

AD_____

Award Number: W81XWH-12-1-0395

TITLE: A Novel Mechanism for the Pathogenesis of Nonmelanoma Skin Cancer Resulting from Early Exposure to Ultraviolet Light

PRINCIPAL INVESTIGATOR: Rebecca Morris

CONTRACTING ORGANIZATION: University of Minnesota
Minneapolis, MN 55912

REPORT DATE: September 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE September 2013		2. REPORT TYPE Annual		3. DATES COVERED 01 September 2012-31 August 2013	
4. TITLE AND SUBTITLE A Novel Mechanism for the Pathogenesis of Nonmelanoma Skin Cancer Resulting from Early Exposure to Ultraviolet Light				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-12-1-0395	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Rebecca Morris E-Mail: rmorris@hi.umn.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Minnesota The Hormel Institute 801 16 th Ave. NE Minneapolis, MN 55912				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We proposed that skin egress, entering the circulation, and traveling throughout the body may be a characteristic of skin stem cells in response to ultraviolet light. We have made four significant findings in this regard: 1) murine epidermal keratinocytes migrate preferentially in vitro to whole living bone marrow with its conditioned medium, 2) keratinocytes migrate towards SDF1 alpha and HMGB1 baits over control attractants such as 3T3, medium without serum, and FBS, 3) CD184 (SDFR1 alpha) is expressed on approximately 27% of CD49f+/CD34+ hair follicle stem cells and suggesting that these cells may have the capacity to follow an SDF1alpha chemokine gradient, and 4) there is an increase in CD49f+/Keratin 14+ cells in blood and bone marrow following solar UV treatment as determined by FACS, immunofluorescence microscopy, and quantitative real-time PCR. The significance of these findings support our hypothesis that: 1) skin egress may be a characteristic of skin stem cells in response to ultraviolet light, and 2) bone marrow may be a long-lived reservoir of sunlight initiated stem cells that can repopulate the skin even years later upon damage caused by petrochemicals, skin wounding, or physical, chemical, or thermal damage to the skin.					
15. SUBJECT TERMS non-melanoma skin cancer, epidermal keratinocytes, stem cells, migration					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	25	19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

INTRODUCTION.....	3
BODY	4
KEY RESEARCH ACCOMPLISHMENTS	7
REPORTABLE OUTCOMES.....	8
CONCLUSIONS	9
REFERENCES.....	10
APPENDIX.....	11
SUPPORTING DATA.....	24

INTRODUCTION:

The epidermal layer of the skin is composed largely of cells called keratinocytes. Keratinocytes in the basal layer are organized into subpopulations based on their proliferative nature and include stem cells (relatively rare) and transit amplifying cells (comprise most of the proliferating cells). When a stem cell divides, one daughter usually remains a stem cell while the other daughter gives rise to transit amplifying cells with limited proliferative potential. Upon completion of their divisions, transit amplifying cells undergo an orderly maturation process called terminal differentiation that includes their outward displacement through the suprabasal layers, production of high molecular weight keratins, loss of their nuclei, and formation of an impermeable outer structure called the cornified envelope. This process is exceptionally orderly and maintains the normal thickness and cellularity, and the normal functions of the epidermis throughout life. This proposal focuses on the stem cells of the hair follicles because they not only serve as a reservoir of epidermal cells, they also possess remarkable regenerative potential and are known to be able to reconstitute a graft, to heal wounds, and even to give rise to non-melanoma skin cancer (1). Therefore, identification of stem cell behavioral characteristics and responses are critical problems in cutaneous biology. We proposed here the novel concept that skin egress, entering the circulation, and traveling throughout the body may be a new behavior of epidermal stem cells. We proposed that sunburn following exposure to sunlight has the capacity to make skin stem cells migrate. In this Discovery award we challenge the existing paradigm of skin cells in response to sunburn. We address the following question: Do hair follicle stem cells migrate from the skin following sunburn as a consequence of ultraviolet light induced inflammation? Our hypothesis is that sunburn makes the hair follicles stem cells leave the skin and enter the blood circulation, and home to the bone marrow. Therefore, we proposed in this pilot study to test the first axiom: that CD34 positive keratinocyte stem cells have the capacity for extracutaneous migration. We will accomplish this with a novel application of standard in vitro assays together with the use of two transgenic mice that are already available including two UV experiments, and a skin grafting experiment. The rationale for the in vitro experiment was that the cellular and molecular players involved may be identified, isolated, and manipulated. The rationale for the in vivo experiments is that the cellular players also may be identified and isolated and explored systemically under their native conditions.

BODY:

In our Discovery proposal, we submitted two Specific Aims to provide evidence whether or not keratinocyte stem cells may leave the skin and migrate to the bone marrow.

MAJOR OBJECTIVES FOR SPECIFIC AIM 1. The first aim was to determine whether keratinocyte stem cells are chemotactic and attracted towards bone marrow and other tissues in culture. **General Approach.** First, we applied a migration co-culture system where whole bone tissue, including hematopoietic and stromal elements were placed as bait beneath keratinocytes including hair follicle stem cells cultivated on a filter. Keratinocytes were attracted to the bait and migrated through the filter and were quantified. Second, we documented the presence of keratin immunoreactive cells in the blood and bone marrow following exposure of mice to solar UV radiation by fluorescence activated cell sorting (FACS), immunofluorescence microscopy, and by quantitative real-time polymerase chain reaction (qPCR).

SPECIFIC RESULTS FOR AIM 1 (YEAR 01). The figures are listed in order as an appendix section.

Specific Aim 1, Task 1. Epidermal keratinocytes were harvested from individual mice and placed in a BD Biocoat Matrigel Migration Assay performed according to the manufacturer's directions. The assays were conducted in growth factor and serum-free medium. Results of pilot experiments were used to determine the number of wells needed to achieve statistically significant results. The positive control was a metastatic keratinocyte cell line, and the negative controls included keratinocytes cultured on the filters without bone tissue in the chamber, "irrelevant" cells such as 3T3 cells as bait, serum-free medium, examination of filters without cells (as a control on the staining procedure), examination of various cell densities to determine the keratinocyte seeding density most appropriate for studying influences on migration (linear portion of scale) and time courses to determine the most appropriate intervals. Keratinocytes were placed on the dish insert and a) whole crushed bone and b) bone marrow conditioned medium were individually placed in the well. **Figure 1a** demonstrates the results of duplicate experiments indicating that 0.5×10^6 cells per well was an optimal seeding density as we wanted to be on an approximately linear range of the seeding dose-response. **Figure 1b** demonstrates the effect of migration time on the number of migrated keratinocytes. Because the 36-hour time point showed the least variance and the greatest reproducibility among experimental replicates and because it was midway between the shortest and longest timepoints, it was chosen as the timepoint for comparison with test baits. Figures 1a and 1b led up to our test experiment comparing the effects of bone marrow and bone marrow conditioned medium on the migration of epidermal keratinocytes (**Figure 1c**). Several interesting findings emerge from this Figure 1c. First, epidermal keratinocytes are strongly attracted to the bone marrow with its conditioned medium as well as to bone marrow conditioned medium alone. Second, the epidermal keratinocytes are minimally attracted to other controls such as 3T3 cells, or even to DMEM with 10% FBS. A third and potentially interesting finding is that bone marrow cells are rather well attracted to epidermal keratinocytes in DMEM without FBS.

