Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Philadelphia College of Osteopathic Medicine

2. **Reporting Period (start and end date of grant award period):** January 1, 2011- June 30, 2014

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Jane Dumsha, PhD

4. **Grant Contact Person's Telephone Number:** 215-871-6783

5. **Grant SAP Number:** 4100054866

6. **Project Number and Title of Research Project:** 1 - Effect of Liposuction Technique on the Viability and Differentiation of Adipose Derived Stem Cells

7. **Start and End Date of Research Project:** January 1, 2011 – June 30, 2014

8. **Name of Principal Investigator for the Research Project:** Marina D'Angelo, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 19,404.81

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
N/A			

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
D'Angelo, Marina	Professor	10%
Adams, Christopher	Associate Professor	10%
Falcone, Victoria	Clinical Collaborator	5% (Y1, Y2) 0% (Y3, Y4)
Moore, Caitlyn	Graduate Student	100% (Y1, Y2)
Murphy, Daniel	Graduate Student	100% (Y2, Y3)
Belogorodsky, Dimitry	Graduate Student	100% (Y3, Y4)
Saunders, Ray	Research Technician	10% (Y1-Y3) 0% (Y4)
Cho, Ellen	Research Technician	0% (Y1) 10% (Y2-Y4)
Chmielewski, Sarah	Research Technician	0% (Y1, Y2) 30% (Y3, Y4)

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
N/A		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes___x_____ No_____

If yes, please indicate the source and amount of other funds:

A pilot grant from the Centers for Chronic Disorders of Aging, PCOM
7/11-6/12 "Effect of BodyJet and VASER Liposuction Technique on the Validity and Differentiation of Adipose Derived Stem Cells."
$7000

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No___x_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal		$	$

	(specify:_____ _____) ☐ Nonfederal source (specify: _____)			
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_____ No____x_____

If yes, please describe your plans:

12. Future of Research Project. What are the future plans for this research project?

We are not continuing. Since the project suffered the loss of our clinician collaborator during Y3, we decided to wrap up what we had completed to pull together a manuscript.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes___x_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male		2	3	
Female		1	1	
Unknown				
Total		**3**	**4**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown		3	4	

Total		**3**	**4**	

	Undergraduate	Masters	Pre-doc	Post-doc
White		3	4	
Black				
Asian				
Other				
Unknown				
Total		**3**	**4**	

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

    Yes_____ No___x_____

    If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

    Yes_____ No_____x____

    If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. **Collaboration, business and community involvement.**

    16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

    Yes___x_____ No_____

    If yes, please describe the collaborations: We partnered with a clinician who runs her own practice for the first two years of the project.

    16(B) Did the research project result in commercial development of any research products?

    Yes_____ No____x_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____x_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project goals, objectives and specific aims as described in the grant proposal:

This proposal will specifically test the hypothesis that the method by which adipose tissue is recovered during liposuction will impact both the quality and differentiation potential of adipose-derived stem cells. There are two Specific Aims: the first is **to determine the role of liposuction technique in the viability of retrieved stem cells from adipose tissue**. Our clinical collaborator, Dr. Falcone, will be providing us with material recovered from liposuction procedures. These procedures will utilize one of five different devices. Viability of isolated cells will be evaluated immediately and normalized by material volume. In addition, the percentage of stem cells among the isolated cells will be determined by examining the expression of three different cell surface markers. The second Specific Aim is **to confirm the potential fates of adipose-derived stem cells as a function of liposuction technique.** Isolated cells will be cultured in fate specific media. Potential of cells to differentiate into one of three different fates will be evaluated. Osteoblastic, chondrocytic and adipocytic cell fates have been chosen as endpoints and will be evaluated using differentiation markers for each cell type. A secondary goal in this work will be to develop preliminary data for future studies, including the examination of roles of anatomical position, repeated procedures and anesthetic composition of the viability and differentiation potential of recovered stem cells.

The primary objective of the project was to determine the role of liposuction method on the ability to isolate stem cells from fat for tissue engineering purposes. We specifically tested the hypothesis that the method by which adipose tissue is recovered during liposuction will impact both the quality and differentiation potential of adipose-derived stem cells.

In Specific Aim 1, we determined that lipoaspirate isolated by Body-Jet Liposuction device, a water-jet assisted liposuction device and/or SmartlipoMPX, a brand of laser lipolysis, provided the most viable cells and the highest number of viable stem cells. In addition, fresh lipoaspirate provided the most viable stem cells, even though viable stem cells could be isolated from frozen lipoaspirate. In Specific Aim 2, we demonstrated that stem cells isolated from cryopreserved lipoaspirate (Body Jet and Smart Lipo isolated) could differentiate into chondrocytes and osteoblasts when cultured in fate-specific media and compared to a commercially available human stem cell line. Accumulated data from the projects allowed us to compare each technique individually and in combination on the ability to recover stem cells from the aspirated material. In addition, the role of anatomical position, repeated procedures and anesthetic composition on the viability and differentiation potential of recovered stem cells was examined.

Demographics

Previous data collected in the pilot study from our lab included all available lipoaspirate samples collected from our clinical collaborator (Moore, 2011). This made for a wide variety of lipoplasty techniques and a wide variety of patient demographics. The results from that study showed patients ranging in age from 15 to over 70. Of the 21 samples analyzed in that study, 5 were taken from male patients. They also included patients who had a previous surgery (of any kind) in the same region, with about 19% of the patients having had previous surgery/surgeries. The lipoplasty methods, in order of largest to smallest proportion of the total samples, included: Vaser; Vaser with SmartLipo; BodyJet with SmartLipo and Custom Acoustic; Vaser with Custom Acoustic; BodyJet with Custom Acoustic; Custom Acoustic; Vaser with Custom

Acoustic; and SmartLipo. Additionally, the adipose tissue was removed from many areas of the body, including, but not limited to, thighs, arms, lower back, and abdomen.

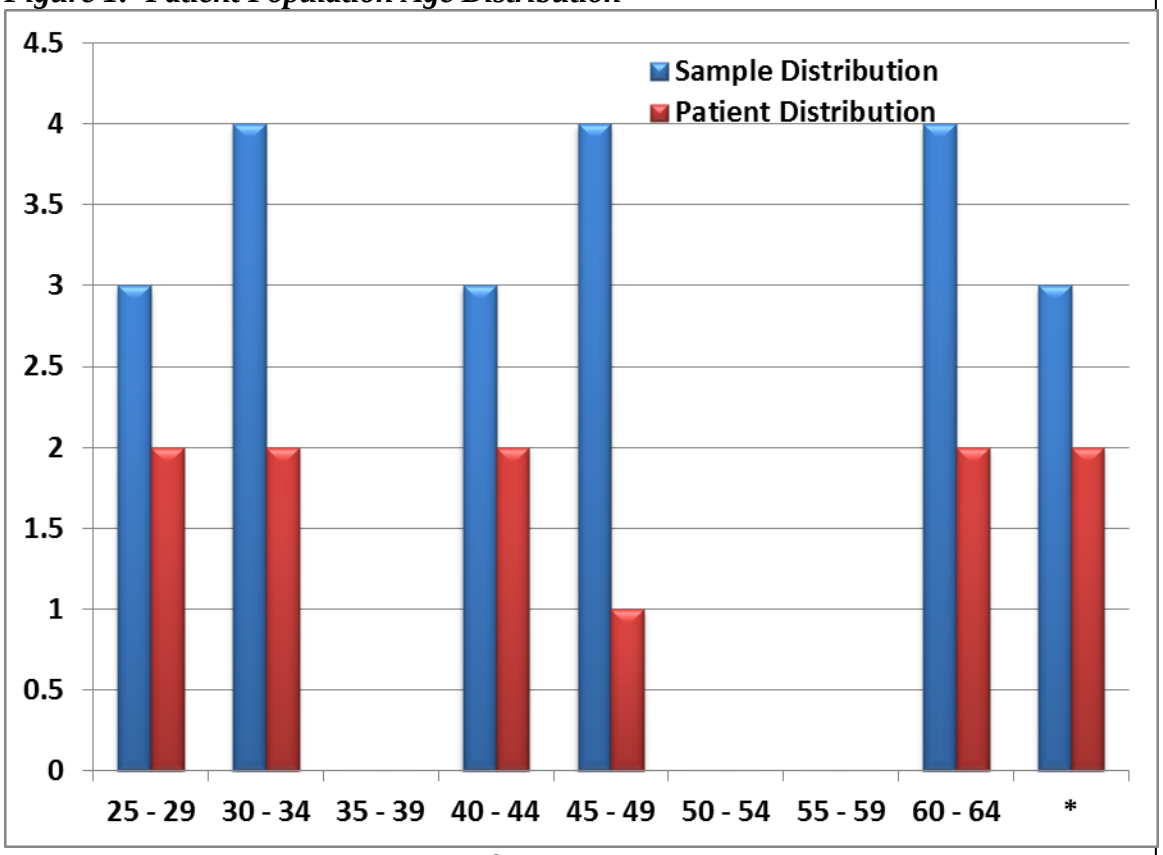
In an attempt to limit the variability in the data, our patient population, thus the sample population, was limited based on the result of the previous experiment and the fact that the majority of this demographic data reflected published adipose-derived stem cells (ADSC) patient data collected by the American Society of Plastic Surgeons (A.S.P.S., 2011). We chose a more narrow age range, limited the number of lipoplasty methods to two, focused on female patients, and accepted only tissue from abdomens with no surgical history. These criteria were chosen to help with interpretation of the data and to take advantage of the majority of samples that would be clinically collected without recruiting patients. The following analysis is based on 21 lipoaspirate samples collected from 11 different patients that met the strict constraints of the protocol.

The age distribution of the patients can be seen in Figure 1, which shows 5-year age ranges starting at 25 to 29 and ending at 60 to 64, with two patients' ages unknown. The age distribution is such that no significant difference was found between the ages of any subsets of the lipoaspirate sample population. Additionally, all tissue was collected from female patients.

Of the samples received, 8 were divided in half, with the first half being isolated immediately (fresh) and the second half being isolated one week later (frozen). A total of 13 samples, or 60% of our samples analyzed, were isolated on the day of surgery. Samples isolated one week later are referred to as frozen samples and account for a total of 8 samples in the analysis (see Figure 2).

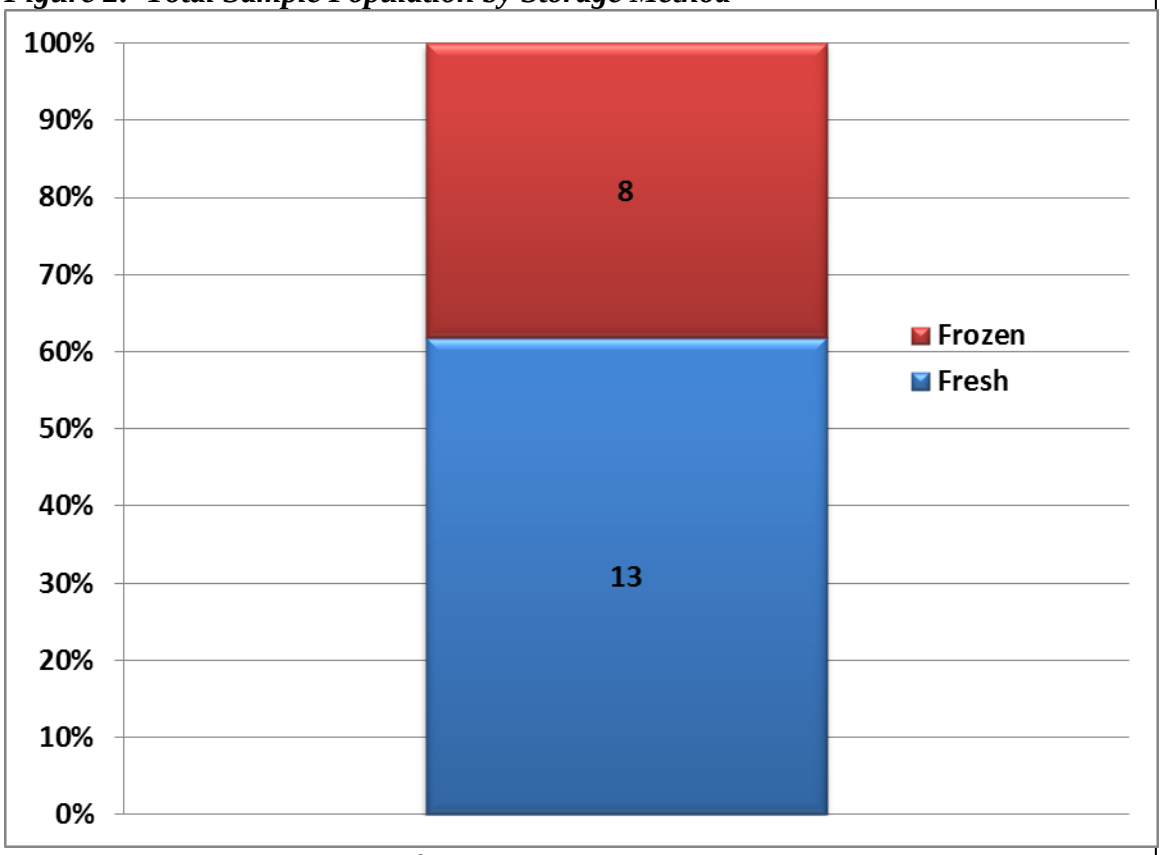
The lipoplasty techniques used by the physician varied depending on the needs of the patient because this study utilized medical waste and patients were not recruited for participation. The samples that we received were collected using mainly two techniques: BodyJet and BodyJet with SmartLipo (Figure 3). BodyJet accounted for 13, or 60%, of our samples and the combination of BodyJet with SmartLipo accounted for 6 samples. However, we also received one sample harvested by Vaser with SmartLipo and two samples harvested with an unknown technique. Although all techniques are included in certain analyses below, only the BodyJet and the BodyJet with SmartLipo samples are analyzed below when comparing lipoplasty techniques.

