Comprehensive Expression Profiling of Tumor Cell Lines Identifies Molecular Signatures of Melanoma Progression

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Background. Gene expression profiling has revolutionized our ability to molecularly classify primary human tumors and significantly enhanced the development of novel tumor markers and therapies; however, progress in the diagnosis and treatment of melanoma over the past 3 decades has been limited, and there is currently no approved therapy that significantly extends lifespan in patients with advanced disease. Profiling studies of melanoma to date have been inconsistent due to the heterogeneous nature of this malignancy and the limited availability of informative tissue specimens from early stages of disease. Methodology/Principle Findings. In order to gain an improved understanding of the molecular basis of melanoma progression, we have compared gene expression profiles from a series of melanoma cell lines representing discrete stages of malignant progression that recapitulate critical characteristics of the primary lesions from which they were derived. Here we describe the unsupervised hierarchical clustering of profiling data from melanoma cell lines and melanocytes. This clustering identifies two distinctive molecular subclasses of melanoma segregating aggressive metastatic tumor cell lines from less-aggressive primary tumor cell lines. Further analysis of expression signatures associated with melanoma progression using functional annotations categorized these transcripts into three classes of genes: 1) Upregulation of activators of cell cycle progression, DNA replication and repair (CDCA2, NCAPH, NCAPG, NCAPG2, PBK, NUSAP1, BIRC5, ESCO2, HELL5, MELK, GINS1, GINS4, RAD54L, TYMS, and DHFR), 2) Loss of genes associated with cellular adhesion and melanocyte differentiation (CDH3, CDH1, c-KIT, PAX3, CITLD1/MSG-1, TYR, MELANA, MC1R, and OCA2), 3) Upregulation of genes associated with resistance to apoptosis (BIRC5/survivin). While these broad classes of transcripts have previously been implicated in the progression of melanoma and other malignancies, the specific genes identified within each class of transcripts are novel. In addition, the transcription factor NF-KB was specifically identified as being a potential “master regulator” of melanoma invasion since NF-KB binding sites were identified as consistent consensus sequences within promoters of progression-associated genes. Conclusions/Significance. We conclude that tumor cell lines are a valuable resource for the early identification of gene signatures associated with malignant progression in tumors with significant heterogeneity like melanoma. We further conclude that the development of novel data reduction algorithms for analysis of microarray studies is critical to allow for optimized mining of important, clinically-relevant datasets. It is expected that subsequent validation studies in primary human tissues using such an approach will lead to more rapid translation of such studies to the identification of novel tumor biomarkers and therapeutic targets.

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INTRODUCTION

The incidence of melanoma is increasing at one of the highest rates for any form of cancer in the United States [1]. At present, there are no systemic agents available that significantly extend the lifespan of patients with advanced disease, and the key to improved survival in all affected individuals remains early diagnosis and treatment. While early stage disease may result in occasional deaths, there are no available tests to predict which early stage tumors have a high likelihood of progression and therefore a worse prognosis. Thus, an urgent need exists for the identification of molecular signatures of melanoma progression which can be used to develop accurate prognostic markers and effective targeted therapies. High-throughput gene expression profiling technologies offer an opportunity to uncover critical molecular events in the development and progression of human melanoma and can be used to design improved prognostic testing and effective treatment strategies. Previous transcriptome analyses in other malignancies have provided valuable information for the assessment of patient group classifications such as subgroups of patients that are likely to respond to a particular therapy [2]. Expression profiling of metastatic melanomas was able to identify previously unrecognized subtypes of disease and predict phenotypic characteristics which may be of importance to melanoma progression [3]. Further studies using serial analysis of gene expression (SAGE) and cDNA arrays have yielded the identification of additional novel molecules and pathways which may be involved in melanoma development[4–7]. Such studies have been limited in utility due to the lack of concordance from one study to the next suggesting tumor heterogeneity [8]. In addition, the identification of molecular signatures of melanoma progression offers an opportunity to uncover critical genetic events in the development and progression of human melanoma and can be used to design improved prognostic testing and effective treatment strategies. Previous transcriptome analyses in other malignancies have provided valuable information for the assessment of patient group classifications such as subgroups of patients that are likely to respond to a particular therapy [2]. Expression profiling of metastatic melanomas was able to identify previously unrecognized subtypes of disease and predict phenotypic characteristics which may be of importance to melanoma progression [3]. Further studies using serial analysis of gene expression (SAGE) and cDNA arrays have yielded the identification of additional novel molecules and pathways which may be involved in melanoma development[4–7]. Such studies have been limited in utility due to the lack of concordance from one study to the next suggesting tumor heterogeneity [8]. In addition, the identification of molecular signatures of melanoma progression offers an opportunity to uncover critical genetic events
Previous studies of primary human melanomas have identified gene signatures associated with tumor progression [9–11]. These signatures included upregulation of cell cycle regulatory proteins, mitotic checkpoint genes, genes involved in DNA replication and repair, and cellular stress response genes in addition to loss of genes promoting apoptosis; however, few of the genes identified in these studies were concordant suggesting limitations due to tumor variability. Since better knowledge of gene expression signatures associated with melanoma progression may identify improved screening tools and therapeutic strategies, we used high density cDNA microarrays for gene expression profiling of genetically well-defined melanoma cell lines isolated from distinctive stages of tumor progression. Novel data reduction algorithms were used to identify gene signatures associated with tumor invasion and metastasis. Here we report unique sets of gene expression signatures that are associated with melanoma progression. Many of these pathways have previously been implicated in melanoma progression; however, the specific signature genes identified are novel. These particular progression-associated genes may reflect the underlying molecular mechanisms of the various phases in the known tumor progression pathways of melanoma. As such, the pathways and molecules identified in this study have the potential to be utilized as therapeutic targets for melanoma as well as novel molecular markers for melanoma progression. Moreover, these studies support the use of renewable sources of tumor cells, such as informative tumor cell lines, for the early identification of genes associated with malignant progression which can be subsequently validated using more precious primary tissue specimens.