Specific Aim 1, Task 2. In the second in vitro experiment, we performed FACS analysis on mouse skin keratinocytes immunostained with antibodies to alpha-6 integrin (CD49f), CD34, and CXCR4 (the receptor for SDF1). Although there is some indication in the literature that epidermal keratinocytes express this receptor, we wanted to determine specifically which subpopulation of keratinocytes (the CD49f+/CD34+ hair follicle stem cells) expressed this receptor. Keratinocytes were harvested from five individual C57BL/6 female mice and processed for FACS analysis as we have previously reported. Positive controls were bone marrow stromal cells. Negative controls were isotype control antibodies. **Figure 2a** details the FACS sorting of freshly harvested epidermal keratinocytes stained with fluorescent antibodies to CD49f (alpha 6 integrin, the external component of the hemidesmosomes found on all basal keratinocytes), CD34 expressed on hair follicle stem cells, and CD184 (also known as CXCR4, the receptor for stromal derived factor 1 alpha (SDF1 alpha)). We note with interest that 10.7% of the CD49+/CD34+ hair follicle stem cells are also positive for CD184. This finding is

interesting to us because, the keratinocytes that have the receptor for CXCL12 would be the most likely cells to be able to respond to it. Here we demonstrate that the CD49f+/CD34 double positive hair follicle stem cells are among those cells. In light of our finding a paper reporting that a subset of lineage-/Sca1+ bone marrow progenitors expresses the PDGFR1 alpha (CD140) and is recruited from the bone marrow to grafted skin where they apparently become epithelial cells (Tamai et al, PNAS, 2010), and because we could find no confirmation of this report, we used some of our leftover cells to determine whether we could also find this population. Our attempts proved fruitful as demonstrated in **Figure 2b** (without lineage depletion) and **Figure 2c** (with lineage depletion). This is significant because these bone marrow cells may be the ones that are attracted to the keratinocyte bait shown in **Figure 1c**. We will apply for permission to pursue this finding in Year 02 of our award.

Specific Aim 1, Task 3. Epidermal keratinocytes were harvested from individual K15EGFP mice will be placed in the Matrigel invasion assay as summarized in Experiment 1. In this experiment we specifically focused on the percentage of the K15EGFP immunoreactive cells. This task was not accomplished in Year 01 because the K15EGFP mice were no longer available. We hope to accomplish this task in Year 02 by using FACS-sorted CD49f+/CD34+ hair follicle stem cells (the same cells that express the keratin 15 marker).

Specific Aim 1, Task 4. In this in vitro experiment, keratinocytes were harvested from individual mice. Keratinocytes were placed on the Matrigel filters, and stromal derived factor-1 was placed in the wells at concentrations of 400 to 800 ng/ml as bait. Positive and negative controls, and analyses were performed as summarized in Experiment 1. **Figure 3a** illustrates that freshly harvested epidermal keratinocytes are attracted to 800 ng/ml of SDF1 alpha (CXCL12) more than they are attracted to 3T3 or DMEM without serum, but almost as much as to DMEM with 10% FBS. We await quantification of our second and third experiments. **Figure 3b** shows our first attempt to assess keratinocyte and bone marrow migration toward HMGB1 bait (HMGB1 is the ligand for the PDGFR1 alpha). We believe that there were problems with the keratinocytes in this determination as the controls were so low. A second determination is illustrated in **Figure 3c** where the controls are as expected. This figure demonstrates that, although epidermal keratinocytes appear not to be attracted to mouse HMGB1 protein above the controls, the bone marrow cells are strongly attracted to it. This observation is consistent with that of **Figure 1c** showing that a subset of lineage depleted bone marrow cells indeed has the PDGFR1 alpha, the receptor for HMGB1.

MAJOR MILESTONES FOR SPECIFIC AIM 1. We found that keratinocyte stem cells migrate through the filters towards the bone marrow as compared with requisite positive and negative controls. We also found a significant increase in the number of CD49f+/Keratin 14+ cells in blood and bone marrow following exposure of the mice to solar UV radiation relevant to Aim 2, Task 5.

MAJOR OBJECTIVES FOR AIM 2 (to be conducted in Year 02). The focus of this in vivo aim is to determine whether hair follicle stem cells or their progeny migrate from the skin during ultraviolet light induced inflammation and carcinogenesis. **General Approach.** We will establish skin grafts of Krt1-15EGFP enriched and depleted subpopulations, and quantify by FACS, fluorescent keratinocytes in the dermis, blood, bone marrow and other tissues.

MAJOR TASKS FOR AIM 2

Specific Aim 2, Task 5. In experiment 2, we will expose mice to artificial sunlight. At the intervals following exposure to artificial sunlight, groups of 2 mice of each genotype will be euthanized, and “green” EGFP-reactive cells in the epidermis and hair follicles, dermis, blood, lymph nodes, and bone marrow will be quantified by light microscopy. Typically, 1000 to 5000 cells are counted.

RESULTS RELEVANT TO SPECIFIC AIM 2, TASK 5

In preparation for Specific Aim 2, Task 5 to be conducted during Year 02, we examined keratinocytes, blood, and bone marrow by FACS to determine whether we could detect keratin 14 immunoreactivity in blood and bone marrow. As illustrated in **Figure 4a**, the percentage of keratin 14 expressing cells is normally quite low in both blood and bone marrow, but increases considerably in response to solar UV treatment. At the moment, we do not yet know whether this UV-dependent increase is a consequence of keratinocyte egress from the skin; however, we also had the opportunity to obtain bone marrow from euthanized mice in ongoing tumor experiments from other investigators at the Hormel Institute, and we looked for keratin 14 immunoreactive cells in blood and bone marrow by light microscopy of smears of nucleated cells. Although we still need more experience to perfect this experimental method, we were able to confirm the presence of keratin 14 immunoreactive cells in both blood and bone marrow as summarized in the table labeled **Figure 4b**. To investigate further this novel observation, we performed qPCR on freshly harvested epidermal keratinocytes, and on mononuclear cells from blood and bone marrow. As expected, both keratins 14 and 15 were detected in epidermal keratinocytes (**Figure 4c**). However, we also detected them in blood and bone marrow from C57BL/6 mice, and keratin 14 only in the blood and bone marrow of BALB/c mice (**Figure 4c**). Therefore, we have confirmed the presence of epidermal cytokeratin 14 by three different methods, by FACS, by immunofluorescence microscopy, and by qPCR.

Specific Aim 2, Task 6. In this experiment epidermal keratinocytes will be harvested from K15EGFP female mice and from wild-type littermates. For the skin reconstitution assay, keratinocytes will be surgically implanted onto a 1 cm diameter area of dorsal fascia using a silicone chamber initially to contain the cells. The grafts will be placed on SCID female mice. These grafts will completely stabilize 1 to 2 weeks following implantation. All surgical procedures will be conducted on anesthetized mice in a laminar flow hood with sterile technique according to procedures of the University of Minnesota Research Animal Resources. Groups of 5 grafts will be harvested at 4 day, 7 day, and 14 day time points. “Green” EGFP reactive keratinocytes will be quantified by fluorescence microscopy or EGFP immunostaining in the grafts as well as in dermis, blood, lymph nodes and bone marrow as described under Specific Aim 1, above.

MAJOR MILESTONES ANTICIPATED FOR AIM 2. In the first experiment (Task 5), if our hypothesis is correct, then we would expect to see a UV dose-dependent increase in the number of skin-derived EGFP-reactive keratinocytes in non-cutaneous tissues. If our hypothesis is partially correct, we should find metastatic cells during the later stages of skin tumor progression. Similarly, in the second experiment, we would find EGFP immunoreactive keratinocytes in non-cutaneous tissues following establishment of the skin grafts. Either positive or negative results in either of these experiments would be significant and would be worth accomplishing.

KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 01:

- Freshly harvested murine epidermal keratinocytes migrate preferentially to whole living bone marrow with bone marrow conditioned medium.
- Freshly harvested murine epidermal keratinocytes migrate to SDF1 alpha, while bone marrow cells migrate to HMGB1 better than freshly harvested epidermal keratinocytes.
- CD184 (SDFR1 alpha) is expressed on approximately 27 percent of CD49f+/CD34+ hair follicle stem cells.
- There is an increase in keratin 14 immunoreactive cells in blood and bone marrow of normal (untreated control) mice as well as a possible increase in keratin 14 expression following solar UV treatment to the dorsum of mice as determined by FACS, immunofluorescence microscopy, and quantitative real-time PCR.