Figure 1: Patient Population Age Distribution



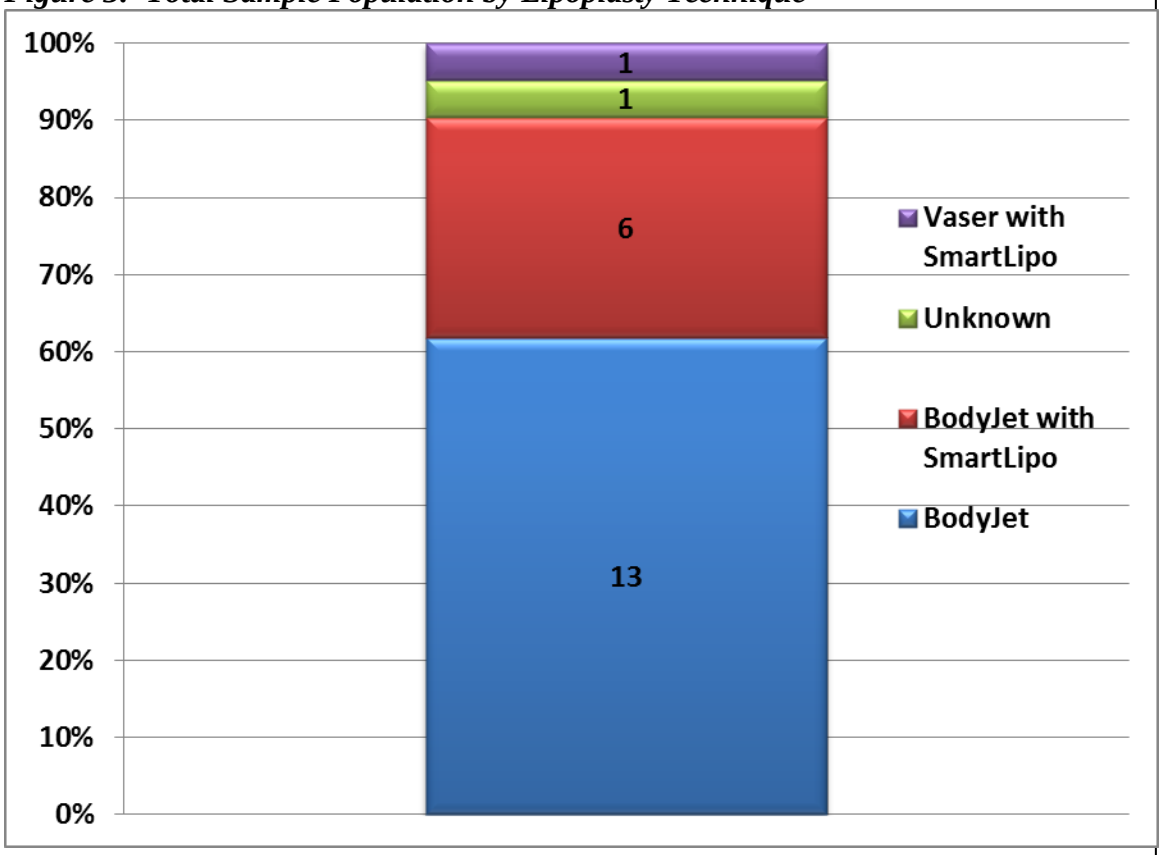
The blue bars represent the number of samples per age group. In some cases, multiple samples were taken from one patient. The red bars represent the number of patients per age group. The last category, labeled "", is composed of patients/samples that were received by the lab without a recorded age.*

Figure 2: Total Sample Population by Storage Method



The blue section represents the fresh sample population, which represents slightly more than 60% of the total samples. The red section represents the "frozen" sample population, which represents slightly less than 40% of the total samples.

Figure 3: Total Sample Population by Lipoplasty Technique



The blue section represents the BodyJet sample population, which represents slightly more than 60% of the total samples. The red section represents the BodyJet with SmartLipo sample population, which represents slightly less than 30% of the total samples. The green section represents 1 unknown sample, and the purple section represents 1 Vaser with SmartLipo sample.

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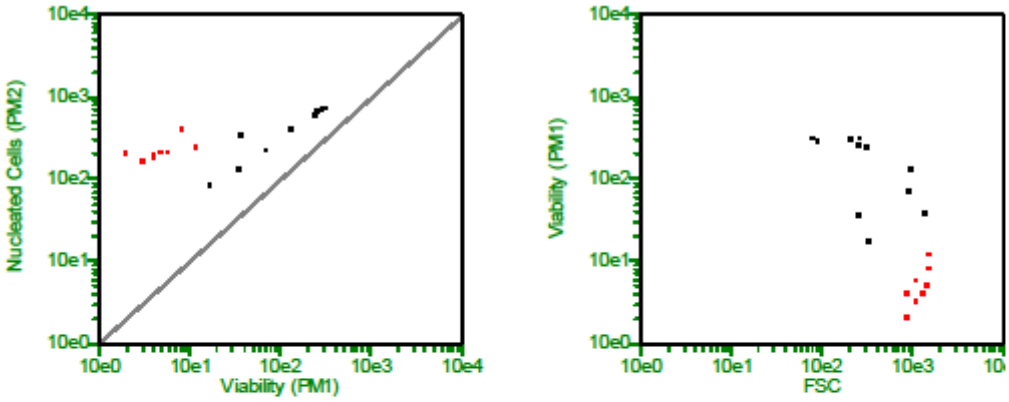
<u>Protocol Optimization – ViaCount Assay</u>

Previously, our lab conducted a pilot study to measure cell viability in lipoaspirate samples (Moore 2011). Cell viability was measured with a Guava EasyCyte Plus® System (Millipore, 2008), which includes: the proprietary live-dead dye, called ViaCount; the Guava EasyCyte Plus flow cytometer; and software which utilizes their EasyFit algorithm that determines cell counts of viable and dead cells. The previous protocol utilized by the lab called for collection of viability data from cells prepared in two different ways: 1) "fresh cells," treated only with ViaCount stain, 2) "fresh-fixed cells," fixed in paraformaldehyde and then treated with Via Count stain. In this study, we recounted the fixed cells and determined that they are not effectively read with Guava's ViaCount Assay (Figure 4). In 1,000 nucleated events counted, only 20 were tallied by the EasyFit algorithm. *The EasyFit debris index indicates that 99.14% of the events counted were debris.* Thus, for this analysis, only unfrozen and unfixed, i.e. fresh cells, were used for the viability assay.

Figure 4: Fixed Cell Guava Viability Assay

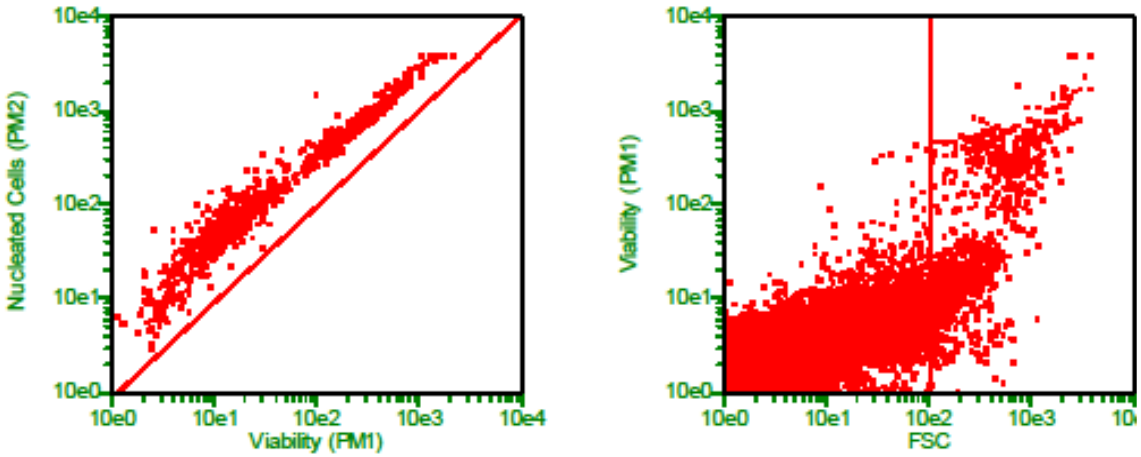


	EasyFit Results		Manual Results	
	Count	% of Total	Count	% of Total
Viable	8	40.00%	941	94.10%
Dead	12	60.00%	59	5.90%
	Viable	Total	Viable	Total
Cells / mL	2.05e04	5.12e04	2.41e06	2.56e06
Cells in Org Sample	2.05e05	5.12e05	2.41e07	2.56e07
Debris Index	99.14%		57.10%	

The dot plot on the left shows brightness of the proprietary "nucleated cell" dye versus the brightness of the "viability dye," both of which are components of Guava's ViaCount stain. The dot plot on the right is showing "viability" versus forward scatter (cell size) in fixed cells. The table lists results as calculated with the EasyFit Algorithm (left) or manual gates (right) that we set. The debris index is calculated as a percentage of the total number of events.

Our previous protocol relied solely on Millipore's EasyFit algorithm to mathematically eliminate debris and determine the living versus apoptotic/dead cellular populations. Figure 5 shows the data collected without the EasyFit algorithm applied, where nearly every (99.6%) nucleated event is counted as living. This artificially high percentage is due to the placement of the gates into default positions, in which the horizontal gate—used for gating by size—was completely removed and the diagonal gate—used for distinguishing the different dyes contained in the ViaCount stain—was placed at a 45 degree angle. Figure 6 shows the same data with EasyFit applied, which is essentially replacing the unused gates mathematically after the actual data collection has already taken place. This is problematic because the amount of debris being counted, as a result of the lack of viability gate settings, is increasing the events counted per microliter to 348% of the manufacturer's recommended maximum, stretching the algorithm beyond its limits. Figure 7 shows EasyFit results with viability gates set manually. Setting these gates prior to data collection greatly reduces the amount of debris being counted by the flow cytometer, bringing the events per microliter well within the recommended range; allowing for a more precise mathematical analysis.

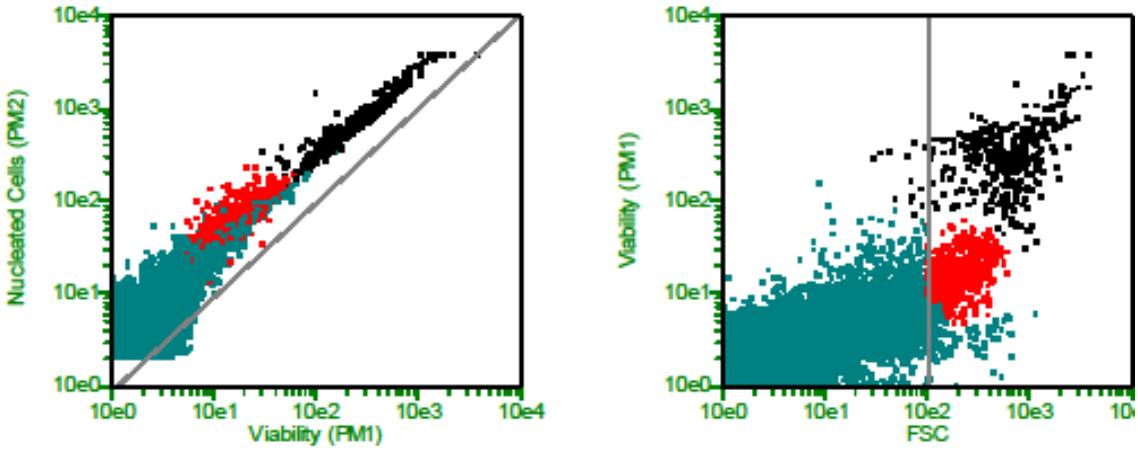
Figure 5: Manual Gate Setting *Guava Viability Assay*



Viable Cells / mL	2.22e06
Viability %	99.60%
Total Cells / mL	2.23e06
Total Viable Cells in Original Sample	2.22e07
Total Cells in Original Sample	2.23e07
Dilution Factor	20
Original Volume (mL)	10

The dot plot on the left shows brightness of the proprietary "nucleated cell" dye versus the brightness of the "viability dye," both of which are components of Guava's ViaCount stain. The diagonal gate in this plot divides viable (to the right of the gate) and dead (to the left of the gate); 99.6% of the events are counted as viable cells. The dot plot on the right is showing "viability" versus forward scatter (cell size). The gate in the middle divides debris (to the left of the gate) and cells (to the right of the gates).

