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Defining Gene Expression in Normal Human Epidermal Keratinocytes and Melanocytes: A Prerequisite for Understanding Hyperplastic and Neoplastic Pathology

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DEFINING GENE EXPRESSION IN NORMAL HUMAN EPIDERMAL KERATINOCYTES AND MELANOCYTES:

A PREREQUISITE FOR UNDERSTANDING HYPERPLASTIC AND NEOPLASTIC PATHOLOGY

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To define pathologic alterations, a reference of "normal" cells is needed in order to interpret genomic methods that study gene expression of melanocytes and keratinocytes in growth-activated or neoplastic skin diseases. Historically, mRNAs isolated from cultured epidermal keratinocytes or melanocytes are used to define normal gene expression patterns. In this study, we profiled global gene expression in human epidermal keratinocytes on Affymetrix U133 plus 2.0 arrays from three different "normal" sources: 1) cultured keratinocytes, 2) FACS keratinocytes from dispase-separated epidermis, and 3) laser-capture microdissected (LCM) epidermis. For melanocytes, the precursor cell of melanoma, the attempt was made to isolate a more physiologically relevant sample source than that of the forced *in vitro* proliferating phenotype. Our results suggested that the best definition of "normal" keratinocyte gene expression is obtained via LCM of normal epidermis. Even short-term suspension culture of KCs (used for FACS) altered gene expression. Established primary KCs in culture express some genes, e.g., keratin 16, at levels found in pathologic states such as psoriasis. Currently limited by LCM methodology, the identification of melanocyte was defined by c-kit$^+$ FACS samples, using the caveats of this technique acknowledged in the keratinocyte comparison. The results of the gene expression analysis show a modulation of many important keratinocyte genes based on whether and how long they were in culture. Overall, our results indicate the need to carefully consider "normal" *in situ* sources of cells in order to properly define normal vs. pathologic gene expression.

This thesis is dedicated

to my father

William J. Billick III

who told me I could do anything I put my mind to

and

to my grandmother

Angela Gates Anderson

who continues to be a resilient pillar of strength at the age of 92

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List of Abbreviations

bFGF	basic fibroblast growth factor
CE	cornified envelope
ET-3	endothelin 3
FACS	fluorescent activated cell sorting
HGF	hepatic growth factor
KC	keratinocyte
LCM	Laser capture microdissection
MM	malignant melanoma
NHEK	Normal human epidermal keratinocytes
NHEM	Normal human epidermal melanocytes
SC	stratum corneum
SCF	stem cell factor

Materials and Methods

Primary cell cultures

Commercially available normal human epidermal melanocytes (PromoCell C-12453 NHEM) were initially isolated from different locations from the epidermis of adult skin, including the face, the breasts, the abdomen, and the thighs. Melanocytes had been isolated using serum-free, phorbol myristate acetate (PMA) free melanocyte growth medium M2. Since PMA is a tumor promoting mitogen that can interfere with experimental approaches, we used cells isolated in melanocyte growth medium M2 and continued to use this medium for cultivation. After isolation, all PromoCell normal human epidermal melanocytes were cryopreserved at passage 2 (P2) by using PromoCell's proprietary, serum-free freezing medium, Cryo-SFM. Each cryovial contained more than 500,000 viable cells after thawing. Proliferating cell cultures were made from 500,000 cryopreserved cells that had been thawed and cultured for three days at PromoCell. Quality control tests were performed for each lot of PromoCell normal human epidermal melanocytes, including cell morphology, adherence rate, and cell viability. Furthermore, immunohistochemical tests for the cell-type specific marker, Mel-5 (tyrosinase), were carried out for each lot. Growth performance was tested through multiple passages up to 15 population doublings (PD) under culture conditions without antibiotics and antimycotics. In addition, all cells were tested for the absence of HIV-1, HBV, HCV, and microbial contaminants (fungi, bacteria, and

mycoplasma). Arrays were performed on 10 independently derived isolates of melanocytes. Values reported are means for all cell strains analyzed.

Normal human epidermal keratinocytes (NHEK) were initially isolated by PromoCell from the epidermis of adult skin [from different locations like the face, the breasts, the abdomen, and the thighs.] C-12004 cells were tested for cell morphology, adherence rate, and cell viability. Furthermore, immunohistochemical tests for the cell-type specific marker cytokeratin were carried out for each lot. >500,000 "passage 2" normal human epidermal keratinocytes (NHEK) from n=10 adult donors were purchased from PromoCell GmbH. Cells were equilibrated at 37°C and 5% CO_2. Grown in PromoCell Keratinocyte Growth Medium 2 (C-20111.) At less than 80% confluency cells were washed with Dulbecco's PBS and lysed in Qiagen RNeasy buffer RLT. Lysates were stored at -80°C before RNA was purified using Qiagen's RNeasy Mini Kit. RNA yield was high from *in vitro* samples, which, were double amplified in the same manner as the *in vivo* LCM samples to reduce technical error resulting from the different protocol of single amplification (H. Mitsui, personal communication.) Arrays were performed on 10 independently derived isolates of keratinocytes. Values reported are means for all cell strains analyzed.

Melanocyte defined media

Using a basal media supplemented only with insulin and hydrocortisone, cell morphology and survival was assessed by microscopy with all possible [individual and synergistic] additions of growth factors: single, double, triple and all four growth factors were compared to PromoCell commercial melanocyte M2 media as 'basal' medium [by using only HC and insulin supplements]. One mg of ET-3 (aSpec, cat # 24524) was diluted in 10 mL of PBS and aliquoted into 39 Eppendorf tubes of 250 μL at 100 μg/mL; the final concentration in media was 10nM. 25 μg of bFGF basic (R&D Systems, Cat# 233-FB/CF) was re-suspended in 250 μL of PBS with 1% BSA/HAS, aliquoted into 25 vials of 10μL, each containing 1 μg of basic FGF at a concentration of 100μg/mL for an end concentration in media of 10ng/mL. 5 μg human HGH (R&D Systems Cat# 294-HGN/CF) was suspended in 250 μL of PBS with 1% BSA/HAS to make 5 10μL aliquots of 100μg/mL for an end concentration in media of 40ng/mL. 50 μg of SCF (R&D systems, Cat#255-SC/CF) was re-suspended in 1 mL of PBS and aliquoted into 5 tubes of 200μL (containing 10μg of SCF at 50 μg/mL) the working medium concentration was 200 ng/mL. All growth factors once re-suspended were stored at -20°C.

RNA extraction and analysis

Lysates were kept at -80°C until samples from the same patient could be pooled for RNA extraction by the RNeasy Micro Kit (Qiagen Valencia, CA) according to manufacturer's protocol with on-column DNase digestion. The amount of RNA was too low to be analyzed by spectroscopy so the quantity was assessed using RT-PCR and a

standard curve created by Hiroshi Mitsui, M.D., Ph.D (personal communication.) Total RNA was extracted into 10.0μl of RNase free water using RNeasy Micro Kit. Quantitative RT-PCR (qRT-PCR) for human acidic ribosomal protein (hARP) was performed using the EZ PCR Core Reagent (Applied Biosystems, Foster City, CA) to calculate the concentration of RNA. Briefly, 1μl of total RNA was diluted into 2 μl of RNase-free water. One μl of diluted total RNA was applied into a well with 49μl of RT-PCR reaction buffer including 3 ul of primers and probe mix. Serial dilutions of total RNA (ranging from 2000 pg/μl to 1 pg/μl) extracted were prepared and used to make a standard curve. The samples were amplified and quantified by using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and the following thermal cycler conditions: 2 minutes at 50°C; 30 minutes at 60°C; 5 minutes at 95°C; and 45 cycles of 15 seconds at 95°C followed by 60 seconds at 62°C. Assays were performed in triplicate. Quality of extracted RNA was then examined using Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Amplification was performed according to the Affimetrix protocol for a two-cycle cDNA synthesis. We made a slight modification on two-cycle cDNA synthesis based on a previous report[1]. First, we used SuperScriptIII (Invitrogen, Carlsbad, CA) instead of SuperScriptII. Second, the total RNA/T7-oligo(dT) mix was incubated at 65°C for five minutes, followed by incubation for one hour at 50°C for first-cycle, first strand cDNA synthesis. In the second cycle, non-labeled cRNA was incubated with Random Primers for 5 minutes at 65°C and SuperScriptIII was used to synthesize first-strand cDNA for one hour at 50°C. Biotin labeling of cRNA transcripts with was performed by using the GeneChip IVT Labeling Kit (Affymetrix). 15 μg of biotin-

labeled RNA were fragmented and hybridized to Human Genome U133 Plus 2.0 arrays (Affymetrix), washed, stained, and scanned according to manufacturer's protocol.

FACS

We used whole tissue: peri-tumoral non-lesional skin which would otherwise be discarded as medical waste after Mohs' surgery. Subcutaneous fat was excised and discarded, and the remaining tissues were washed twice with PBS. The dermal layers were heavily scored with a scalpel and digested in 2.4 U/mL Dispase II (Roche Diagnostics) overnight at 4°C. Epidermis and dermis were separated with forceps. We modified the method originally reported in 1983 [2], which retrieved over 95% viable keratinocytes as determined by trypan blue staining. Epidermal sheets were subsequently cut in small pieces and incubated with 0.25% trypsin (Invitrogen) for 10 min at 37°C. After washing, epidermal fragments were incubated in complete media consisting of RPMI 1640 (Invitrogen) supplemented with 5% pooled human serum (Mediatech), 0.1% gentamicin (Invitrogen), and 1% 1 M Hepes buffer (Sigma Aldrich) overnight (approximately 16-18 hours) at 37°C. As even one hour of dispase abolished detection of many extracellular proteins [3] and trypsin cleaves exposed peptides, it was necessary to give the cells time (~16 hours) to regenerate their cell surface antigens. Afterwards, a single cell suspension was obtained by filtration through 40 μm pore nylon cell strainers (BD Biosciences.) Single cell suspensions from the epidermis were stained with the following mouse anti-human monoclonal antibodies: HLA-DR-Alexa Fluor 700 (IgG2a clone L243; BioLegend at the dilution of 1:1,000), CD207-Phycoerythrin (IgG1 clone

DCGM4; Immunotech at the dilution of 1:100), and CD117-Allophycocyanin (IgG1 clone YB5.B8; BD Biosciences at the dilution of 1:100) and sorted on a FACSAria. Appropriate isotype controls were used. Populations of melanocytes were collected by sorting CD-117^{+} (c-kit^{+}) cells and populations of Langerhans cells were collected by sorting for HLA-DR^{+}CD207^{+} cells. The isolated HLA-DR^{-}CD207^{-}CD117^{-} cell populations of keratinocytes, collected from a significant number of patients (n=10), were lysed for RNA extraction which was then amplified and hybridized to Affymetrix chips.

LCM

Laser capture microdissection (LCM) of normal human skin was performed on 10 patients samples with Rockefeller University's IRB consent. Samples were frozen in OTC medium (Sakura) and stored at -80° before and after being cut into 8μm sections in a -20°C cryostat. Two or three sections of skin were mounted on each membrane mounted metal frame slide (Molecular Machines and Industries, Haslett, MI), then stored at -80°C. Five such slides were used to acquire enough starting material for further analysis. An abbreviated Hematoxylin & Eosin (H&E) staining technique was used to minimize time and damage to the RNA. LCM was performed following the manufacturer's protocol for CellCut system (Molecular Machines and Industries). The area of the epidermis was defined on a computer screen display and then cut by laser. In the same fashion the papillary and reticular dermis were isolated. Papillary dermis was completely dissected and captured separately to minimize contamination of reticular dermis with any epidermal components. Special isolation cap tubes with adhesive lids and diffusors (from Molecular Machines and Industries) were used to capture respective

sections of epidermis, papillary dermis and reticular dermis. The tubes were kept on ice until lysis by 100 µl of RLT buffer (Qiagen, Valencia, CA) and 1% β-mercaptoethanol (Fisher Scientific) within 2 hours from the initial staining step to minimize RNA degradation.

Melanoma cell lines

We collaborated with scientists in the Department of Dermatology and Allergy Skin Cancer Center at Charité Mitte in Berlin, Germany who had developed cell lines from metastatic melanoma lesions. These cell lines are not immortalized and their growth *in vitro* varies greatly. In fact, prior attempts to grow them in our laboratory had failed; only one out of six cryo-preserved vials were able to proliferate in the hands of very capable technicians. In 2007, I traveled to Charité where they were able to grow these cell lines, also to variant degrees of success, in Dr. Uwe Trefzer's facilities at the Charité-University of Medicine. Time to confluency was mixed amongst the tumor-derived cell lines; but in less than two weeks I was able to extract at least 19 out of 30 (T-25 or some T-75 flasks) different tumor cell lines and lyse them [according to Qiagen's protocol] in RLT + β-ME. The samples were then shipped on dry ice to The Rockefeller University, where we stored them at -80°C until which time the RNA was isolated, many of the cell lines that had not proliferated well (7 of the 19) did not yield enough quality RNA that could be used. RNA was then amplified and hybridized to Affymetrix Plus 2.0 gene chips as previously described.

Statistcal analysis

Affymetrix CEL files were scanned for spatial artifacts by using software packages Harshlight [4] and arrayQualityMetrics from R/Bioconductor. *Harshlight* uses a combination of statistic and image processing methods to identify three different types of defects: localized blemishes affecting a few probes, diffuse defects affecting larger areas, and extended defects which may invalidate an entire chip [4]. arrayQualityMetrics is a Bioconductor package that provides a report with diagnostic plots for one or two colour microarray data. The quality metrics assess reproducibility, identify apparent outlier arrays and compute measures of signal-to-noise ratio. Expression values were obtained by using the GC-RMA (GC Robust Multi-Chip Average) algorithm. To identify differentially expressed genes (DEGs), a moderated t-test was used in the *limma* package framework. Resultant P-values were adjusted for multiple hypotheses by using the Benjamini-Hochberg procedure, which controls for the False Discovery Rate (FDR). Expression values were converted to log2 to approximate a normal distribution. All through the experiments we will identify the FDR and Fold Change cut-off used to define the DEG lists for each table (normally log2 of 4, except for heatmaps for which fewer genes and a higher threshold was often used.) Principal Components Analysis (PCA) was used to represent the high dimensionality of the data along the direction of maximal variance.

Chapter 1. Introduction to the epidermis

Melanoma, malignant transformation of melanocytes, is increasing now more than ever before. The only cure is early detection and complete excision. There are no specific chemotherapies for metastatic melanoma that have proven to increase life expectancy, even those currently in clinical trials targeting specific BRAF mutations in the patients of relevance; visually tumours seem to regress, but to no significant decrease in mortality. It can therefore only be hoped that drug development will yield more effective treatments and that dermatologic screening can be improved upon in a disease that is approaching epidemic proportions; both ends depend on a better molecular understanding of pathology. The majority of melanoma arise de novo; so for these cases it would be advantageous to be able to identify high-risk individuals on the genomic level. Similarly, for melanoma that arise from congenital nevi (20-30%,) it would be of use to appreciate the steps of transformation.

The majority of adults have some form of pigmented lesions. There are many different types of moles or nevi, the most common being characterized by melanocytes in the dermis or epidermis. Nevus & melanoma cells differ from the typical solitary dendritic melanocyte in that they have undergone proliferation to lie in contiguity with each other. Nevi can be either benign or malignant. It has been shown that melanoma is not related to the total number of nevi on any given patient, but rather the number of dysplastic nevi present. It is therefore of interest to examine the molecular changes that accompany dysplasia and malignant transformation. It would be extremely useful to be

able to identify new targets for drugs and to identify high-risk individuals on the basis of genomic data.

Melanocytes are cells with dendrites that naturally touch three dozen keratinocytes [5]. However these cells become bipolar when grown in minimal media culture systems. Under extreme conditions of starvation, such as media supplemented with only insulin and hydrocortisone, their cell bodies become deflated and morphologically spindle-shaped. Melanocytes have co-evolved to protect the replicating cells from the continual exposure of the skin to the sun. The ozone layer partially absorbs harmful ultraviolet light; but, in the face of persistent sun exposure and the recent degeneration of this atmospheric layer, the mutagenic effects correlate with an increased rate of skin cancer.

The mammalian epidermis is a continually renewing structure that provides the interface between the organism and an innately hostile environment. Melanin absorbs sunlight, thereby protecting keratinocytes from damage from UV irradition. Melanocytes near the stratum basale make and export melanin through their dendrites to local keratinocytes. Melanized keratinocytes are constantly shed during desquamation, creating a need for continual synthesis of melanin, and transfer to new keratinocytes that are still alive. Adequate pigmentation of the skin is dependent on many factors within this unit. Melanocytes need to receive the proper signals to synthesize melanin as well as the melanosomes that package, transport and transfer it from the cell body to the periphery of the dendrite. Whole organelle donation from one cell to a heterologous cell is unique to the melanocyte-keratinocyte unit [6]. Melanophores (referred to as melanocytes in humans) have thousands of melanosomes compartmentalizing melanin

into vesicles that range from 0.3 to 1 μm. Of neural crest origin, differentiated melanocytes have protrusions that radiate from the cell body that give the distinctive dendritic or stellate appearance. In humans, the primary role of pigment is to protect against ultraviolet radiation from the sun, but may also play an important role in the immune system. Mammals have two types of melanin, both synthesized from dopaquinone, which is a derived from oxidized tyrosine. Humans have black/brown eumelanin and also red/yellow pheomelanin, unique to upper vertebrates.

Importantly, melanocytes exist *in vivo* in intimate association with keratinocytes. The keratinocyte is the principal cell component. Keratinocyte proteins form a physical epithelial barrier, protect against microbial damage, and prepare immune responses to danger. In human epidermis, functional symbiosis requires homeostatic balance between keratinocytes and melanocytes. Compelling evidence from co-culture studies demonstrated a sophisticated, multileveled regulation of normal melanocytic phenotype orchestrated by undifferentiated, basal-type keratinocytes. Keratinocytes control cell growth and dendricity, as well as expression of melanoma-associated cell surface molecules of normal melanocytes. In contrast, melanoma cells are refractory to the keratinocyte-mediated regulation.

Thus in order to understand melanocyte gene expression, it is essential to consider keratinocyte gene expression. *In vivo,* keratinocytes exist in stratified layers within the epidermis. The stratum corneum (SC), the uppermost layer of the human epidermis, provides the vital divider between the organism and its environment. It is generated by keratinocytes that migrate from the basal layer to the surface of the epidermis while terminally differentiating via a calcium-dependent process. Keratins are bundled into

macrofibrils through their association with filaggrin, while the cornified envelope is being assembled and cross-linked by Ca^{++}-dependent transglutaminase enzymes to form the epidermal barrier. The final steps in keratinocyte demarcation are most pronounced by the alterations in cell structure and morphology. Keratins are aligned in an ordered array, condensed by the matrix protein filaggrin. Low concentrations of calcium in the basal layer of the epidermis favor keratinocyte proliferation, and an increasing calcium gradient toward the surface influences the complex delineation process. Thus, calcium-binding proteins, which mediate calcium signals by interacting with and modulating specific target proteins, are pertinent for epidermal differentiation.

Keratins are the major gene product of keratinocytes and form the intermediate filament cytoskeletal network in these cells of the epidermis. Intermediate filament proteins consist of a central alpha-helical rod domain flanked by non-helical sequences of varying size and composition. Keratin proteins fall into two classes based of their electrophoretic properties and sequence similarities. One member of each class is required to form the heterodimeric coiled-coil precursor, which, through lateral and longitudinal associations, forms the mature keratin intermediate filament. The major epidermal keratins of the type I class are KRT9, KRT10, KRT14 and KRT16; the major epidermal keratins of the type II class are KRT1, KRT2, KRT5, KRT6A and KRT6B. The expression of individual keratins is both specific for the particular body site as well as for the exact stage of differentiation of the epidermal keratinocyte. In addition to their importance for the structural integrity of the epidermis, keratin networks function in inter-cellular signaling.

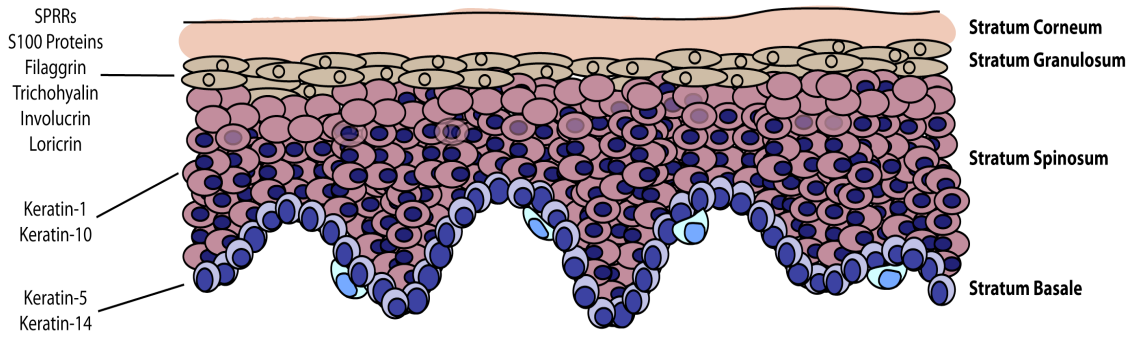


Figure 1. Diagram of the epidermis

Keratinocytes in the basal layer express KRT5 and KRT14. This layer of the epidermis represents the stem cell-like feature of the skin as only these cuboidal, columnar cells undergo continuous replication. Upon differentiation and migration to the spinous layer, these keratin genes are downregulated and the expression of KRT1 and KRT10 is induced, and these gene products become the predominant filamentous network in the subsequent cellular layer. Named because of the abundance of desmosomes, it is within the stratum spinosum of polygonal 'prickle-cells'[7] where the process of cornification begins. In cells of the upper spinous layer, KRT2 and KRT9 are expressed. The other major epidermal keratins, KRT6 and KRT16, are normally expressed in the outer root sheath of the hair follicle and in palmoplantar epidermis (the latter was not examined in this study.)

The stratum granulosum cells acquire keratohyaline granules that are predominantly filled with profilaggrin, which is the 400 kD precursor of the ~37 kD fillagrin peptides that aggregate keratin filaments into tight bundles that eventually lead to the collapse of the cell into flattened squames [8] that are anucleated and have lost

unnecessary organelles. The “grainy” third layer is also the location of loricrin deposition. Loricrin comprises the vast majority of the cornified envelope: 70-80% of the total protein mass[8].

The corneocytes are tightly opposed to each other. The barrier to water, though, is not absolute. Transepidermal water loss (TEWL) is the normal passage of water through the stratum corneum into the atmosphere. Pronounced changes in lipid composition accompany keratinocyte differentiation. Lamellar granules, termed keratinosomes, are visible [most prominently in the granular cell layer] by transmission electron microscopy of the epidermis. These membrane-bound organelles contain stacked lamellae composed of phospholipids, cholesterol, and glucosylceramides. During transition of a granular cell into a corneocyte the keratinosome fuses with the membrane to release lipids and acid hydrolases that break down the phospholipids and convert glucosylceramide to ceramides. The result is an equimolar mixture of ceramides, free fatty acids and cholesterol plus <5% [by weight] cholesterol sulfate.

Over a decade ago, in 1996, the phrase "epidermal differentiation complex" (EDC) was coined in the *Journal of Investigative Dermatology* to describe the almost 2-Mb locus on human chromosome 1q21 that contains at least 45 genes [9] encoding both epidermal cornification and S100 proteins[10]. The first family of the EDC consists of 13 genes, including involucrin, loricrin, and 3 classes of small proline rich proteins: 2 SPRR1 genes 8 SPRR2 genes, and 1 SPRR3 gene. These genes encode structural proteins of the human epidermis, which the transglutaminase enzymes crosslink to yield the cornified cellular envelope. The second family of the EDC consists of profilaggrin and trichohyalin. These genes encode intermediate filament-associated proteins

synthesized in the granular layer of the epidermis that conjoin with the keratin filaments of keratinocytes during cornification. The third family of genes in the EDC consists of 10 genes of the S100 family, S100A1 through S100A10. These encode small calcium-binding proteins with 2 EF-hands.

For the past four decades, the study of skin has often been conducted *in vitro* where skin is supplemented with many growth factors and hormones. Terminally differentiated cells like keratinocytes or even melanocytes have been grown in cultured media and assumed to be "normal" cells, though, these cell types are largely non-proliferative *in vivo*. In turn the predominant, *in vitro* characterization of a normal skin cell is somewhat skewed and may differ significantly from the *in vivo* state. It has long been acknowledged that keratinocytes in culture often resemble pathogenic stages.

In wound healing and many pathologic conditions, keratinocytes become activated: they turn into migratory, hyperproliferative cells that produce and secrete extracellular matrix components and signaling polypeptides. At the same time, their cytoskeleton is also altered by the production of specific keratin proteins [11]. These changes are orchestrated by growth factors, chemokines, and cytokines produced by keratinocytes and other cutaneous cell types. The responding intracellular signaling pathways activate transcription factors that regulate expression of keratin genes. Keratinocytes are activated by the release of IL-1. Subsequently, they maintain the activated state by autocrine production of proinflammatory and proliferative signals. Keratins K6 and K16 are markers of the active state of regenerative maturation [11]. Signals from the lymphocytes, in the form of interferon-gamma, induce the expression of K17 and make keratinocytes contractile. This enables the keratinocytes to shrink the

provisional fibronectin-rich basement membrane. Signals from the fibroblasts, in the form of TGF-beta, induce the expression of K5 and K14, revert the keratinocytes to the healthy basal phenotype, and thus complete the activation cycle.