REPORTABLE OUTCOMES YEAR 01:

- No reportable outcomes were made in Year 01.
- Submission of a Manuscript is anticipated by the end of 2014.
- We anticipate submitting an R01 proposal on the subject of this DOD award by the end of Year 02 (2014).
- We expect to make a presentation of this research at the International Skin Carcinogenesis Conference in June of 2014.

CONCLUSIONS FROM OUR RESEARCH IN YEAR 01:

We conclude that the data presented herein support our novel hypothesis that (mouse) skin keratinocytes can leave the cutaneous epithelium and enter the blood and bone marrow. Should these findings hold true in Year 02, the medical implications would be that the bone marrow could be a reservoir of transformed keratinocytes with the potential to migrate back to the skin as cancer initiating cells upon further damage to the skin. The implications for the US Military would be 1) the need to prevent the first damaging exposure to the skin, and 2) the need to prevent the re-recruitment of the initiated keratinocytes back to the skin by manipulating the cytokine pathways used (possibly SDF1alpha or HMGB1?) upon further damage to the skin such as by combat wounds. Further, if our findings are substantiated by the in vivo studies proposed in Year 02 and by complementary findings in humans that we will propose in an R01 next year, they would suggest that the number of keratin 14-expressing cells in the blood could provide a simple test for persons at risk for developing non-melanoma skin cancer.

REFERENCES:

1. Li, S., Park, H., Trempus, C.S., Gordon, D., Liu, Y., Cotsarelis, G., and **Morris, R.J.** Keratin 15 expressing stem cells from the hair follicles contribute to papilloma development in the mouse. *Mol. Carcinogenesis* 2012, Epub ahead of print, Li, Park, and Trempus share equally as first author.
2. Tamai, K., Yamazaki, T., Chino, T., Ishii, M., Otsuru, S., Kikuchi, Y., Iinima, S., Kaga, K., Nimura, K., Shimbo, T., Umegaki, N., Katayama, I., Miyazaki, J., Takeda, J., McGrath, J.A., Uitto, J., and Kaneda, Y. PDGFR1 alpha-positive cells in bone marrow are mobilized by high mobility group box 1 (HMGB1) to regenerate injured epithelia. *Proc. Natl. Acad. Sci. USA* 108: 6609-6614, 2011.

APPENDIX:

The Figures representing the principal findings during Year 01 follow here in order as one page per figure together with descriptive legends. Twelve (12) pages of Appendix Figures follow.

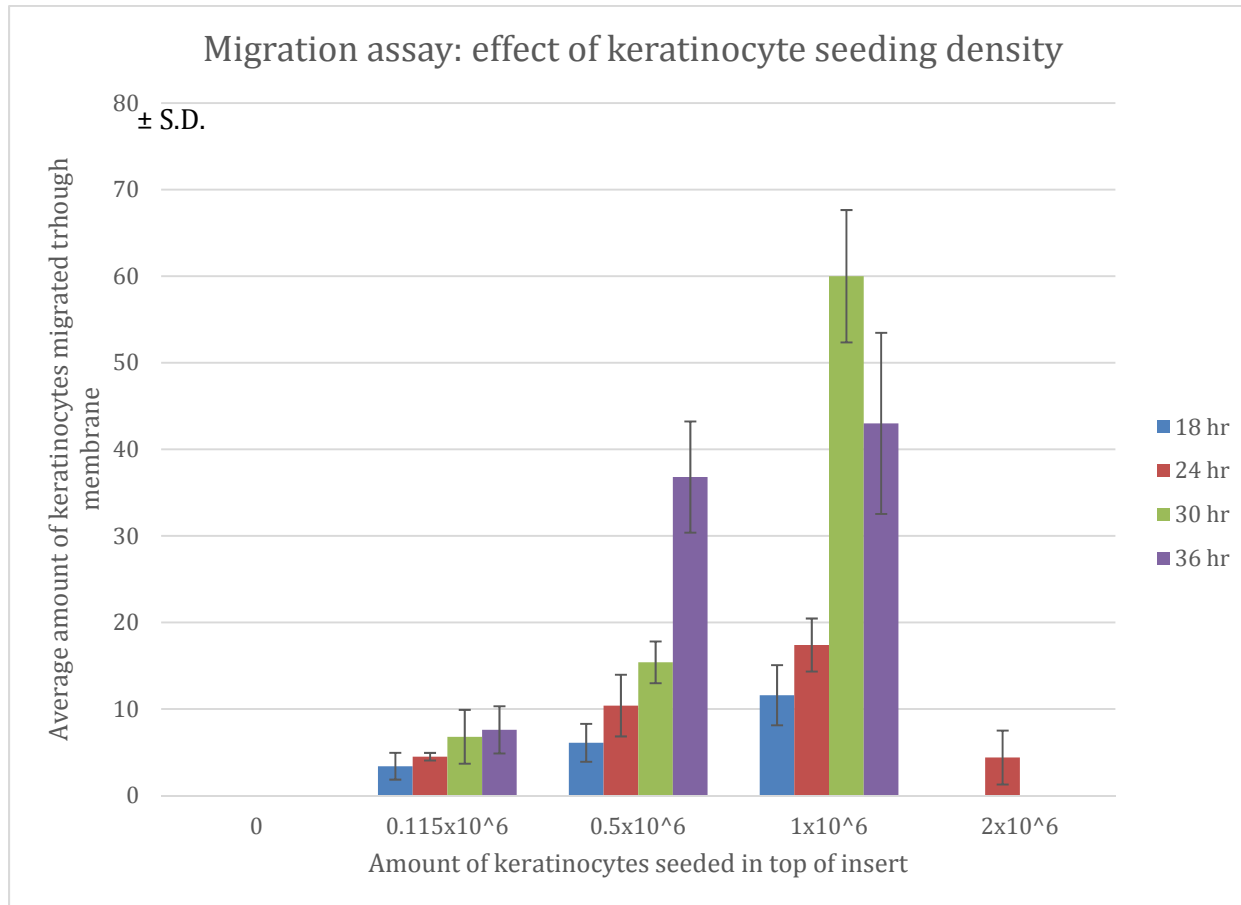


Figure 1a. Keratinocyte dose response Optimal keratinocyte seeding density was determined to be 0.5×10^6 keratinocytes per insert. This chart is a representative assay of 2 performed. Each assay consisted of 3 wells per time point for each condition.

