasks were carried out under a laminar flow hood. A passage Zero SCAP cell vial stored at -80°C was thawed in a 37°C warm water bath and the contents transferred to a T-75 flask (BD Falcon, San Jose, CA) with 10ml of minimal essential medium alpha Eagle (α -MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Hereinafter “10% FBS media”) and incubated at 37°C in 5% CO_2 and 100% humidity. After 24 hours, the media was removed by vacuum pipetting and 10ml of fresh media was added. Cells were allowed to grow until reaching 80% confluence. At that point the media was removed by vacuum pipetting and the cells were passaged by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C . The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 6ml of 10% FBS media. Three 2ml aliquots of re-suspended cells were then placed in 3 T-75 flasks each containing 10ml of 10% FBS media and incubated at 37°C in 5% CO_2 and 100% humidity. Cells were used between passages 3 and 5.

MTA Preparation

White MTA ProRoot (Dentsply Endodontics, Tulsa, OK) was mixed per the manufacturer's recommendations. Specifically, 100mg of WMTA was mixed with 35 μ l of sterile water under a laminar flow hood in a sterile Dappen Dish (Keystone Industries, Cherry Hill, NJ) for approximately 1 minute. Ten milligram pellets were formed by placing the mixed WMTA into the large groove of a Lee MTA Pellet Forming Block (G. Hartzell & Son, Concord, CA) with the mixing spatula. The pellets were then placed in a 100 mm Cell Culture Dish (BD Falcon, San Jose, CA) and allowed to set for 1 hour or 24 hours at 37°C in 5% CO₂ and 100% humidity.

Calcium Enriched Media Preparation

The calcium enriched media was prepared by making a 1M CaCl₂ stock solution by dissolving 5.549g of anhydrous CaCl₂ in 50ml of plain α -MEM media. The desired serial dilutions of 3.0mmol, 0.3mmol, and 0.03mmol free calcium ions were then made by taking 5 μ l of 1M stock solution into 5ml of plain α -MEM media to form the 3.0mmol Ca media. Then 500 μ l of the 3.0mmol Ca media was added to 4.5ml of plain α -MEM to form the 0.3mmol Ca media. Finally, 500 μ l of the 0.3mmol Ca media was added to 4.5ml of plain α -MEM to form the 0.03mmol Ca media.

Transwell Migration Assay

SCAP cell migration was assessed by using a Transwell Migration Assay. SCAP cells were prestained with Cell Tracker Green (CTG) (Invitrogen Corp., Carlsbad, CA) as follows: the media from a T-75 flask containing passage 4 SCAP cells at 80% confluence

was removed by vacuum pipetting and 3ml of plain α -MEM media (Hereinafter “plain media”) plus 6 μ l of 10mM CTG stock solution were added to the flask (a 2 μ M final concentration of CTG). The flask was incubated at 37°C in 5% CO₂ and 100% humidity for 30 minutes. The plain media with CTG was removed by vacuum pipetting and 10ml of 10% FBS media was added to the flask and incubated for 30 minutes at 37°C in 5% CO₂ and 100% humidity. The plain media was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100 μ l of re-suspended cells in 900 μ l of plain media into a 2ml Eppendorf tube. 1×10^5 cells in 250 μ l of plain media were seeded into the upper chambers of transwell 8- μ m-size pore FluoroBlok 24 well multiwell system membrane filters (BD Falcon, San Jose, CA) (Hereinafter “FluoroBlok inserts”). The lower chambers of the 24 well multiwell system contained 500 μ l of plain media. The FluoroBlok inserts were placed into the lower chambers of the 24 well multiwell system and incubated for 4 hours at 37°C in 5% CO₂ and 100% humidity to allow for attachment of the cells to the membrane filter. After 4 hours, the FluoroBlok inserts were transferred to a new set of lower chambers of the 24 well multiwell system which contained the following test conditions: plain media with 1 hour set WMTA, plain media with 24 hour set WMTA, plain media, α -MEM

supplemented with 2% FBS and 1% penicillin/streptomycin (Hereinafter “2% FBS media”) with 1 hour set WMTA, 2% FBS media with 24 hour set WMTA, 3.0mmol Ca media, 0.3mmol Ca media, 0.03mmol Ca media, 2% FBS media, and 10% FBS media. One pellet of WMTA was used in each well that contained WMTA and 500µl of media was used in each well. The plate was incubated at 37°C in 5% CO₂ and 100% humidity. Cells that migrated to the bottom of the FluoroBlok inserts were read by fluorescence at 485/585 nm using a microplate reader (Genius; Tecan, Grödig, Austria) at 30 minutes, 60 minutes, 3, 6, 12, 24, 48, and 72 hours.