Keratinocytes have long been studied in culture. For differentiation to occur upon reaching confluency in culture conditions, removal of exogenous growth factors is in fact necessary, which demonstrated their autocrine capabilities. Keratinocytes provide themselves with the growth factors necessary for their proliferative phenotype. The three-dimensionality of the epidermis is not recapitulated but some stratification can be observed. Though grown in an artificial environment, late makers of differentiated keratinocytes such as loricrin, filaggrin and transglutaminase-1 are produced in addition to cytokeratins 10. Keratin 14 and involucrin are detected at lower confluency. Aside from studies in animals like mice, culture, however artificial, has been the norm for the study of keratinocytes. Yet the physiological relevancy of *in vitro* experimentation has never been detailed by large genomic study.

The idea that cancer develops through progressive mutations/alterations in normal cells is at the heart of the Vogelstein model of colon cancer pathogenesis. In this model, normal colonic epithelial cells evolve to become benign proliferative neoplasms (adenoma) and then those lesions acquire additional alterations that convert benign neoplasms to malignant ones [12]. This model seems well suited for the skin in that keratinocyte neoplasms seem to progress from dysplastic lesions (acanthomas, actinic keratoses), to *in situ* carcinomas, to invasive carcinomas. Likewise, melanocytes undergo benign proliferative conversion to nevi, and progressive dysplasia in nevi leads to melanomas, which also seem to progress from *in situ* to invasive lesions.

This thesis project is directed at identifying critical gene expression pathways that are associated with early steps in melanocyte pathology, i.e., the conversion of a stable, non-proliferative melanocyte within the epithelium to a proliferative phenotype. In addition, there is critical cross-regulation of melanocyte growth by epidermal keratinocytes, which supply numerous growth factors for the melanocytes. Keratinocytes are plastic in human skin in that they can exist in a low-proliferative homeostatic growth state (with low production of growth factors) or in a state of accelerated growth/differentiation called 'regulated maturation' where production of relevant growth factors is increased.

It can be postulated that genes activated or suppressed in culture may be mutated in cancer. Genes that are gained with culture are generally more interesting than those lost (unless they are tumor suppressors.) In this thesis, there is thus an effort to define growth-activated expression pathways in both melanocyte and keratinocytes, since both sets of pathways may influence benign or malignant transformation. Thus a critical starting point is to define the "normal" genomic profiles of keratinocytes and melanocytes in normal human skin before induced proliferation occurs.

To this end we set out to study and compare the gene expression of keratinocytes: *in vitro*, *ex vivo* as collected by FACS and *in situ* by LCM. The staining of human tissue for LCM greatly damages the RNA needed for analysis of gene expression. We are able to collect pieces of the epidermis and dermis as stained by a simple H&E method; but, it is currently not possible to stain melanocytes specifically for dissection. We compared the gene expression of melanocytes isolated by their expression of CD-177, or c-kit, with commercial 'normal human epidermal melanocytes' in culture using a

proprietary medium sold by PromoCell. The use of serum and other unknown elements (eg pituitary homogenate) affect gene expression such that typical ingredients of melanocyte growth media must be excluded. The best methodology at this point to examine true physiological qualities of *in vivo* melanocytes is by FACS of epidermal cell suspensions and by the study of primary cultures in defined medium, which was created as detailed in Chapter 6.

As a way to look at progression we analyzed cultured cells from metastatic melanoma tumors. We compared the gene expression of a significant number (n=7) of melanoma cell lines, normal human epidermal melanocytes *in vitro* and the FACS derived melanocytes.

This thesis therefore takes a close look at the genomics of normal keratinocytes and melanocytes in the attempt to determine how precisely they represent normal cells *in vivo* through a variety of techniques. Our conclusions confirm the artifacts created by tissue culture and attempt to categorize the changes precisely so that all the studies conducted with this model system can be examined with the appropriate stipulations. We also provide insight into hyperplastic and neoplastic growth amongst these two cell types in the epidermis.

Chapter 2. Laser capture microdissection of skin

Theory and aims

Growth and differentiation are tightly linked processes in the epidermis that must be balanced precisely. If there are too many dividing cells, hyperproliferative disorders of the skin can result (eg psoriasis, basal- or squamous-cell carcinomas.) The epidermis controls this balance partly by an orchestrated transcriptional program that creates temporally and spatially distinct epidermal cells: each carrying out functions necessary for their specific position within the skin. In the basal layer are the only cells actively undergoing replication. At some point the keratinocytes will stop dividing, detach from the basement membrane and enter a path of terminal differentiation that will ultimately allow them to form the barrier necessary for the proper function of the skin. Keratinocytes form a three-dimensional lattice that can only be replicated in culture with the addition of exogenous extracellular matrix proteins to provide for the scaffolding necessitated by such structural complexity. In addition cultured cells are highly proliferative, whereas those in normal skin are not [unless activation signals are sensed such as those necessary for wound healing.] It therefore stands to reason that cultured cells are not an appropriate model system from which to study the epidermis at the molecular level.

This chapter is based on the hypothesis that primary cells in culture, though not immortalized, should not be treated as 'normal' *ex vivo* representations. In order to prove this idea, it was necessary to find a source of epidermal keratinocytes that had yet to be

isolated and influenced by exogenous culture milieu. Using laser capture microdissection it was possible to isolate the epidermis (98% keratinocyte cells) directly from human tissue samples. Analysis of extracted RNA expressed from cultured primary cell lines, as compared with the "*in vivo*" epidermal LCM-captured samples, showed extreme genetic variation from what the current 'gold standard' of primary normal human epidermal keratinocytes *in vitro*.

Dermis versus epidermis gene sets

In order to show the validity of LCM to detect differential gene expression in human skin components, the top genes expressed in the epidermal and dermal samples captured, and amplified through Affymetrix assays were examined. Cut-off fold change (FCH) of 4 was used and a p value of 0.05; though, for the sake of brevity, 10 representative genes were chosen for the tables in this chapter (as the Affymetrix U133 Plus 2.0 chips detect over 47,000 transcripts.)

Genes highly expressed in dermis include many types of collagens, as expected for their function in scaffolding, as well as endothelial cell products such as complement factors and PECAM1 (Table 1a). Of note is CXCL12, a chemokine present here in non-inflamed tissue; though, previously reported to be secreted by aging stromal fibroblasts [13]. Table 1a. columns of "Dermis" and "Epi" highlights mean log base 2 values of gene expression as detected on arrays (n=10) of the fold elevation in the dermis collected versus epidermis.

Table 1a.

Genes highly expressed in normal human dermis/epidermis by Fold Change (FC)

Symbol	Description	FC	log_2FC	P	fdr	Dermis	Epi
CXCL12	chemokine ligand 12	2231.8	-11.1	0	0	13.6	2.5
C3	complement component 3	1250.2	-10.3	0	0	13.4	3.1
CFH	complement factor H	903.9	-9.8	0	0	12.5	2.6
COL1A2	collagen, type I, alpha 2	762.2	-9.6	0	0	13.7	4.1
COL3A1	collagen, type III, alpha 1	717.6	-9.5	0	0	12.5	3.0
COL6A3	collagen, type VI, alpha 3	775.0	-9.6	0	0	13.1	3.5
PECAM1	platelet/endothelial cell adhesion molecule	721.6	-9.5	0	0	13.0	3.5
FBN1	fibrillin 1	466.9	-8.9	0	0	12.7	3.8
PDGFRA	platelet-derived growth factor receptor, alpha	429.3	-8.7	0	0	12.5	3.8
VCAN	versican	426.4	-8.7	0	0	11.5	2.8

Conversely, the genes expressed most highly in the epidermis (Table 1b) play a large role in maintaining the strength and integrity of epithelial tissue, including several members of the EDC. Marenholz et al. (1996) noted that calcium levels tightly control epidermal differentiation and expression of EDC genes [17]. Due to the importance of calcium in the regulation of keratinocyte differentiation, it is rational to expect to see certain Ca^{++} binding protein in the epidermis. Accordingly, calmodulin-like skin protein (CLSP) was highly differentially expressed in the epidermal list. The high expression levels of the late cornified envelope 1B gene (LCE1B) and the keratinocyte differentiation-associated protein (KRTDAP) are perhaps the most obvious tissue-

specific markers of the epidermis, as they are keratinocyte products that would not be expressed in dermal tissue.

Table 1b. highlights the columns designated "Dermis" and "Epi" represent mean ln values of gene expression as detected on arrays (n=10) of the fold elevation in the epidermis collected versus dermis. Cut-off fold change (FCH) of 4 was used with a p value of 0.05.

Table 1b.

Genes highly expressed in normal human epidermis/dermis by Fold Change (FC)

Symbol	Description	Fold elevation in epidermis	log_2FC	P	fdr	Dermis	Epi
DSC1	desmocollin 1	647.679	9.3	0	0	3.7	13.1
DSG1	desmoglein 1	620.481	9.3	0	0	3.8	13.1
KRT2	keratin 2	479.378	8.9	0	0	5.4	14.3
CALML5	calmodulin-like 5	420.781	8.7	0	0	3.1	11.8
LCE1B	late cornified envelope 1B	358.767	8.5	0	0	5.2	13.7
SBSN	suprabasin	343.175	8.4	0	0	5.2	13.6
PKP1	plakophilin 1 (ectodermal dysplasia/skin fragility)	320.515	8.3	0	0	4.0	12.3
KRTDAP	keratinocyte differentiation-associated protein	298.7	8.2	0	0	6.4	14.7
LOR	loricrin	297.698	8.2	0	0	5.3	13.5
CST6	cystatin E/M	279.39	8.1	0	0	3.2	11.3

Keratin 2, of the spinous layer is highly expressed in the "*in vivo*" epidermis collected by LCM as opposed to the dermis. Suprabasin has been identified as an epidermal differentiation marker and potential cornified envelope precursor, expressed only in the suprabasal layers of epidermis with differentiating keratinocytes [14]. Loricrin is a major component of the crosslinked cell envelope of the epidermis, otherwise known as cornified cell envelope (CE), marginal, or peripheral band. Cystatin M/E has a restricted expression pattern in humans largely limited to cutaneous epithelia [15,16]. It has also been suggested that cystatin M/E is required for viability and for correct formation of cornified layers in the epidermis and hair follicles.

There are many unique ectodermal cell:cell junctional proteins that make up the desmosomes in the epidermis, such as desmoglein and desmocollin. Another desmosomal component is plakophilin, also expressed at high levels in the epidermis. The detectable expression of these molecules is consistent with an isolated epidermis, confirming the LCM methodology.

In vivo v*ersus in vitro primary keratinocytes*

After we were confident that we had correctly dissected the epidermis, we were then able to compare its gene expression to that of 'primary' keratinocytes growing *in vitro*. It is important to note that *in vitro* keratinocytes grown in culture do go through stages of differentiation, though, they appear to differ from *in vivo* stages of differentiation. For example, *in vitro* keratinocytes have been shown to express wound healing and psoriatic markers [11] which suggest alternative genomic circuits at play. In this evaluation we can consider the varying levels of cytokines and growth factors (both paracrine and autocrine) as well as their receptors, cell cycle and epidermal differentiation complex genes expressed *in vitro* in normal human epidermal keratinocytes (NHEK) which have been considered the norm for the past several decades.

Epidermal differentiation complex (EDC) and related genes of the epidermal barrier

Though the EDC is strictly defined as genes in one locus on the same chromosome, some relevant genes from other loci are included in this analysis as many of the EDC genes may not be expressed *in vitro*. Table 2a. highlights the mean fold elevation (FC) *in vitro* (n=10) as compared to the LCM epidermis (n=10).

Table 2a. Genes up-regulated *in vitro* [as compared to LCM]

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	LCM
PI3	peptidase inhibitor 3, skin-derived	85.3	6.42	0	0	10.15	3.74
S100A6	S100 calcium binding protein A6	29.0	4.86	0	0	12.84	7.98
ACER3	alkaline ceramidase 3	16.2	4.02	0	0	8.69	4.68
ACSL3	acyl-CoA synthetase long-chain family 3	14.9	3.90	0	0	12.01	8.12
ACSL4	acyl-CoA synthetase long-chain family 4	13.1	3.71	0	0	8.10	4.39
SPRR1B	small proline-rich protein 1B (cornifin)	7.5	2.91	0	0	14.63	11.73
SCARB1	scavenger receptor class B, member 1	6.1	2.60	0	0	9.78	7.18
ST3GAL5	ST3 beta-galactoside α-2,3-sialyltransferase 5	5.8	2.53	0	0	8.44	5.92
TGM1	transglutaminase 1 (K epidermal type I)	4.7	2.23	0	0	11.03	8.80
SPRR4	small proline-rich protein 4	4.4	2.15	0	0	4.67	2.52

PI3, or SKALP, peptidase inhibitor 3 has been identified in psoriatic patient skin but is absent in normal human epidermis. It is therefore of interest that it was expressed at extremely high levels in culture as compared to LCM epidermis [18]. Expression of S100A6, a member of the S100 calcium binding proteins that typically regulate the epidermis, has been documented in a variety of human cancer tissues [19] and is diffusely expressed in Spitz nevi and in melanoma to a lesser extent [20]. The exact function of ST3GAL5 remains to be elucidated, other than its role in the sphingolipid metabolism

that has been implicated in cellular cross-talk in cell survival and resistance to apoptosis[21]. EDC gene SPRR1B has been reported to quench reactive oxygen species in wound healing [22]. SPRR4 was only recently identified as a cornified envelope precursor, due to its low expression levels *in vivo*, induced by UV light and concomitant thickening of the stratum corneum [23]. Transglutaminase 1 expression *in vitro* was increased, probably because it is produced primarily by keratinocytes [8] and there are many transglutaminases present in skin creating somewhat of a functional redundancy.

The epidermis is an active site of cholesterol synthesis. SCARB1, up-regulated 6-fold *in vitro*, has been reported to be at its greatest expression level in undifferentiated keratinocytes [24]. Altered expression of lipid metabolism is also seen in keratinocytes *in vitro* which goes along with the down-regulation of EDC the gene expression [as seen in Table 2b.] ACSL3 and ACSL4 were both up-regulated in culture relative to *in vivo* epidermis. The proteins encoded by these gene are isozymes of the long-chain fatty-acid-coenzyme A ligase family. Although differing in substrate specificity, subcellular localization, and tissue distribution, all isozymes of this family convert free long-chain fatty acids into fatty acyl-CoA esters, and thereby play a key role in lipid biosynthesis and fatty acid degradation. The ACSL3 isozyme is highly expressed in brain, and preferentially utilizes myristate, arachidonate, and eicosapentaenoate as substrates. The ACSL4 isozyme preferentially utilizes arachidonate as substrate. It is unusual to see these isozymes expressed in keratinocytes as there have been no other such reports; and, arachidonate is not the usual substrate in the human epidermis.

Table 2b. Epidermal barrier genes up-regulated in LCM [as compared to *in vitro*]

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	LCM
LOR	loricrin	231	-7.851	0	0	6.37	14.22
CD1A	CD1a molecule	57	-5.84	0	0	2.27	8.11
LCE2B	late cornified envelope 2B	25	-4.651	0	0	6.04	10.69
CLDN23	claudin 23	24	-4.609	0	0	4.07	8.68
FLG	filaggrin	23	-4.509	0	0	9.83	14.34
SCEL	sciellin	16	-3.98	0	0	8.09	12.07
LCE1B	late cornified envelope 1B	15	-3.863	0	0	10.11	13.97
NSMAF	neutral sphingomyelinase activation factor	11	-3.465	0	0	3.16	6.62
CDSN	Corneodesmosin	8	-2.962	0	0	7.94	10.90
EVPL	Envoplakin	5	-2.335	0	0	7.87	10.20

Loricrin, upregulated 231-fold in the epidermis as compared to in culture, appears initially in the granular layer of human epidermis and forms composite keratohyalin granules with profilaggrin (upregulated 23-fold); along with involucrin it is a major component of the crosslinked cell envelope (CE) of the epidermis. Late cornified envelope proteins 1B and 2B were expressed 15- and 25-fold higher in the epidermis, respectively. Sciellin, a component of the CE, was also relatively upregulated in LCM. NSMAF is a crucial component of epidermal barrier repair [25]. Corneodesmosome is unique to anucleated corneocytes, and along with enveloplakin, and claudin 23 have roles in cell adhesion; these molecules were all relatively highly expressed in epidermis as compared to the lateral keratinocyte structures seen in culture.

CD1a is a Langerhans cell marker, expressed in the epidermis but detected at low levels in keratinocyte cultures. This is just a caveat of LCM of the epidermis, it is not a pure keratinocyte population, and contains a small number of Langerhans cells as well as melanocytes. Further discussion of the benefits and drawbacks of methodology can be found in Chapter 3.

Growth factors, cytokines/chemokines and their receptors

The differential expression of growth factors, cytokines, chemokines and their receptors is perhaps the most obvious testimony of how differently the cells in variant surroundings are being operated with regards to genomic circuitry. *In vivo* LCM samples show high expression of a variety of chemokine receptors indicating cellular response to the immune components of the natural environment of the skin (Table 4a.) Cells grown *in vitro* on the other hand show more hormone and growth factor receptors exogenous to the normal human milieu, with more of a response to elements in the growth media. A set of representative gene is shown on the next page in Tables 3a and 3b with a $\log_2$ fold elevation cut-off *in vitro* of 4 and p value less than 0.01.

Table 3a.

Growth factors and chemokines up-regulated *in vitro* [as compared to LCM]

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	LCM
AREG	amphiregulin	862	9.752	0	0	13.96	4.21
IL1A	interleukin 1, alpha	383	8.58	0	0	11.07	2.49
VEGFC	vascular endothelial growth factor C	216	7.758	0	0	10.03	2.27
IL1B	interleukin 1, beta	98	6.621	0	0	9.58	2.96
CXCL1	GRO alpha (melanoma growth stimulating activity, alpha)	37	5.212	0	0	8.11	2.89
IL8	interleukin 8	36	5.18	0	0	7.56	2.38
PXN	paxillin	27	4.744	0	0	9.46	4.72
SOCS2	suppressor of cytokine signaling 2	21	4.418	0	0	7.85	3.44
HBEGF	heparin-binding EGF-like growth factor	21	4.4	0	0	9.10	4.70
STAT3	signal transducer and activator of transcription 3	14	3.765	0	0	7.35	3.59
PDGFC	platelet derived growth factor C	6	2.609	0	0	10.88	8.27
IRS1	insulin receptor substrate 1	5	2.281	0	0	6.14	3.86
GRB10	growth factor receptor-bound protein 10	5	2.191	0	0	5.32	3.13
NRP1	neuropilin 1	4	2.074	0	0	5.74	3.66
VEGFA	vascular endothelial growth factor A	4	2.064	0	0	8.36	6.29

Table 3b. Growth factor and chemokine receptors up-regulated *in vitro*

[compared to LCM epidermis] p<0.01 Fold change (FC)>4

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	LCM Epi
TNFRSF21	tumor necrosis factor receptor superfamily, member 21	182	7.51	0	0	11.05	3.54
NR2F2	nuclear receptor subfamily 2, group F, member 2	84	6.39	0	0	9.63	3.24
TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	54	5.77	0	0	10.85	5.08
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	24	4.61	0	0	12.58	7.97
FOLR3	folate receptor 3 (gamma)	22	4.48	0	0	7.58	3.09
GRB10	growth factor receptor-bound protein 10	16	4.04	0	0	8.79	4.75
IL4R	interleukin 4 receptor	15	3.90	0	0	10.20	6.30
TRAF4	TNF receptor-associated factor 4	14	3.77	0	0	6.32	2.55

The mammalian members of the epidermal growth factor (EGF) family of growth factors include EGF itself, transforming growth factor-alpha (TGFA) amphiregulin (AREG) heregulin (HGL) and betacellulin (BTC). These act as autocrine & paracrine factors. AREG is an autocrine factor for keratinocytes and is expressed at increased levels in psoriatic epidermis. It is also has been shown that AREG enhances protection against nematodes in a mouse model and was expression was increased in parallel with typical Th2 cytokines. In keratinocytes grown *in vitro* AREG is up almost 3 logs (base 10) compared to expression levels in LCM *in vivo* samples [26]. It is therefore of interest that Th2 cytokine receptors IL4R, IL13RA1 and IL13RA2 are also both increased *in vitro* along with heparin-binding EGF-like growth factor (HBEGF.)

The insulin-like growth factor 1 receptor (IGF1R) plays a critical role in transformation events. It is highly over-expressed in most malignant tissues where it functions as an anti-apoptotic agent by enhancing cell survival. The p53 gene, the most frequently mutated gene in human cancer, is a nuclear transcription factor that blocks cell cycle progression and induces apoptosis. Werner et al. (1996) reported results of experiments that indicated that mutant p53 proteins have a stimulatory effect on promoter activity, whereas wildtype p53 suppresses the activity of the IGF1R promoter [27]. These effects of p53 seemed to involve its interaction with components of the basal transcription machinery. Due to the central role of IGF1R in cell cycle progression and transformation, derepression of IGF1R promoter by mutant p53 may constitute an important paradigm in tumorigenesis. (Most of the cancer-related mutations of p53 occur in the central domain of the p53 molecule.) SOCS2 negatively regulates cytokine receptor signaling via the Janus kinase /signal transducer and activation of transcription pathway (the JAK/STAT pathway) [28]. It interacts with IGF1R and thus may play a regulatory role in IGF1 receptor signaling.

Many polypeptide mitogens, such as basic fibroblast growth factor (FGFB) and platelet-derived growth factors are active on a wide range of different cell types. In contrast, vascular endothelial growth factor (VEGF) is a mitogen primarily for vascular endothelial cells. It is, however, structurally related to platelet-derived growth factor. Platelet-derived growth factors, such as PDGFC, trigger mitosis and entry into the cell cycle. Frantz et al. (1997) found that, upon insulin stimulation, the 65-kD form of GRB10 translocated from the cytosol to the membrane [29]. GRB10 also bound activated platelet-derived growth factor receptor (PDGFRB) and epidermal growth factor

receptor, suggesting that GRB10 functions downstream from activated insulin and growth factor receptors. PDGFC, VEGFA, VEFGC and GRB10 are all up-regulated in culture relative to LCM. VEGF overexpression by basal keratinocytes has been reported in psoriasis [30], therefore, suggesting a hyperplastic state of keratinocytes *in vitro*.

STAT3 is activated by a variety of cytokines and growth factors such as interleukin-6, epidermal growth factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor, and granulocyte colony-stimulating factor. STAT3 also regulates the phosphorylation of paxillin (PXN), upregulated here in culture, known to affect cell migration. Focal adhesion kinase ($p125^{FAK}$) is a cytosolic kinase that is concentrated in focal contacts[31]. Due to its localization, $p125^{FAK}$ has been thought to be involved in regulating cell morphology and cell migration in response to cell adhesion to extracellular matrix proteins. $p125^{FAK}$ is rapidly phosphorylated following cell attachment to fibronectin-coated surfaces or integrin clustering by antibodies. $p125^{FAK}$ is also considered a focal adhesion docking protein capable of facilitating the recruitment and activation of other tyrosine-phosphorylated signaling molecules, such as $pp60^{src}$ and paxillin. Paxillin is another signaling molecule that localizes to focal adhesions and becomes tyrosine phosphorylated either during integrin-mediated or growth factor-induced adhesion; and, is highly upregulated in culture conditions. Paxillin contains a domain that interacts with the C-terminus of $p125^{FAK}$, and $p125^{FAK}$ recruitment to focal contacts appears to require paxillin binding. Paxillin has been demonstrated to be a substrate for $p125^{FAK}$ phosphorylation in both *in vitro* [32] and *in vivo* systems [33,34]. Recently, treatment with several peptide hormones such as prolactin, insulin-like growth factor I (IGF-I), hepatocyte growth factor, vascular endothelial growth factor, and

platelet-derived growth factor (PDGF) have been shown to augment the tyrosine phosphorylation of $p125^{FAK}$, the paxillin-MAPK complex serves as a central regulator of HGF-stimulated focal adhesion kinase and Rac activation in the vicinity of focal adhesions, thus promoting the rapid focal adhesion turnover and lamellipodia extension that are required for migratory and tubulogenic responses [35].

Oncostatin M (OSM) is a member of the IL6 family of cytokines. Functional receptors for IL6 family cytokines are multisubunit complexes involving members of the hematopoietin receptor superfamily. Many IL6 cytokines utilize gp130 (IL6ST) as a common receptor subunit. OSM binds to the gp130 receptor subunit and, in association with leukemia inhibitory factor receptor (LIFR), induces a proliferative response in permissive cells. OSMR expression was upregulated with lipopolysaccharide (LPS) treatment.

Several genes highly expressed *in vitro* are known associates of cancers. Levels of cell membrane-associated folate receptors (FOLR) are elevated in a variety of malignant tissues compared to their normal counterparts. Using PCR, Shen et al. (1994) detected FOLR3 mRNA in certain carcinomas and in tissues that are sources for hematopoietic cells such as normal or malignant spleen, bone marrow, and thymus [36]. Progesterone receptor membrane component 1 (PGRMC1) is related to cytochrome b5, binds to heme, and is associated with DNA damage resistance and apoptotic suppression. PGRMC1 is induced by carcinogens, including dioxin, and is up-regulated in multiple types of cancer. In a recent study, it was found that Pgrmc1 increased in vivo tumor growth, anchorage-independent growth, and migration [37]. Zou et al. (2007) reported that the hepatocyte growth factor receptor (HGFR) MET proto-oncogene plays an

important part in preventing FAS-mediated apoptosis of hepatocytes by sequestering FAS [38]; so, it may add some anti-apoptotic elements to keratinocytes in culture.