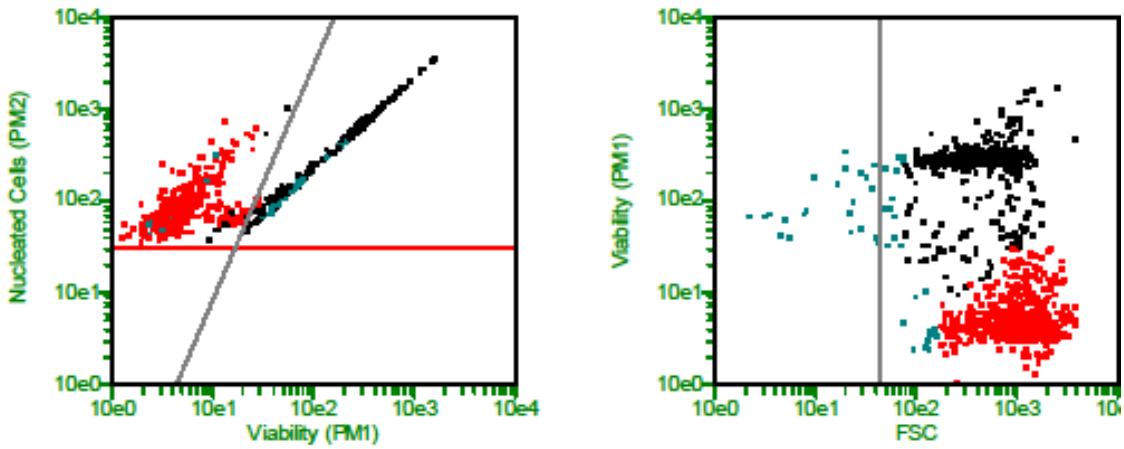
Figure 6: Easy Fit Algorithm: Guava Viability Assay



	EasyFit Results		Manual Results	
	Count	% of Total	Count	% of Total
Viable	507	56.84%	996	99.60%
Dead	385	43.16%	4	0.40%
	Viable	Total	Viable	Total
Cells / mL	1.13e06	1.98e06	2.21e06	2.22e06
Cells in Org Sample	1.13e07	1.98e07	2.21e07	2.22e07
Debris Index	94.30%		93.61%	

This graph shows the same data as Figure 5 with the same gates, but colored by the EasyFit algorithm. The table compares the EasyFit results (seen in the dot plots above) versus the manual results seen in Figure 5.

Figure 7: Manual Gating and Easy Fit Algorithm: ***Guava Viability Assay***



	EasyFit Results		Manual Results	
	Count	% of Total	Count	% of Total
Viable	451	46.40%	472	47.20%
Dead	521	53.60%	528	52.80%
	Viable	Total	Viable	Total
Cells / mL	1.90e05	4.09e05	1.99e05	4.21e05
Cells in Org Sample	1.90e06	4.09e06	1.99e06	4.21e06
Debris Index	5.26%		2.53%	

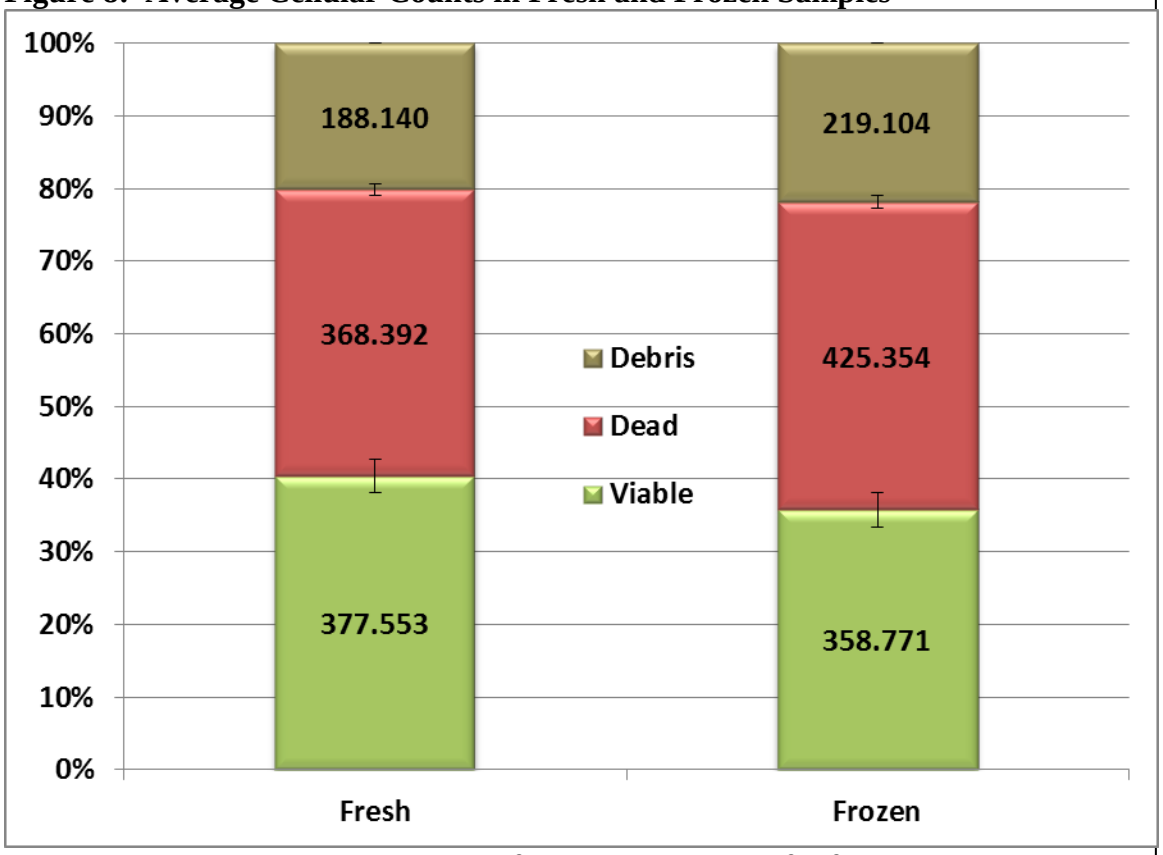
Similar to Figures 5 and 6, this figure shows the same two dot plots, with the same gates, except the left dot plot shows the addition of a manually set gate that eliminates events with lower amounts of "nucleated cells" dye brightness.

The entire procedure, from surgery to flow cytometry and tissue culture, was completed within 12 hours. All viability data were recorded immediately following the isolation, which was performed as soon as possible after the surgery. All surface marker data were recorded at least 24 hours after the isolation using fixed cells. Due to these time restrictions, fluorescence duration, mechanical malfunction, and in rare cases, technician error, not all lipoaspirate samples received from the physician were able to be evaluated.

Viability – Fresh versus Frozen

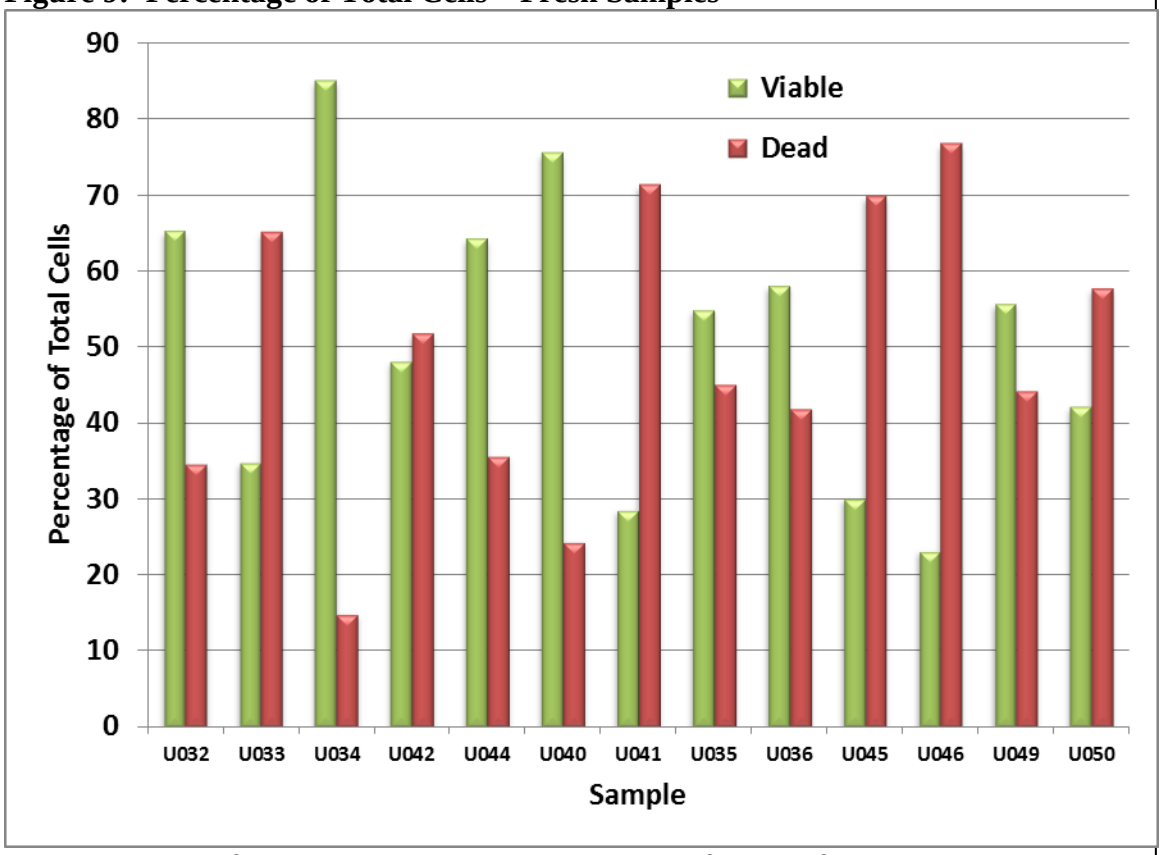
We compared the ViaCount data collected from fresh and frozen preparations. The average number of events, divided into viable cells, dead cells, and debris, are displayed as a percentage of the whole for all ViaCount assays (n = 21, 13 fresh, 8 frozen) in Figure 8. This chart is of aggregate data averaged from the raw number of events counted in the cellular suspensions isolated from individual samples. *The debris averages about 20% of the total events for both frozen and fresh samples.* A comparison of viable versus dead cells, as a percentage of total cells, for individual samples is shown for fresh isolates (Figure 9) and frozen isolates (Figure 10) to demonstrate the individual variability of samples.

Figure 8: Average Cellular Counts in Fresh and Frozen Samples



This chart shows the average number of events (cell counts) for frozen samples compared to the average cell counts for the fresh samples by percentage of the average total cell count.