WST-1 Proliferation Assay

SCAP cell proliferation was evaluated after exposure to 1h or 24 hr set WMTA in plain or 2% FBS medium by WST-1 proliferation assay method. The media from a T-75 flask containing passage 4 SCAP cells at 80% confluence was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100µl of re-suspended cells in 900µl of plain media into a 2ml Eppendorf tube. 4×10^3 cells were seeded into 12 well companion plates (BD Falcon, San Jose, CA) with 1.5ml of media in the following test conditions: 3 sets of 3 wells containing plain media, 3 sets

of 3 wells containing 2% FBS media, 1 set of 3 wells containing 3.0 mmol Ca media, 1 set of 3 wells containing 0.3 mmol Ca media, 1 set of 3 wells containing 0.03 mmol Ca media, and 1 set of 3 wells containing 10% FBS media. Cell culture transwell inserts, 3- μ m-size pore (BD Falcon, San Jose, CA), were placed into each well. Two WMTA pellets were placed into the transwell inserts of the test groups as follows: plain media with 1 hour set WMTA, plain media with 24 hour set WMTA, 2% FBS media with 1 hour set WMTA, and 2% FBS media with 24 hour set WMTA. The plates were incubated at 37°C in 5% CO₂ and 100% humidity and the media changed every 3 days. The plates were evaluated at 1, 3, 5, 7, 9, 11 and 14 days via WST-1 Proliferation Assay.

The WST-1 proliferation assay was performed as follows: the transwell inserts were removed and discarded, the media was removed via vacuum pipetting and 300 μ l of new media containing 30 μ l of WST-1 reagent (Roche, Mannheim, Germany) (1:10 dilution) was added to each well. The plates were incubated at 37°C in 5% CO₂ and 100% humidity for 1 hour. One-hundred microliters of media plus WST-1 reagent was transferred from each well in the 12 well plate to a single well in a 96 well plate. Controls of WST-1 reagent in a 1:10 dilution with plain, 2% FBS, and 10% FBS media were also placed in wells of the 96 well plate. The absorbance (450nm-685nm) was determined in a microplate reader (Genius; Tecan, Grödig, Austria).

RT-PCR Analysis

SCAP cell differentiation was evaluated using RT-PCR analysis and gel electrophoresis. The media from a T-75 flask containing passage 4 SCAP cells at 80% confluence was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025%

Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100µl of re-suspended cells in 900µl of plain media into a 2ml Eppendorf tube. Four-thousand cells were seeded into the wells of 6 well companion plates (BD Falcon, San Jose, CA) with 3ml of media in the following test conditions: 3 sets of 3 wells containing plain media, 3 sets of 3 wells containing 2% FBS media, 1 set of 3 wells containing 3.0mmol Ca media, 1 set of 3 wells containing 0.3mmol Ca media, 1 set of 3 wells containing 0.03mmol Ca media, and 1 set of 3 wells containing 10% FBS media. Three micrometer-size pore transwell cell culture inserts (BD Falcon, San Jose, CA) were placed into each well. Three WMTA pellets were placed into the transwell inserts of the test groups as follows: plain media with 1 hour set WMTA, plain media with 24 hour set WMTA, 2% FBS media with 1 hour set WMTA, and 2% FBS media with 24 hour set WMTA. The plates were incubated at 37°C in 5% CO₂ and 100% humidity and the media changed every 3 days. The plates were evaluated at 7, 14 and 21 days via RT-PCR Analysis.

The RNA isolation was performed with the use of an RNeasy Mini Kit (Qiagen, Valencia, CA) The media was removed from all wells in the 6 well plate by vacuum pipetting and the cells were harvested by adding 200µl of freshly mixed RLT lysis buffer (15ml RLT lysis buffer + 150µl 2-mercaptoethanol) to each well and vigorously scraping

with a cell scraper (Corning Inc., Corning, NY) for 2 cycles of 2 minutes per well. The contents of the wells were then agitated by gentle pipetting and transferred to a collection tube supplied in the kit. All 3 wells for each condition were combined into a single collection tube (600µl total volume). The collected sample was placed in a QIAshredder (Qiagen, Valencia, CA) and centrifuged at 13,000 RPM for 2 minutes at 4°C. The QIAshredder insert was discarded and 600µl of 70% alcohol was added to the tube. Half of the sample was placed in a Mini Spin Column from the RNeasy kit and centrifuged at 13,000 RPM for 2 minutes at 4°C. The remaining sample was passed through the same Mini Spin Column. The solution that passed into the collection tube was discarded and 700µl of Buffer RW1 was added to the Mini Spin Column and centrifuged at 13,000 RPM for 15 seconds at 4°C. The solution that passed into the collection tube was discarded and 500µl of Buffer RPE was added to the Mini Spin Column and centrifuged twice at 13,000 RPM for 15 seconds at 4°C. The collection tube was discarded. The Mini Spin Column placed on a new collection tube centrifuged at 13,000 RPM for 2 minutes at 4°C. The Mini Spin Column was rotated 180° and centrifuged at 13,000 RPM for 1 minute at 4°C. The Mini Spin Column transferred to a new Eppendorf tube and 35µl of RNase-Free Water was added to the middle of the Mini Spin Column, which was then centrifuged at 13,000 RPM for 1 minute at 4°C. The solution that passed into the Eppendorf tube was collected by pipette and placed again in the middle of the Mini Spin Column and centrifuged at 13,000 RPM for 1 minute at 4°C. The Mini Spin Column was discarded and the purified RNA sample was contained in the Eppendorf tube. The purity of the RNA sample was measured on cuvette/spectrophotometer by placing 1µl of RNA