In mouse models MET and VEGF have a synergistically positive effect on cell proliferation. IL6 seems to increase expression of VEGF, and VEGF plus HGF has been shown to increase levels of IL-6, GRB10, PDGF, VEGFC and neuropilin (NRP1) [39]. Overall a similar picture to what is observed in *in vitro* keratinocytes.

Scavenger receptor BI (SR-BI) mediates the selective uptake of high density lipoprotein (HDL) cholesteryl ester (CE), a process by which HDL CE is taken into the cell without internalization and degradation of the HDL particle. Low density lipoprotein-related protein 12 (LRP12) overexpression has been noted in oral squamous cell carcinomas[40]. This gene was identified by its differential expression in cancer cells. The product of this gene is predicted to be a transmembrane protein. The level of this protein was found to be lower in tumor derived cell lines compared to normal cells. This gene was thus proposed to be a candidate tumor suppressor gene.

Table 4a. Receptors up-regulated in LCM [as compared to *in vitro*]

Symbol	Description	FC	$\log_2$ FC	P	fdr	in vitro	LCM
THRA	thyroid hormone receptor, alpha	5.8	-2.55	0	0	3.69	6.23
THRB	thyroid hormone receptor, beta	4.8	-2.26	0	0	5.56	7.81
PRLR	prolactin receptor	5.4	-2.43	0	0	2.26	4.69
INSR	insulin receptor	5.2	-2.39	0	0	4.63	7.01
FGFR3	fibroblast growth factor receptor	8.8	-3.14	0	0	10.85	13.99
IL11RA	interleukin 11 receptor, alpha	8.6	-3.11	0	0	4.15	7.26
IL20RA	interleukin 20 receptor, alpha	8.6	-3.11	0	0	3.54	6.65
FGFR2	fibroblast growth factor receptor	4.1	-2.05	0	0	7.11	9.16
IL1R1	interleukin 1 receptor, type I	15.3	-3.94	0	0	6.82	10.75
IFNGR1	interferon gamma receptor 1	4.5	-2.17	0.001	0	3.57	5.73

From the samples collected from frozen human skin by LCM we get a very different impression of the cytokine and growth factor milieu *in vivo* (Table 4b) than conclusions based on *in vitro* samples (Table 3a) would lead you to believe. It is obvious that the keratinocytes are no longer within a living organism: their transcriptional circuits have been re-wired to survive in culture and the cells have lost much of the former gene expression that is critical to the proper functioning of the epidermis. *In vivo* there are receptors for thyroid hormone (THRA, THRB,) that also function as transcription factors, prolactin (PRLR) which can play a role in apoptosis, and insulin (INSR,) a player in the MAPK pathway, that are all down-regulated *in vitro* by at least 5-fold compared to *in vitro* expression. Several fibroblast growth factor receptors, FGFR2 and FGFR3, are

differentially expressed by about a log [of 10,] intimating their importance *in vivo*. This data suggests a less important role for these receptors and their hormones when keratinocytes are cultured; the same seems to be true for cytokine receptors. The receptor for IL-1 (IL1R1) is expressed 15 times higher in LCM samples as compared to the primary NHEK cell line. The receptors for both IL-11 (IL11RA,) and IL-20 (IL20RA) are expressed at levels of almost 10-fold higher *in vivo* over the cultured cells. In addition, the interferon gamma receptor (IFNGR1) expression level is 4 times higher in the dissected epidermis than in isolated keratinocytes that have only been passaged twice *in vitro*. This shows how important *in vivo* study is for assessing paracrine, autocrine and immune system signals in the epidermis because any culture model, even primary cells that are not immortalized, creates many modifications due to the profound disparity in cellular environs.

Table 4b.

Growth factors and chemokines up-regulated in LCM [as compared to *in vitro*]

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	LCM
CCL27	chemokine ligand 27	269	-8.074	0	0	3.81	11.88
CXCL14	chemokine ligand 14	222	-7.794	0	0	2.54	10.33
MAF	musculoaponeurotic fibrosarcoma oncogene	214	-7.742	0	0	4.68	12.43
BTC	betacellulin	136	-7.085	0	0	2.38	9.47
IL1F7	interleukin 1 family, member 7	35	-5.116	0	0	2.27	7.38
CCRL1	chemokine receptorlike1	31	-4.967	0	0	2.42	7.38
IL7	interleukin 7	29	-4.863	0	0	2.40	7.26
BMP7	bone morphogenetic protein 7	24	-4.565	0	0	2.27	6.84
KIT	Hardy-Zuckerman feline sarcoma viral oncogene	13	-3.687	0	0	2.98	6.67
IL33	interleukin 33	12	-3.538	0	0	2.56	6.10
NOTCH3	Notch homolog 3	5	-2.377	0	0	5.82	8.19

In terms of chemokines and other ligands for the cellular receptors there is a significant difference between what is produced in the skin versus in culture. The primary receptor for BTC is the EGFR. We see betacellulin (BTC) and MAF, which has positive regulatory functions on RNA polymerase II production, are both expressed more than 2 logs(base 10) higher in samples collected by LCM compared to the primary keratinocytes grown *in vitro*. Expression of the multi-functional Notch homolog 3 gene

is also significantly elevated *in vivo* by 5-fold where it not only functions in Notch signaling, but also calcium binding, transcription and development. IL-7, important in the humoral immune response and positively regulating proliferation of both B and T cells, is decreased by almost 30-fold in culture. Similarly, IL-33, another important cytokine is down 12-fold in culture as compared to LCM samples. CXCL14, which has signal transduction, intercellular communication and chemotaxis functionalities, is decreased by more than 200 times the LCM expression level *in vitro*. CCL27, also important in chemotaxis, cell-cell signaling and in the immune response is detected at almost 300 times the amount found in culture. The gene expression of CCRL1, interleukin 1 family, member 7(zeta) (IL1F7) which has IL-1R antagonistic abilities, is down-regulated *in vitro* by 35-fold. Additionally, in cultured keratinocytes, growth factors such as bone morphogenetic protein 7 (BMP7) and KIT are each relatively expressed about 20 times lower than in normal human epidermis.

Cell cycle and related genes of interest

Genes expressed highly in LCM captured epidermis suggest a more regulated growth environment *in vivo* (Table 5b.) Relative to the *in vitro* samples we see many more genes involved in cell cycle arrest as well as those that promote cell death (apoptosis) and negative regulators of transcription.

Table 5a. Top cell cycle-related genes differentially expressed in culture

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	LCM
KRT18	keratin 18	608	9.25	0	0	12.38	3.13
DKK1	dickkopf homolog 1	605	9.24	0	0	11.51	2.27
G0S2	G0/G1switch 2	303	8.24	0	0	11.47	3.22
TNFRSF21	TNF receptor superfamily, 21	229	7.84	0	0	11.61	3.77
NRG1	neuregulin 1	160	7.32	0	0	9.96	2.64
INHBA	inhibin, beta A	131	7.04	0	0	9.46	2.43
IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3	130	7.02	0	0	9.55	2.53
RAC2	ras-related C3 b.tox substrate 2	128	7.00	0	0	10.01	3.02
LAMB3	laminin, beta 3	88	6.46	0	0	13.44	6.99
FOSL1	FOS-like antigen 1	82	6.36	0	0	9.29	2.93
CCND2	cyclin D2	72	6.16	0	0	10.83	4.67
ODC1	ornithine decarboxylase 1	71	6.15	0	0	13.02	6.87
RRAD	Ras-related associated diabetes	70	6.13	0	0	8.76	2.63

Looking specifically at genes related to cell cycle we see a distinct trend of less differentiated keratinocyte genes and architechture of the epithelium, upregulation of cyclins and genes that promote cell proliferation. In addition to inhibition of apoptotic pathways including specifically Wnt antagonism, disregulation of many proto-oncogenes. members of the RAS superfamily and JUN family. Many of these genes have already been reported in association with various cancers.

In regards to less differentiated genes we see KRT18, embryonic keratin most different N-terminal and C-terminal domains and location on chromosome 12q13 suggests early divergence from other human type I keratins [41], clustered on chromosome 17. Wnt signals are required for the initiation on hair follicle[42] and taste papilla development[43]. However *in vitro* we see high expression of DKK1, which is a Wnt antagonist[44] upregulated in serum of patients with multiple myeloma by playing a role in the inhibition of osteoclast differentiation[45]. Dkk1 has also been shown to progressively destroy the gastrointestinal epithelial architecture in mice[46]. NRG1 plays critical roles in the growth and development of multiple organ systems. IGF2BP3 is the first biomarker of prognostic significance in ovarian clear cell carcinoma that has been validated in an independent case series[47].

G0S2 acts as an inhibitor of ATGL activity and ATGL-mediated lipolysis[48]. The G0S2 protein regulates apoptosis, while INHBA overexpression may promote cell proliferation[49]. Death domains like those of TNFRSF21 control cell apoptosis; and, highly expressed in many tumors and their cell lines[50]. The protein encoded by this RAC2 is a GTPase which belongs to the RAS superfamily of small GTP-binding proteins. Members of this superfamily appear to regulate a diverse array of cellular events, including the control of cell growth, cytoskeletal reorganization, and the activation of protein kinases. The expression levels of LAMB3 mRNAs were higher in malignant tissues than in the corresponding normal tissues[51]. The Fos gene family consists of 4 members: FOS, FOSB, FOSL1, and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the

transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation [provided by RefSeq].

Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. Cyclin D2 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. CCND2 has been shown to interact with and be involved in the phosphorylation of tumor suppressor protein Rb. Knockout studies of the homologous gene in mouse suggest the essential roles of this gene in ovarian granulosa and germ cell proliferation. High level expression of this gene has been observed in ovarian and testicular tumors [provided by RefSeq]. ODC1 encodes the rate-limiting enzyme of the polyamine biosynthesis pathway which catalyzes ornithine to putrescine. The activity level for the enzyme varies in response to growth-promoting stimuli and exhibits a high turnover rate in comparison to other mammalian proteins [provided by RefSeq].

Table 5b. Top cell cycle-related genes differentially expressed *in vivo*

P-value cut-off of 0.01 $\log_2$ of Fold Change>4 false discovery rate (fdr)

Symbol	Fold Elevation in LCM	$\log_2$FC	P	fdr	*in vitro*	LCM
CRIP1	662	9.37	0	0	3.11	12.48
DAPL1	648	9.34	0	0	3.41	12.75
ID4	481	8.91	0	0	2.32	11.23
GAS7	89	6.48	0	0	4.67	11.15
EGLN3	63	5.98	0	0	2.84	8.82
HLF	56	5.80	0	0	2.26	8.06
GAS1	53	5.72	0	0	4.71	10.43
ST7L	36	5.16	0	0	2.72	7.88
SGEF	30	4.93	0	0	5.21	10.14
BCL2	26	4.70	0	0	3.17	7.87
BNIPL	25	4.67	0	0	7.96	12.63
FGD2	23	4.53	0	0	5.01	9.54
C13orf15	22	4.47	0	0	3.46	7.93
RRAGD	22	4.45	0	0	4.28	8.73
RTN4	22	4.45	0	0	3.87	8.32
PRKRA	21	4.42	0	0	3.52	7.94
PER2	21	4.41	0	0	5.40	9.81
GATA3	21	4.41	0	0	7.07	11.48
WNT4	17	4.07	0	0	2.26	6.33

Cysteine-rich intestinal protein 1 (CRIP1) has been identified as a novel marker for early detection of cancers. CRIP is highly expressed in intestine and immune cells. Increased CRIP expression seen in response to LPS suggests that CRIP may play a role in immune cell activation or differentiation or in processes associated with cellular repair[52].

The death-associated protein-like 1 or DAPL1 is an early epithelial differentiation-associated protein also involved in apoptosis. ID4 is a negative regulator of transcription and therefore a potential tumour suppressor gene. GAS1 overexpression is able to block cell proliferation in many cancer cell lines[53]. The effect is mediated by p53[54] and GAS1 is a positive regulator of the Sonic hedgehog signaling pathway[55] and has been proposed as a potential melanoma metastasis suppressor[56]. The Gas 7 protein increases markedly during growth arrest of NIH3T3 cells and persists transiently at high levels upon re-entry of cells into the cell cycle. Growth arrest-specific (gas) genes are expressed preferentially in cells that enter a quiescent state. gas7, which was identified in serum-starved murine fibroblasts, was reported to be expressed *in vivo* selectively in neuronal cells of the mature cerebral cortex, hippocampus, and cerebellum. gas7 transcripts encode a 48-kDa protein containing a structural domain that resembles sequences of OCT2, a POU transcription factor implicated in neuronal development and synapsins, which have a role in modulating neurotransmitter release[57]. EGLN2, Rat Sm-20 is a homologue of the Caenorhabditis elegans gene egl-9 and has been implicated in the regulation of growth, differentiation and apoptosis in muscle and nerve cells. HLF is a well known proto-oncogene. Wild-type Hlf is able to bind DNA specifically as a homodimer or as a heterodimer with other PAR factors. Structural alterations of the E2a-

Hlf fusion protein markedly impair its ability to bind DNA as a homodimer compared with wild-type Hlf[58]. ST7L gene was identified by its similarity to the ST7 tumor suppressor gene, which is clustered with WNT2 gene in the chromosome 7q31 region. Because allelic loss or rearrangements of human chromosome 1p13 region are reported in breast cancer, germ cell tumors, squamous cell carcinoma of head and neck, non-small cell lung cancer, gastrointestinal stromal/smooth muscle tumors (GIST), meningioma, melanoma, acute megakaryoblastic leukemia (M7), and Kaposi's sarcoma, ST7R might be a novel tumor suppressor gene on human chromosome 1p13[59]. Src homology 3 domain-containing guanine nucleotide exchange factor (SGEF). A particularly interesting characteristic of the SGEF gene is that it produces two transcripts, one of which encodes a protein with the structural features typical of other Rho GEFs. The second transcript encodes a much shorter protein that could function as a modulator of Rho GEF activity[60]. BCL2 deregulation prolongs cell survival as it normally regulates apoptosis in cells and is considered to be a proto-oncogene; though, it does not play a role in proliferation[61]. BNIPL or BCL2/Adenovirus E1B 19-KD Protein-Interacting Protein 2-Like also suppresses cell growth by inducing apoptosis[62]. FGD2 is expressed in antigen-presenting cells, including B lymphocytes, macrophages, and dendritic cells. Response Gene To Complement 32 (RGC32) or C13ORF15 is a cell cycle regulatory factor that mediates cell proliferation, both as an activator and substrate of CDC2[63]. RRAGD is a monomeric guanine nucleotide-binding protein, or G protein. By binding GTP or GDP, small G proteins act as molecular switches in numerous cell processes and signaling pathways. The product of the RTN4 gene is a potent neurite outgrowth inhibitor that may also help block the regeneration of the central nervous

system in higher vertebrates. Alternatively spliced transcript variants derived both from differential splicing and differential promoter usage and encoding different isoforms have been identified [RefSeq]. Reticulons are, so far, the only molecules to participate in all three apoptosis signaling pathways. PRKRA encodes a protein kinase activated by double-stranded RNA, which mediates the effects of interferon, particulatly in response to viral infection. Cellular protein may be a stress-activated, physiologic activator of PKR that couples transmembrane stress signals and protein synthesis[64]. The expression of Per1 and Per2 in glioma cells was much lower than in the surrounding non-glioma cells. Therefore, disturbances in Per1 and Per2 expression may result in the disruption of the control of normal circadian rhythm, thus benefiting the survival of glioma cells. The growth of stable PER2 transformants *in vivo* was significantly and dose-dependently suppressed according to the amount of PER2 expressed, indicating that PER2 plays a role in the growth suppression of sarcoma cells[65]. The GATA3 protein contains two GATA-type zinc fingers and is an important regulator of T-cell development and plays an important role in endothelial cell biology. GATA3 drives invasive breast cancer cells to undergo the reversal of epithelial-mesenchymal transition, leading to the suppression of cancer metastasis[66]. WNT4 is a member of the wingless-type MMTV integration site family that encodes secreted signaling proteins that have been implication in regulation of cell fate. Proliferation-related to Ki-67 antigen (MKi67) expression was also most highly expressed in *in vitro* keratinocyte cultures, probably due to the constitutive activation by growth media sold from PromoCell.

Conclusions

Taken together we can see that the epidermis, precisely isolated from the dermis by LCM, provides a much more accurate genomic picture of human keratinocytes than primary cell lines. The caveat of this methodology, however, is that you also isolate melanocytes and Langerhans cells present at low levels in the epidermis.

Chapter 3. FACS to isolate skin cells before culture

Theory and aims

The gene expression of a normal, "resting" keratinocyte is difficult to define due to the limitations of the available methods. Cultured keratinocytes are exposed to conditions so dissimilar from their normal environment that their gene expression does not represent their *in vivo* counterparts. Laser capture microscopy (LCM) mitigates this problem because the keratinocytes are processed immediately after they are isolated from frozen tissue. Unfortunately, LCM is limited by the contamination by other cells in the epidermis such as melanocytes, Langerhans cells, and Merkel cells. In order overcome the above limitations, we employed fluorescence activated cell sorting (FACS) to isolate keratinocytes from human skin for gene analysis and comparison. This method virtually eliminates the possibility that other cells from the epidermis will contaminate the gene expression signatures of the samples. The keratinocytes are cultured for only a short period of time before analysis, and therefore, there are fewer variations in gene expression compared to traditionally cultured cells.

Results

Table 6b shows gene expression levels in FACS-derived keratinocytes relative to LCM epidermis and to cultured keratinocytes. The table is organized to show genes up-regulated in FACS-derived keratinocytes relative to LCM epidermis or to show genes up-regulated in LCM epidermis relative to FACS-derived keratinocytes (Table 6a).

Briefly, it is expected that genes specific to the outermost non-viable cell layers of the epidermis would not be detected in FACS samples, and only basal and lower spinous level of keratinocytes would be collected after tissue dissociation. Therefore, proteins characteristic of the upper spinous, granular and corneocytic layers (e.g. late cornified envelope proteins, loricrin, filaggrin, desmoglein, sciellin, enveloplakin and decorin) are highly upregulated in LCM epidermis as compared to FACS keratinocytes. Similarly, tight junctions and any other intercellular connections would be disrupted by the creation of a single cell suspension, as is necessary for proper FACS experimental function. Tight junctions regulate movement of solutes and ions through the paracellular space and prevent mixing of proteins and lipids in the outer leaflet of the apical and basolateral plasma membrane domains. Claudins, such as CLDN8 (whose RNA is almost 20 times higher in epidermis than in FACS cell suspension), are components of epithelial cell tight junctions.

EGFR ligands are autocrine-acting growth factors for keratinocytes, playing a central role in controlling proliferation of these cells [67,68] [69] [70,71,72]. Member of the EGF family [which is known to play an important role in skin morphogenesis, homeostasis and repair] growth factor betacellulin (BTC) is highly expressed in LCM as

compared to FACS, while its primary receptor EGFR is not differentially expressed with any significance. There is abundant evidence that normal development and homeostasis of the skin and its appendages, specifically of hair follicles, depend on the correct expression and activity of the EGFR and its ligands; so, our data that EGFR is not significantly up or down-regulated seems to fit in normal skin LCM samples. Over-expression of the EGFR has been detected in epithelial squamous cell carcinomas [73,74] and in psoriasis [75,76], and substantial evidence implicates ErbB signaling as a major component in the pathogenesis of non-melanoma skin cancer[77] [78]; and, overexpression of multiple EGFR ligands occurs in psoriatic epidermis [70,79,80,81] but these LCM samples are from non-lesional skin. LRP4, a protein that contains multiple EGF-like domains[82], which are believed to play a critical role in a number of extracellular events, including cell adhesion and receptor-ligand interactions, is also detected *in vivo*.

Many molecules that interact with the organism as a whole (eg immunity) are detected *in situ*. Chemoattractant signal transducer ITPR2 is expressed more than 25 times higher in LCM epidermis than in FACS keratinocytes. Selective monocyte chemoattractant CXCL14 [83] is high in LCM epidermis: expression is 60 times lower in FACS keratinocytes. RORC, a member of the nuclear receptor gene superfamily that encodes transcriptional regulators that play critical roles during homeostasis, is detected in LCM epidermis. In particular the decision of antigen-stimulated cells to differentiate into either TH-17 or T(reg) cells depends on the cytokine-regulated balance of RORC and Foxp3[84]. S1P (a lipid mediator and regulator of cell proliferation) degrading enzyme SGPP2 is present almost 50 times higher in LCM samples.

Table 6a. Genes up-regulated in LCM epidermis v. FACS keratinocytes

P-value cut-off of 0.01 $\log_2$FC of Fold Change>4 false discovery rate (fdr)

Symbol	Description	FC	lgFC LCM/FACS	P	fdr	FACS	LCM
FLG2	filaggrin family member 2	4860	12.25	0	0	3.14	15.38
LOR	loricrin	783	9.61	0	0	4.60	14.22
FLG	filaggrin	446	8.80	0	0	5.53	14.34
LCE2B	late cornified envelope 2B	137	7.10	0	0	3.59	10.69
HLA-DQB2	major histocompatibility complex, class II, DQ beta 2	108	6.76	0	0	2.66	9.41
BTC	betacellulin	100	6.65	0	0	2.82	9.47
BCL11A	B-cell CLL/lymphoma 11A	83	6.38	0	0	3.09	9.47
CXCL14	chemokine ligand 14	61	5.92	0	0	4.41	10.33
CD1A	CD1a molecule	57	5.84	0	0	2.27	8.11
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha	53	5.74	0	0	2.28	8.01
LCE1B	late cornified envelope 1B	49	5.62	0	0	8.35	13.97
HLA-DPB1	major histocompatibility complex, class II, DP beta 1	49	5.62	0	0	4.08	9.69
CD207	CD207 molecule, langerin	49	5.62	0	0	2.31	7.92
SGPP2	sphingosine-1-phosphate phosphotase 2	48	5.58	0	0	3.16	8.74
COL21A1	collagen, type XXI, alpha 1	42	5.40	0	0	3.42	8.82
KRT73	keratin 73	38	5.24	0	0	2.26	7.50
CRIP1	cysteine-rich protein 1 (intestinal)	35	5.12	0	0	7.37	12.48
CIRBP	cold inducible RNA b.p.	34	5.09	0	0	2.94	8.03

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
IL1F7	interleukin 1 family, 7 (zeta)	30	4.92	0	0	2.27	7.20
KLF8	Kruppel-like factor 8	29	4.84	0	0	6.33	11.16
KRT77	keratin 77	28	4.82	0	0	7.58	12.40
RORC	RAR-related orphan receptor	27	4.77	0	0	3.20	7.97
LRP4	low density lipoprotein receptor-related protein 4	27	4.76	0	0	7.42	12.19
HLA-DRA	major histocompatibility complex, class II, DR alpha	27	4.74	0	0	2.70	7.44
TNFRSF19	tumor necrosis factor receptor sf, member 19	26	4.71	0	0	7.76	12.48
ITPR2	inositol 1,4,5-triP receptor, 2	26	4.71	0	0	6.52	11.24
RASA4	RAS p21 protein activator 4	23	4.52	0	0	3.20	7.72
CD74	HLA-DR-gamma	21	4.39	0	0	5.55	9.94
HYMAI	hydatidiform mole associated	21	4.38	0	0	3.19	7.57
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	21	4.37	0	0	3.92	8.29
LTB4R	leukotriene B4 receptor	20	4.34	0	0	5.10	9.44
CPVL	carboxypeptidase, vitellogenic-like	20	4.31	0	0	2.74	7.05
BCHE	butyrylcholinesterase	19	4.25	0	0	4.58	8.83
CLDN8	claudin 8	18	4.21	0	0	4.46	8.67
SGEF	Src homology 3 guanine nucleotide exchange factor	18	4.20	0	0	5.93	10.14
POSTN	periostin, osteoblast factor	18	4.14	0	0	3.13	7.27
HLF	hepatic leukemia factor	18	4.13	0	0	4.71	8.84
OSTF1	osteoclast stimulating factor1	17	4.12	0	0	4.82	8.94
SCAI	suppressor cancer invasion	17	4.11	0	0	3.88	7.98