Figure 9: Percentage of Total Cells – Fresh Samples



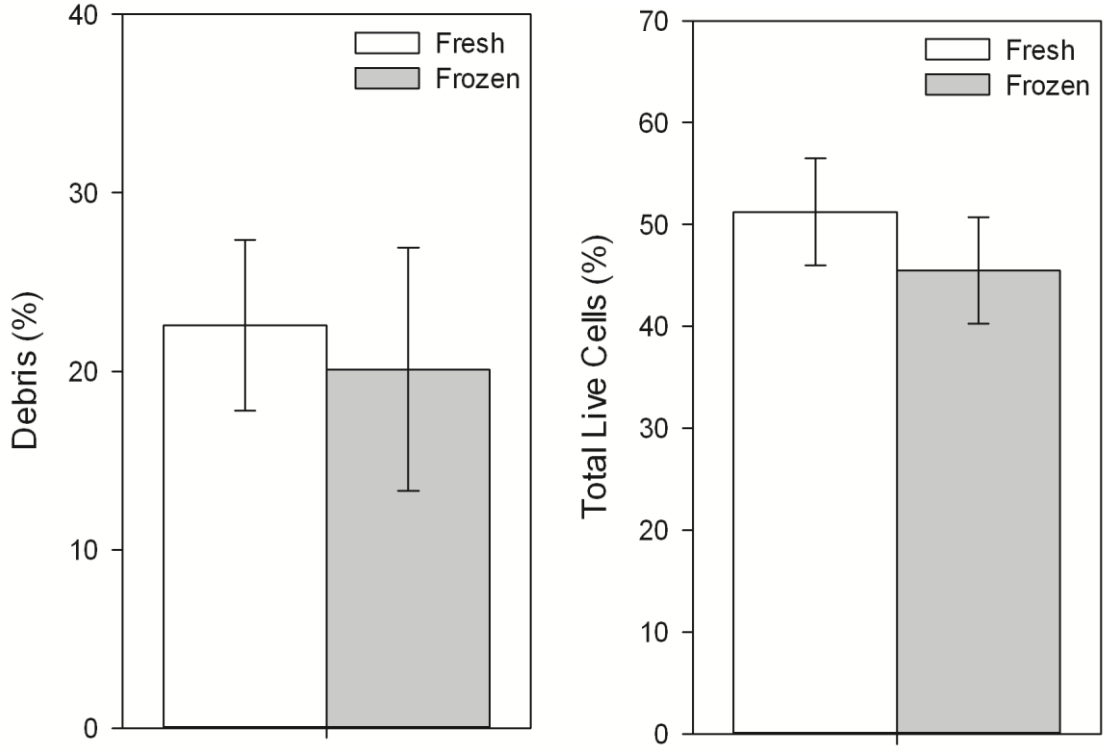
The percentage of total cells is displayed in this chart for each fresh sample. The majority of samples have a higher green (percentage of viable cells) than red (percentage of dead cells) bar; however, there is still a noteworthy inter-sample variation.

Figure 10: Percentage of Total Cells – Frozen Samples

The percentage of total cells is displayed in this chart for each frozen sample. The majority of samples have a higher red (percentage of dead cells) than green (percentage of viable cells) bar; however, there is still a noteworthy inter-sample variation.

Figure 8 shows the number of events for viable, dead, and debris as a percentage of the total events (ideally 1,000 events). Figures 9, 10, and 11 compare only cells. The final viability graph for frozen versus fresh samples (Figure 11B) compares the viable cell numbers as a percentage of total cells. As indicated, the freezing of the lipoaspirate at -80°^{C} for 1 week has no significant difference in the percentage of viable cells isolated from the tissue. Graphs 9 and 10 focus only on the nucleated events, i.e. cells, because, as Figure 11a shows, there is no significant difference in the amount of debris present in the cellular suspension isolated from fresh samples versus the amount of debris present in the cellular suspension isolated from frozen samples.

Figure 11: Comparison of Fresh and Frozen Samples by Percentage of Debris or Live Cells

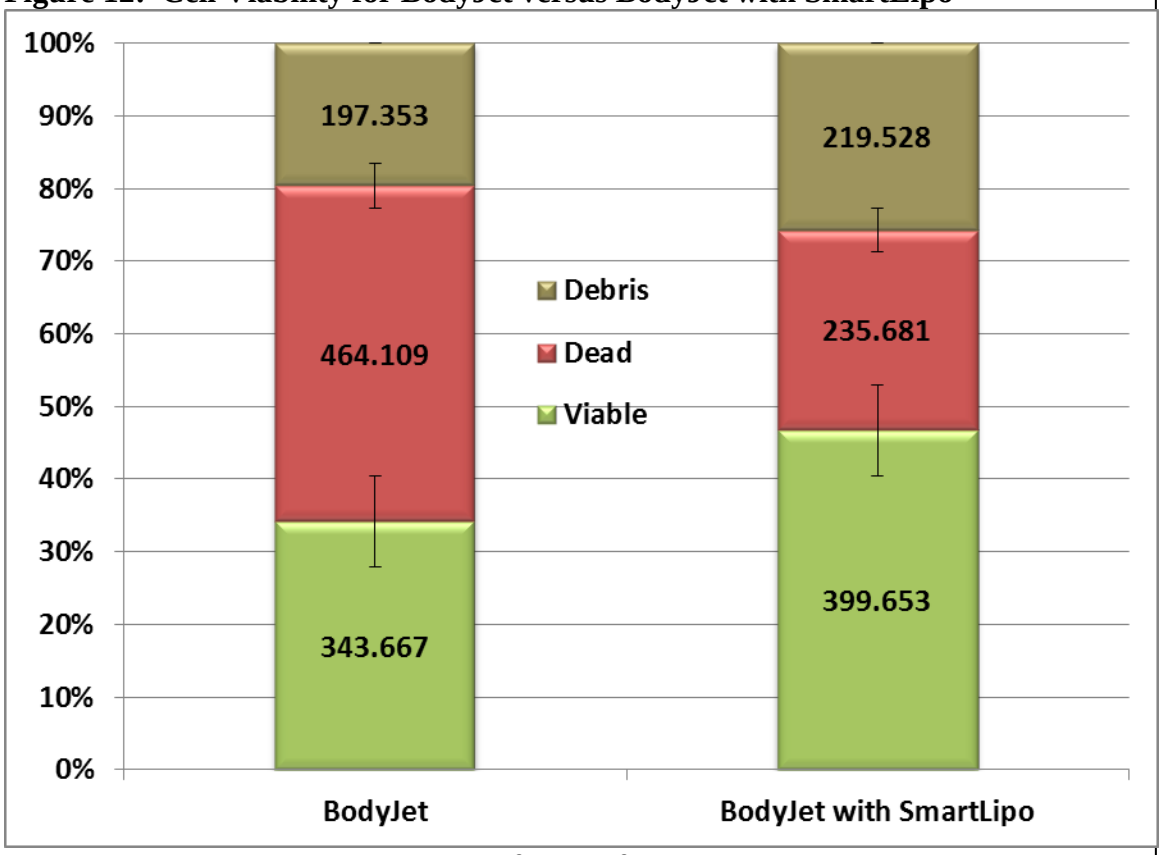


(A) **Debris as a Percentage of Total Events** *The bar graph shows the average debris as a percentage of total events counted from fresh (white) and frozen (grey) samples. There is no statistically significant difference.* (B) **Live Cells as a Percentage of Total Cells** *The bar graph shows the average events counted as viable cells as a percentage of the total nucleated events from fresh (white) and frozen (grey) samples. There is no statistically significant difference.*

Viability – BodyJet versus BodyJet with SmartLipo

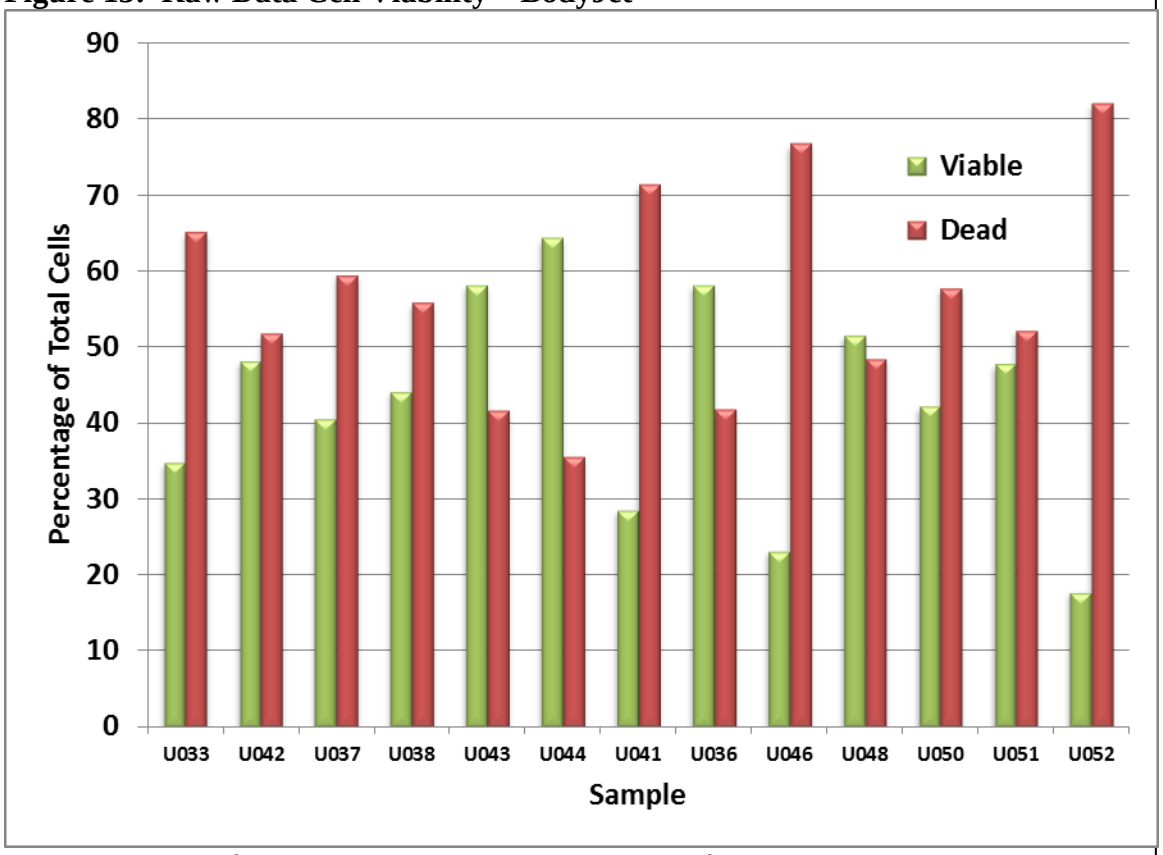
The average number of events, divided into viable cells, dead cells, debris and separated by BodyJet or BodyJet with SmartLipo, are displayed as a percentage of the whole for all ViaCount assays in Figure 12. This chart is of aggregate data averaged from the raw number of events counted in the cellular suspensions isolated from individual samples. The data shows 52% of the total samples were live cells. A raw data comparison of viable versus dead cells, as a percentage of total cells, for individual samples is shown in Figure 13 and Figure 14 to demonstrate the individual variability of samples.

Figure 12: Cell Viability for BodyJet versus BodyJet with SmartLipo



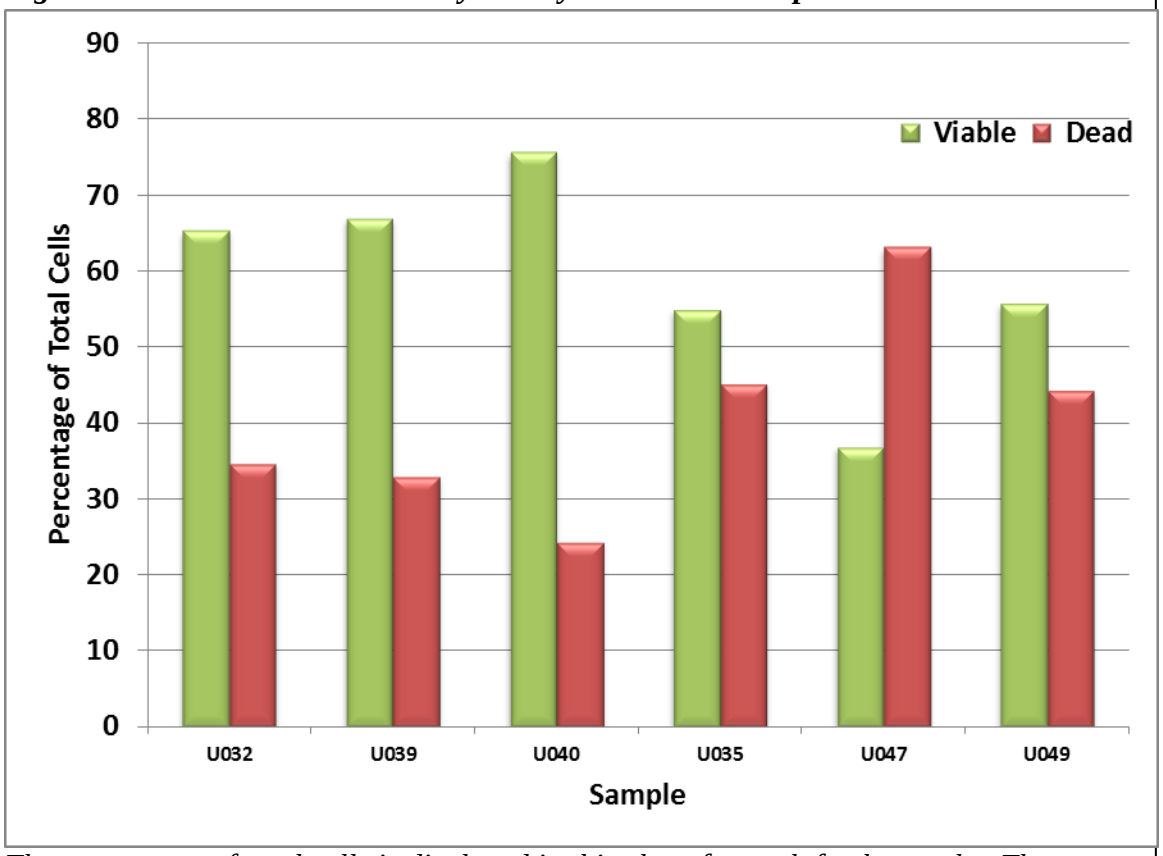
This chart shows the average number of events for the BodyJet samples compared to the average cell counts for the BodyJet with SmartLipo samples by percentage of the average total cell count. The average debris index is slightly higher in BodyJet with SmartLipo samples.