sample into 100 μ l of RNase-free water and reading the optical density. Working solutions were standardized to 100ng for the RT-PCR reactions.

RT-PCR was performed using SuperScript III Platinum kit (Invitrogen Corp., Carlsbad, CA). The primer sequences used were as follows: GAPDH (sense 5' gacccttcattgacctcaact 3'; antisense 5' caccaccttcttgatgtcatc 3'; 683-bp amplicon); DSPP (sense 5' gacccttcattgacctcaact 3', antisense 5' tgccatttgcgtgatgttt 3'; 181-bp amplicon); DMP-1 (sense 5' caggagcacaggaaaaggag 3', antisense 5' ctggtggtatcttgggcact 3'; 213-bp amplicon); and MEPE (sense 5' gcaaaagcacccatcgtatt 3', antisense 5' ctgcctctacaaggetgac 3'; 385-bp amplicon). For each sample, 5 μ l of 100ng working solution was placed in a 0.2ml PCR tube with 25 μ l of 2X Reaction Mix, 2 μ l sense primer, 2 μ l anti-sense primer, 1 μ l Taq enzyme, and 15 μ l of RNase-free water. The PCR tube was tightly capped and placed in a Mastercycler gradient machine (Eppendorf North America, Hauppauge, NY). Polymerase chain reaction (PCR) was performed with 35 cycles of denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec, and extension at 72°C for 60 sec.

The RT-PCR products were evaluated by electrophoresis in 2% agarose gels stained with ethidiumbromide. The 2% agarose gels were made by combining 2g UltraPure agarose (Invitrogen Corp., Carlsbad, CA) with 100ml TAE (1X) buffer in a 125ml Erlenmeyer flask and microwaving for 1-1.5 minutes until the solution was clear, then adding 5 μ l of ethidiumbromide to the flask and swirling to mix thoroughly. The gel mixture was then poured into a 15cm x 10 cm Gel Caster (Bio-Rad, Hercules, CA) with a 20 well comb and allowed to set for 45 minutes. Well #1 in each gel was loaded with 5 μ l of loading dye and 10 μ l of 100kb Plus DNA Ladder (Life Technologies, Grand Island, NY), 15 μ l of

sample and 5µl of dye were loaded into the sample wells. The gels were connected to a Bio-Rad Power Pak 300 (Bio-Rad, Hercules, CA) and run at 80V for 45 minutes. Gel photographs were taken with a Kodak EDAS 290. A densitometric analysis was performed using ImageJ software (NIH). This experiment was repeated 3 times in triplicate.

Flow Cytometric Analysis

SCAP cells from passages 2 and 10 were evaluated by Flow Cytometry to characterize the cell population of each passage. A buffer solution of 2% FBS in PBS (hereinafter “buffer”) was made for use in the dilution of the antibodies. The media from a T-75 flask containing passage 2 and 10 SCAP cells at 80% confluence was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100µl of re-suspended cells in 900µl of plain media into a 2ml Eppendorf tube. 5×10^5 cells were seeded into 5ml polystyrene round-bottom tubes (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The supernatant was discarded via gentle vacuum pipetting and the pellet of cells was re-suspended in 100µl of 5% bovine serum albumin (BSA) and incubated on ice for 30

minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting. The pellet of cells was re-suspended in 1ml of PBS, centrifuged for 5 minutes at 1000 RPM at 4°C, the supernatant was discarded via gentle vacuum pipetting, and this step was repeated with an additional 1ml of PBS (Hereinafter “wash cycle”). A control sample from each passage was then re-suspended with 100µl of buffer and incubated in the dark on ice until read by flow cytometry. The supernatant from the remaining tubes was discarded via gentle vacuum pipetting and the samples divided into 3 groups based on the antibody staining received. In the CD146 group, the pellet of cells was re-suspended in 100µl of 1:11 (1µl antibodies/11µl buffer) human CD146-APC antibodies (Milenyi Biotec, Auburn, CA) and incubated in the dark on ice for 30 minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting and a wash cycle performed. The supernatant was discarded via gentle vacuum pipetting and samples from each passage were then re-suspended with 100µl of buffer and incubated in the dark on ice until read by flow cytometry. Next, in the CD24 group the pellet of cells was re-suspended in 100µl of 2:10 (2µl antibodies/10µl buffer) PE mouse anti-human CD24 antibodies (BD Pharmingen, San Jose, CA) and incubated in the dark on ice for 30 minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting and a wash cycle performed. The supernatant was discarded via gentle vacuum pipetting and samples from each passage were then re-suspended with 100µl of buffer and incubated in the dark on ice until read by flow cytometry. Then, in the STRO-1-FITC group the pellet of cells was re-suspended in 100µl of 1:25 (1µl antibodies/25µl buffer) mouse anti-STRO-1

primary antibodies (Invitrogen Corp., Carlsbad, CA) and incubated in the dark on ice for 60 minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting and a wash cycle performed. The supernatant was discarded via gentle vacuum pipetting and a control sample from each passage was then re-suspended with 100µl of buffer and incubated in the dark on ice until read by flow cytometry. The pellet of cells in the remaining tubes were re-suspended in 100µl of 1:100 (1µl antibodies/100µl buffer) FITC rabbit anti-mouse IgG conjugate antibodies (Invitrogen Corp., Carlsbad, CA) and incubated in the dark on ice for 30 minutes. The samples were then centrifuged for 5 minutes at 1000 RPM at 4°C and the supernatant was discarded via gentle vacuum pipetting and a wash cycle performed. The supernatant was discarded via gentle vacuum pipetting and samples from each passage were then re-suspended with 100µl of buffer and incubated in the dark on ice until read by flow cytometry. A control tube of cells stained with only the FITC rabbit anti-mouse IgG conjugate antibodies for each passage was also made. All samples were re-suspended with a pipette prior to flow cytometry. The samples were processed at the University of Michigan Life Science Center Flow Cytometry Core on a BD FACSAria3 Flow Cytometry machine using the BD FACSDiva software and 10,000 events.

Gray MTA Pilot Studies

SCAP cell proliferation in the presence of gray MTA (GMTA) (Gray MTA ProRoot Dentsply Endodontics, Tulsa, OK) was evaluated using the WST-1 proliferation assay method. The media from a T-75 flask containing passage 4 SCAP cells at 80% confluence was removed by vacuum pipetting and the cells were harvested by adding 3ml of 0.025% Trypsin-EDTA to the flask and incubating it for 5 minutes until the cells

detached from the flask and were visualized floating in the media. Five milliliters of 10% FBS media was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1000 RPM at 4°C. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 5ml of plain media. Cells were counted using a hemocytometer with the sample created by placing 100µl of re-suspended cells in 900µl of plain media into a 2ml Eppendorf tube. 4×10^3 cells were seeded into the wells of 12 well companion plates (BD Falcon, San Jose, CA) with 1.5ml of media in the following test conditions: 7 sets of 3 wells containing 2% FBS media, 1 set of 3 wells containing plain media, and 1 set of 3 wells containing 10% FBS media. 3-µm-size pore transwell cell culture inserts (BD Falcon, San Jose, CA) were placed into each well. Twenty-four hour set GMTA and WMTA pellets were placed into the transwell inserts of 6 sets of the wells containing 2% FBS media as follows: 3 pellets of GMTA, 6 pellets of GMTA, 9 pellets of GMTA; and 3 pellets of WMTA, 6 pellets of WMTA, 9 pellets of WMTA. Additionally, 4×10^3 cells were seeded into the wells of 12 well companion plates (BD Falcon, San Jose, CA) with 1.5ml of media in the following test conditions: 7 sets of 3 wells containing 2% FBS media, 1 set of 3 wells containing plain media, and 1 set of 3 wells containing 10% FBS media. Prior to cell seeding, GMTA and WMTA pellets were freshly mixed and placed wet into the 6 sets of the wells that would contain 2% FBS media as follows: 3 pellets of GMTA, 6 pellets of GMTA, 9 pellets of GMTA; and 3 pellets of WMTA, 6 pellets of WMTA, 9 pellets of WMTA. The wet GMTA and WMTA pellets were spread over the bottom surface of the well to provide a uniform thickness covering the bottom of the well. The plates were then placed in an incubator

and allowed to set for 24 hours at 37°C in 5% CO₂ and 100% humidity. The plates were incubated at 37°C in 5% CO₂ and 100% humidity and the media changed every 3 days. The plates were evaluated 7 days via WST-1 proliferation assay.

The WST-1 proliferation assay was performed as previously described in the **WST-1 Proliferation Assay** protocol.