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
IGFBP5	insulin-like gf bp 5	16	3.98	0	0	4.83	8.81
LY75	lymphocyte antigen 75	16	3.98	0	0	4.45	8.43
ORMDL1	ORM1-like 1 (S. cerevisiae)	16	3.97	0	0	3.23	7.20
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	16	3.96	0	0	2.77	6.73
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene	15	3.95	0	0	4.58	8.53
PLAGL1	ZAC tumor suppressor	15	3.93	0	0	6.12	10.06
RHOBTB3	Rho-related BTB domain containing 3	15	3.91	0	0	4.07	7.98
FGFR3	fibroblast gf receptor 3	15	3.87	0	0	10.13	13.99
HLA-DQA1	major histocompatibility complex, class II, DQ alpha1	14	3.79	0	0	2.26	6.06
EML5	echinoderm microtubule associated protein like 5	14	3.79	0	0	2.44	6.23
TGFBR3	transforming growth factor, beta receptor III	14	3.76	0	0	6.38	10.14
TNNT2	troponin T type 2 (cardiac)	13	3.73	0	0	3.69	7.42
TPD52	tumor protein D52	13	3.69	0	0	4.03	7.72
PSORS1C2	psoriasis susceptibility 1 candidate 2	13	3.67	0	0	2.39	6.06
DNAJC24	DnaJ (Hsp40) homolog, subfamily C, member 24	13	3.66	0	0	2.46	6.12
MUC15	mucin 15, cell surface ass.	12	3.64	0	0	4.29	7.93
TPRG1	tumor protein p63 regulated1	12	3.63	0	0	6.79	10.42
MAGEE1	melanoma antigen family E,1	12	3.62	0	0	3.16	6.78
PBX1	pre-B-cell leukemia homeobox	12	3.59	0	0	3.03	6.62
AGR3	anterior gradient homolog 3	12	3.58	0	0	2.26	5.84

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
POF1B	premature ovarian failure, 1B	12	3.57	0	0	3.37	6.94
IL20RA	interleukin 20 receptor, alpha	12	3.55	0	0	3.10	6.65
IL7	interleukin 7	12	3.55	0	0	3.72	7.26
PIK3C2G	phosphoinositide-3-kinase, class 2, gamma polypeptide	12	3.54	0	0	4.08	7.61
RICS	Rho GTPase-activating	12	3.53	0	0	6.38	9.92
RORA	RAR-related orphan receptor	11	3.52	0	0	8.78	12.30
MAP2K5	mitogen-activated protein kinase kinase 5	11	3.49	0	0	2.40	5.89
DSG1	desmoglein 1	11	3.48	0	0	10.24	13.72
RAB7B	RAB7B, member RAS oncogene family	11	3.48	0	0	5.43	8.91
MYCBP2	MYC binding protein 2	11	3.48	0	0	9.24	12.72
PCDH21	protocadherin 21	11	3.47	0	0	2.90	6.37
DCN	decorin	11	3.47	0	0	4.98	8.45
TCEA3	transcription elongation factor A (SII), 3	11	3.46	0	0	7.72	11.19
ST7L	suppression of tumorigenicity 7 like	11	3.45	0	0	4.44	7.88
KCND3	potassium voltage-gated channel, member 3	11	3.45	0	0	4.21	7.66
MAP3K2	mitogen-activated protein kinase kinase kinase 2	11	3.44	0	0	7.18	10.62
SCEL	sciellin	11	3.42	0	0	8.65	12.07
CDSN	corneodesmosin	11	3.40	0	0	7.50	10.90
GATA3	GATA binding protein 3	10	3.38	0	0	8.09	11.48
CAT	catalase	10	3.38	0	0	8.88	12.26
KRT2	keratin 2	10	3.36	0	0	11.17	14.53

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
ALDH3A2	aldehyde dehydrogenase 3 family, member A2	10	3.34	0	0	4.73	8.07
COL3A1	collagen, type III, alpha 1	10	3.34	0	0	2.53	5.87
DAPL1	death ass protein-like 1	10	3.34	0	0	9.41	12.75
GPRIN2	G protein regulated inducer of neurite outgrowth 2	10	3.31	0	0	2.48	5.79
HLA-DRB4	major histocompatibility complex, class II, DR beta 4	10	3.30	0	0	6.59	9.89
COBL	cordon-bleu homolog	10	3.29	0	0	7.12	10.42
PTGER3	prostaglandin E receptor 3	10	3.29	0	0	5.87	9.16
NOL3	nucleolar protein 3 (apoptosis repressor)	10	3.29	0	0	5.01	8.29
CHD1L	chromodomain helicase DNA binding protein 1-like	10	3.28	0	0	3.89	7.17
IDE	insulin-degrading enzyme	10	3.28	0	0	6.17	9.45
SYTL2	synaptotagmin-like 2	10	3.26	0	0	5.43	8.69
ALOX12	arachidonate 12-lipoxygenase	9	3.23	0	0	3.87	7.10
HCRP1	hepatocellular carcinoma-related HCRP1	9	3.22	0	0	2.44	5.66
TIA1	TIA1 cytotoxic granule-associated RNA bp	9	3.21	0	0	6.60	9.81
STAR	steroidogenic acute regulatory protein	9	3.18	0	0	2.68	5.86
PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	9	3.15	0	0	7.90	11.05
BCL11B	B-cell CLL/lymphoma 11B	9	3.15	0	0	7.74	10.89
PGRMC2	progesterone receptor membrane component 2	9	3.14	0	0	8.75	11.89
ATL2	atlastin GTPase 2	9	3.10	0	0	6.46	9.56

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
HSPC159	galectin-related protein	8	3.08	0	0	10.84	13.92
RCOR3	REST corepressor 3	8	3.08	0	0	6.89	9.98
BCLAF1	BCL2-associated t.f.1	8	3.06	0	0	2.76	5.82
KIN	KIN, antigenic determinant of recA protein homolog	8	3.05	0	0	2.63	5.68
MCF2L	MCF.2 cell line derived transforming sequence-like	8	3.05	0	0	4.39	7.43
ANKH	ankylosis (mouse)	8	3.03	0	0	4.76	7.79
CLNS1A	chloride channel, nucleotide-sensitive, 1A	8	3.02	0	0	4.88	7.90
AHSA2	activator of heat shock 90kDa protein ATPase 2	8	3.02	0	0	3.04	6.06
NSMAF	neutral sphingomyelinase activation associated factor	8	3.01	0	0	3.61	6.62
POU2F3	POU class 2 homeobox 3	8	2.99	0	0	4.13	7.12
SYT17	synaptotagmin XVII	8	2.99	0	0	2.29	5.27
ACER1	alkaline ceramidase 1	8	2.96	0	0	2.51	5.47
ACBD5	acyl-Coenzyme A binding domain containing 5	8	2.96	0	0	5.13	8.09
BCOR	BCL6 co-repressor	8	2.94	0	0	5.25	8.19
RHOT1	ras homolog gene family, T1	7	2.91	0	0	8.39	11.29
PTGS1	prostaglandin-endoperoxide synthase 1	7	2.88	0	0	7.85	10.73
BMS1P5	BMS1 pseudogene 5	7	2.87	0	0	3.10	5.97
EVL	Enah/Vasp-like	7	2.87	0	0	3.59	6.45
ELL3	elongation factor RNA polymerase II-like 3	7	2.84	0	0	9.18	12.01
NFASC	neurofascin homolog	7	2.83	0	0	2.64	5.46

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
TAF15	TAF15 RNA polymerase II, TATA (BP)-associated factor	7	2.83	0	0	5.49	8.32
GRTP1	growth hormone regulated TBC protein 1	7	2.81	0	0	7.84	10.65
KIFC2	kinesin family member C2	7	2.81	0	0	7.24	10.04
TNFSF10	TNF (ligand) 10	7	2.80	0	0	6.99	9.79
MEF2A	myocyte enhancer factor 2A	7	2.80	0	0	6.39	9.18
RAB12	RAS oncogene family	7	2.79	0	0	4.36	7.15
NMNAT3	nicotinamide nucleotide adenylyltransferase 3	7	2.78	0	0	4.81	7.60
EVPL	envoplakin	7	2.78	0	0	7.42	10.20
TP53AIP1	tumor protein p53 regulated apoptosis inducing protein 1	7	2.76	0	0	2.65	5.42
NPY1R	neuropeptide Y receptor Y1	7	2.76	0	0	4.97	7.73
FGL2	fibrinogen-like 2	7	2.73	0	0	2.88	5.61
PDCD5	programmed cell death 5	7	2.72	0	0	4.54	7.26
BMP7	bone morphogenetic protein7	7	2.72	0	0	4.12	6.84
MNDA	myeloid cell nuclear differentiation antigen	7	2.71	0	0	2.63	5.35
GAS1	growth arrest-specific 1	7	2.70	0	0	7.72	10.43
AGBL3	ATP/GTP bp-like 3	7	2.70	0	0	2.37	5.08
TET2	tet oncogene family 2	6	2.67	0	0	4.36	7.02
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene	6	2.67	0	0	3.85	6.52
CCRL1	chemokine receptor-like 1	6	2.67	0	0	4.72	7.38
CCL27	chemokine ligand 27	6	2.65	0	0	9.23	11.88
RAB2A	RAS oncogene family	6	2.65	0	0	2.86	5.51

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
EPPK1	epiplakin 1	6	2.65	0	0	6.64	9.29
MSTP9	macrophage stimulating, pseudogene 9	6	2.64	0	0	5.47	8.10
KIAA1704	KIAA1704	6	2.59	0	0	3.79	6.38
KRT72	keratin 72	6	2.58	0	0	2.28	4.87
POLR2J4	polymerase (RNA) II J4	6	2.56	0	0	5.43	7.99
KIAA0494	KIAA0494	6	2.55	0	0	3.90	6.46
E2F2	E2F transcription factor 2	6	2.54	0	0	4.09	6.63
MAP3K1	MAPK kinase kinase 1	6	2.53	0	0	3.56	6.10
MAP3K6	mitogen-activated protein kinase kinase kinase 6	6	2.53	0	0	7.37	9.91
RAB27B	RAS oncogene family	6	2.53	0	0	10.16	12.69
RASAL1	RAS protein activator like 1	6	2.53	0	0	2.48	5.01
SP8	Sp8 transcription factor	6	2.53	0	0	3.29	5.82
DNAJB2	DnaJ (Hsp40) homolog, B, 2	6	2.48	0	0	8.02	10.51
MCTS1	malignant T cell amplified sequence 1	6	2.48	0	0	3.47	5.95
BNIPL	BCL2/adenovirus E1B 19kD interacting protein like	5	2.46	0	0	10.18	12.63
HRASLS	HRAS-like suppressor	5	2.44	0	0	3.86	6.30
CFD	complement factor D (adipsin)	5	2.43	0	0	5.43	7.86
FARP2	FERM, RhoGEF and pleckstrin domain protein 2	5	2.42	0	0	5.40	7.82
CHD9	chromodomain helicase DNA binding protein 9	5	2.41	0	0	8.46	10.87
CNGA1	cyclic nucleotide gated channel alpha 1	5	2.39	0	0	2.98	5.37
KLK1	kallikrein 1	5	2.39	0	0	5.19	7.58

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
KIAA1712	KIAA1712	5	2.38	0	0	7.67	10.05
GAS7	growth arrest-specific 7	5	2.37	0	0	8.78	11.15
DIO2	deiodinase, iodothyronine, type II	5	2.37	0.001	0	4.95	7.31
CD36	(thrombospondin receptor)	5	2.33	0.001	0	2.78	5.11
TNRC6A	trinucleotide repeat containing 6A	5	2.33	0	0	3.10	5.43
CD47	CD47 molecule	5	2.33	0	0	5.51	7.83
CLCA4	chloride channel accessory 4	5	2.33	0	0	5.73	8.06
KCNJ2	potassium inwardly-rectifying channel J 2	5	2.32	0	0	5.41	7.73
EFNA4	ephrin-A4	5	2.32	0	0	5.72	8.04
FABP7	fatty acid binding protein 7	5	2.31	0	0	2.83	5.13
LST1	leukocyte specific transcript 1	5	2.30	0	0	2.58	4.87
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	5	2.28	0	0	8.01	10.29
MYCL1	v-myc myelocytomatosis 1, lung carcinoma derived	5	2.28	0	0	5.76	8.04
DCAF4	DDB1/CUL4 ass factor 4	5	2.27	0	0	3.06	5.33
NAIP	apoptosis inhibitory protein	5	2.27	0	0	4.18	6.45
BAG1	BCL2-associated athanogene	5	2.26	0	0	7.76	10.02
MST1	macrophage stimulating 1 (HGF-like)	5	2.25	0	0	6.32	8.57
THRA	thyroid hormone receptor, a (erythroblastic leukemia)	5	2.24	0	0	4.00	6.23
VIPR1	vasoactive intestinal peptide receptor 1	5	2.21	0	0	3.94	6.15
KIAA1543	KIAA1543	5	2.20	0	0	4.19	6.39

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
PRLR	prolactin receptor	5	2.20	0	0	2.49	4.69
CHI3L1	chitinase 3-like 1	5	2.18	0	0	7.98	10.15
CIDEB	cell death-inducing effector b	4	2.17	0	0	4.93	7.10
IL11RA	interleukin 11 receptor, alpha	4	2.16	0	0	5.10	7.26
MLL3	myeloid/lymphoid or mixed-lineage leukemia 3	4	2.15	0	0	6.68	8.83
VPRBP	Vpr (HIV-1) binding protein	4	2.15	0	0	4.83	6.98
POU6F1	POU class 6 homeobox 1	4	2.15	0	0	3.20	5.34
CIR1	corepressor with RBPJ, 1	4	2.14	0	0	4.92	7.06
RABGAP1	RAB GTPase activating protein 1-like	4	2.13	0	0	4.14	6.27
CEP68	centrosomal protein 68kDa	4	2.12	0	0	6.72	8.84
ARRB1	arrestin, beta 1	4	2.11	0	0	4.39	6.51
PAQR5	progestin and adipoQ receptor family member V	4	2.10	0	0	4.23	6.33
STAG1	stromal antigen 1	4	2.09	0	0	2.88	4.97
HLA-DMB	major histocompatibility complex, class II, DM beta	4	2.09	0	0	3.36	5.45
EPHA4	EPH receptor A4	4	2.07	0	0	6.11	8.18
NOTCH3	Notch homolog 3	4	2.07	0	0	6.13	8.19
RPRML	reprimo-like	4	2.06	0	0	2.31	4.37
CUL4A	cullin 4A	4	2.02	0	0	4.43	6.46
BOC	Boc homolog (mouse)	4	2.01	0	0	4.35	6.35

There are caveats to the technique of LCM. Though we attempt to avoid collecting hair follicles and other skin appendages in LCM there is some contamination; for example, keratin 73 (type II keratins that are specifically expressed in the inner root sheath of hair follicles) is still detected at relatively high levels. Similarly, Keratin 77 (or K1b,) strongly expressed in the ducts of eccrine sweat glands [85,] was also detected in LCM where the deeper parts of the gland would have been captured unintentionally. CIRBP is a cold-shock protein in human cells[86] that wa elevated, which could be induced during the freezing process of skin samples in preparation for LCM. It was also not surprising to see melanocyte products as well as many different major histocompatibility genes, CD207, LY75 and CD1a detected in LCM epidermis as the samples are not a pure keratinocyte cells due to inherent technological error. The resolution of the microscope with H&E staining may not be of high enough quality to precisely segregate the basal layer of keratinocytes; this may also be due to the limitation of precision in the laser and the exactitude with which it dissects. This shows the limitation of the method, although, the keratinocyte gene expression can still be readily distinguished from that of Langerhans cells due to their vastly variant biology.

Surprisingly, many inflammation-related and growth growth genes became highly up-regulated after keratinocytes were dissociated and subjected to short-term incubation of suspension cultures. Table 6b. is organized by high expression in FACS keratinocyte samples, but fold change (FC) is also listed. CCL20, arguably the most important molecule in cutaneous inflammation, was up-regulated 3 logs in FACS over LCM. Expression levels of S100A9, an extremely imflammatory protein of innate immunity, and its heterodimeric protein complex partner, S100A8 were both increased over 100-

fold in FACS samples as contrasted to LCM. The production of many of these immunologic transcripts detected have been ascribed to other types of resident skin cells; so, this unique view point shows the true potential of the keratinocyte. IL1RL1, associated with atopic dermatitis, was detected here 3-logs higher in FACS samples than LCM. Expressed at similarly high levels relative to '*in vivo*' LCM was neutrophil chemotaxic cytokine IL-8. Heat shock proteins 70 (Hsp70) and 90 (Hsp90) recently identified in tape-stripping experiments were observed[87] here as well. The FACS technique could be argued as a non-physiological equivalent to the wound healing response and reveals previously un-established aspects of keratinocyte plasticity.

Keratin 6B, which dimerizes with keratin 17, is associated with hyperproliferative states and its expression was increased almost 3 logs in FACS keratinocytes as compared to LCM epidermis. Among the members of the cytokeratin subfamily of intermediate filament (IF) proteins, keratin 17 is remarkable since it is normally expressed in the basal cells of complex epithelia, but not in stratified or simple epithelia; therefore indicative of a certain type of epithelial 'stem cell.' Here it is remarkable because it was rapidly induced in wounded stratified epithelia. SPRR1A is a member of the EDC induced in differentiating keratinocytes, here it was notable for its 20-fold increased expression level in FACS over LCM '*in situ*' conditions, potentially suggesting an attempt at regeneration within the dissociated keratinocyte suspension.

There are many growth and transcription factors up-regulated as well which support the idea of early activation response in FACS samples. AREG and HBEGF expression is augmented approximately 400-fold in FACS as contrasted to LCM epidermis. CXCL1 and CXCL2 are postulated melanocyte growth factors, showing the

attempt of the individual keratinocyte to regenerate the epidermis. ATF3 and JUN especially are known for their potent transcriptional activation. Adrenomedullin has been reported to be induced under hypoxic conditions[88] and has roles in vasodilation, angiogenesis as well as growth modulation.

Table 6b. Genes up-regulated in FACS keratinocytes v. LCM epidermis

P-value cut-off of 0.01 Log_2 of Fold Change>4 false discovery rate (fdr)

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
S100A8	S100 calcium binding protein A8	129	7.01	0	0	14.20	7.19
HSPA6	heat shock protein 6 HSP70B	89	6.47	0	0	14.03	7.56
KRT17	keratin 17	73	6.19	0	0	14.03	7.84
IL1RL1	interleukin 1 receptor-like 1	2659	11.38	0	0	13.98	2.60
ATF3	activating transcription factor 3	93	6.55	0	0	13.92	7.37
ADM	adrenomedullin	25	4.65	0	0	13.82	9.17
SPRR1A	small proline-rich protein 1A	21	4.36	0	0	13.77	9.41
CCL20	chemokine ligand 20	1909	10.90	0	0	13.76	2.86
KRT6B	keratin 6B	883	9.79	0	0	13.70	3.91
IGFBP3	insulin-like growth factor bp 3	6	2.56	0	0	13.59	11.03
JUN	jun oncogene	125	6.96	0	0	13.43	6.46
IL8	interleukin 8	2005	10.97	0	0	13.35	2.38
AREG	amphiregulin	431	8.75	0	0	13.23	4.48
GADD45A	growth arrest and DNA-damage-inducible, alpha	25	4.64	0	0	13.21	8.57
GADD45B	growth arrest DNA-damage-inducible, beta	72	6.18	0	0	13.20	7.02
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	97	6.61	0	0	12.96	6.35
HSPA6	heat shock protein 6 (HSP70B)	368	8.52	0	0	12.89	4.36
S100A7	S100 calcium binding protein A7	57	5.84	0	0	12.86	7.02

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
S100A9	S100 calcium binding protein A9	187	7.55	0	0	12.65	5.10
DDIT4	DNAdamage-inducible transcript	31	4.96	0	0	12.51	7.55
KRT16	keratin 16	39	5.30	0	0	12.46	7.16
IL1F9	interleukin 1 family, member 9	36	5.15	0	0	12.42	7.27
IRF6	interferon regulatory factor 6	27	4.76	0	0	12.33	7.57
FOS	FBJ osteosarcoma viral oncogene	11	3.43	0	0	12.22	8.79
MAP3K8	mitogen-activated protein kinase kinase kinase 8	6	2.67	0	0	12.20	9.53
NFIL3	nuclear factor, IL3 regulated	51	5.67	0	0	12.13	6.46
ISG20	IFN stimulated gene	134	7.07	0	0	12.09	5.02
HBEGF	heparin-binding EGF-like GF	342	8.42	0	0	12.08	3.67
HSPA8	heat shock 70kDa protein 8	26	4.71	0	0	11.98	7.27
FOSB	FBJ osteosarcoma oncogene B	67	6.06	0	0	11.77	5.70
IFRD1	IFN-related dev regulator 1	30	4.89	0	0	11.72	6.84
IL13RA2	interleukin 13 receptor, alpha 2	613	9.26	0	0	11.71	2.45
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene F	28	4.82	0	0	11.66	6.84
EIF5	eukaryotic translation initiation 5	38	5.25	0	0	11.65	6.39
BCL10	B-cell CLL/lymphoma 10	487	8.93	0	0	11.64	2.72
EDN1	endothelin 1	20	4.33	0	0	11.49	7.16
KRTAP3-2	keratin associated protein 3-2	567	9.15	0	0	11.42	2.27
ACTB	actin, beta	32	5.02	0	0	11.41	6.39
CXCL2	GRO2 Oncogene	323	8.33	0	0	11.36	3.03
CHD2	chromodomain helicase DNA binding protein 2	36	5.18	0	0	11.30	6.12

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
IL1R2	interleukin 1 receptor, type II	31	4.96	0	0	11.30	6.34
SGMS2	sphingomyelin synthase 2	173	7.43	0	0	11.18	3.75
CD83	CD83 molecule	52	5.71	0	0	11.04	5.34
CXCL3	GRO3 oncogene	123	6.94	0	0	10.71	3.77
IRAK2	IL-1 receptor-associated kinase 2	25	4.62	0	0	10.54	5.92
RBBP6	retinoblastoma binding protein 6	8	2.96	0	0	10.53	7.57
CXCR4	chemokine receptor 4	176	7.46	0	0	10.43	2.97
ANXA1	annexin A1	96	6.59	0	0	10.26	3.67
IL6ST	IL6 signal transducer (oncostatin M receptor)	28	4.78	0	0	10.24	5.46
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	24	4.61	0	0	10.24	5.63
FST	follistatin	208	7.70	0	0	10.18	2.48
HSPA13	heat shock protein 70kDa, 13	62	5.96	0	0	10.12	4.16
PDK4	pyruvate dehydrogenase kinase, 4	11	3.44	0	0	10.08	6.64
KRTAP19-1	keratin associated protein 19-1	202	7.66	0	0	9.97	2.32
S100P	S100 calcium binding protein P	34	5.07	0	0	9.82	4.75
EGR4	early growth response 4	178	7.48	0	0	9.77	2.29
IRF1	interferon regulatory factor 1	32	5.02	0	0	9.75	4.74
CD44	CD44 molecule	107	6.74	0	0	9.55	2.82
SPRR3	small proline-rich protein 3	25	4.63	0	0	9.55	4.91
SOCS3	suppressor of cytokine signaling 3	33	5.02	0	0	9.48	4.45
KLF7	Kruppel-like factor 7 (ubiquitous)	62	5.95	0	0	9.36	3.41
MICB	MHC class I polypeptide-related sequence B	48	5.60	0	0	9.28	3.68

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
TSLP	thymic stromal lymphopoietin	43	5.44	0	0	9.11	3.67
TNFRSF21	tumor necrosis factor receptor 21	40	5.33	0	0	9.10	3.77
EGR1	early growth response 1	46	5.54	0	0	9.10	3.56
SOCS2	suppressor of cytokine signaling 2	48	5.58	0	0	9.03	3.45
TNC	tenascin C	45	5.48	0	0	9.01	3.53
INHBA	inhibin, beta A	95	6.58	0	0	9.00	2.43
TGFB2	transforming growth factor ß 2	96	6.58	0	0	8.97	2.39
OCLN	occludin	30	4.90	0	0	8.87	3.97
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1	87	6.44	0	0	8.82	2.38
CXCL1	chemokine ligand 1 (melanoma growth stimulating activity,α)	59	5.89	0	0	8.78	2.89
GADD45G	growth arrest and DNA-damage-inducible, gamma	6	2.59	0	0	8.78	6.19
PI3	peptidase inhibitor 3, skin-derived	36	5.15	0	0	8.77	3.62
TNFRSF10D	TNF receptor superfamily10d, decoy w. truncated death domain	31	4.97	0	0	8.65	3.68
EDNRB	endothelin receptor type B	9	3.12	0	0	8.59	5.47
HLA-F	major histocompatibility class I, F	5	2.18	0	0	8.42	6.24
G0S2	G0/G1switch 2	33	5.04	0	0	8.27	3.22
RRAS2	related RAS oncogene homolog 2	28	4.81	0	0	7.99	3.18
IL24	interleukin 24	30	4.93	0	0	7.73	2.80
ELF3	E74-like factor 3 (ets domain TF, epithelial-specific)	33	5.06	0	0	7.71	2.65
CDC23	cell division cycle 23 homolog	27	4.73	0	0	7.71	2.98
ITGA5	integrin, α5; fibronectin receptor	22	4.48	0	0	7.61	3.14

Symbol	Description	FC	log_2FC	P	fdr	FACS	LCM
MAP3K8	mitogen-activated protein KKK 8	5	2.28	0	0	7.33	5.05
NRG1	neuregulin 1	26	4.69	0	0	7.33	2.64
HIST1H2AE	histone cluster 1, H2ae	26	4.72	0	0	7.31	2.59
TACSTD2	tumor-assoc signal transducer 2	27	4.78	0	0	7.27	2.50
PTHLH	parathyroid hormone-like	26	4.71	0	0	7.05	2.34
ABL2	v-abl oncogene homolog 2	25	4.65	0	0	6.97	2.32
TNF	tumor necrosis factor	26	4.69	0	0	6.96	2.26
VNN1	vanin 1	21	4.40	0	0	6.94	2.54
IL20	interleukin 20	22	4.47	0	0	6.77	2.30
IL1A	interleukin 1, alpha	19	4.22	0	0	6.71	2.49
KRT75	keratin 75	21	4.37	0	0	6.64	2.28
VEGFC	vascular endothelial G.F. C	20	4.30	0	0	6.57	2.27
MET	proto-oncogene (HGF receptor)	12	3.60	0	0	6.47	2.87
FGFR1	fibroblast growth factor receptor1	13	3.72	0	0	6.17	2.45

The following table 7. Shows genes that are significantly up-regulated in keratinocytes obtained by FACS as compared to cultures *in vitro*. In addition LCM values are listed [in grey.] From this list of genes one can appreciate which alterations in gene expression are due to artifacts of culture as opposed to early activation genes triggered by disassociation and short term *ex vivo* incubation. The LCM epidermal values are included to allow the reader the opportunity to appreciate the effect of inflammatory activation due to FACS methodology as compared to long or short-term culture 'homeostasis', as there are many genes whose expression vary vastly from the *in situ* to the *ex vivo* and *in vitro* environments.