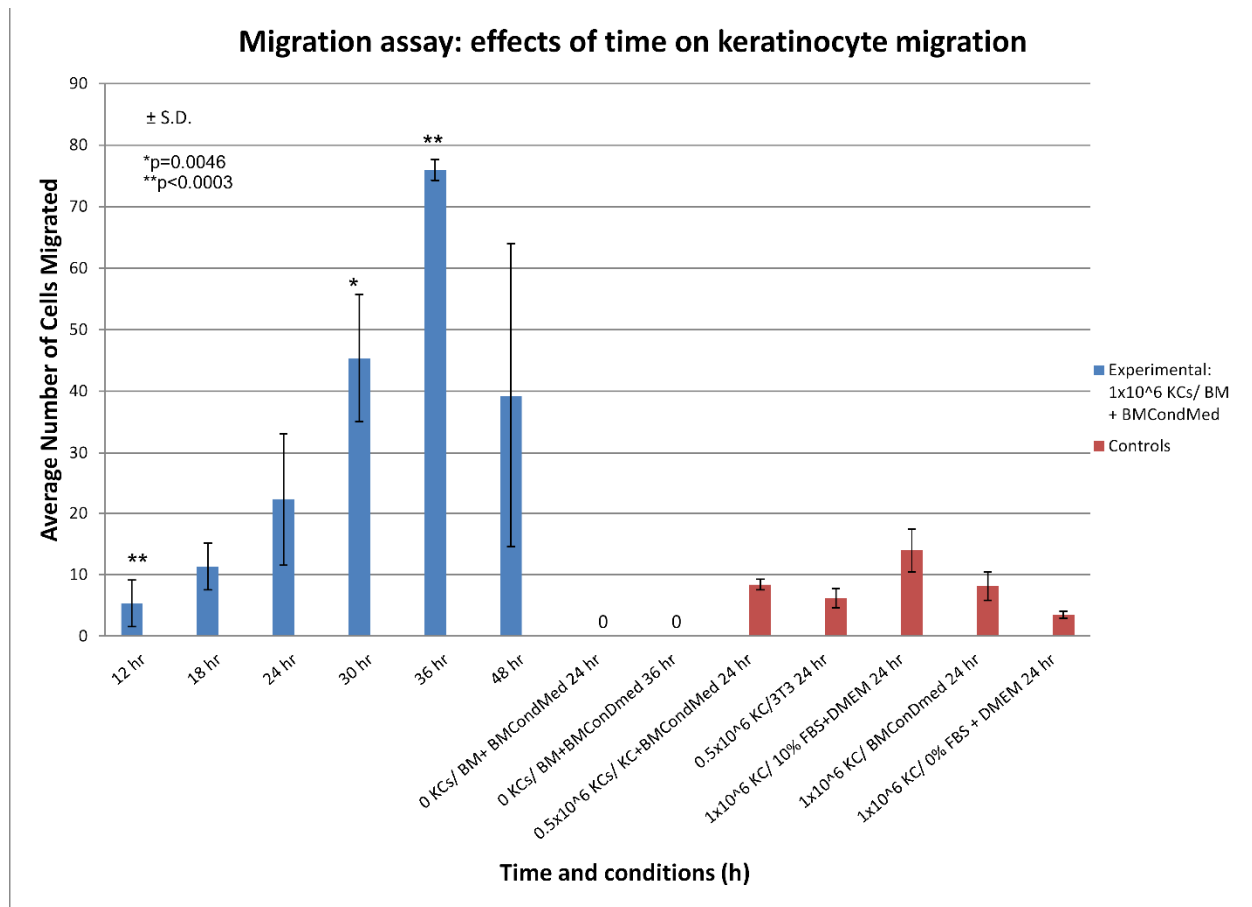


Figure 1b. Keratinocyte time response. This chart shows that the optimal migration time was determined to be 36 hours. This chart is the compiled data of 3 assays performed, each consisting of 3 wells per condition.

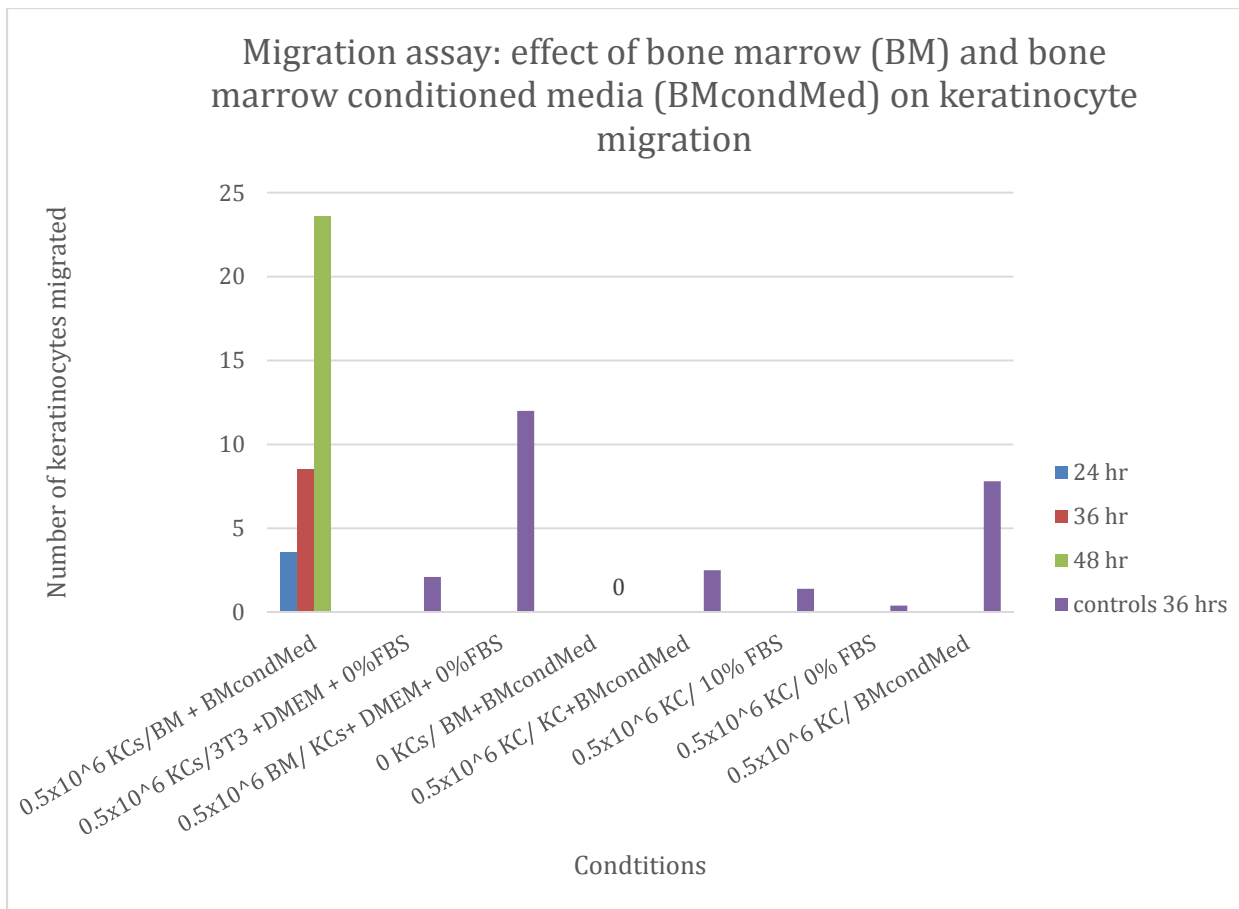


Figure 1c. Keratinocyte Migration with Bone Marrow or Bone Marrow Conditioned Media as chemo-attractant. These data are representative of 2 assays with each assay consisting of 3 wells for each condition except for the experimental well of 0.5x10⁶ KCs over BM+BMcondMed at 36 hours which used 6 wells. The point of this figure is that freshly harvester epidermal keratinocytes migrate through Matrigel and a filter towards bait consisting of bone marrow cells and their conditioned medium. These results also demonstrate that bone marrow cells migrate towards epidermal keratinocytes.