RESULTS
In order to define gene expression patterns during the course of melanoma development and progression, we evaluated a series of primary and metastatic melanoma cells derived from lesions of discrete phases of melanoma progression as well as pools of primary human melanocytes. Tumor cell lines derived from three radial growth phase (RGP) melanomas (WM35, Sbc12, and WM1552C), four vertical growth phase (VGP) melanomas (WM902B, WM278, WM983A, and WM793), and three metastatic melanomas (WM852, WM983B, 1205Lu) were evaluated. These cell lines possess a notable ability to recapitulate the clinical stages of disease from which they were derived [12,13] and have been characterized with respect to tumorigenicity and metastasis [14–17]; cellular growth characteristics including life span, growth factor dependency, anchorage-independent growth [12]; and pigmentation and morphology [18]. In addition, cytogenetic analyses in these cell lines including non-random abnormalities such as deletions, translocations, and amplifications have been well-documented and suggest high relevance to the primary tumor of origin [reviewed in [12]].

Global gene expression patterns were obtained using Affymetrix gene chips and comparison of gene expression profiles was performed using hierarchical clustering analysis. This clustering analysis identified two distinct groups of melanoma cell lines based on the similarity of their expression patterns, separating radial growth phase (RGP) and metastatic melanomas [MM (Figure 1); however, vertical growth phase (VGP) melanomas failed to form a distinctive cluster (Figure 1A). The first group, which we characterized as “less-aggressive” primary melanomas (designated as Group1), included all three RGP melanomas (WM35, Sbc12, and WM1552C) and two VGP melanomas (WM902B and WM793). The second group, which we characterized as “more-aggressive” melanomas (designated as Group2), included all three metastatic melanomas (WM853, WM983B, and 1205Lu) and two VGP melanomas (WM983B and WM793). Additional cluster analyses with different linkage matrices produced similar results (data not shown). Of note, only 2 of the 10 cell lines (Sbc12 and WM1552C) were found to be wildtype for BRAF kinase and this genotype failed to demonstrate a notable cluster in our hierarchical analyses.