Table 7. Genes upregulated in FACS keratinocytes compared to *in vitro* cultured

P-value cut-off of 0.01 Log_2 of Fold Change>4 false discovery rate (fdr)

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	FACS	LCM
CCL20	chemokine ligand 20	390	8.61	0	0	5.15	13.76	2.86
IL13RA2	IL13 receptor, alpha 2	117	6.88	0	0	4.84	11.71	2.45
IL8	interleukin 8	55	5.79	0	0	7.56	13.35	2.38
CXCL2	chemokine ligand 2	36	5.17	0	0	6.19	11.36	3.03
SGMS2	sphingomyelin synthase2	19	4.22	0	0	6.96	11.18	3.75
MMP1	matrix metallopeptidase1	15	3.88	0	0	9.75	13.63	2.66
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	14	3.84	0	0	9.75	13.59	6.91
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	12	3.64	0	0	9.32	12.96	6.35
HBEGF	heparin-binding EGF-like growth factor	12	3.59	0	0	9.10	12.69	4.70
TGFB2	TGF, beta 2	12	3.55	0	0	5.42	8.97	2.39
CD44	CD44 molecule	11	3.47	0	0	6.09	9.55	2.82
NFIL3	nuclear factor, IL3 reg'd	10	3.32	0	0	8.81	12.13	6.46
PLAUR	plasminogen activator, urokinase receptor	9	3.17	0	0	9.36	12.53	2.95
IL7R	interleukin 7 receptor	9	3.17	0	0	5.49	8.66	3.13
SOD2	superoxide dismutase 2, mitochondrial	8	2.93	0	0	9.33	12.25	6.63
OSMR	oncostatin M receptor	7	2.88	0	0	7.48	10.37	4.83

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	FACS	LCM
NEDD4L	neural precursor cell expressed, developmentally down-regulated 4-like	7	2.87	0	0	8.77	11.65	3.13
GPRC5A	G protein-coupled receptor, family C, group 5, member A	7	2.86	0	0	9.25	12.11	2.58
HIST1H4B	histone cluster 1, H4b	7	2.83	0	0	5.99	8.82	3.24
FOSL1	FOS-like antigen 1	7	2.78	0	0	9.29	12.07	2.93
JUN	jun oncogene	7	2.72	0	0	10.71	13.43	6.46
NEDD9	neural precursor cell, developmentally down-regulated 9	7	2.71	0	0	5.87	8.59	3.75
IL24	interleukin 24	6	2.70	0	0	5.03	7.73	2.80
SESN2	sestrin 2	6	2.64	0	0	7.54	10.18	4.86
CFLAR	CASP8 and FADD-like apoptosis regulator	5	2.44	0	0	8.35	10.79	6.36
ADM	adrenomedullin	5	2.31	0	0	11.51	13.82	9.17
CTTN	cortactin	5	2.29	0	0	8.67	10.96	6.43
CREB5	cAMP responsive element binding protein 5	5	2.24	0	0	6.78	9.01	4.48
FOXC1	forkhead box C1	5	2.21	0	0	9.83	12.04	4.69
TMEFF1	transmembrane w. EGF-like & follistatin-like 1	4	2.16	0	0	6.57	8.73	2.62
SYNJ2	synaptojanin 2	4	2.14	0	0	6.46	8.61	4.21
JMJD6	jumonji domain 6	4	1.98	0	0	8.26	10.23	5.78
ERRFI1	ERBB receptor feedback inhibitor 1	4	1.91	0	0	12.09	14.00	10.06
TLE1	transducinlike enhancer1	4	1.82	0	0	8.20	10.03	5.31

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	FACS	LCM
KPNA4	karyopherin alpha 4	3	1.75	0	0	7.82	9.57	5.19
PATL1	protein assoc with topoisomerase II 1	3	1.73	0	0	5.18	6.91	2.85
SOX9	SRY box 9	3	1.68	0	0	8.60	10.28	2.99
PCOLCE2	procollagen C-endopeptidase enhancer 2	3	1.65	0	0	6.10	7.75	2.99
MCL1	myeloid cell leukemia seq1 (BCL2-related)	3	1.63	0	0	8.60	10.24	5.63
EIF5	eukaryotic translation initiation factor 5	3	1.54	0	0	10.10	11.65	6.39
CDC23	cell division cycle 23	3	1.48	0	0	6.23	7.71	2.98
NEU1	sialidase 1 (lysosomal)	3	1.47	0	0	7.90	9.37	5.42
RAP2B	RAS oncogene family	3	1.43	0	0	6.75	8.18	4.21
HIC2	hypermethylated in cancer 2	3	1.43	0	0	7.79	9.22	5.20
DNAJA1	DnaJ (Hsp40)A,member1	3	1.40	0	0	11.73	13.13	9.33
S100A9	S100 calcium b.p. A9	3	1.39	0	0	11.26	12.65	5.10
IRF6	IFN regulatory factor 6	3	1.37	0	0	10.96	12.33	7.57
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	3	1.36	0	0	12.13	13.49	9.54
HSPA13	heat shock protein 70kDa family, member 13	3	1.33	0	0	7.72	9.05	5.33
CD164	CD164 molecule, sialomucin	2	1.29	0	0	7.94	9.23	5.69
KPNA1	karyopherin alpha 1 (importin alpha 5)	2	1.20	0	0	6.18	7.38	3.09
HIST1H2BK	histone cluster 1, H2bk	2	1.19	0	0	10.60	11.79	8.29
HIST1H2BC	histone cluster 1, H2bc	2	1.19	0	0	6.36	7.55	3.72

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	FACS	LCM
CLU	clusterin	2	1.18	0	0	7.71	8.89	5.72
LCE3D	late cornified envelope	2	1.16	0	0	12.40	13.55	9.50
FGD6	FYVE, RhoGEF and PH domain containing 6	2	1.10	0	0	8.60	9.70	5.93
PVRL2	poliovirus receptor-related 2 (herpesvirus B)	2	1.10	0	0	6.41	7.51	4.31
SOCS2	suppressor of cyt sig 2	2	1.10	0	0	7.93	9.03	3.45
CCNE1	cyclin E1	2	1.09	0	0	7.89	8.98	5.35
KIAA1949	KIAA1949	2	1.04	0	0	8.77	9.80	5.13
VEGFA	vascular endothelial GFA	2	1.04	0	0	8.46	9.49	6.46
MAP7D1	MAP7 domain contain1	2	1.03	0	0	9.17	10.21	6.47
TGIF1	TGFB-induced factor homeobox 1	2	1.03	0	0	9.79	10.82	7.32
PGF	placental growth factor	2	0.96	0	0	7.06	8.02	4.39
TOB1	transducer of ERBB2, 1	2	0.93	0	0	12.55	13.49	10.27
ETS1	v-ets erythroblastosis virus E26 oncogene 1	2	0.93	0	0	12.55	13.48	9.08
PRNP	prion protein	2	0.90	0	0	6.09	6.99	3.43
GADD45A	growth arrest and DNA-damage-inducible, alpha	2	0.89	0	0	12.31	13.21	8.57
BAIAP2L1	BAI1-assoc 2-like 1	2	0.87	0	0	8.40	9.27	4.80
DDIT4	DNAdamage-inducible 4	2	0.87	0	0	11.64	12.51	7.55
OSBPL10	oxysterol b.p.-like 10	2	0.86	0	0	4.68	5.54	2.43
NFATC2IP	NF of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting	2	0.84	0	0	8.72	9.56	6.64
CD47	CD47 molecule	2	0.84	0	0	10.20	11.04	7.47

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	FACS	LCM
HSPH1	heat shock 105kDa /110kDa protein 1	2	0.80	0	0	11.78	12.58	8.63
SMAD7	SMAD family member 7	2	0.79	0	0	7.15	7.94	4.96
UCA1	urothelial cancer associated 1	2	0.78	0	0	5.14	5.92	2.55
GMFB	glia maturation factor, β	2	0.77	0	0	10.07	10.84	6.64
H2BFS	H2B histone, member S	2	0.77	0	0	7.95	8.71	4.68
PMEPA1	prostate TM protein, androgen induced 1	2	0.75	0	0	10.43	11.18	4.50
ARHGEF2	Rho/Rac (GEF) 2	2	0.74	0	0	9.63	10.37	6.20
TRIP10	thyroid hormone receptor interactor 10	2	0.73	0	0	7.20	7.93	4.79
EIF4E	eukaryotic translation initiation factor 4E	2	0.71	0	0	9.75	10.46	6.32
STIP1	stress-induced-phosphoprotein 1	2	0.70	0	0	4.89	5.59	2.74
ANXA1	annexin A1	2	0.70	0	0	13.92	14.62	11.25
CANX	calnexin	2	0.69	0	0	9.43	10.12	5.99
CXCL1	melanoma growth stimulating activity α	2	0.68	0	0	8.11	8.78	2.89
TNFRSF10D	TNF receptor, 10d, decoy truncated death domain	2	0.68	0	0	7.98	8.65	3.68
ABL2	v-abl murine leukemia oncogene homolog 2	2	0.64	0	0	8.55	9.19	5.92
S100A8	S100 calcium b.p. A8	2	0.64	0	0	13.56	14.20	7.19
PDS5A	PDS5, regulator of cohesion maintenance, A	2	0.60	0	0	8.45	9.05	5.26
NRAS	neuroblastoma RAS viral oncogene homolog	1	0.58	0	0	8.87	9.45	5.49

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	FACS	LCM
ROD1	ROD1 regulator of differentiation 1	1	0.57	0	0	9.00	9.56	6.87
MED21	mediator complex 21	1	0.56	0	0	7.75	8.31	5.46
TNC	tenascin C	1	0.53	0	0	8.48	9.01	3.53
DSC2	desmocollin 2	1	0.53	0	0	9.50	10.03	5.72
G2E3	G2/M-phase specific E3 ubiquitin ligase	1	0.52	0	0	6.67	7.20	4.67
PEX13	peroxisomal biogenesis factor 13	1	0.52	0	0	7.18	7.70	5.14
DSG3	desmoglein 3	1	0.49	0	0	10.33	10.82	4.96
LPIN1	lipin 1	1	0.49	0	0	8.94	9.43	5.68
MPZL2	myelin protein zero-like2	1	0.48	0	0	7.00	7.48	3.57
CCNYL1	cyclin Y-like 1	1	0.47	0	0	7.62	8.09	3.61
PLK2	polo-like kinase 2	1	0.47	0	0	12.55	13.02	10.55
ILF2	IL enhancer factor 2	1	0.44	0	0	10.10	10.55	7.22
PGRMC1	progesterone receptor membrane component 1	1	0.44	0	0	6.16	6.60	3.82
MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4	1	0.44	0	0	6.76	7.20	3.69
GLS	glutaminase	1	0.41	0	0	6.70	7.11	3.11
TNFRSF12A	TNF receptor, 12A	1	0.36	0	0	10.62	10.98	4.59
AKT3	v-akt murine thymoma oncogene homolog 3	1	0.35	0	0	5.04	5.39	2.88
TGFA	TGF alpha	1	0.34	0	0	11.38	11.72	7.68
CDK2AP2	cyclin-dependent kinase 2 associated protein 2	1	0.33	0	0	7.85	8.18	5.00

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	FACS	LCM
CDYL	chromodomain protein, Y-like	1	0.32	0	0	8.07	8.39	6.06
IER3	Immediate early response	1	0.32	0	0	13.71	14.03	10.48
GRB10	g.f. receptor-b.p. 10	1	0.29	0	0	7.23	7.52	4.14
FGG	fibrinogen gamma chain	1	0.29	0	0	11.06	11.35	8.96
ZAK	zipper kinase AZK	1	0.29	0	0	10.19	10.47	7.93
HIST1H2BD	histone cluster 1, H2bd	1	0.27	0	0	8.95	9.22	6.66
MAP2K1	mitogen-activated protein kinase kinase 1	1	0.25	0	0	9.56	9.81	6.49
HUS1	HUS1 checkpoint	1	0.24	0	0	8.14	8.38	5.66
SYPL1	synaptophysin-like 1	1	0.24	0	0	8.81	9.05	5.67
PAK2	p21 protein (Cdc42/Rac)-activated kinase 2	1	0.23	0	0	7.28	7.51	3.91
MAOA	monoamine oxidase A	1	0.23	0	0	9.70	9.93	7.01
ANXA5	annexin A5	1	0.19	0	0	12.08	12.27	9.84
PNLIPRP3	pancreatic lipase-related protein 3	1	0.19	0	0	11.73	11.92	4.78
STAT3	acute-phase response fac	1	0.18	0	0	7.35	7.53	3.59
CKS2	CDC28 protein kinase regulatory subunit 2	1	0.17	0	0	11.47	11.64	8.68
RABL3	RAS oncogene -like 3	1	0.15	0	0	6.16	6.30	3.58
TICAM2	TLR adaptor molecule 2	1	0.13	0	0	7.90	8.04	4.22
MYO1B	myosin IB	1	0.13	0	0	10.43	10.56	5.17
MSN	moesin	1	0.13	0	0	11.92	12.05	9.82
TRAF4	TNF rec-assoc factor 4	1	0.12	0	0	7.86	7.98	5.17
TCEB1	transcription elongation factor B polypeptide 1	1	0.12	0	0	10.33	10.45	8.14

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	FACS	LCM
TNIP1	TNFAIP3 protein 1	1	0.11	0	0	11.54	11.65	9.18
HMGA1	high mobility AT-hook 1	1	0.07	0	0	10.72	10.79	8.58
CD59	complement regulatory	1	0.06	0	0	11.24	11.30	7.45
CD24	CD24 molecule	1	0.05	0	0	9.56	9.61	5.31
CTGF	connective tissue G.F.	1	0.04	0	0	7.49	7.53	2.73
MALT1	mucosa associated lymphoma translocation1	1	0.02	0	0	9.08	9.11	6.43
ARHGAP21	Rho GTPase activating protein 21	1	0.02	0	0	10.16	10.18	7.29

This data is perhaps the most difficult to interpret; though, the *ex vivo* cells isolated by FACS were more similar to the *in situ* LCM samples than keratinocytes cultured *in vitro*, they have been taken out of their natural environment and processed to create a single cell suspension necessary for this technique. Regulation of gene expression in isolated keratinocytes was therefore affected by the mere methodology of dissociating cells from whole tissue and subsequent short-term incubation to restore surface antigen after enzymatic processing.

Keratins 1 and 10, typically co-expressed in suprabasal terminally differentiated cells of epidermis, were seen at lowest levels *in vitro* and found at high levels *in vivo* especially from samples collected by LCM. Keratin 2 has been shown to be associated with the upper spinous layer and was seen to be at low levels in *in vitro* culture. CDK inhibitor 1B was also absent in keratinocytes in culture, showing negative regulation of cell proliferation only from samples collected most directly from tissue.

Though we try to avoid dissecting hair follicles with the epidermis using LCM, they would obviously be absent from cells in culture, as was also seen in single-cell suspensions from FACS by the lack of Keratin 72 and Keratin 73 expression. KRT75 a.k.a. Keratin 6 is predominately seen in cells that have been induced to hyperproliferate by disease, injury, and in epithelial cell culture conditions; so, it is not a surprise to see its highest level *in vitro* and even some of the FACS samples that were incubated overnight. Ki-67 is commercially available to detect proliferating cells but not in quiescent ones. Expression of this antigen occurs preferentially during late G1, S, G2 and M phases of the cycle, but cannot be detected in those that have entered G0, which includes the majority of the cellular residents in normal epithelium.

Chapter 4. Comparison of Melanocyte vs. Langerhans cells by FACS

Theory and aims

In the previous chapter we examined genes expressed in cultured and FACS Keratinocytes, along with LCM epidermis, which showed a wide variability of expression depending on the conditions from which the cells were extracted. In this part of the thesis the goal was to examine FACS samples of epidermal suspensions to establish melanocyte-selective genes. It seemed necessary to extract melanocytes directly from human skin instead of using primary cell lines that had already been subject to exogenous mitogens. The first aim was to segregate the major cell types of the epidermis: keratinocytes from the morphologically dendritic melanocyte and Langerhans cells.

It has been widely accepted that c-kit is present on melanocytes, necessary during embryogenesis for proper migration of melanoblasts from the neural crest and subsequent differentiation. Though typically associated with B cells, published literature and staining of c-kit in mice and humans demonstrates a strong co-stain with melanocytes, especially robust in hair follicle sheaths. I therefore hypothesized that the antibody to this surface membrane receptor could isolate melanocytes from the epidermis using FACS. Figure 2 is an illustration of the staining of c-kit in skin from our lab. In a collaborative effort with Dr. Hideki Fujita, who studies Langerhans cells, the dendritic cell population in the epidermis, we were able to use the same skin samples sorting for c-kit or Langerin (the CD207 antigen) from the epidermis. In this manner we were able to

get the cells necessary for our respective experiments, as well as verifying each others' data.

Prior to analyzing the cells obtained for genomics, it was pertinent to establish that they were in fact accurately isolated by this novel technique. Thus, the two populations of cells that make up the minority of the epidermis, $CD1a^{+}/CD207^{+}$ Langerhans cells & $CD117^{+}$ melanocytes, were compared and scrutinized for their characteristic gene expression. We decided that this was an acceptable protocol for isolating melanocytes, as typical cell-specific genes were present, including many of the enzymes from the pigmentation pathway that are lost in culture. In addition, the $CD207^{+}$ population contained many of the gnomic markers of Langerhans cell including HLA and other dendritic cell genes, further confirming the methodology.

It is of note that subsequently, another group reported the used of antibodies to c-kit to isolate murine melanocytes from mixed dermal cell suspensions (murine melanocytes are follicular and not epidermal.) Their determination that these cells were indeed melanocytes serves to validate this technique; but, their methods, however, included 10 days of *in vitro* culture prior to FACS in media containing FBS, bFGF, PMA and transferrin among other undefined elements in exogenous media [89]. Their resulting primary cell population is therefore more akin to the commercial PromoCell primary cells used in our work, with several additional mitogenic effects in their melanocyte growth medium.

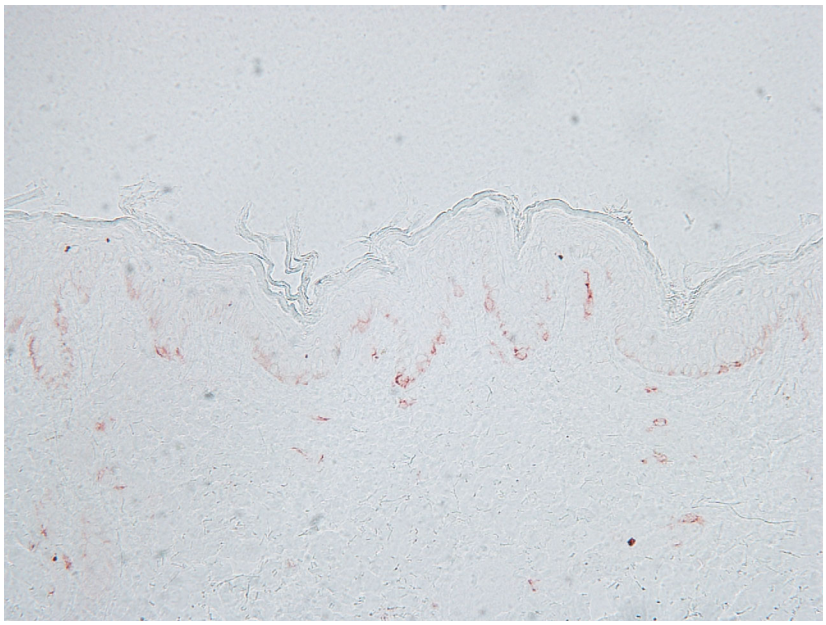


Figure 2. 20x image of c-kit staining human skin. Staining is seen primarily in the basal cell layer of the epidermis, though some mast cells etc are also stained in the dermis (though this layer is removed prior to cell suspension for staining and FACS.)

Picture courtesy of Hiroshi Mitsui, M.D., Ph.D.

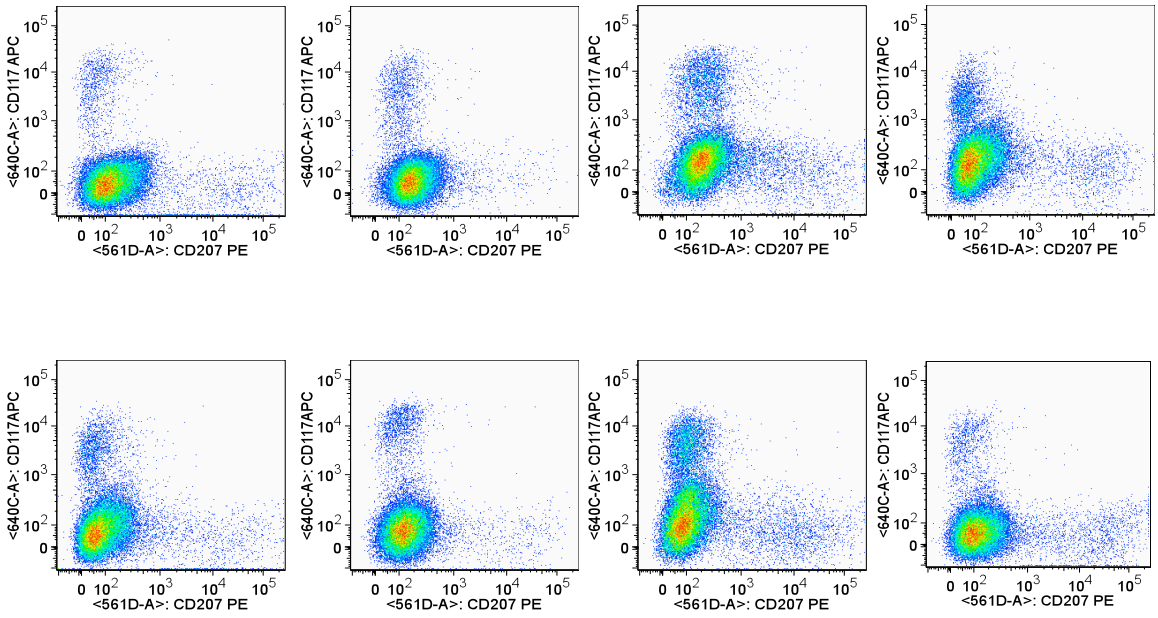


Figure 3. FACS single positive populations of CD117$^+$ (c-kit) melanocytes and CD207$^+$ Langerhans cells were used for analysis in this chapter.

Collaboration with Hideki Fujita, M.D., Ph.D.

Langerhans cell populations

Upon inspection of the top 80 genes differentially expressed from the CD1a$^+$ partition, as compared to the CD117$^+$ population, one can be confident that they are indeed Langerhans cells. They expressed to the highest degree the lysosomal-associated membrane protein 3, otherwise known as DC-LAMP. Indoleamine-pyrrole 2,3 dioxygenase (INDO,) which is an immunomodulating tryptophan-catabolizing enzyme, was also among this highly expressed group of genes unique to dendritic cells of the skin. Other surface molecules detected at elevated levels include the major histocompatibility

complexes HLA-DQalpha1 and HLA-DQbeta1, CD86, CD205 (LY75,) CCR7 and colony-stimulating factor 2 receptor alpha (CSF2RA.) Inflammatory chemokine IL-1β was also expressed. Prostaglandin receptor EP4 (PTGER4) is essential for migration of Langerhans cells to regional lymph nodes in mice [90]. Ten genes highly expressed in the $CD117^{+}$ population representative of Langerhans cells are shown in Table 8.