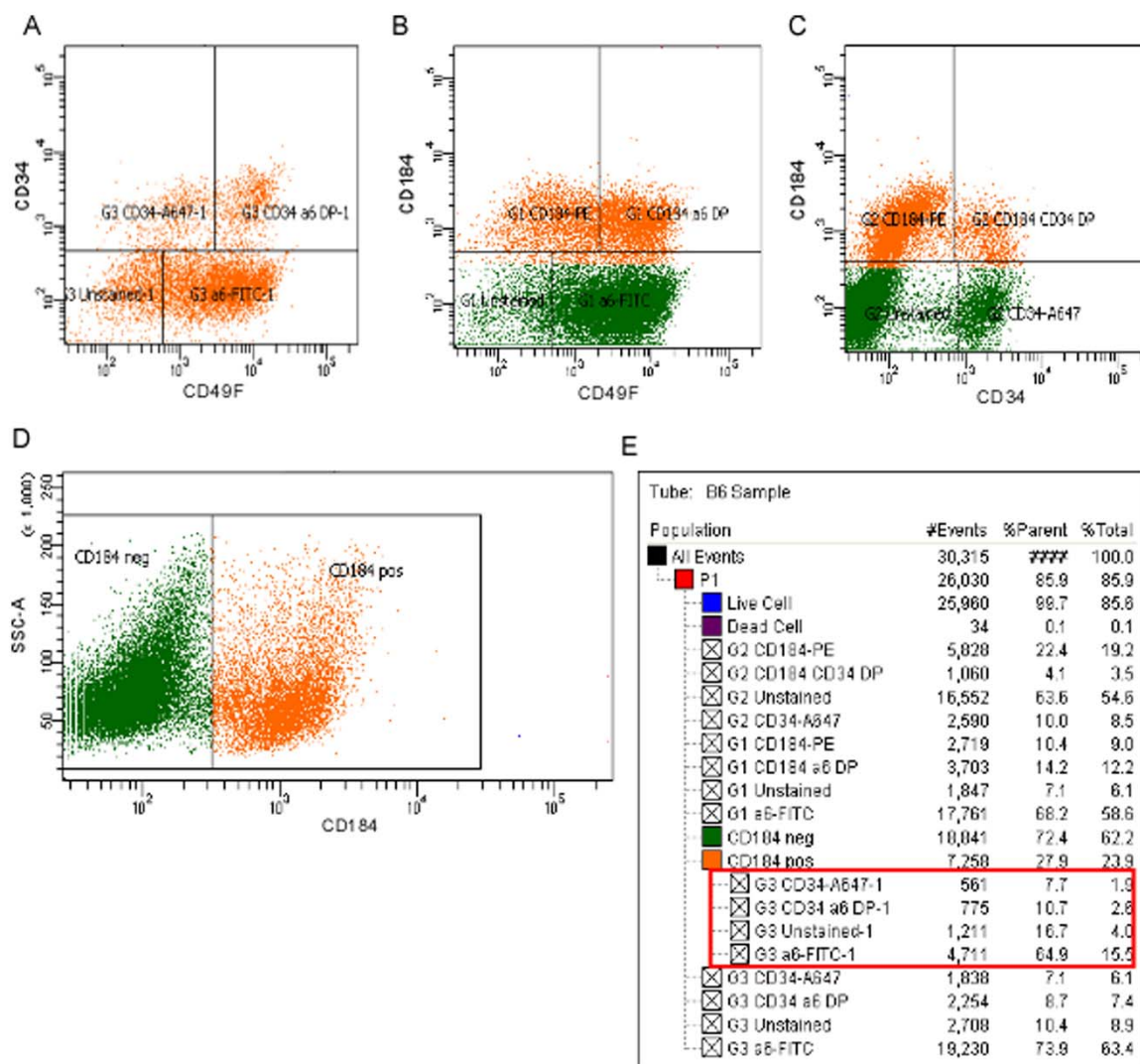


Figure 2a. FACS plot of a6+/CD34+/CXCR4+. FACS report from analysis of CD34, CD49f, and CD184 (Sdf-1 alpha receptor) (also known as CXCR4) on freshly harvested murine epidermal keratinocytes.

A: Cells that are CD34+ and CD49f+ (a6+) double positive; 8.7% of the parent population.

B: Cells that are CD184+ (sdf1R+) and CD49f+ (a6+) double positive; 14.2% of the parent population.

C: Cells that are CD184+ (sdf1R+) and CD34+; 4.1% of the parent population.

D: Total count of cells that are positive for CD184+ (sdf1R+); 27.9% of the parent population.

E: Table of the percentages of cells in different gated populations during flow cytometry. Cells that are CD34+, CD49f+ (a6+), and CD184+ (sdf1R+) triple positive account for 10.7% of the parent population.

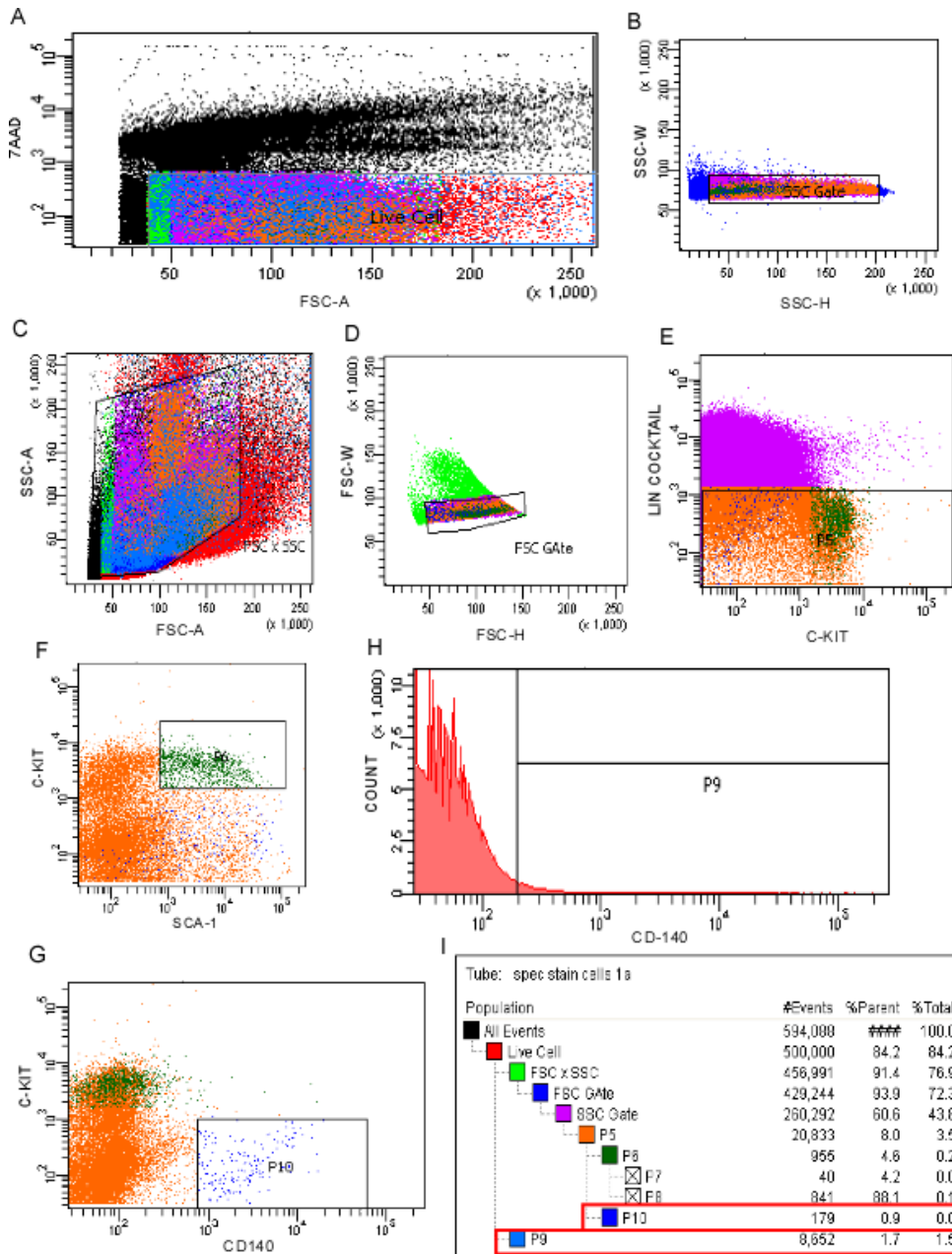


Figure 2b. FACS plot of a6+/CD34+/PDGFRa+, No lineage depletion. FACS analysis on harvested bone marrow cells without lineage depletion using the BD hematopoietic stem cell and progenitor cell kit in conjunction with CD140 (PDGFRa).

A: Gating for live cells of interest.