In order to identify a cohort of genes differentially expressed between our defined groups of melanomas, the gene expression array dataset was subjected to the microarray data analysis program Significance Analysis of Microarray (SAM) [19]. This analysis resulted in 142 differentially expressed probesets with a 3.5% false discovery rate (Figure 1B and C). In total, 89 probe sets representing 65 well-defined genes were found to be upregulated in more-aggressive (group 2) versus less-aggressive (group 1) melanomas and 53 probe sets representing 37 well-defined genes were found to be downregulated (Table S1). When more stringent criteria were applied (well-characterized genes which are differentially expressed greater than 4-fold) to this signature, we identified 21 upregulated and 5 downregulated genes in the more-aggressive melanoma cells (Figure 1C). Of note, the set of genes highly expressed in more-aggressive melanomas includes many novel genes with reported functional roles in cell cycle regulation and proliferation such as ZWINT, CDC2A, NAPAP, NCAPI, NAPI, PBK, NUSAP1, BIRC5, ESCO2, HELLS, MELK, and CDK12C [20–31] as well as genes that are involved DNA replication and repair processes including GINS1, GINS4, RAD54L, TTMS, and DHFR [32–34] (Table 1). Differential expression of these genes was validated by quantitative real-time RT-PCR (Figure 1D).

Since we were interested in defining melanoma progression signatures, and all melanomas are initiated in primary human melanocytes, we evaluated our expression profiling data in the context of cultured neonatal primary human melanocytes (Figure 2). Surprisingly, when two pools of short-term cultured primary human melanocytes (HPM1 and HPM2) were included in the previously employed hierarchical clustering protocol, the global gene expression pattern of the normal melanocytes was found to be more similar to that of the more-aggressive melanomas (Group 2) than the less-aggressive melanomas (Group 1) (Figure 2A). Since early cultures of primary human melanocytes derived from neonatal foreskins divide rapidly yet possess a normal differentiation program, we reasoned that the similarities of these cells to more aggressive tumors was likely due to their proliferative potential. In order to test this hypothesis we compared gene expression profiles of more-aggressive melanoma cells (Group 2) to those of short-term cultured primary human melanocytes. Expression profiles were subjected to SAM analysis which identified a cohort of differentially expressed genes with a 0.85% false discovery rate. Remarkably, all differentially expressed genes were found to be down-regulated genes in aggressive melanoma cells versus primary human melanocytes suggesting that loss of specific gene signatures may be a key event in the development of advanced melanomas (Figure 2B). Further assessment of all melanoma expression profiles using TreeView revealed that the majority of these melanoma-associated genes are also down-regulated in the less-aggressive primary melanomas (Group1) (Figure 2C). This gene signature is comprised of critical mediators of cellular adhesion and melanocyte development and differenti-
ation and includes: CDH3, CDH1, c-KIT, PAX3, CITED1/MSG-1, TYR, MELANA, MC1R, and OCA2 [35–42] (Table 2). While such a loss of cellular adhesion by E-cadherin and P-cadherin has been extensively documented in melanoma (reviewed in [43]), and the loss of differentiation-associated genes is not wholly surprising, this signature does notably identify specific defects in the intrinsic melanocyte development program that may contribute to melanoma development. In addition, genes with tumor suppressor and metastasis suppressor functions (DPP4, SYK) are included in this melanoma signature [44,45]. Significant down-regulation of these genes in the aggressive metastatic melanoma cells was validated using semi-quantitative duplex RT-PCR (Figure 2D). Furthermore, this differentially expressed “melanoma signature” contains many genes whose functional roles in melanoma progression have not been well characterized and may provide novel insights into the early development of melanoma from primary melanocytes.

A current melanoma progression model suggests the sequential evolution of primary in situ tumors and minimally invasive tumors which are termed “radial growth phase”, followed by a subsequent conversion to a more aggressive “vertical growth phase”, in which tumor cells are programmed to cross the epidermal basement membrane and invade vertically into the dermis. It has been postulated that the VGP is the critical stage in which a tumor gains metastatic capacity. We therefore compared the gene expression profiles of RGP and VGP melanomas using a uniquely designed data reduction algorithm in order to identify genes that are likely to be relevant to this critical invasive phenotype (Figure 3A). Our
Table 1. Genes with altered expression in aggressive melanoma cells are involved in cell cycle control, cell proliferation, DNA repair and replication.