Table 8. Langerhans genes highly expressed in FACS-derived populations

Fold change (LC - Mel)	Gene Symbol	Gene Title
522	LAMP3	lysosomal-associated membrane protein 3
419	CCR7	chemokine (C-C motif) receptor 7
358	INDO	indoleamine-pyrrole 2,3 dioxygenase
207	HLA-DQA1	major histocompatibility complex, class II, DQ α1
170	CSF2RA	colony stimulating factor 2 receptor, alpha,
167	CD86	CD86 molecule
153	HLA-DQA1	major histocompatibility complex, class II, DQ α1
149	PTGER4	prostaglandin E receptor 4 (subtype EP4)
103	LY75	lymphocyte antigen 75
85	IL1B	interleukin 1, beta

Melanocyte cell populations

What is most interesting and exciting upon examination of the top 100, shown in table 9, differentially expressed transcripts in the c-kit^{+} pool is how many unknown and hypothetical proteins were identified, because these represent novel, putative melanocyte-specific genes. At the top of the list is TRPM1, also known as melastatin-1, a relatively novel gene that seems to correlate directly with melanin content. Typical melanocyte-associated genes such as melan-A and tyrosinase are increased by 23-fold relative to the Langerhans cell isolate. N.B. there may be some genes represented more than once, due to the array technique which has several probes per gene, duplication is only further verification that the gene is truly expressed and was accurately detected.. The detection of preciously reported melanocyte-specific genes further indicted the successful isolation of melanocyte by this method.

Table 9. Top 100 Genes expressed in Melanocytes as compared to Langerhans cells

Fold Change (M-LC)	Gene Symbol	Gene Title
98	TRPM1	transient receptor potential cation channel subfamily M,1
83	LOC100129562	hypothetical protein LOC100129562
78	BCHE	butyrylcholinesterase
69	ABCA8	ATP-binding cassette, sub-family A (ABC1), member 8
69		CDNA clone IMAGE:4791597
66		CDNA clone IMAGE:4791597
63		Transcribed locus
61		CDNA clone IMAGE:5311370
57	GPM6B	glycoprotein M6B
53	PAX3	paired box 3
53	PCSK2	proprotein convertase subtilisin/kexin type 2
49	GMPR	guanosine monophosphate reductase
49	SGCE	sarcoglycan, epsilon
49		Transcribed locus
48	SLC6A15	solute carrier family 6, member 15
44	SRPX	sushi-repeat-containing protein, X-linked
44		Transcribed locus
42	LPPR4	plasticity related gene 1
42	GPM6B	glycoprotein M6B
42	ASPA	aspartoacylase (Canavan disease)
41	ZNF521	zinc finger protein 521
41	GPR155	G protein-coupled receptor 155

Fold Change (M-LC)	Gene Symbol	Gene Title
41	EPHA5	EPH receptor A5
39	SORBS2	sorbin and SH3 domain containing 2
38	PDE10A	phosphodiesterase 10A
38	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
38	LZTS1	leucine zipper, putative tumor suppressor 1
37	STXBP6	syntaxin binding protein 6 (amisyn)
36	LOC493869	Similar to RIKEN cDNA 2310016C16
36		Homo sapiens, clone IMAGE:4214654, mRNA
36	RHOBTB3	Rho-related BTB domain containing 3
36	CDH19	cadherin 19, type 2
36	GYG2	glycogenin 2
36	TRIM2	tripartite motif-containing 2
35	PLSCR4	phospholipid scramblase 4
34	PAG1	phosphoprotein associated with glycosphingolipid 1
34	TRPM1	transient receptor potential cation channel subfamily M,1
34	CSPG4	chondroitin sulfate proteoglycan 4
34	SORBS1	sorbin and SH3 domain containing 1
34	TRIM63	tripartite motif-containing 63
33	TFAP2B	transcription factor AP-2 beta
33	CAPN3	calpain 3, (p94)
33	GPM6B	glycoprotein M6B
32	PPP1R9A	protein phosphatase 1, regulatory (inhibitor) subunit 9A
32		MRNA from chromosome 5q31-33 region
32		CDNA FLJ39330 fis, clone OCBBF2016405

Fold Change (M-LC)	Gene Symbol	Gene Title
32	DPP6	dipeptidyl-peptidase 6
32	AEBP1	AE binding protein 1
31	VEPH1	ventricular zone expressed PH domain homolog 1
31	TIMP2	TIMP metallopeptidase inhibitor 2
31	RHOJ	ras homolog gene family, member J
31	GPM6B	glycoprotein M6B
31		CDNA FLJ90705 fis, clone PLACE1007591
31	SPTBN1	spectrin, beta, non-erythrocytic 1
30	PKNOX2	PBX/knotted 1 homeobox 2
30	TBC1D16	TBC1 domain family, member 16
30		Homo sapiens, clone IMAGE:3618365, mRNA
30	DIP2C	DIP2 disco-interacting protein 2 homolog C (Drosophila)
29		MRNA, clone ICRFp507B0451
29		Transcribed locus
29	CABLES1	Cdk5 and Abl enzyme substrate 1
29	C6orf218	Chromosome 6 open reading frame 218
28	DLC1	deleted in liver cancer 1
28	SLC6A15	solute carrier family 6, member 15
28	MAP1B	microtubule-associated protein 1B
28		CDNA clone IMAGE:5284125
27	LEF1	lymphoid enhancer-binding factor 1
27		Transcribed locus

Chapter 5. FACS Melanocytes vs. Melanocyte lines in culture

Theory and aims

After showing how many alterations accumulate in culture with keratinocytes it was even more pertinent to examine melanocytes, as they do not replicate under non-pathologic conditions, and therefore *in vitro* models are likely to be extremely misleading. Melanocytes can progress to different types of dysplasia, including nevi, dysplastic nevi and lentigo simplex. In turn, these can develop into melanoma *in situ* or lentigo maligna which then may have migratory potential and become invasive malignant melanoma. It is therefore pertinent for pathology to discover which growth pathways allow melanocytes to proliferate, even if only transiently (eg BRAF expressed transitorily.) If a growth factor or growth-regulated pathway identified in resting melanocytes that trigger the non-proliferative cells to grow, then the question arises as to what is the dependence of melanoma on such pathways. Is there a growth addiction or do they become superfluous? Looking at *in vitro* cultured melanocytes may show what the genomic pathways are that underlie conversion from hyperplasia to neoplasia.

Preparations of ten FACS melanocytes were compared with 12 NHEM established in serum-free culture using gene array. Some of the genes in the FACS were due to some keratinocyte contamination that could be removed by additional computational subtraction, but are included here as they are an artifact inherrant to the FACS technique.

Results

The two sources of melanocytes differed especially in cell-cycle related genes (as shown in tables 10a and 10b,) but the typical pigmentation genes were not differentially expressed. Table 11 shows some pigmentation related genes and many melanosome genes were detected at high levels *in vitro* as compared to FACS.

Table 10a. Genes expressed at high levels in c-kit$^+$ FACS Melanocytes as compared to 1° Melanocytes cells *in vitro* (probable role in cell proliferation.)

Symbol	Description	FC	log_2FC	P	fdr	Vitro	FACS
FOS	FBJ murine osteosarcoma viral oncogene homolog	17.29	4.11	0	0	6.02	10.13
BCL2	B-cell CLL/lymphoma 2	9.90	3.31	0	0	8.14	11.45
JUN	jun oncogene	8.99	3.17	0	0	7.84	11.01
FGF9	fibroblast growth factor 9 (glia-activating factor)	8.93	3.16	0	0	2.27	5.43
EDN1	endothelin 1	8.48	3.08	0	0	2.34	5.42
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha	8.40	3.07	0	0	4.38	7.45
KRAS	v-Ki-ras2 rat sarcoma viral oncogene homolog	6.36	2.67	0	0	2.34	5.01
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	6.35	2.67	0	0	4.62	7.29
PTEN	phosphatase and tensin homolog	5.17	2.37	0	0	4.37	6.74
MAPK8	mitogen-activated protein kinase 8	4.01	2.00	0	0	4.92	6.92

Table 10b. Genes expressed in 1° Normal Human Melanocytes cells *in vitro* as compared to c-kit$^+$ FACS Melanocytes (probable role in cell proliferation.)

Symbol	Description	FC	log_2FC	P	fdr	*in vitro*	FACS
BAX	BCL2-associated X protein	78	6.28	0	0	10.44	4.16
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)	46	5.51	0	0	8.32	2.81
CDC2	cell division cycle 2, G1 to S and G2 to M	29	4.85	0	0	7.80	2.95
IGF2R	insulin-like growth factor 2 receptor	29	4.85	0	0	9.73	4.88
TOP2A	topoisomerase (DNA) II alpha 170kDa	22	4.46	0	0	7.74	3.28
TP53I3	tumor protein p53 inducible protein 3	19	4.25	0	0	9.71	5.46
MCF2	MCF.2 cell line derived transforming sequence	19	4.25	0	0	6.65	2.41
MAP2K2	MAPK kinase 2	19	4.23	0	0	7.30	3.07
CCNB2	cyclin B2	17	4.06	0	0	7.66	3.60
RAC1	ras-related C3 botox substrate 1	16	3.97	0	0	10.93	6.96
CDK2	cyclin-dependent kinase 2	15	3.88	0	0	8.00	4.12
MET	met proto-oncogene (hepatocyte growth factor receptor)	11	3.45	0	0	10.83	7.38
FGFR1	fibroblast growth factor receptor	8	2.93	0	0	11.23	8.29
AKT1	v-akt murine thymoma viral oncogene homolog 1	6	2.67	0	0	9.68	7.01
BRCA1	breast cancer 1, early onset	6	2.67	0	0	8.81	6.14
STAT3	(acute-phase response factor)	6	2.49	0	0	8.86	6.37

Table 11.

Melanocyte-associated genes up-regulated *in vitro* over c-kit$^+$ FACS (P<0.01)

Symbol	Description	log_2FC	FC	P	fdr	*in vitro*	FACS
ABCB5	ATP-binding cassette 5	9.33	645	0	0	12.25	2.92
SLC45A2	solute carrier fam 45, 2	5.67	51	0	0	9.43	3.76
GNAQ	guanine nucleotide b.p.(G protein), q polypeptide	5.33	40	0	0	9.98	4.65
PPIB	peptidylprolyl isomerase B (cyclophilin B)	4.53	23	0	0	12.78	8.25
RAB5C	RAS oncogene family	4.39	21	0	0	10.47	6.08
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	4.20	18	0	0	10.70	6.50
PLDN	pallidin homolog (mouse)	4.15	18	0	0	8.46	4.32
GANAB	glucosidase, alpha; AB	4.11	17	0	0	10.44	6.33
SERPINF1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	4.08	17	0.001	0	11.66	7.58
CALU	calumenin	4.01	16	0	0	8.68	4.67
DDT	D-dopachrome tautomerase	3.92	15	0	0	10.26	6.34
LAMP1	lysosomal-associated membrane protein 1	3.91	15	0	0	7.04	3.13
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B 1	3.86	15	0	0	10.99	7.13
YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon	3.81	14	0	0	7.75	3.94
MMP14	matrix metallopeptidase 14 (membrane-inserted)	3.77	14	0	0	8.05	4.28

Chapter 6.

Using receptor information to investigate putative growth factors (and/or other new pathways not previously identified)

Theory and aims

A plethora of information regarding melanocyte growth factors have been published, but most accounts were derived from cells grown in undefined media. It was thus necessary to determine which factors are necessary for melanocyte survival and proliferation in defined medium. Results of initial genomic profiling of NHEM receptor expression as compared to the vast list of receptors indicated *in silico* as being essential in melanocyte growth in undefined media yielded an overlapping list of receptors that we aimed to target using specific ligands. Our hypothesis being that each of the growth factors may be able to stimulate their receptor to independently promote proliferation of melanocytes. Comparison of *in vitro* receptor expression with current methods of melanocytic culture *in silico* led to the consensus that there are at least 4 main growth factors may be needed for proper signaling: bFGF, HGF, SCF and ET-3.

Basic fibroblast growth factor, also known as FGF2, is a wide-spectrum mitogenic, angiogenic, and neurotrophic factor that is expressed at low levels in many tissues and cell types and reaches high concentrations in the brain and pituitary gland. FGF2, or bFGF, has been implicated in a multitude of physiologic and pathologic processes, including limb development, angiogenesis, wound healing, and tumor growth. bFGF is important for melanocyte survival and is produced in the skin by keratinocytes

and fibroblasts. While bFGF is not known to be expressed by normal melanocytes, it has been shown to be expressed by melanoma cells[91] acting via its specific transmembrane receptor (FGFR) and activating the subsequent intracellular second messenger cascades.

The MET proto-oncogene encodes for the hepatocyte growth factor (HGF) receptor, a plasma membrane tyrosine kinase that is involved in melanocyte growth and melanoma development. MET is expressed on melanocytes and HGF is a growth factor for cultured melanocytes [92]. HGF/MET has been shown to influence melanocyte transformation and the acquisition of the metastatic phenotype [93]. The role of MET in growth and motility, often called "invasive growth" places MET at a center stage of tumor and metastasis development. Indeed, MET overexpression is very common in human cancer.

Stem cell factor (SCF), also known as steel factor or c-kit ligand, stimulates migration, proliferation, and differentiation of mouse melanocytes. Loss of SCF in mice leads to the absence of melanoblasts.[94] SCF is expressed in endothelial cells and keratinocytes but not frequently in basal melanocytes[95,96] and is highly mitogenic for human melanoblasts and melanocytes in culture and for melanocytes *in vivo*.[97,98]

Among the major paracrine factors that are involved in melanocyte biology, the endothelins play significant roles during the early development of these cells, in their response to ultraviolet (UV) radiation, and in pathological conditions including melanoma. A role for ET-3 as a survival and proliferation factor for committed precursors has been established in both avian and mouse models. Waardenburg syndrome type 4 (WS4), or Shah-Waardenburg syndrome, is also known as Hirschsprung

disease Type II (HSCR II) and is characterized by an absence of epidermal melanocytes and enteric ganglia. Mutations in the genes encoding the endothelin type-B receptor (EDNRB) and its physiological ligand endothelin 3 (ET-3) are now known to account for the majority of HSCR II patients. Little is known about the mechanisms underlying the regulation of the endothelin receptor B (EDNRB) expression in melanocytes. EDNRB and ET-3 are transiently expressed in crest-derived melanoblast and neuroblast precursors, and in the surrounding mesenchymal cells, respectively. The influence of EDNRB-mediated signaling on the emigration, migration, proliferation, and differentiation of melanocyte and enteric neuron precursors, *in vivo* and *in vitro* has recently been the subject of great scrutiny.

There was then the need to create a defined media in which to examine different combinations of these growth factors at known concentrations minus the pituitary homogenate and serum typically present *in vitro*. We sought to establish mitogenic potential of single growth factors and also combinations thereof in defined medium.Hence, individual and synergistic effects of growth factors bFGF, HGF, SCF and ET-3 were determined on melanocytes cultured in defined medium.

Results

Conventional methods of cell proliferation are not readily adaptable to assessing melanocyte growth as the primary cells are quite delicate, so any staining protocols such as CFSE labeling had to be vastly modified, ie reducing incubation time and concentration of dye used. In addition, melanocytes *in vivo* do not replicate under non-

pathologic conditions, hence doubling time *in vitro* is around three days. Some of the defined media conditions (eg basal media alone) do not support cell survival let alone proliferation. Simple methods of cell counts amongst growth media conditions only show changes in survival, and therefore do not give clear or absolute information pertaining to growth signals. Kits such as those sold by Invitrogen are often subject to variation in metabolism and thus could not be relied upon for this particular assay.

Melanocytes were highly influenced by the different media, as visualized by microscopic analysis of cell morphology, but cell number counts alone cannot distinguish between simple survival *in vitro* and withdrawal from active proliferation. It was clear that different conditions of growth factors and combinations thereof did indeed affect growth, but in the search for "normal", distinct changes in morphology that accompanied growth precluded the use of raw cell counts alone. Figure 5 includes photographic examples of primary human melanocytes in the various defined culture medium conditions as well as brief descriptive analysis of growth and morphology after one cell doubling (3 days) in Table 12. As melanocyte medias are proprietary, the documentation of the pure effects of individual growth factor on cell survival *in vitro* is novel.

Table 12.

Defined Media conditions for 1° Melanocyte & description after 3 days *in vitro*

	Insulin	HC	FGF	HGF	SCF	ET3	Visual description by microscopy
1							many dead cells, some spread
2							survival, bi-polar cells; senescent ?
3							survival; similar to 2
4							proliferation is marked; similar to 3
5							proliferation is marked
6							very short bipoles; survival questionable
7							not as good as 3; shorter bipoles
8							proliferation is marked ≅ 3 (and 5)
9							proliferation is marked; better than 3
10							very short bipoles, less growth than 9
11							short/intermediate bipoles, growth<9
12							growth with abundance of short bipoles
13							growth=9, morphology better than 13
14							decent growth, ≅ 9 and 14
15							large cell bodies + long fragile dendrites
16							very proliferative
17							very proliferative
M2							very proliferative. Cells tripolar.

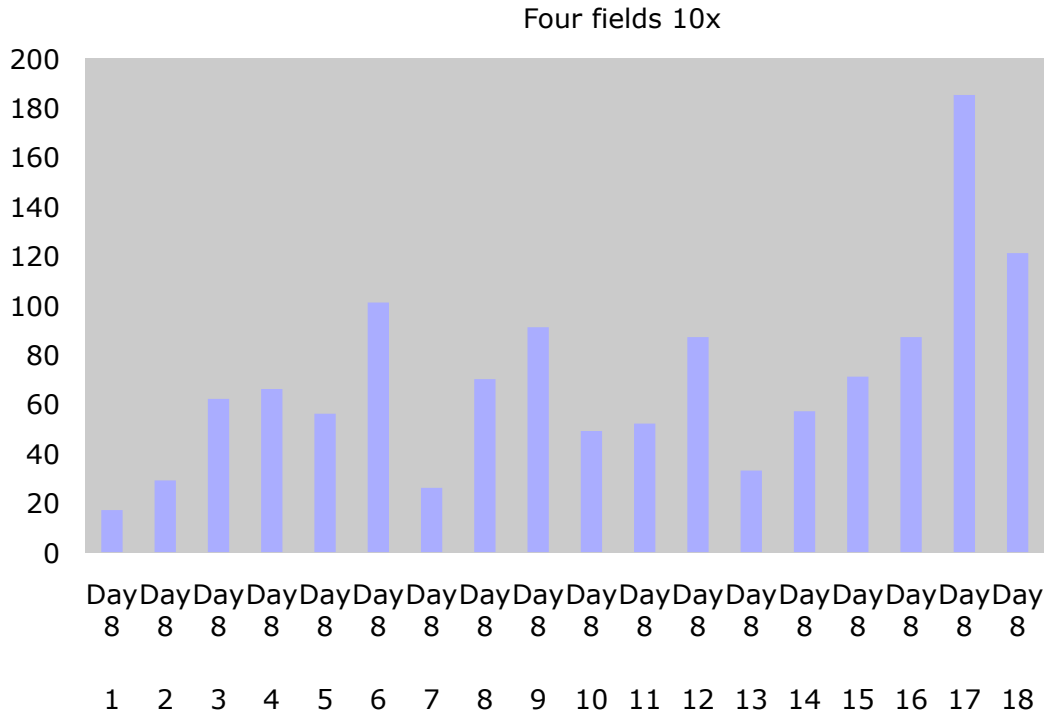


Figure 4. Cell numbers in culture at day 8.

Numbers in the bottom row correspond to media conditions in Table 12.

Figure 5. Microscopic photographs of NHEM under various growth conditions

1.24×10^5 cells were seeded in each well of a 6-well plate at 4 days of incubation

Figure 5

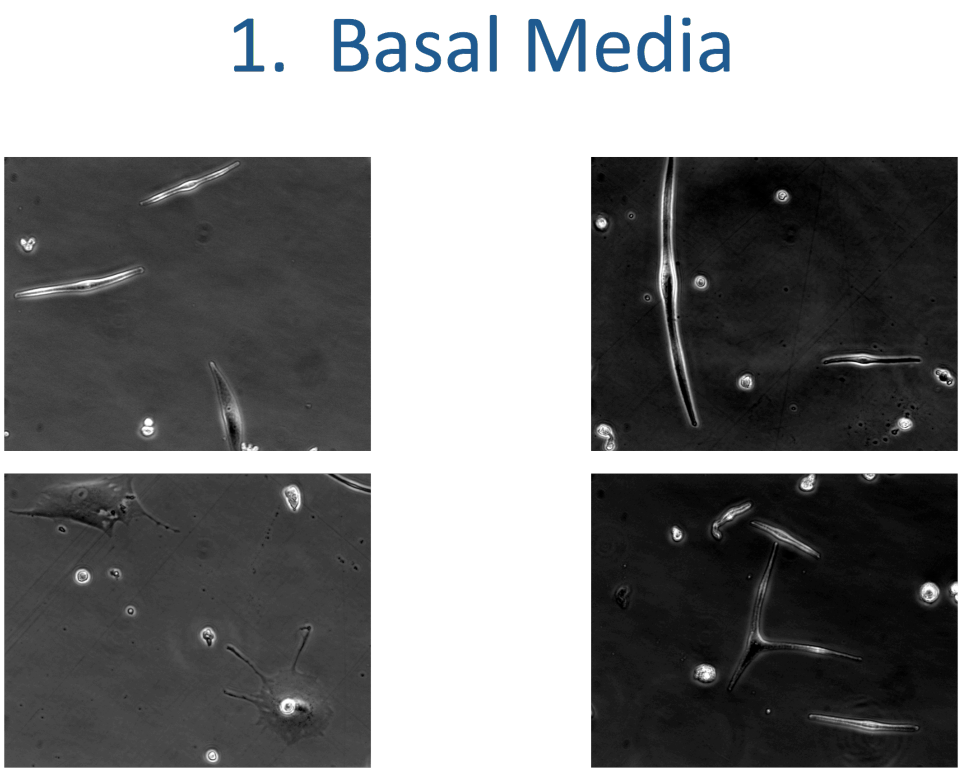
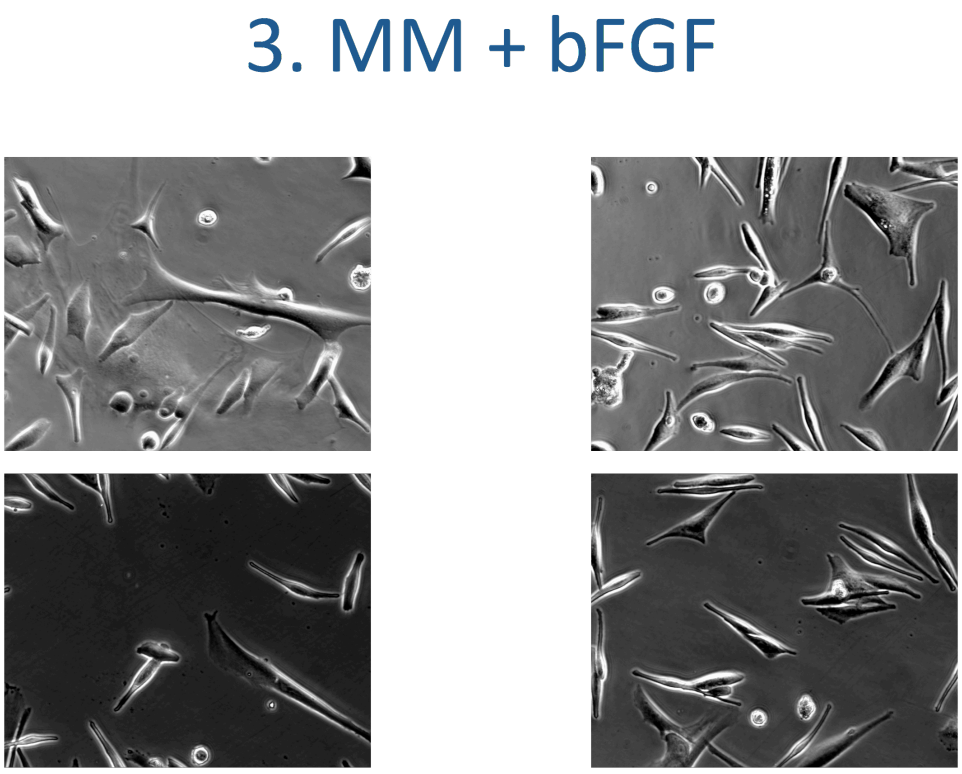


Figure 5 (continued)