Figure 13: Raw Data Cell Viability – BodyJet



The percentage of total cells is displayed in this chart for each BodyJet sample. The majority of samples have a higher red (percentage of dead cells) than green (percentage of viable cells) bar; however, there is still a noteworthy inter-sample variation.

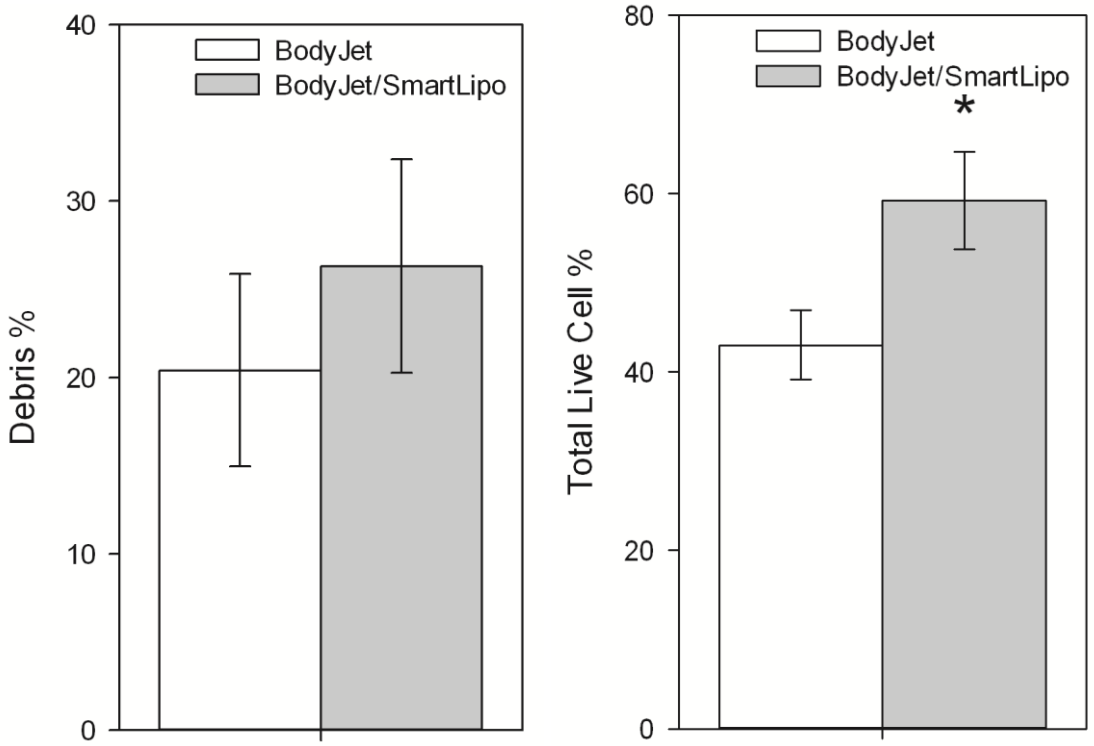
Figure 14: Raw Data Cell Viability – BodyJet with SmartLipo



The percentage of total cells is displayed in this chart for each fresh sample. The majority of samples have a higher green (percentage of viable cells) than red (percentage of dead cells) bar; however, there is still a noteworthy inter-sample variation.

Figure 12 shows the number of events for viable, dead, and debris as a percentage of the total events (ideally 1,000 events). However, all other graphs focus only on the nucleated events, i.e. cells because, as Figure 15A shows, there is no significant difference in the amount of debris present in the cellular suspension isolated from BodyJet samples versus the amount of debris present in the cellular suspension isolated from BodyJet with SmartLipo samples.

Figure 15: Comparison of BodyJet and BodyJet with SmartLipo Samples by Percentage of Debris or Live Cells



(A) **Debris as a Percentage of Total Events** *The bar graph shows the average debris as a percentage of total events counted from BodyJet (white) and BodyJet with SmartLipo (grey) samples. There is no statistically significant difference.* (B) **Live Cells as a Percentage of Total Cells** *The bar graph shows a statistical difference ($p < 0.05$) in the average events counted as viable cells as a percentage of the total nucleated events from BodyJet (white) versus BodyJet with SmartLipo (grey) samples.*

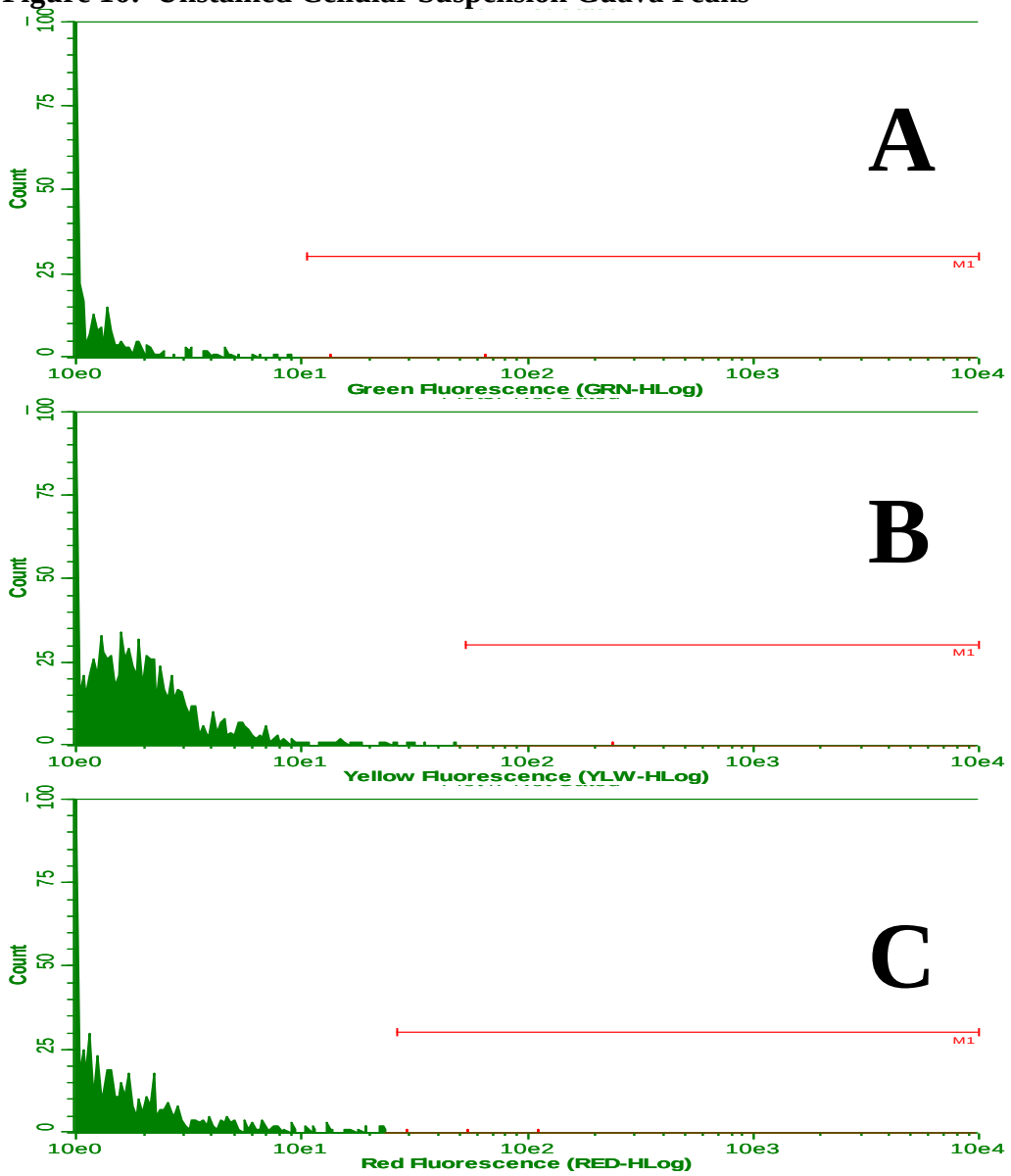
Focusing on cells, the final viability graph for BodyJet versus BodyJet with SmartLipo samples compares the viable cell numbers as a percentage of total cells. As indicated in Figure 15B, the data indicates that there is a significant difference in the percentage of viable cells contained in lipoaspirate harvested with BodyJet versus lipoaspirate harvested using both BodyJet and SmartLipo technologies. Although the difference may not be large, the cellular suspensions isolated from BodyJet with SmartLipo samples do contain more viable cells than the cellular suspensions isolated from BodyJet only samples.

Surface Markers – Guava Express Pro Assays

Stem cell populations were determined by using the Guava Express Pro assay. For every sample, the cellular solution isolated from the tissue was gated based on size alone, then corrected for background fluorescence after the data collection took place. Figure 16 shows the histograms from a representative unstained cellular suspension. Any peaks seen in this figure are background noise and are therefore not included in the markers. The histogram markers define the four quadrants of the dot plots for stained cells. A representative dot plot is shown in Figure 17 with the remaining data converted to graphs for easier analysis. Unfortunately, the Guava

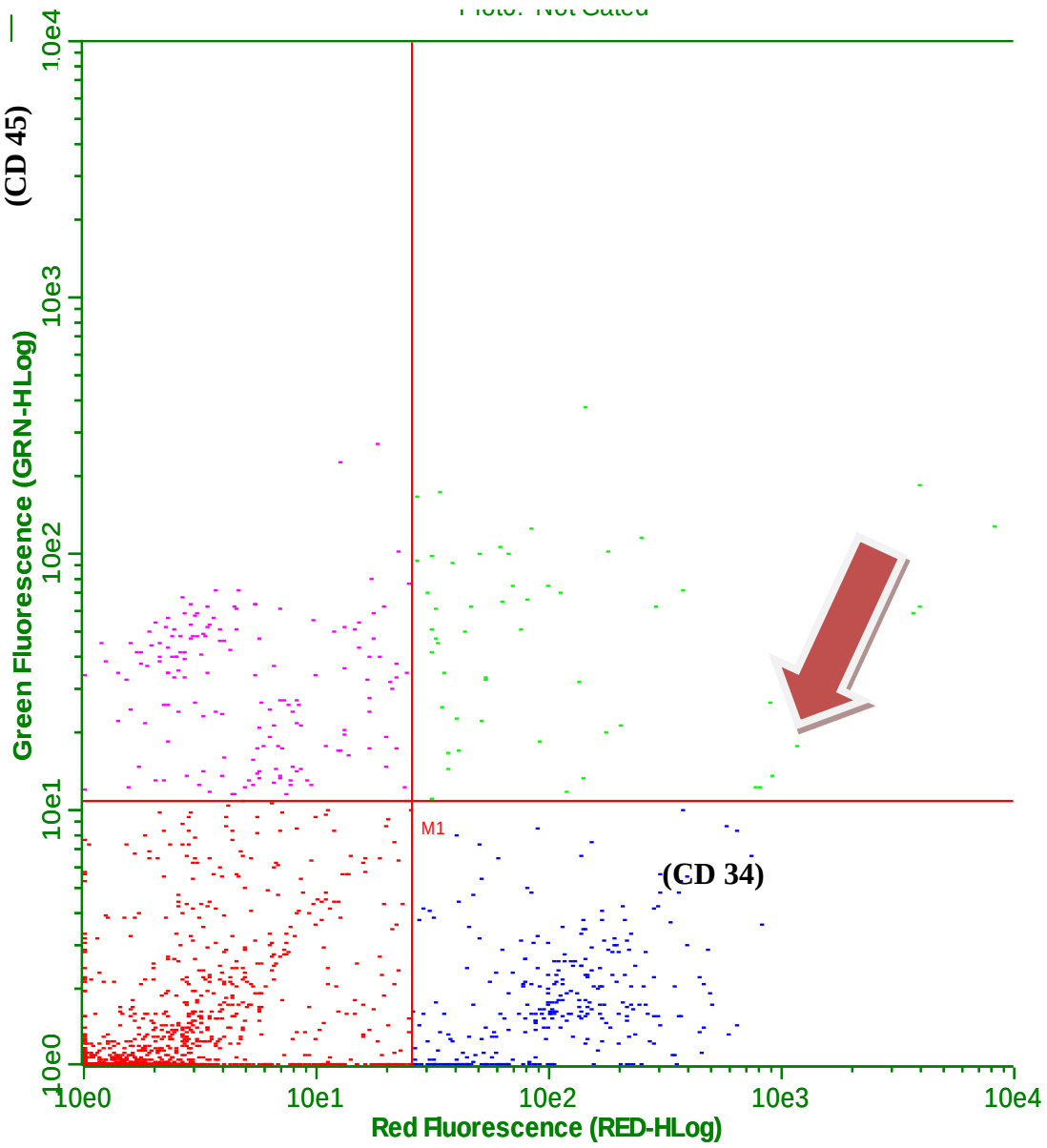
EasyCyte Flow cytometer and accompanying software does not allow for three-dimensional analysis. All analyses of surface marker data included CD34 (surface marker for stem cells) versus CD45 (surface marker for leukocytes) and CD34 versus CD31 (surface marker for leukocytes, red blood cells, and platelets.)