B-D: Forward and side scatter gating

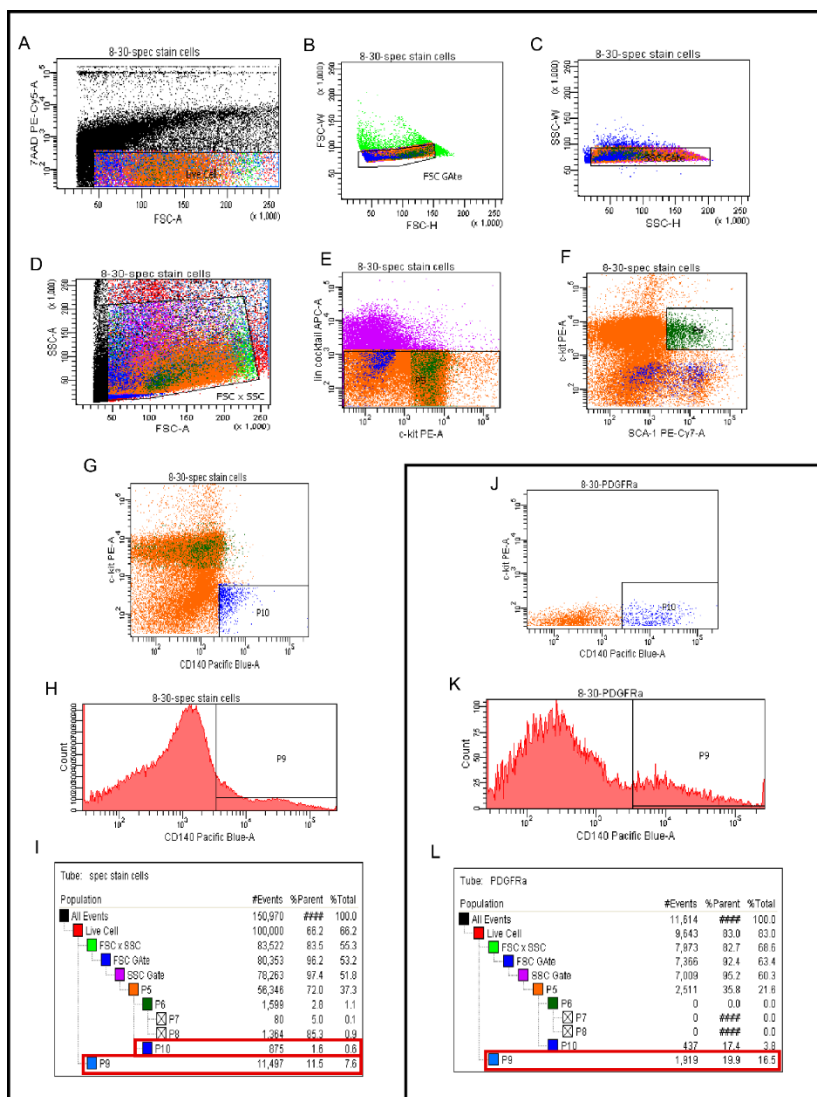
E: dot plot with P5 representing the population of target hematopoietic stem cells and progenitor cells.

F: P6 is the population of cells that are progenitor cells.

G: P10 represents the population of CD140 positive cells from the progenitor cells.

H: Histogram of progenitor cells with P9 representing the gate for CD140 positive cells of the progenitor cells.

I: Table showing percent of cell populations. P10 shows that 0.9% of the parent population of progenitor cells are CD140 positive. P9 shows that 1.7% of all cells are CD140 positive.



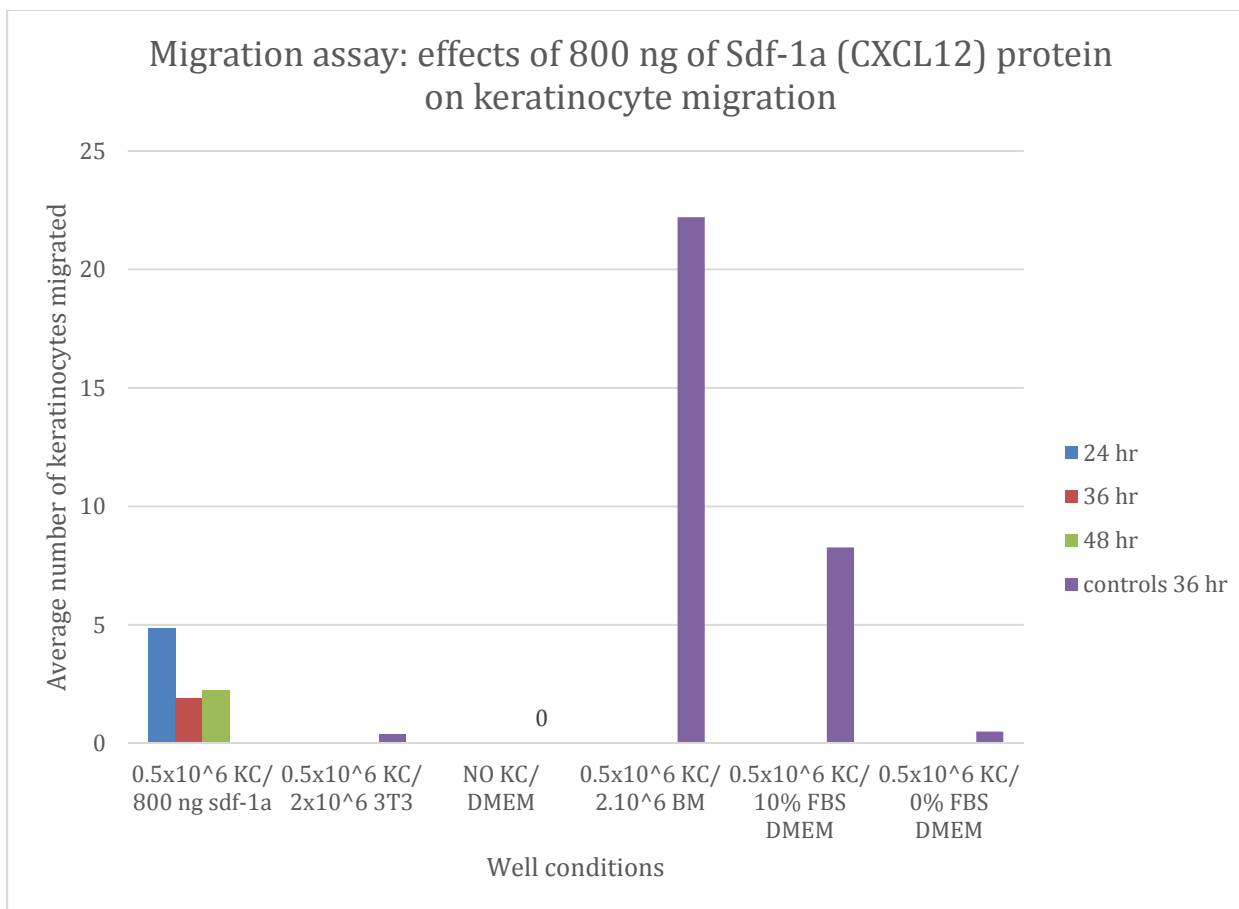


Figure 3a. CXCL12 Migration Assay Data for the first migration assay using Sdf-1a (CXCL12) as a chemoattractant was using the literature determined 800 ng as the optimal concentration is shown here. This experiment was run using the BD Biocoat matrigel migration assay with 3 wells seeded per control condition and 6 wells seeded for the experimental group. The second repetition of this assay was with a gradient of concentrations of 2000 ng, 800 ng, and 200 ng of Sdf-1a protein. These data are still being processed. The third repetition of this assay was a repeat of the first assay and is still being counted.