| Probe Set ID | Gene Title | Gene Symbol | Fold b | Function                          |
|--------------|------------|-------------|--------|-----------------------------------|
| 227350_at    | Helicase, lymphoid-specific | HELLS  | 8.5    | cell proliferation                |
| 202599_at    | thymidylate synthetase   | TYMS    | 7.2    | DNA replication, DNA repair       |
| 204558_at    | RADS4-like (S. cerevisiae) | RADS4L  | 6.2    | DNA repair, response to DNA damage |
| 204625_at    | maternal embryonic leucine zipper kinase | MELK    | 5.9    | Mitosis, protein phosphorylation  |
| 204159_at    | cyclin-dependent kinase inhibitor 2C (p18) | CDKN2C  | 5.8    | cell cycle                        |
| 212949_at    | barren homolog 1 (Drosophila)/NCAPH |   | 5.8    | cell cycle, chromosome condensation |
| 219588_s_at  | leucine zipper protein 5 | NCAPG2  | 4.6    | cell cycle, chromosome condensation |
| 218663_at    | chromosome condensation protein G | HCAPG1  | 4.5    | cell cycle, chromosome condensation |
| 211767_at    | GINS complex subunit 4 (Sld5 homolog) | GINS4    | 5.6    | DNA replication, cell proliferation |
| 206102_at    | GINS complex subunit 1 (Psf1 homolog) | GINS1   | 4.7    | DNA replication, cell proliferation |
| 202095_s_at  | baculoviral IAP repeat-containing 5 | BIRC5   | 5.1    | G2/M transition cell cycle         |
| 219148_at    | PDZ binding kinase | PBK     | 5.1    | mitosis, protein phosphorylation   |
| 235178_x_at  | establishment of cohesion 1 homolog 2 | ESCO2   | 5.1    | cell cycle                        |
| 226661_at    | cell division cycle associated 2 | CDC2  | 4.9    | cell cycle                        |
| 204026_s_at  | ZW10 interactor | ZWINT    | 4.7    | cell cycle, spindle organization   |
| 202534_x_at  | dihydrofolate reductase | DHFR   | 4.5    | DNA replication, nucleotide metabolism |
| 218039_at    | nucleolar and spindle associated protein 1 | NUSAP1  | 4.1    | cell cycle, chromosome condensation |
| 1555788_a_at | tribbles homolog 3 (Drosophila)/TRIB3 |   | −6.7   | anti-proliferation, apoptosis      |

*Genes with greater than four-fold differential expression are shown.

bFold represents average expression ratio of group 2 over Group1.

* Genes with multiple probesets are shown with data from a single representative probeset.

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malignancies including melanoma and are felt to be essential components of tumor invasion and metastasis. In addition, many of these invasion-specific signature genes are also upregulated in metastatic melanomas (Figure 3B). These genes and their relative expression profiles are depicted in Figure 3B. These cellular processes have previously been implicated in tumor progression for a wide variety of malignancies including melanoma and are felt to be essential components of tumor invasion and metastasis. In addition, many of these invasion-specific signature genes are also upregulated in metastatic melanomas (Figure 3B). The differential expressions were validated on the selected genes using semi-quantitative duplex-PCR analysis (Figure 3C).

Since the melanoma invasion-specific signature was associated with common functions of matrix invasion/inflammation/cell migration we sought to determine whether a common upstream regulatory pathway might link these signature genes. The eight most highly up-regulated genes from our melanoma invasion-specific signature were selected for further evaluation and gene promoter sequences were analyzed to identify transcription factor binding cis elements. This promoter analysis yielded a profile of transcription factors with common sequence elements in the signature genes. The most ubiquitous cis elements among the gene promoters evaluated were E12, E47, GCN4, GR, HES-1, IL-6, MEF-2, NF-kB, N-Oct-3, PU.1, RAR-alpha1, SRF, and the basal gene transcriptional complex components of TFIIID, TBP, and TBF1. We notably identified the NF-kB binding sequence in 7 out of 8 of the most upregulated invasion-specific signature genes (Figure 3D). We were particularly interested in the NF-kB pathway as a mediator of melanoma invasion since our most highly upregulated invasion-specific genes, CXCL1, CXCL2, IL-8, and IL6, cell adhesion (HINT, ITGA4, ITGB8, CSPG2, ZP4, and FLRT3), and extracellular matrix organization (MMP-1, COL1A1, COLA2, and COL5A2) (Table 3). These genes and their relative expression profiles are depicted in Figure 3B. These cellular processes have previously been implicated in tumor progression for a wide variety of malignancies including melanoma and are felt to be essential components of tumor invasion and metastasis. In addition, many of these invasion-specific signature genes are also upregulated in metastatic melanomas (Figure 3B). The differential expressions were validated on the selected genes using semi-quantitative duplex-PCR analysis (Figure 3C).