Figure 16: Unstained Cellular Suspension Guava Peaks



These three histograms are taken directly from the Guava software. Each histogram shows the number of positive events by intensity. The red line, labeled M1 for Marker 1, was drawn on each histogram to eliminate background fluorescence. These markers were drawn using an unstained cellular suspension and were redrawn for each sample. (A) green fluorescence, which corresponds with CD31; (B) yellow fluorescence, which corresponds with CD3; and (C) red fluorescence, which corresponds with CD34+.

Figure 17: Stem Cell Population Dot Plot

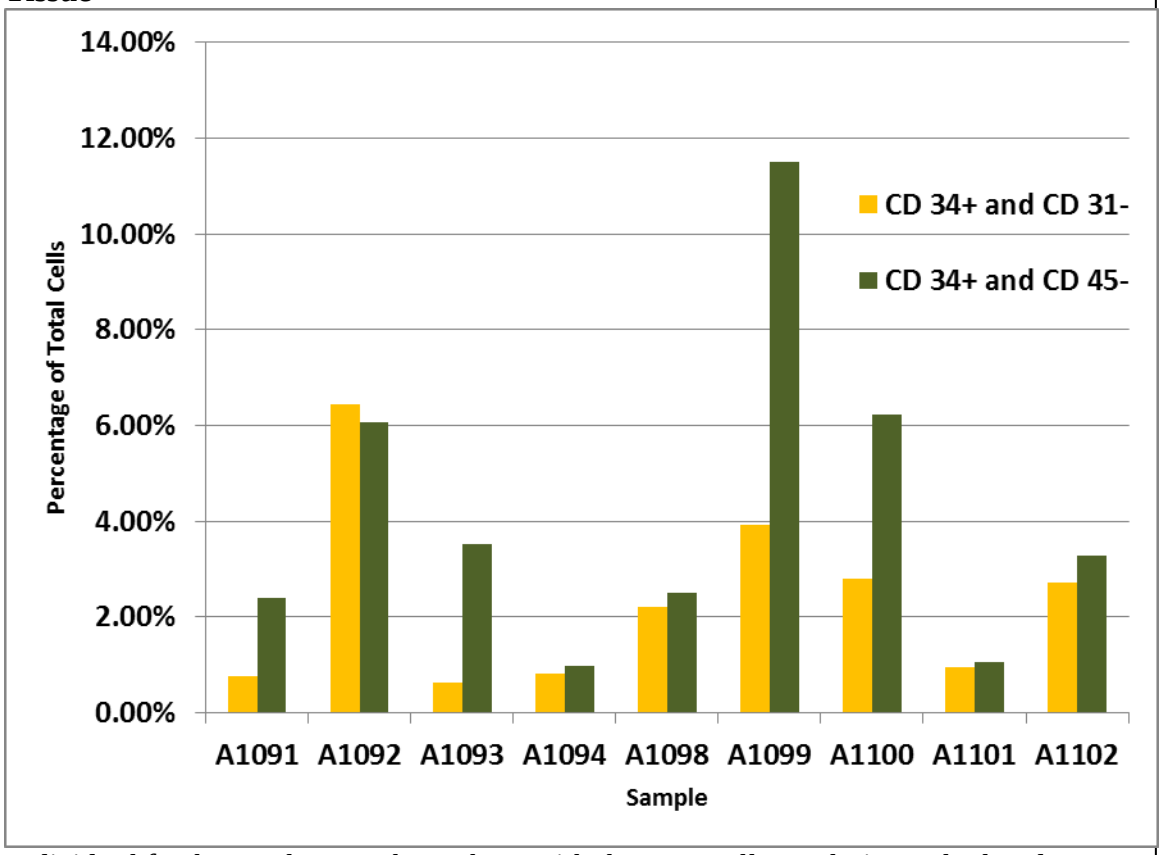


This dot plot was created by applying the histogram marker settings to the 2-dimensional plot. The upper-right quadrant represents cells that are positive for CD45 and CD34 antibodies, which are leukocytes. The upper-left quadrant represents cells that are only positive for CD45, and are also some subtype of leukocytes. The lower-left quadrant represents the majority of cells, which are not positive for CD45 or CD34, and are most-likely adipocytes. The lower-right quadrant (arrow) represents cells that are positive for CD34, but negative for CD45. This quadrant is where stem cells would be found.

Surface Markers – Frozen versus Fresh

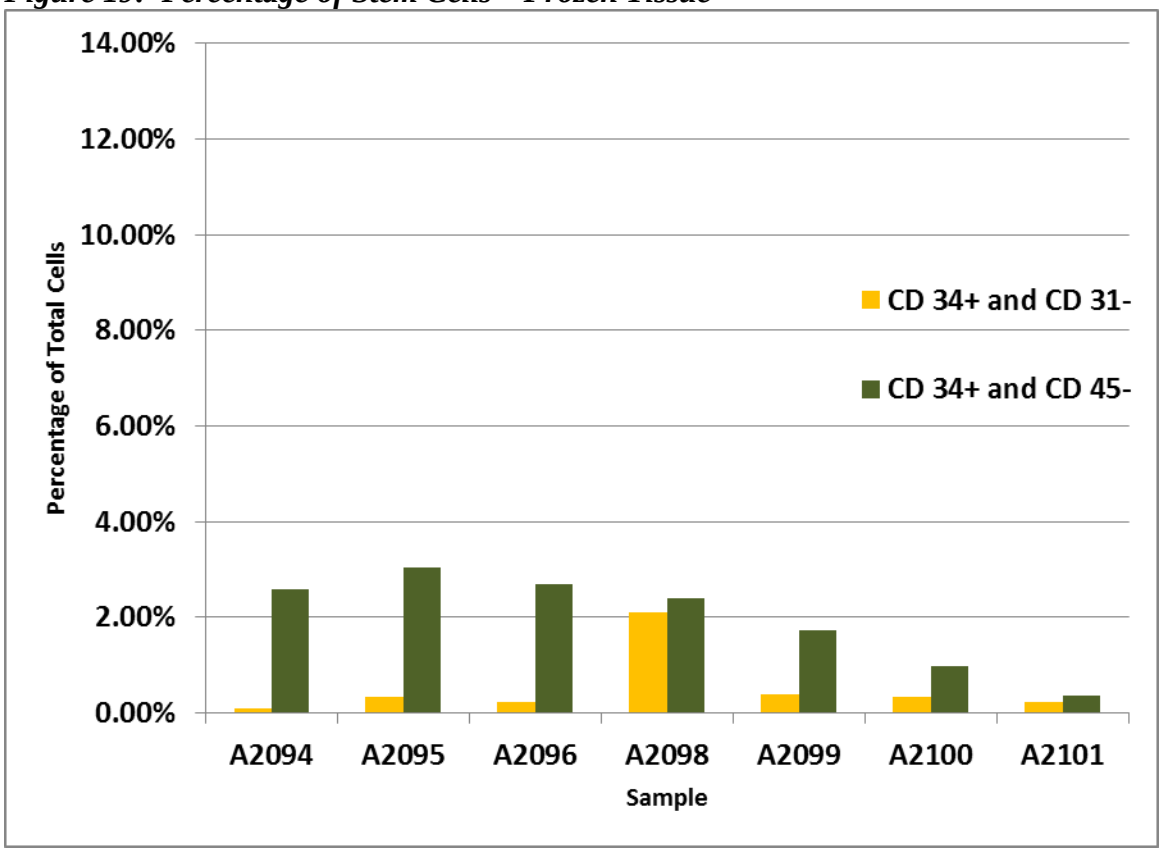
For most cellular suspensions isolated from fresh samples, stem cells—cells stained positively for CD34, but not CD31 or CD45 (Figure 18)—account for approximately 1% to 4% of the cells, with a few individual samples outside of that range. However, in cellular suspensions isolated from frozen samples (Figure 19), stem cells account for a much lower range, with no individual samples surpassing 3.5%.

Figure 18: Percentage of Stem Cells – Fresh Tissue



Individual fresh samples are shown here with the stem cell population calculated as CD34+, CD31- (yellow) and as CD34+, CD45- (green) as a percentage of the total cells. The yellow bar represents the number of CD34+, CD31- cells as a percentage of total cells.

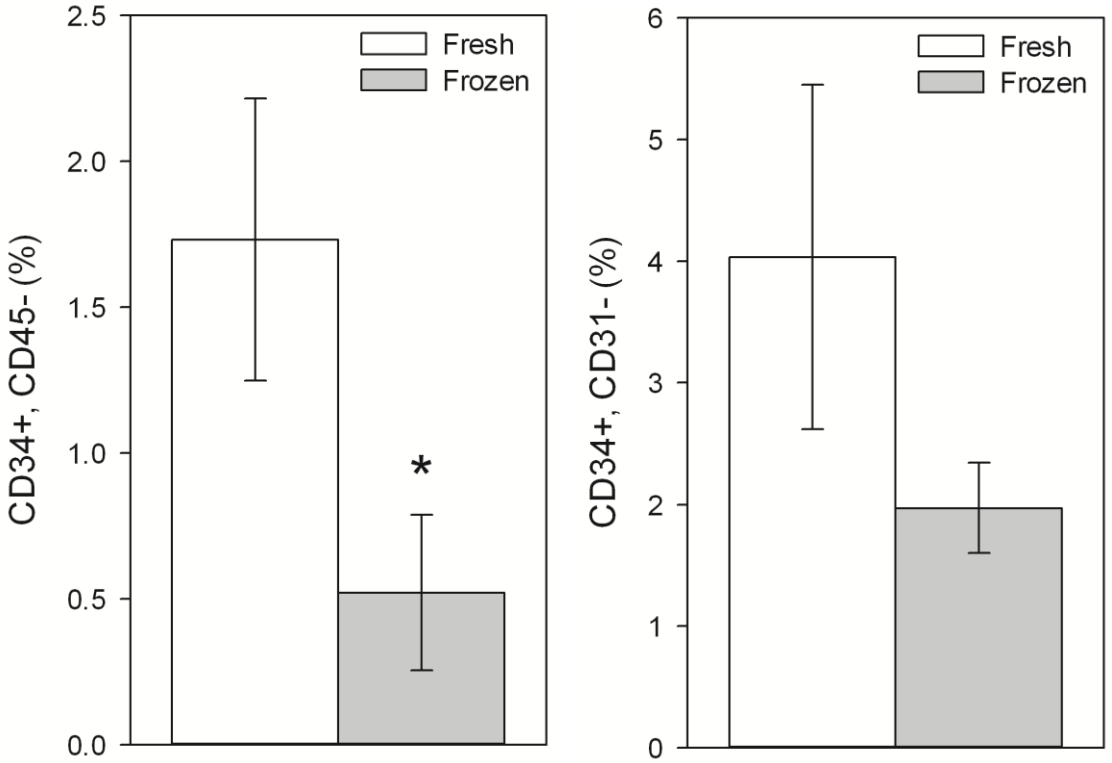
Figure 19: Percentage of Stem Cells – Frozen Tissue



Individual frozen samples are shown here with the stem cell population calculated as CD34+, CD31- (yellow) and as CD34+, CD45- (green) as a percentage of the total cells. The yellow bar represents the number of CD34+, CD31- cells as a percentage of total cells.