Migration assay: effects of mHMGB1 dose response on migration of keratinocytes and bone marrow cells

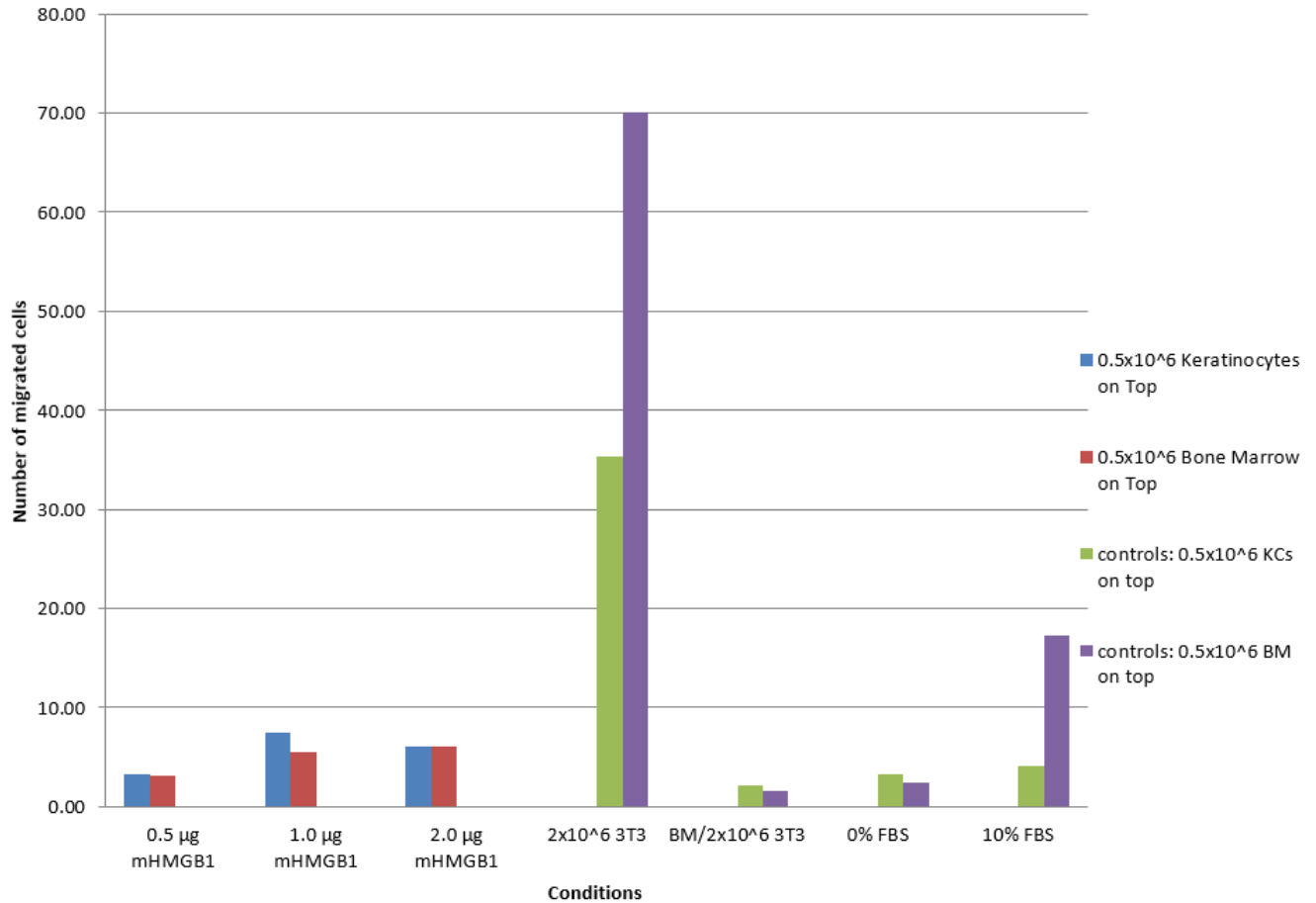


Figure 3b. HMGB1 Migration Assay First trial using mHMGB1 as chemoattractant. 3 wells were seeded for all conditions except for the 1.0 ug mHMGB1 dose in which 6 wells were seeded and all wells were run for 36 hours. We believe there was a problem with the quality of the KCs in this experiment as the KC over BM control wells have far less migration than seen in previous trials.

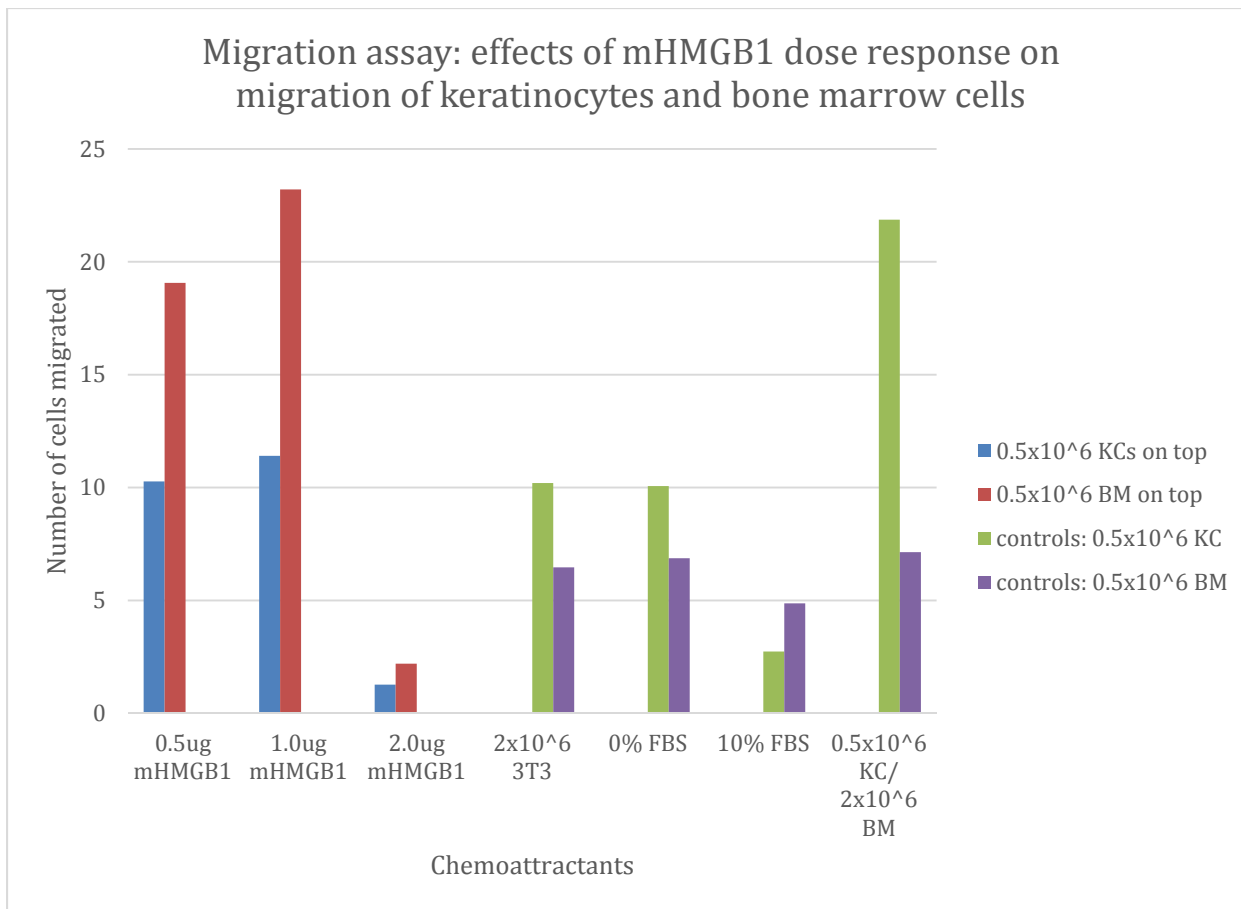
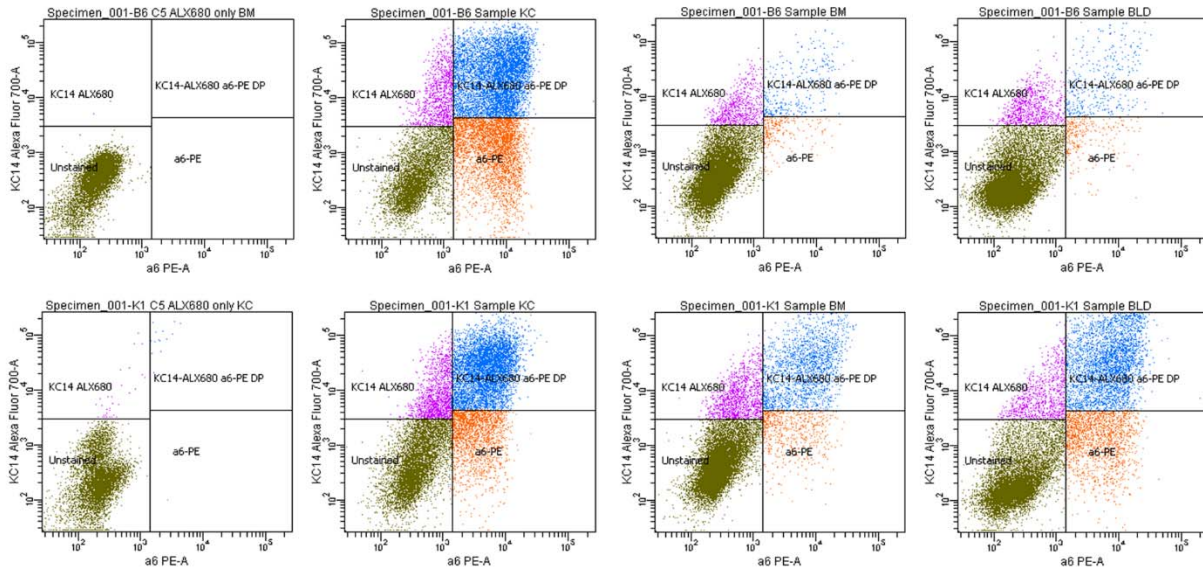