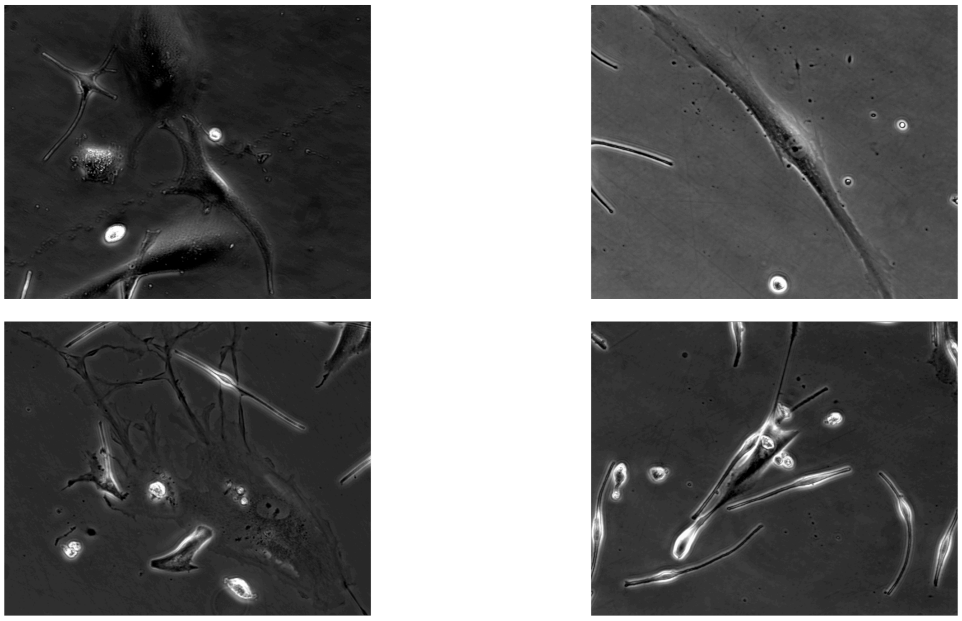
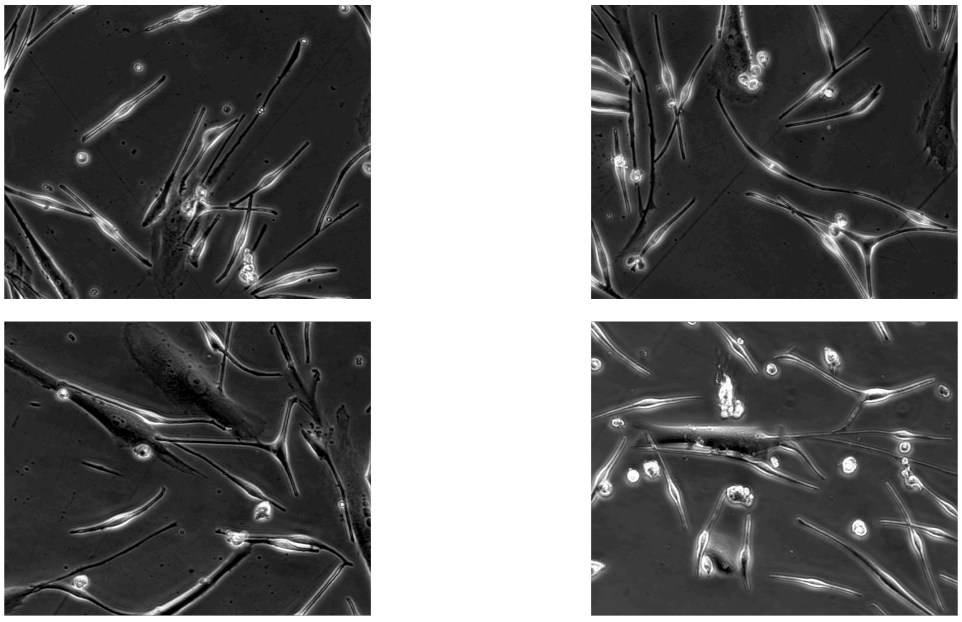


Figure 5 (continued)

5. MM + SCF



6. MM + ET-3

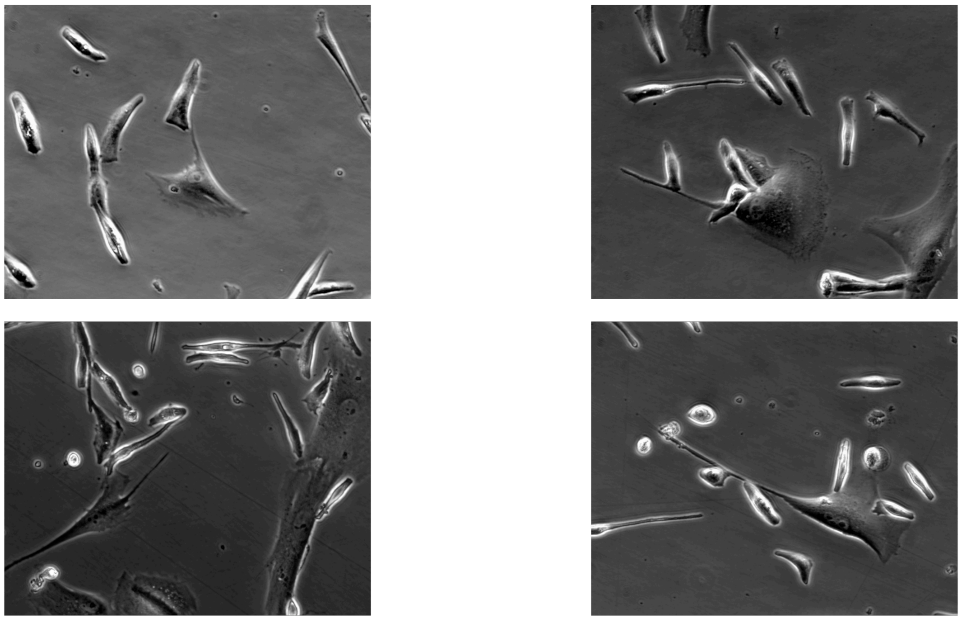
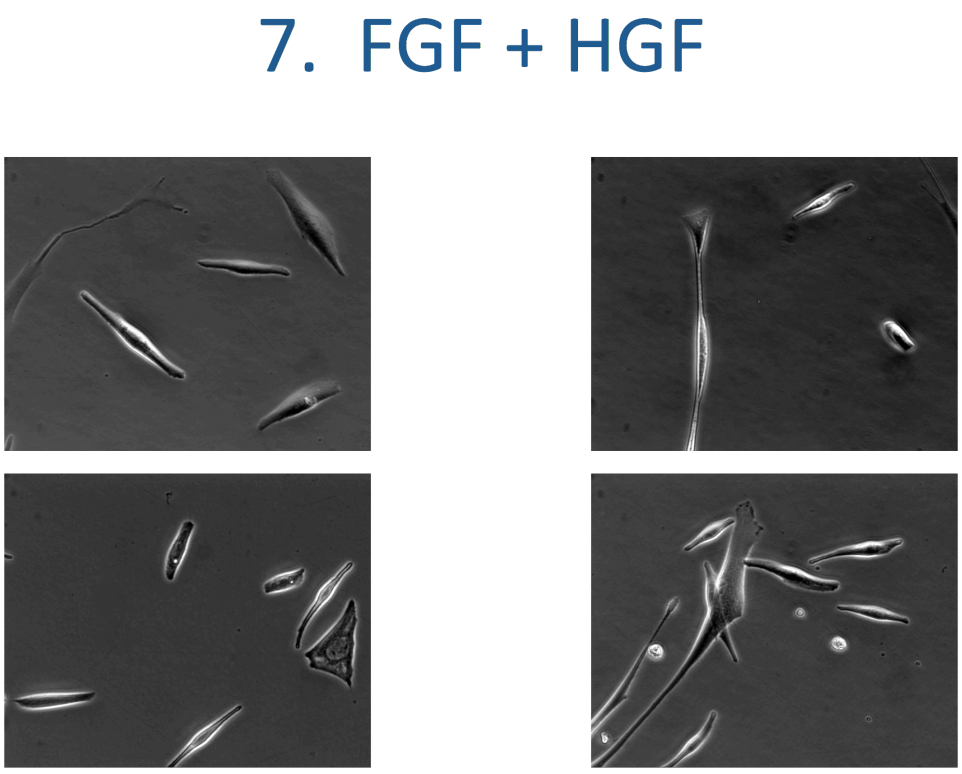
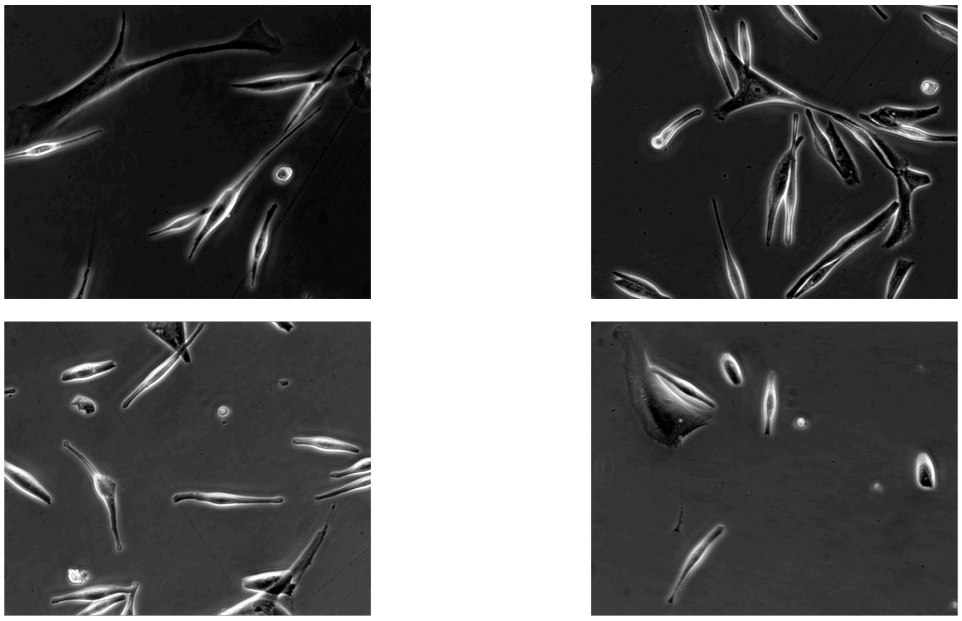


Figure 5 (continued)





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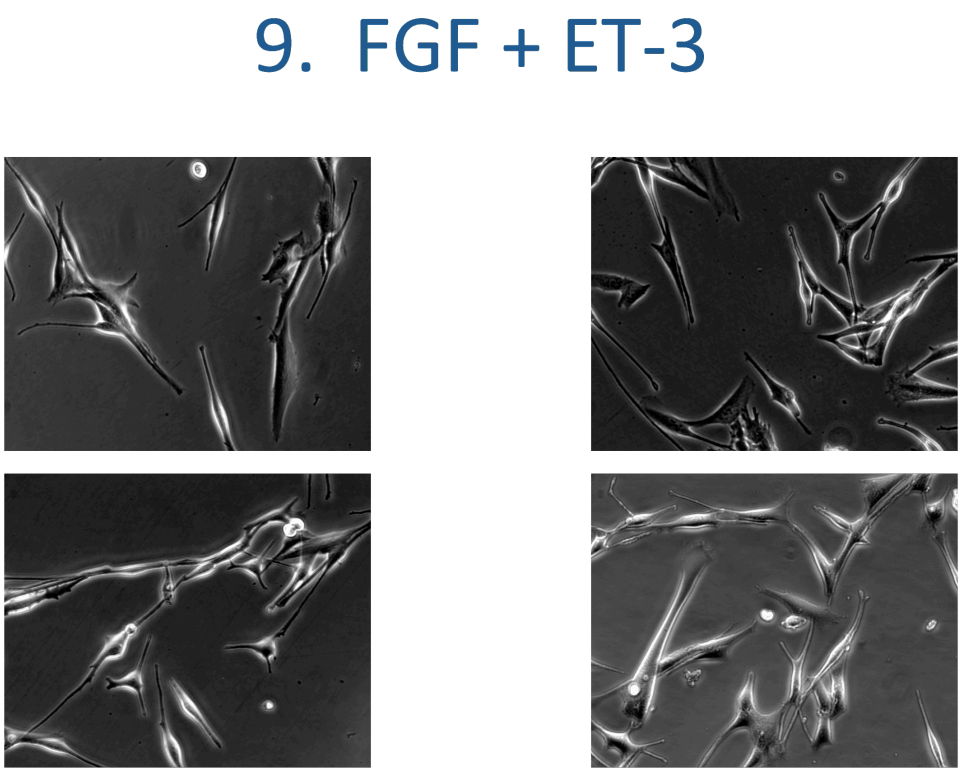


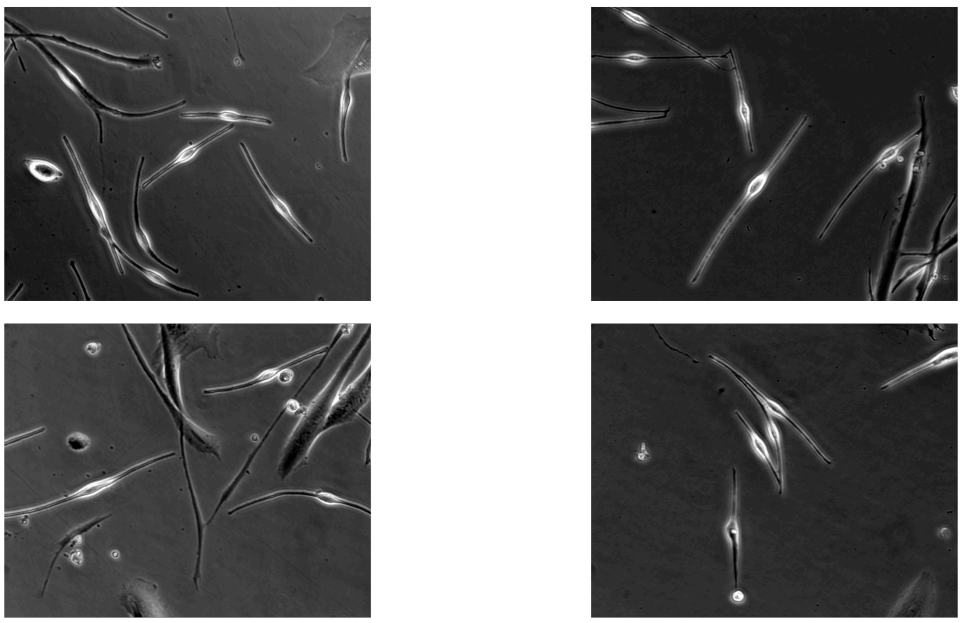
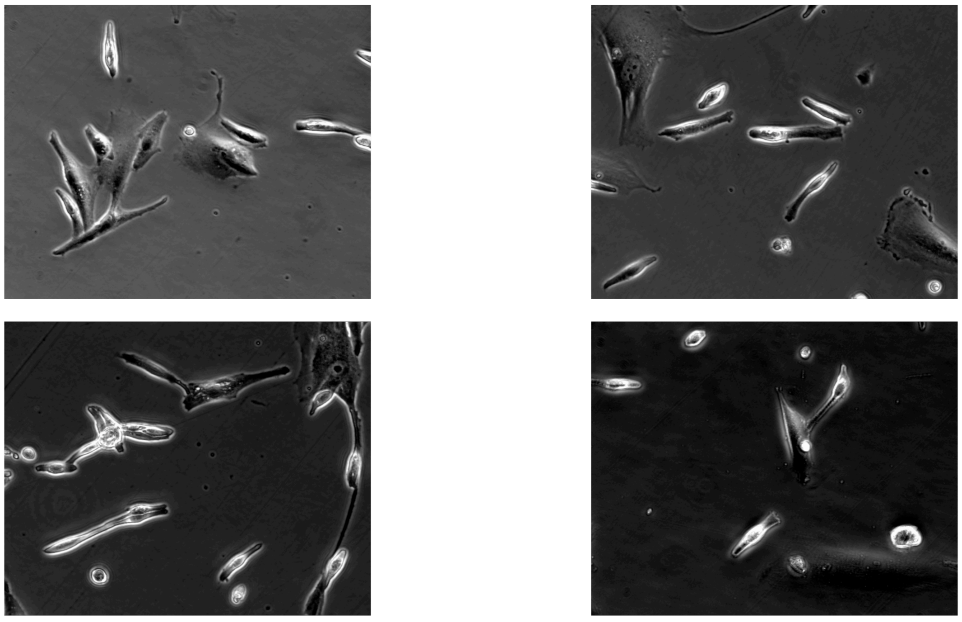


Figure 5 (continued)

11. HGF + ET-3



12. SCF + ET-3

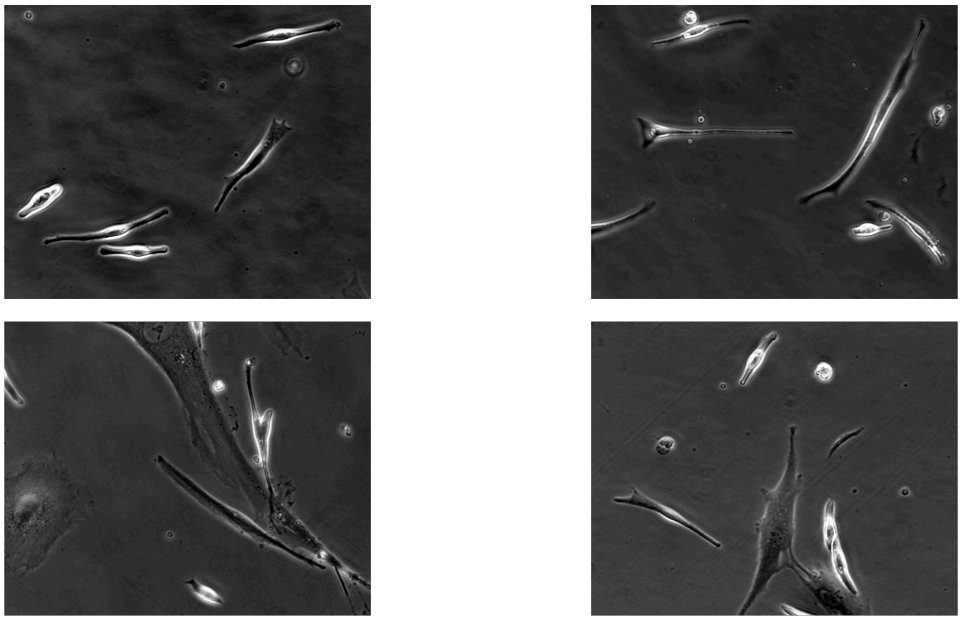
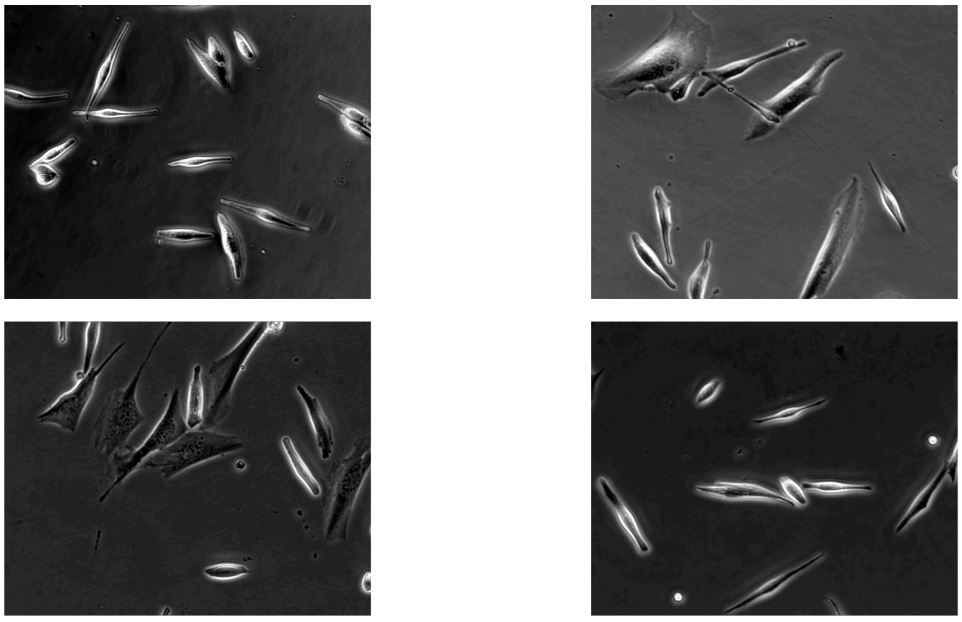


Figure 5 (continued)

13. FGF + HGF + SCF



14. ET-3 + FGF + HGF

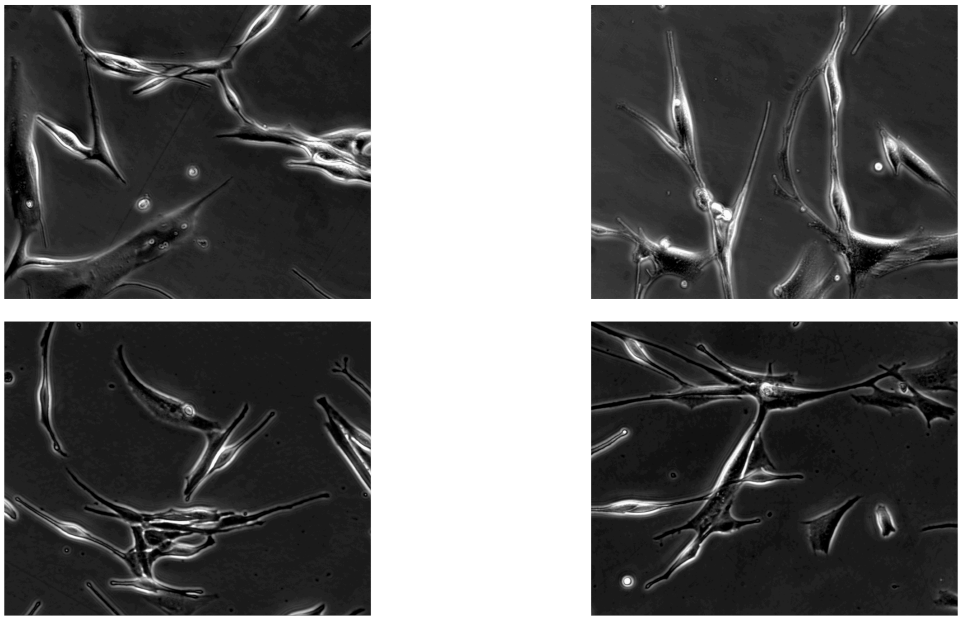
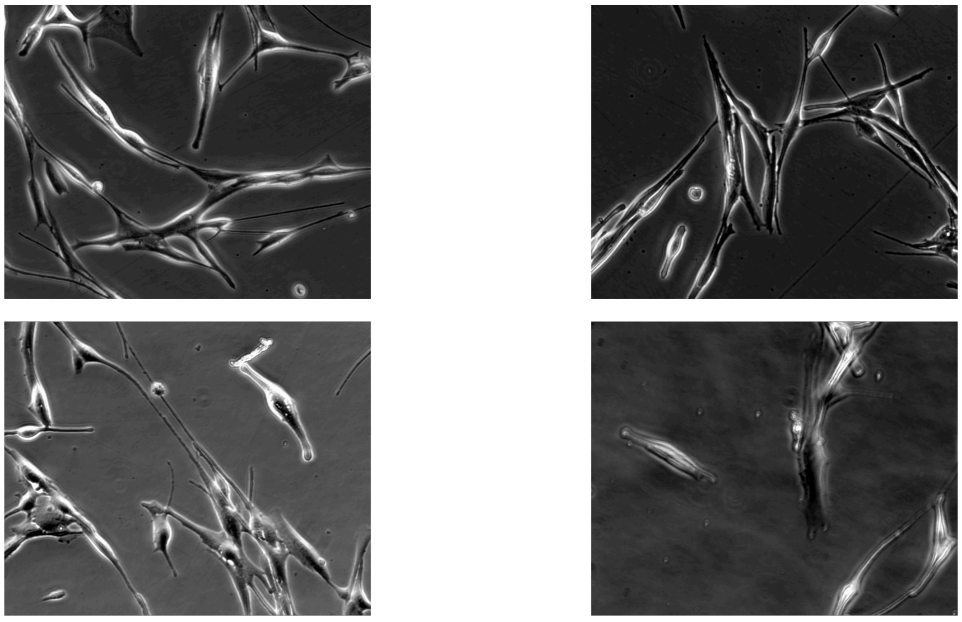


Figure 5 (continued)