For statistical analysis, the individual samples were combined and compared. Figure 20A shows the comparison of CD34+, CD45- cells, as a percentage of the total cells, from fresh samples and frozen samples. The difference of about 1.25% is significant. This means that while the isolation process is still successful in pulling viable cells from frozen tissue, a significantly lesser number of the cells being isolated are actually stem cells. The comparison CD34+, CD31- cells from fresh and frozen samples mirrors that of the CD34+, CD45- comparison, although the CD34+, CD31- difference is very close, but not quite statistically significant.

Figure 20: Stem Cells as a Percentage of Total Cells: Fresh vs. Frozen

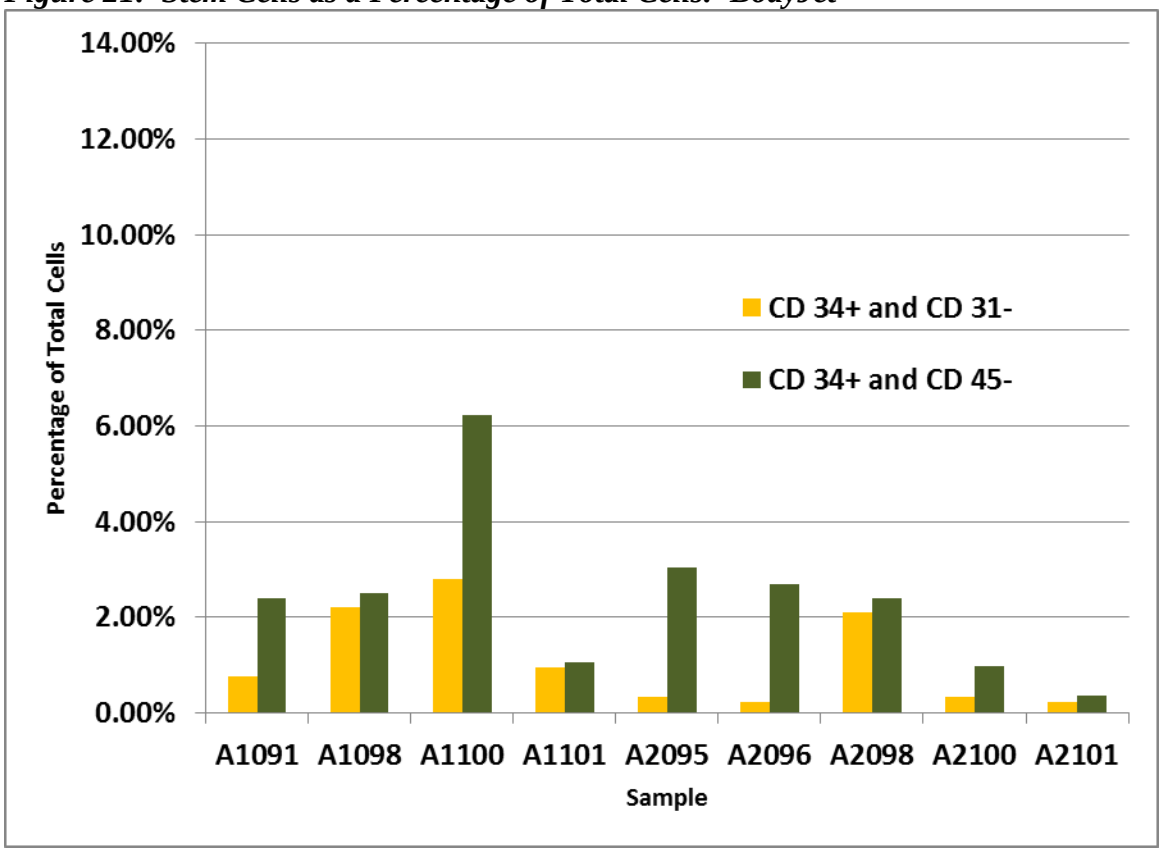


(A) *The bar graph shows the CD34+,CD45- cells as a percentage of total events counted from fresh (white) and frozen (grey) samples. There is a statistically significant difference ($p < 0.05$).*
(B) *The bar graph shows the CD34+,CD31- cells as a percentage of total events counted from fresh (white) and frozen (grey) samples.*

<u>Surface Markers – BodyJet versus BodyJet with SmartLipo</u>

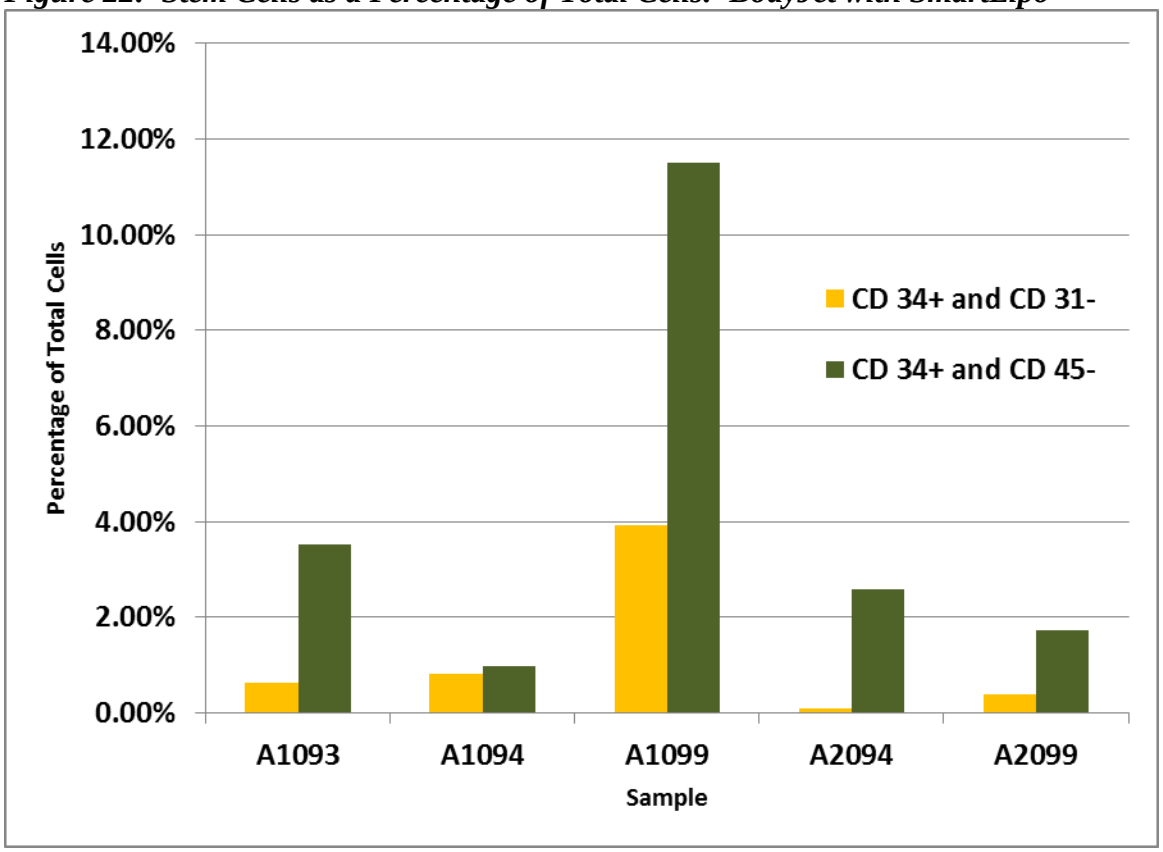
For most cellular suspensions isolated from BodyJet and from BodyJet with SmartLipo samples, stem cells account for approximately 1% to 4% of the cells, with a few individual samples outside of that range, as seen in Figure 21.

Figure 21: Stem Cells as a Percentage of Total Cells: BodyJet



Individual BodyJet samples are shown here, with the stem cell population calculated as CD34+, CD31- (yellow) and as CD34+, CD45- (green) as a percentage of the total cells.

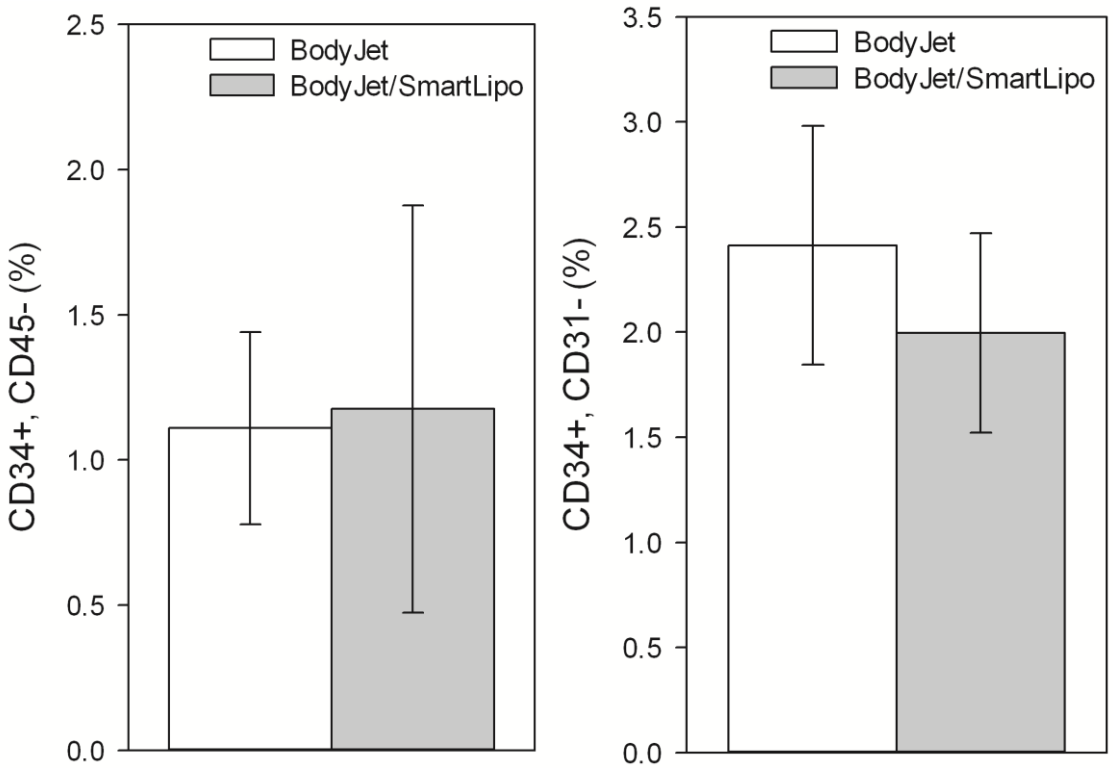
Figure 22: Stem Cells as a Percentage of Total Cells: BodyJet with SmartLipo



Individual BodyJet with SmartLipo samples are shown here with the stem cell population calculated as CD34+, CD31- (yellow) and as CD34+, CD45- (green) as a percentage of the total cells.

For statistical analysis, the individual samples were combined and compared. Figure 23A shows the comparison of CD34+, CD45- cells, as a percentage of the total cells, from BodyJet and BodyJet with SmartLipo samples. With both techniques averaging just over 1%, there is little to no difference in the percentage of stem cells retrieved from either set of lipoaspirate samples. This finding is confirmed with the comparison of CD34+, CD31- cells seen in Figure 23B.

Figure 23: Stem Cells as a Percentage of Total Cells: BodyJet vs. BodyJet with SmartLipo



(A) *The bar graph shows the CD34+,CD45- cells as a percentage of total events counted from BodyJet (white) and BodyJet with SmartLipo (grey) samples. There is no statistically significant difference.* (B) *The bar graph shows the CD34+,CD31- cells as a percentage of total events counted from BodyJet (white) and BodyJet with SmartLipo (grey) samples. There is no statistical significance.*

Differentiation Fate of Cryostored Lipoaspirate

A. Isolation of Body Jet and Body Jet/SmartLipo Cryostored Stem Cells

We wanted to determine whether Body Jet or Body Jet with SmartLipo lipoaspirate cryostored can yield ADSCs that differentiate along the chondrocyte and osteoblast differentiation pathways. We utilized fat-only samples of lipoaspirate that had been stored in our lab at -80ºC in 50mL centrifuge tubes since April 2010. We isolated the cell population from these samples using the isolation technique optimized in our lab (Murphy et al., 2012). Based on our previously published findings, we expected approximately 3% of the retrieved cells to be stem cells, whether from the Body Jet procedure or the Body Jet in combination with SmartLipo.