Figure 3c. Second trial using mHMGB1 as chemoattractant. 3 wells were used for all conditions except for the 1.0 ug mHMGB1 dose in which 6 wells were used. These results demonstrate that although keratinocytes are not significantly attracted to mHMGB1 as bait, the bone marrow cells are attracted to the HMGB1 as bait.



		K14-/a6-	K14+/a6-	K14+/a6+	K14-/a6+
KC	Control	31.6	6	38.1	24.3
	UV	41.6	9.3	35.7	13.7
BM	Control	93	3.9	1.7	1.5
	UV	78.7	7.1	10.5	3.6
BLD	Control	90.5	5.2	2.3	1.9
	UV	61	5.4	21.9	11.7

Figure 4a (FACS dot plot) and (table). FACS of KCs in blood and bone marrow. The percent distribution of K14 and a6 subpopulations among KCs, BM, and BLD samples in solar UV treated and control C57BL/6 mice. In BM (red boxes) and BLD (blue boxes) there is an increase in the K14+/a6+ subpopulation after UV treatment. There is also a decrease in the K14-/a6- subpopulation in BM and BLD samples and an increase in the K14-/a6+ subpopulation in only BLD samples.

Keratin 14 immunoreactive cells are found in bone marrow

Mouse Strain	Tissue Type	Treatment Duration	Treatment Type	%K14+ cells
C57BL/6	KC	0	none	+++
C57BL/6	KC	0	none	+++
C57BL/6	BM	1x	UV	++
C57BL/6	BM	1x	UV	++
SKH-1	BM	12 wk	UV	+++
C57BL/6	BM	24 wk	UV	++
C57BL/6	BM	31 wk	DMBA/TPA	++

+ Low ++ Medium +++ High

Figure 4b. Percentage of K14 in immunofluorescence microscopy (table). Mice underwent UV or chemical treatment to induce tumor formation. At different intervals of carcinogenesis bone marrow was collected, smears were made and then stained with fluorescent antibodies to keratin 14. Keratin 14 immunoreactive cells and negative nucleated cells were counted and the percentage positive calculated. This table shows that keratin 14 is seen not only at a greater presence than most of the keratinocytes as expected in untreated mice, but that at a middle time point of 12 weeks of UV treatment from an SKH-1 mouse with papilloma development there is also a high incidence of keratin 14 in the bone marrow. These results confirm the FACS results in figure 4a that keratin 14 immunoreactive cells are found in the bone marrow both before and during cutaneous carcinogenesis.

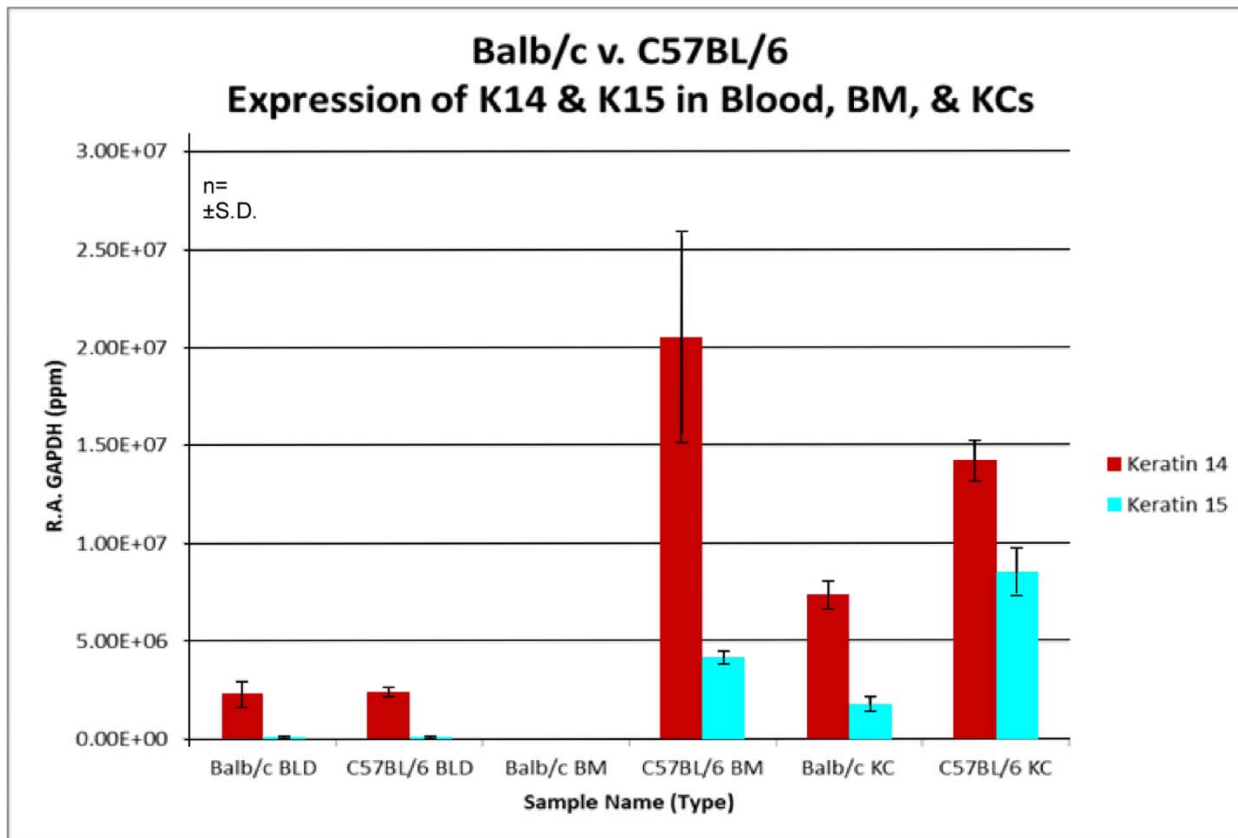


Figure 4c. QPCR for KCs in blood and bone marrow of normal, untreated mice. This graph is the compiled data from 3 replicate assays in which each assay had triplicate wells for each condition. Keratin 14 and 15 expression in the blood and bone marrow of BALB/c and c57BL/6 mice in terms of relative abundance. Keratin 14 is known to be present in all basal keratinocytes whereas keratin 15 is present in the CD49f+/CD34+ hair follicle stem cells. The surprising thing we see here is that keratin 14 is expressed in both blood and bone marrow of C57BL/6 mice and in blood of BALB/c mice. We were also surprised to detect keratin 15 in the bone marrow of C57BL/6 mice.

SUPPORTING DATA:

- Figures relevant to each Task are presented as Appendix pages of this Progress Report but are described following the presentation of each Task and the Methods used to achieve it.