15. ET-3 + FGF + SCF



16. ET-3 + HGF + SCF

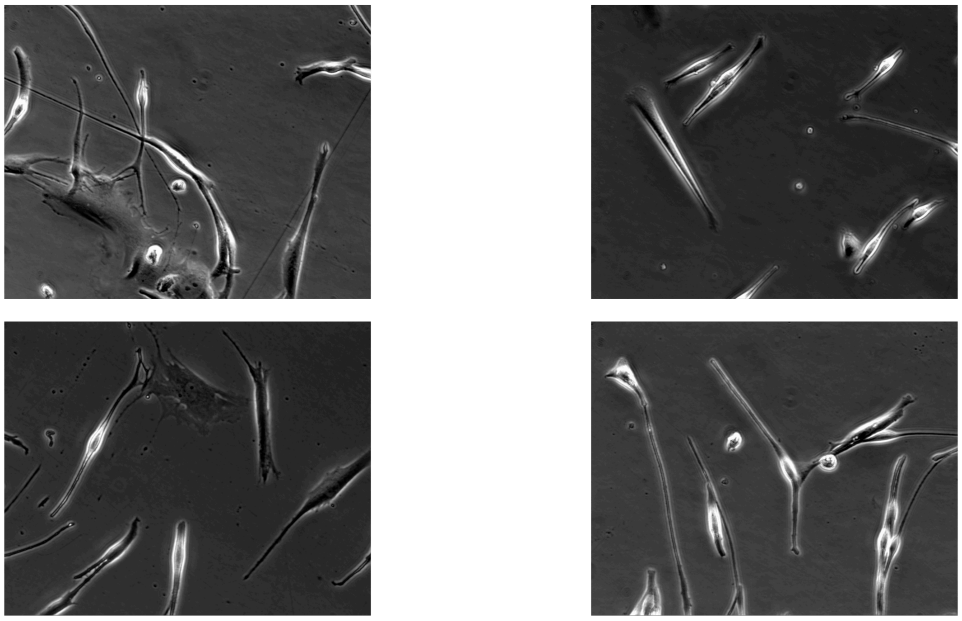
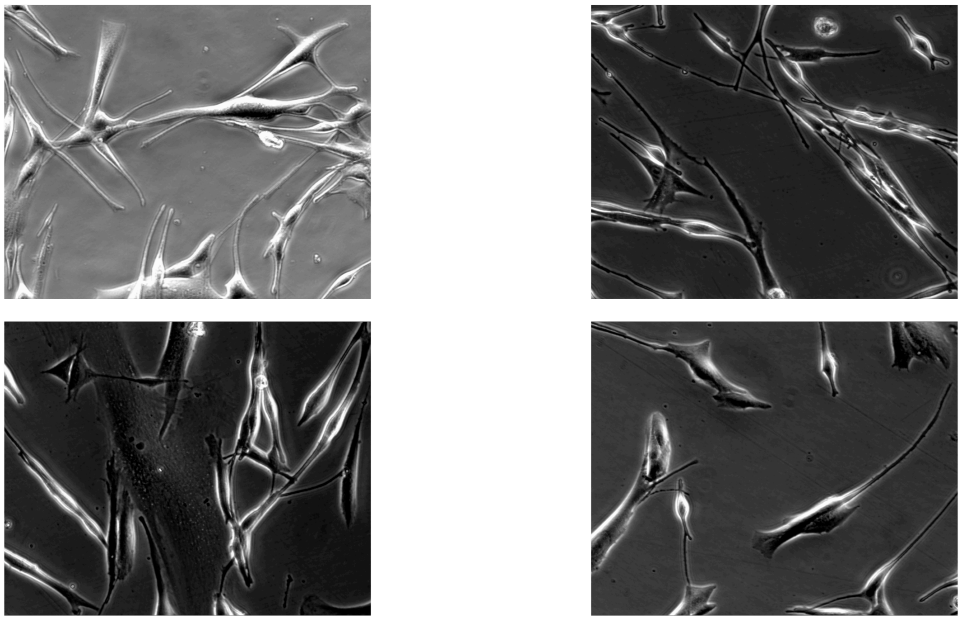
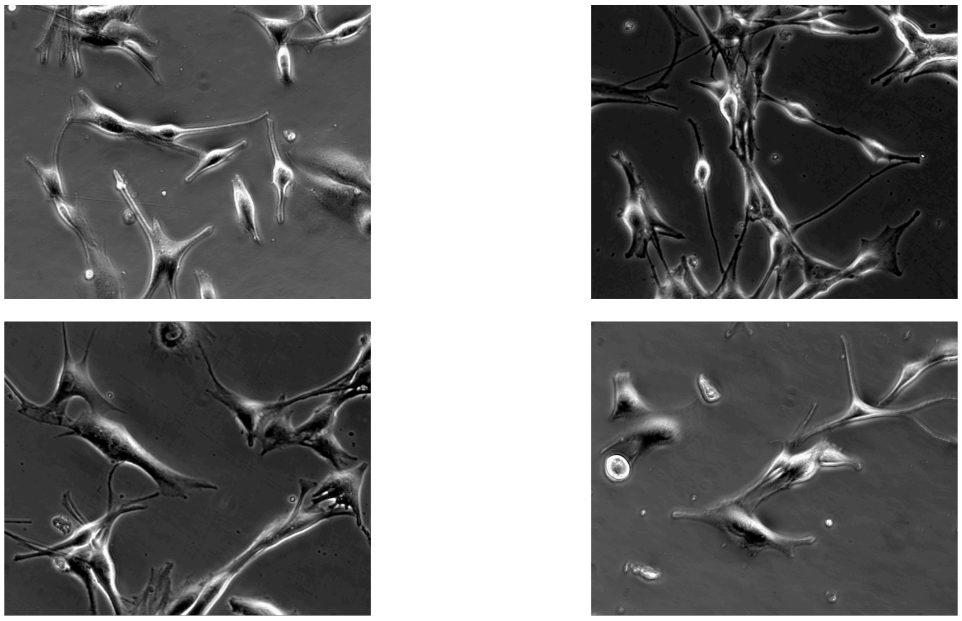


Figure 5 (continued)

17. bFGF + ET-3 + HGF + SCF



18. Complete M2 Media

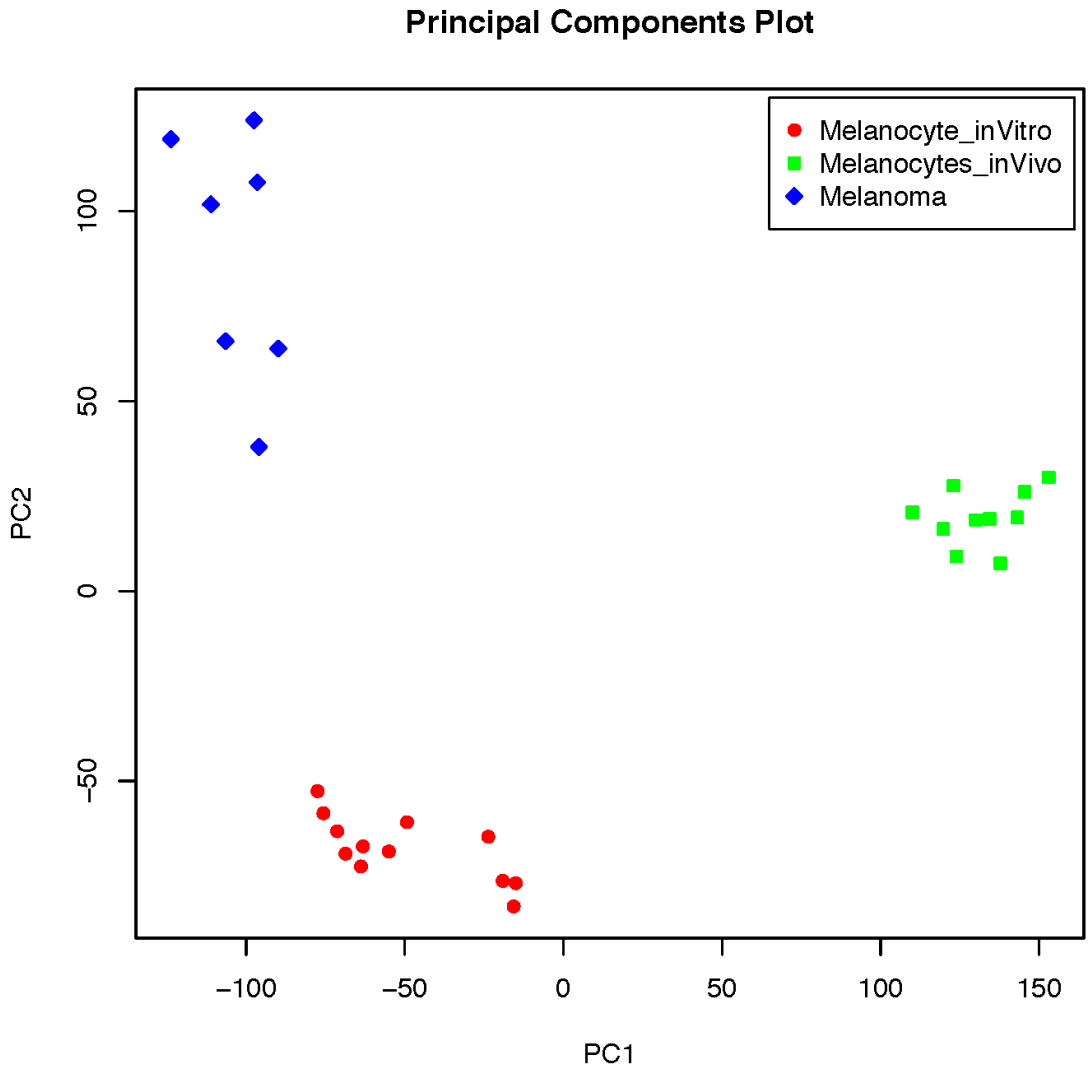


The more growth factors added may increase the cell number or survival, but, it was condition #9 with only bFGF and ET-3 that was determined to be the best growth condition for viability and morphology. Currently the laboratory continues to extract melanocytes by FACS from normal human skin for further study in the defined media conditions detailed in this thesis. Dr. Wang has since shown distinct proliferation using time lapse photography, where she was able to make note of dividing cells and keep track of the balance between cell death and growth that made simple quantification of these cells impossible by methods previously employed. Dr. Wang is using this defined media to grow FACS melanocytes for short and long term culture, analyzing gene expression, growth and metabolism under varying times in culture. Future directions and future analyses that would depend on basic information contained within that data-set.

Chapter 7. Extending information to study of melanoma

Theory and aims

Malignant melanoma is a potentially life-threatening tumor whose incidence is increasing worldwide because of increasing exposure to solar ultraviolet radiation. It is extremely resistant to chemotherapy and has a dismal prognosis once it has spread to lymph nodes or beyond. Many experimental protocols take an immunologic approach, trying to stimulate cytotoxic T cells to destroy the tumor. These strategies, although not yet effective, have the advantage of narrowly targeting tumor antigens in order to avoid excessive toxicity to other organs. Melanoma is a particularly good system for immunologic therapy since it expresses differentiation antigens restricted to the melanocyte lineage. These proteins are involved in the synthesis and transport of the pigment melanin. However, advanced melanomas often become amelanotic as they dedifferentiate. As tumors downregulate antigen expression they may no longer be responsive to targeted therapy. To this end, we analyzed several cell lines that were derived from metastatic melanoma. We expected them to have variant expression from normal melanocytes; but, what was shown was that they actually clustered much closer to the *in vitro* primary PromoCell NHEM. As shown in the Principal components (PCA) plot in figure 6, and the heatmap in figure 7, the melanocytes isolated from "in vivo" skin by FACS are the most variant from both of the *in vitro* NHEM and the samples collected from melanoma cell lines at Charité. The difference is so great that one would almost look to the long term *in vitro* cultured melanocytes as a model for secondary site malignancy and not as the "norm".



<u>Figure 6.</u> Melanoma cell lines cluster with NHEM, not "in vivo" FACS melanocytes. The x-axis accounts for 67% of the variation amongst the samples, while the y-axis 17% so, the clustering of the principal components of melanoma cell lines with the melanocytes *in vitro* on PC1 relative to the *in vivo* melanocytes was unexpected.

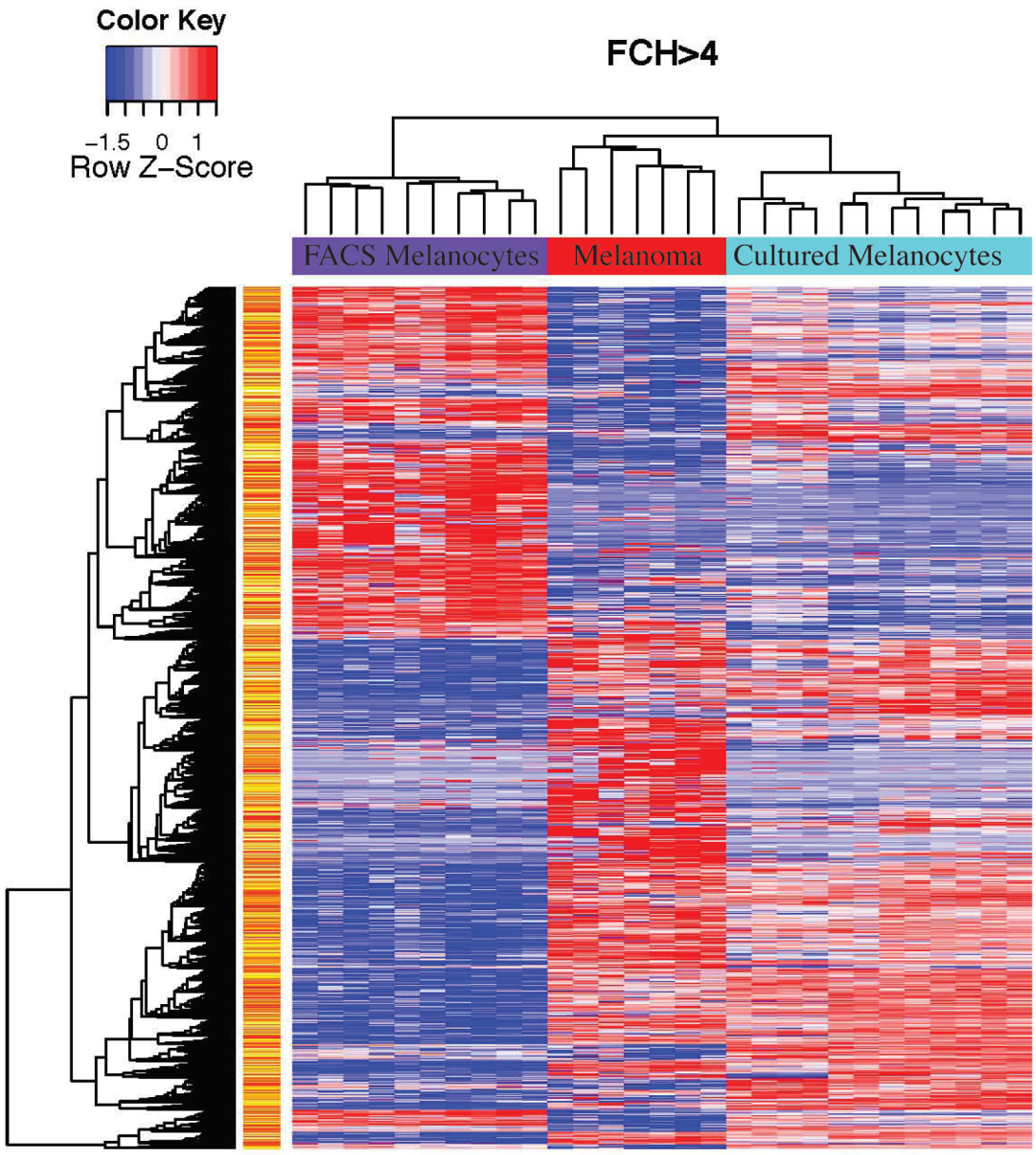


Figure 7. Heatmap with a cut-off of fold change greater than 4 and p value of 0.01

Unsupervised hierarquical clustering (Euclidian distance) FACS v Melanoma v *in vitro* again showed clustering of NHEM with melanoma cell lines, both grown *in vitro*.

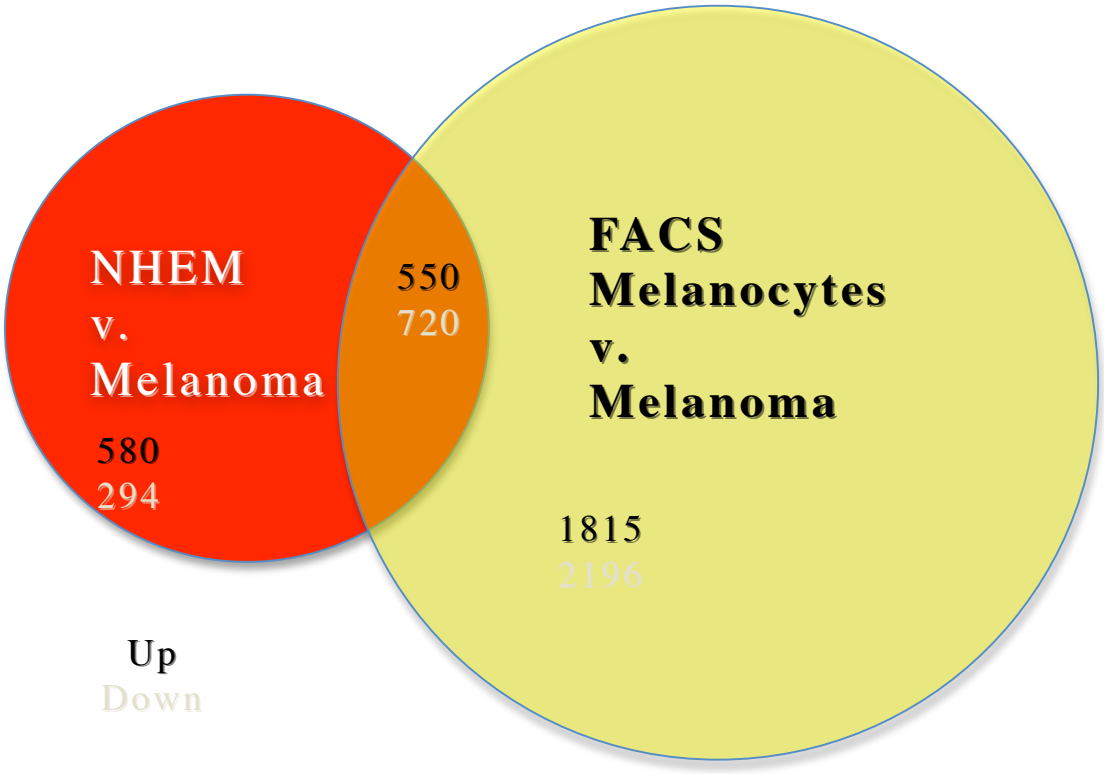
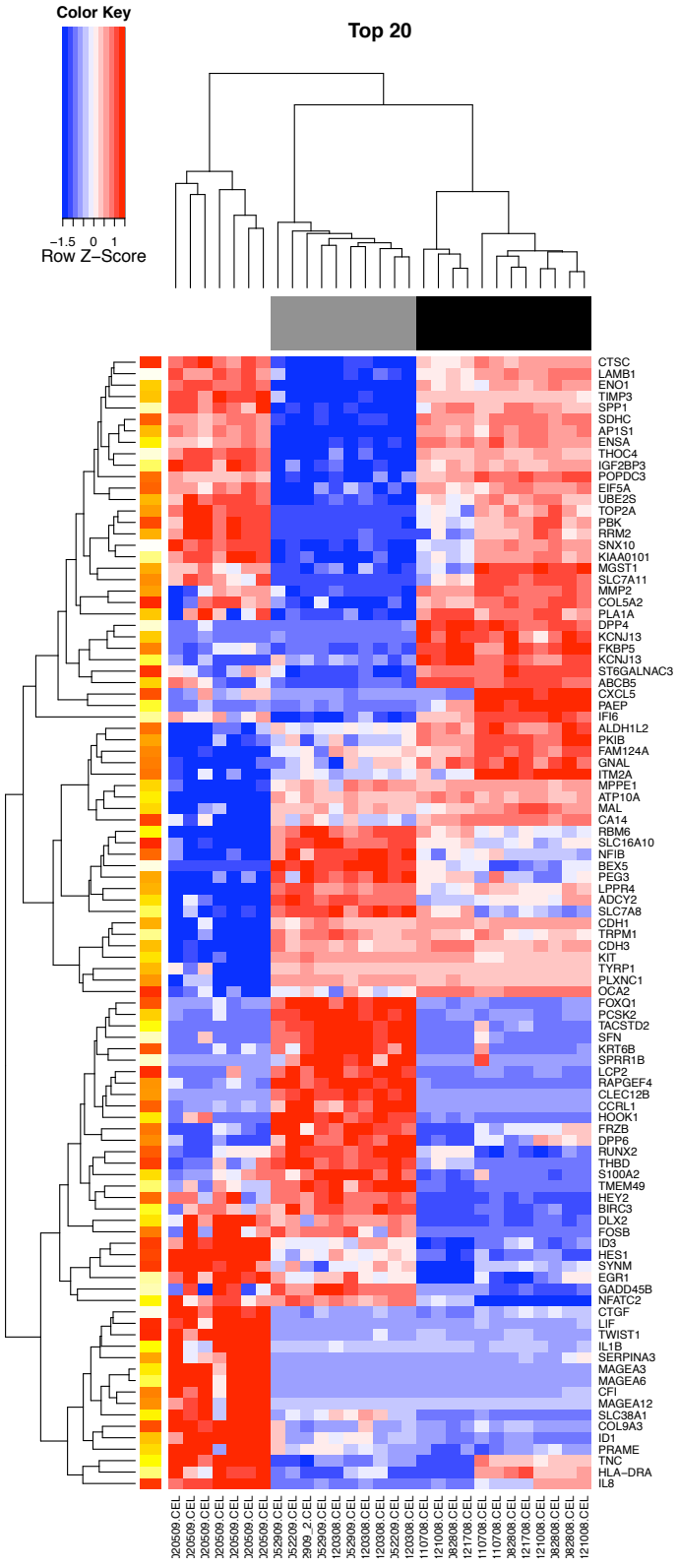


Figure 8. Venn diagram representing overlap of variation in gene expression. Number of genes differentially up- (in black) or down-regulated (in tan) are depicted in each circle, as well as those whose relative expression is shared in common between *in vitro* and FACS melanocytes as compared to the melanoma cell lines *in vitro*.

The difference in gene expression was not very great between NHEM and melanoma cell lines, represented in scale as compared to the difference in gene expression between FACS melanocytes and melanoma cell lines in the Venn diagram in Figure 8. In comparing the two *in vitro* groups: NHEM to melanoma, there are not that many differentially expressed genes, especially when you take into consideration the number of differentially expressed genes from the melanoma cell lines and the FACS derived melanocytes. This data shows the importance of the focus on melanocytes *in vitro* and how they are equivalent to melanoma. Figure 9 on the following page depicts the top 20 differentially expressed genes from each comparison (FAC v. *in vitro, in vitro* v. melanoma cell lines, melanoma cell lines v FACS) that takes a closer look at the genes that are highly expressed *in vitro* NHEM, FACS melanocytes and melanoma cell lines. (Melanoma cell line n= 7, FACS n= 10, NHEM n= 12.) This type of comparison is flawed however, by the artifacts produced in culture as well as those of FACS methodology. Some of the genes up-regulated in FACS only are keratinocyte contaminants (e.g. KRT6B.) IL-8 and IL-1B are both up-regulated, while c-kit is down-regulated in melanoma cell lines. Further analysis of a greater number of melanoma cell lines along with primary and metastatic melanomas needs to be done in this manner to determine the genomic phenotype that distinguishes malignant melanoma.



<u>Figure 9.</u> Heatmap of top 20 genes differentially expressed from each type of melanocyte compared. Melanoma cell line (n= 7) FACS (n= 10) NHEM (n= 12)

Chapter 8. Discussion

Though culture models of primary cells are not entirely accurate for *in vivo* comparison, there are still many cell specific genes. To this end primary cell culture of epidermal and dermal components were analyzed for unique genes that could then be used to analyze complex tissue samples and derive the cellular components. As we move forward into the era of molecular diagnosis, however, it is important to establish a more accurate genomic profile of "normal" before pathology can be examined. Therefore, diseased skin can be assessed for the purpose of understanding the underlying molecular problem so that treatment can be improved upon, handling disease with more appropriate treatments and perhaps even by rational chemotherapy in drug development.

We hypothesize that melanoma, like other cancers, is based on a progression of normal cells to dysplastic cells then to neoplastic cells, acquiring additional changes that eventually lead to the ability to invade into the dermis and metastasize distantly. Under a Rockefeller IRB protocol we will be studying progression of melanocytes at each of the different stages from normal "resting" to transition states to benign proliferations (e.g. nevi) to dysplastic states (dysplastic nevi) to melanoma.

The laboratory will continue to define the properties and molecules that distinguish melanocytes from other cell types and to understand the process by which normal melanocytes are converted to neoplastic melanocytes and, in particular, acquire cellular characteristics that permit them to invade into dermals tissues (vertical growth phase) and further metastasize. Of particular importance is determining the fundamental proliferative factors that regulate growth of normal melanocytes, and to establish the

extent to which melanoma cells are dependent on these factors for survival and growth. Further LCM on pigmented lesions will focus on pathology by focal selection of melanocytes from different parts of dysplastic nevi to examine step by step changes in gene expression.

We believe that future strategies for treatment of melanoma will be based on either boosting anti-tumour immunity or specific disruption of signaling pathways upon which the malignancies depend. The first approach could use either immunization with defined antigens associated with melanoma, melanogenesis etc., or by *ex vivo* expansion of lymphocytes isolated from melanoma patients. The information on proteins expressed in melanocytes at different growth stages could be important for the identification of melanocyte-specific proteins that are not part of the recognized pigmentation pathway. The second approach is also directly related to the studies that began in this thesis. The principle would be rational chemotherapy based on the identification of key growth, differentiation, migration and cell survival pathways that are traceable to specific molecules and genomic programs within normal or malignant cells. The most notable example of targeted treatment is PLX4032 which inhibits the elevated kinase activity in the RAS-RAF-MEK-ERK pathway created by the B-RAF^{V600E} mutation, present in approximately 50% of malignant melanoma (MM) [99]. This drug unfortunately has not been shown to increase life expectancy despite clinical benefits in morbidity and visible regression of metastases [100]. Other drugs need to be formulated that can act synergistically to attack the cancer in a "cocktail" format, as well as the other half of the MM population which does not have this specific mutation. Hence, the pursuit of

genomic information to find common growth-regulatory pathway for normal and malignant melanocytes needs to be continued.

In addition, our FACS experiments yielded some very interesting results. Though initially intended to isolate melanocytes for study, the population of keratinocytes also revealed novel gene expression potential. The transcriptome analysis of keratinocytes after they had been enzymatically separated from the epidermis revealed many similarities to published gene expression during early would healing. This data indicated a plasticity inherent to the keratinocyte previously unreported.

In summary, *in vitro* cultures are useful models; but, it is important to recognize that they cannot be relied upon for translational research in dermatology because of the growth inducing conditions that are so altered from *in vivo* as to generate gene expression profiles which resemble pathologic phenotypes. These data sets should serve as a baseline for future analysis of malignant epidermal states on the transcriptional level.

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