B. Body Jet and Body Jet/SmartLipo Stem Cell Plating

We isolated cells from the 50ml lipoaspirates and counted total cell number and viability with the Countess Automated Cell Counter (Table 1). Based on the total number of cells isolated, the expected percentage of ADSCs in that cell number and the percent viability, cells were plated at different densities to better optimize growth and differentiation.

Lipoaspiration Isolation	Technique	Total Cell Number	% Viability	Plating Density
1	BJ/SL	10,000	9%	10,000 per $25cm^2$
	BJ	70,000	18%	70,000 per $25cm^2$
2*	BJ/SL	5,000	3%	5,000 per 48-well plate
	BJ	50,000	12%	5,000 per 48-well plate
3*	BJ/SL	20,000	11%	5,000 per 48-well plate
	BJ	45,000	9%	5,000 per 48-well plate
4*	BJ/SL	55,000	10%	10,000 per 48-well plate

Table 1. Total cells were isolated from frozen lipoplasty samples BJ = BodyJet technique only and BJ/SL = BodyJet in combination with SmartLipo lipoplasty. Plating densities were as follows; 20,000 cells per cm^2, 5,000 cells per well in a 48 well plate. **Number*** indicates isolations used to obtain Olympus Fluoview FV1000 confocal microscopy pictures.

Isolation 1 was not placed in any differentiation media because of the low cell density when plated. Isolations 2-4 were placed at varying plating densities (Table 1) into maintenance media for 48 hours. The media was then changed to either Osteoblast Differentiating Media (ODM) or Chondrocyte Differentiating Media (CDM) for two weeks and tested via immunocytochemistry for the presence of osteocalcin and chondroitin sulfate proteoglycan. Immunocytochemistry of the Body Jet and Body Jet/SmartLipo samples revealed the presence of proteins indicative of extracellular matrix protein formation (Figure 24).

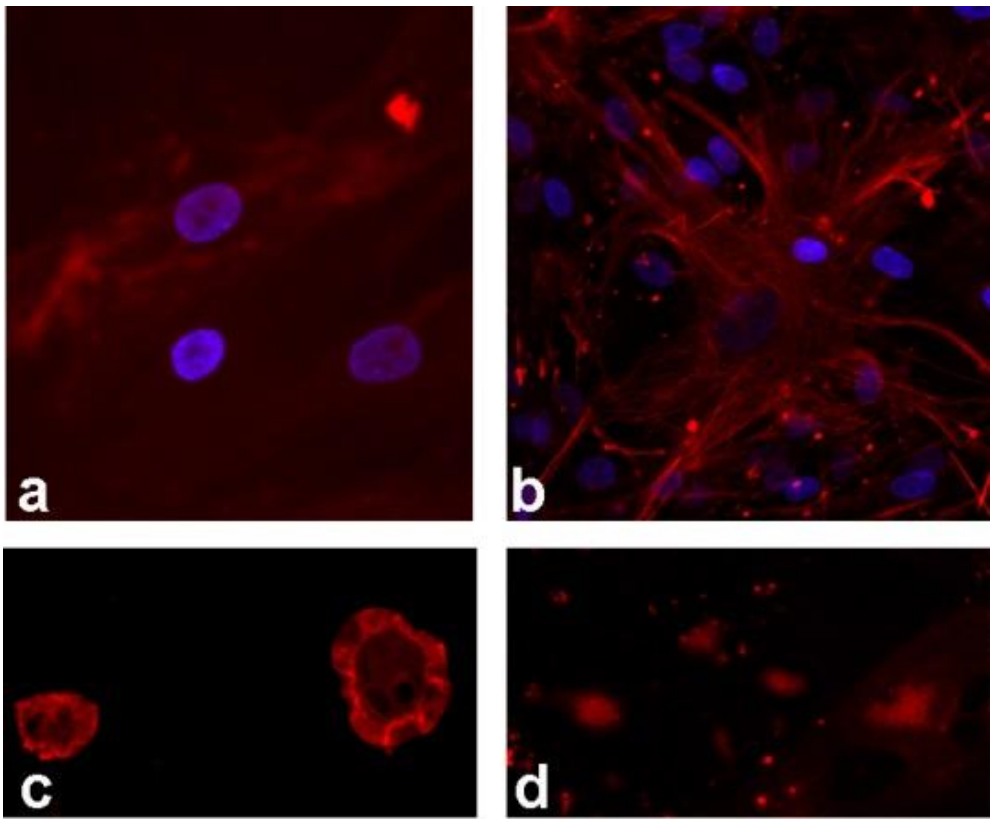


Figure 24. Immunocyology of extracellular matrix proteins produced by cultured, previously frozen lipoaspirate. Total cells were isolated from frozen lipoaspirate (Table 1) and reared in Osteoblast and Chondrocyte Differentiation Media for 2 weeks. a.) PCS-500-011 ATCC stem cell line stained for osteocalcin; b.) PCS-500-011 ATCC stem cell line stained for chondroitan sulfate proteoglycan (CSPG); c.) cells isolated from Body Jet with Smart Lipo cryostored lipoaspirate reared in ODM and stained for osteocalcin; d.) cells isolated from Body Jet cryostored lipoaspirate reared in CDM and stained for CSPG. Osteocalcin antibody (Abcam, Cambridge, MA) (secondary = Alexa Fluor 594 (red) (Invitrogen, Grand Island, NY)) Chondroitin sulfate proteoglycan antibody (CSPG) (Millipore, Billerica, MA) (secondary = Alexa Fluor 594 (red) (Invitrogen, Grand Island, NY)). All panels photographed with Olympus Fluoview FV1000 confocal microscope at 400x magnification, except panel d, which was 200x magnification.

REFERENCES:

American Society of Plastic Surgeons. "Report of the 2011 Plastic Surgery Statistics." 2012. http://www.plasticsurgery.org/News-and-Resources/2011-Statistics-.html.

Belogorodsky, D; Genoese, A; Debo, M; Chmielewski, SE; Murphy, D; Cho, E; Adams, CS and **M. D'Angelo** "Comparison of lipoplasty technique effects on the cellular fate potential of adipose derived stem cells" 2014 FASEBJ 28: 732.7

Moore, Caitlyn M., D'Angelo, Marina, Falcone, Victoria, and Adams, Christopher S. 2011 "The effect of liposuction technique on the number and viability of adipose derived stem cells (ADSC)." 1st Annual Research Day, April 27, 2011, PCOM, Philadelphia, PA.

Murphy, D; Moore, C; Genoese, A; Debo, M; Falcone, F; Adams, C and M D'Angelo "Lipoplasty technique affects the cellular fate potential of adipose derived stem cells: A comparison of Body Jet and Body Jet with Smart Lipo techniques" 2012 FASEB J 26: lb

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__x___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__x___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.) United States of America

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__x___ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
__x___ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.				☐Submitted ☐Accepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes__x_____ No_____

If yes, please describe your plans: We are currently preparing a manuscript for submission to the American Journal of Cosmetic Surgery

"A PRELIMINARY STUDY OF THE EFFECTS OF LIPOSUCTION TECHNIQUE ON ADIPOSE-DERIVED STEM CELL VIABILITY AND ABUNDANCE." Adams, C; Belogorodsky, D; Murphy, D; Moore, C; Cho, E; Chmielewski, S; Falcone, V and Marina D'Angelo.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

NONE

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

NONE

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___x_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____x_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Marina D'Angelo, PhD	Professor
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Drexel University	B.S.	1986	Biology
Thomas Jefferson University	Ph.D.	1992	Developmental Biology

A. Positions and Honors

Assistant Research Scientist (1992-1994) Pathology Department, Dr. Leslie Gold--Advisor -- NYU Medical Center, NYC, NY.
NRSA Postdoctoral Researcher (1994-9/98) Anatomy and Histology Department, Dr. Maurizio Pacifici--Advisor-- University of Pennsylvania School of Dental Medicine, Philadelphia, Pa.
Postdoctoral Fellow (9/98-6/99) Anatomy and Histology Department, Dr. Maurizio Pacifici – Advisor - University of Pennsylvania School of Dental Medicine, Philadelphia, Pa.
Lecturer/Postdoctoral Fellow (7/99-9/00) Biochemistry Department, Dr. Phoebe Leboy – Advisor – University of Pennsylvania School of Dental Medicine, Philadelphia, Pa.
Associate Scientist (9/00-7/01) Department of Anatomy and Cell Biology, Dr. Steven Popoff– Advisor – Temple University School of Medicine, Philadelphia, Pa.
Assistant Professor (7/01-6/05) Department of Anatomy, Philadelphia College of Osteopathic Medicine, Philadelphia, Pa.
Adjunct Assistant Professor (7/05-6/08) Department of Anatomy, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA.
Adjunct Associate Professor of Physical Therapy (7/05-Present) Institute for Physical Education Therapy, Widener University, Chester, Pa.
Associate Professor (7/05-6/11) Department of Anatomy, Philadelphia College of Osteopathic Medicine, Philadelphia, Pa.
Visiting Scholar (10/11-9/12) Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA
Professor (7/11-Present) Department of Anatomy, Philadelphia College of Osteopathic Medicine, Philadelphia, PA.

People's Choice Award 2012, Wharton Business Plan Competition: Consultant/Advisor for ChondroPro team.
University City Science Center QED Proof-of-Concept Program Top Ten Finalist 2010: "Metalloprotease peptide derivative therapeutics for osteoarthritis."
East Coast Connective Tissue Society Young Investigator Award 1999: "MMP-13 expression during chondrocyte maturation."
Orthopaedic Research Society New Investigator Recognition Award 1997: "Changes in TGFß activation and isoform profiles accompany endochondral ossification."

Patents: USPTO #61/391,446 "Compositions and methods for inhibition of matrix metalloprotease (MMP); M D'Angelo and A. Selim. Granted 12/13/2013.

Start-Up Company: Co-Founder, ProteaPex Therapeutics, Inc (March 2011-Present) Innovations in Animal Health; www.proteapextherapeutics.com

C. Peer-reviewed publications or manuscripts in press (in chronological order)

Selected Publications:

Herati, RS; Knox,VW; Ma, X; O'Donnell, P; **D'Angelo, M**; Haskins, ME and Katherine P. Ponder (2008) "Radiographic Evaluation of Mucopolysaccharidosis I and VII Dogs After Neonatal Gene Therapy" *Molecular Genetics and Metabolism* 95(3):142-151.

Simonaro, C.M.; **M. D'Angelo**; X. He; E. Eliyahu; N. Shtraizent; M.E. Haskins and E.H. Schuchman (2008) "Mechanism of glycosaminoglycan-mediated bone and joint disease: Implications for the Mucopolysaccharidoses and other connective tissue diseases." *The American Journal of Pathology* 172(1):112-122

Journal Cover:
Glyn D. Palmer, Alejandro H. Piton, Lwin Mon Thant, Serafim M. Oliveira, **Marina D'Angelo**, Mukundan G. Attur, Steven B. Abramson, Cristina C. Teixeira (2010) "F-spondin regulates chondrocyte terminal differentiation and endochondral bone formation" *Journal of Orthopaedic Research* 28(10):1323

D. Research Support

Grants Funded as PI:
CCDA Grant 10/07-10/08 "Hypertrophic chondrocytes produce a large latent TGFβ complex that includes MMP13"
CCDA Grant 7/08-6/09 "The protease sensitive hinge region of LTBP1 is a substrate for MMP-13"
CCDA Grant 7/09-6/10 "Efficacy of collagenase 3 peptides in abrogation of tissue destruction in an animal model of osteoarthritis"
CCDA Grant 7/10-6/11 "Novel Metalloprotease Peptide Derivative Therapeutics for Osteoarthritis"
CCDA Grant 7/12-6/13 "Assaying Endogenous Matrix Metalloproteases (MMPs) in Acid-etched, Dentinal Cavity Walls of an Extracted Tooth"
CCDA Grant 7/13-6/14 "Development of an *in vitro*, whole tooth model to study effects of endogenous matrix metalloprotease activity at dentinal cavity walls of restoration"

Grants Funded as Co-Investigator/Collaborator:
National MPS Society Grant 7/03 to 6/05 "Joint pathology in MPS VI " – collaborator
National MPS Society Grant 7/05 to 6/07 "Biomarkers and treatments for joint pathology in MPS VI" - collaborator
NIDDK 2/02 to 1/07 "MPS VII canine model" – collaborator
NIDDK 5/05 to 4/09 "MPS VI and I feline model"- collaborator
CCDA Pilot Grant 7/10-6/11 "Effect of liposuction technique on the validity and differentiation of adipose derived stem cells"