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DISCUSSION

Molecular profiling studies of melanoma to date have been variably successful and often inconsistent. Much of this inconsistency has been attributed to the heterogeneous nature of this malignancy and the lack of significant sources of meaningful archived tissue specimens for analysis. In addition, variable sample preparation techniques are also likely to lead to disparate results between investigators. Here we have used a series of well-defined melanoma cell lines from varying stages of malignant progression to assess molecular signatures associated with disease progression. These cell lines have undergone extensive characterization of their tumorigenic potential and invasion capacity [14,47], and have been shown to possess a remarkable ability to recapitulate the clinical stages of disease from which they were derived. We show that unsupervised hierarchical clustering of global gene expression profiles of melanoma cell lines allows for the classification of tumor cells into 2 groups (Figure 1A) that we have defined as less-aggressive (Group 1) and more-aggressive (Group 2) melanomas. While all radial growth phase melanomas clustered in Group 1, and all metastatic melanomas clustered in Group 2, vertical growth phase melanomas failed to form a distinctive cluster suggesting that vertical growth phase melanomas may be considered to be a transient or transition phase within the current melanoma progression model [48].

Aggressive (Group 2) melanomas were characterized by upregulation of genes associated with cell cycle progression, DNA replication and repair, and altered expression of apoptosis-related genes including upregulation of the antiapoptotic gene BIRC5/survivin [49] and downregulation of the novel stress-associated apoptosis inducer TRIB3 [50] (Table 1). These signature genes for melanoma progression are remarkably similar to those obtained from recent large-scale studies using primary human melanomas and suggest high correlation with alterations seen in primary tumor specimens [10]. Notably, we did not identify a dominant signature associated with BRAF kinase mutations which may be reflective of the relative infrequency of wildtype BRAF in these cell lines. As a whole, this gene signature suggests a series of molecular alterations occur in aggressive melanomas that promote melanoma cell growth, survival and apoptotic resistance which contribute to the unresponsiveness of melanomas to traditional chemotherapeutic agents [51].
Table 2. Differential expression of genes that are downregulated in aggressive melanoma cells (Group2) compared to primary human melanocytes. Genes with greater than five-fold differential expression are shown.

| Probe Set ID | Gene Title | Gene Symbol | Fold | Function |
|--------------|------------|-------------|------|----------|
| 203256_at    | cadherin 3, type 1, P-cadherin (placental) | CDH3 | -161.9 | cell adhesion |
| 201131_s_at  | cadherin 1, type 1, E-cadherin (epithelial) | CDH1 | -35.1 | cell adhesion |
| 205051_s_at  | v-kit viral oncogene homolog | KIT | -94.0 | signal transduction |
| 1565162_s_at | microsomal glutathione S-transferase 1 | Mgst1 | -92.5 | — |
| 203716_s_at  | dipeptidyl-peptidase 4 (CD26) | DPP4 | -74.0 | proteolysis |
| 206039_at    | RAB33A, member RAS oncogene family | RAB33A | -70.4 | signal transduction |
| 226311_at    | CDNA clone IMAGE: 30924414 | — | -64.3 | — |
| 236901_at    | ADAM metallopeptidase | ADAMTS2 | -62.1 | proteolysis |
| 229095_s_at  | similar to LIM | LOC440895 | -54.6 | — |
| 227705_at    | transcription elongation factor A (SII)-like 7 | TCEAL7 | -49.1 | — |
| 1556698_a_at | hypothetical protein LOC285513 | LOC285513 | -35.0 | — |
| 213924_at    | metallophosphoesterase 1 | MPPE1 | -33.5 | — |
| 209569_x_at  | DNA segment on chromosome 4 | D4S234E | -31.6 | dopamine receptor signaling |
| 202283_at    | serpin peptidase inhibitor, clade F, member 1 | SERPINF1 | -29.2 | development/proliferation |
| 204570_at    | cytochrome c oxidase subunit VIII polypeptide 1 | COX7A1 | -28.8 | electron transport |
| 202546_at    | vesicle-associated membrane protein 8 | VAMP8 | -27.9 | vesicle-mediated transport |
| 208017_s_at  | MCF.2 cell line derived transforming sequence | MCF2 | -27.8 | intracellular signaling cascade |
| 217248_s_at  | solute carrier family 7, member 8 | SLC7A8 | -27.1 | amino acid transport |
| 206479_at    | transient receptor potential cation channel | TRPM1 | -25.8 | cation transport |
| 205114_s_at  | chemokine (C-C motif) ligand 3 | CCL3 | -25.6 | chemotaxis/inflammatory |
| 220813_at    | cysteinyi leukotriene receptor 2 | CYSLTR2 | -25.3 | signal transduction |
| 206355_at    | G protein, alpha activating activity polypeptide | GNAL | -23.5 | signal transduction |
| 237472_at    | SRY (sex determining region Y)-box 1 | SOX1 | -23.4 | regulation of transcription |
| 203815_at    | glutathione S-transferase theta 1 | GSTT1 | -22.1 | response to stress |
| 212599_at    | autism susceptibility candidate 2 | AUTS2 | -20.3 | — |
| 206426_at    | melan-A | MLANA | -20.3 | — |
| 225407_at    | myelin basic protein | MBP | -19.7 | CNS development |
| 219436_s_at  | endomucin | EMCN | -19.4 | — |
| 1556427_s_at | similar to hypothetical protein | LOC221091 | -17.9 | — |
| 201425_at    | aldehyde dehydrogenase 2 family (mitochondrial) | ALDH2 | -17.8 | carbohydrate metabolism |
| 207144_s_at  | Cbp/p300-interacting transactivator 1 | CITED1 | -17.7 | regulation of transcription |
| 212338_at    | myosin ID | MYO1D | -17.7 | — |
| 21935_s_at   | chromosome 16 open reading frame 30 | C16orf30 | -17.0 | — |
| 227532_at    | leucine rich repeat containing 39 | LRRC39 | -16.5 | — |
| 206569_at    | interleukin 24 | IL24 | -16.1 | apoptosis/immune response |
| 220668_at    | spleen tyrosine kinase | SYK | -15.9 | signal transduction |
| 213122_at    | TSPY-like 5 | TSPYL5 | -15.8 | nucleosome assembly |
| 204187_at    | guanosine monophosphate reductase | GMPR | -14.7 | nucleic acid metabolism |
| 204880_at    | O-6-methylguanine-DNA methyltransferase | MGMT | -14.3 | DNA repair |
| 204777_s_at  | mal, T-cell differentiation protein | MAL | -14.2 | cell differentiation |
| 1555505_a_at | tyrosinase (oculocutaneous albinism IA) | TYR | -13.6 | melanin biosynthesis |
| 226778_at    | chromosome 8 open reading frame 42 | C8orf42 | -13.1 | — |
| 225792_at    | hook homolog 1 (Drosophila) | HOOK1 | -12.5 | cytoskeleton organization |
| 209924_at    | chemokine (C-C motif) ligand 18 | CCL18 | -11.8 | chemotaxis/inflammatory |
| 211427_s_at  | potassium inwardly-rectifying channel, | KCNJ13 | -11.7 | ion transport |
| 213556_at    | similar to R28379-1 | LOC390940 | -11.7 | — |
| 240173_at    | transcribed locus | — | -11.5 | — |
While gene signatures associated with aggressive melanomas provide insights into molecular pathways important for tumor progression, further analysis of these tumor cell lines in conjunction with expression profiles from primary human melanocytes using SAM analysis revealed a striking signature characterized exclusively by gene loss in melanomas and primarily by loss of cellular adhesion and melanocyte differentiation-associated genes (Figures 2B, 2C). We suggest that this melanoma-associated signature defines critical molecular mechanisms involved in melanocyte development and differentiation which distinguish these tumor cells from their primary cell of origin. In fact, the identification of several genes in this signature with established functional roles in melanocyte differentiation and melanin biosynthesis such as CDH3, CDH1, CITED1/MSG-1, TYR, MELANA, MC1R, and OCA2 [35–42], supports this notion. Moreover, this signature has identified two tumor suppressor genes, DPP4 and SYK, whose downregulation has previously been implicated in melanoma development [52–54].

Finally, evaluation of an invasion-specific signature for melanoma identified dominant gene activation by the transcription factor, NF-κB. Constitutive activation of NF-κB and an inflammatory response is an emerging hallmark of various tumor types [55]. In addition, NF-κB has specifically been implicated in the development of invasive aggressive melanomas through autocrine and paracrine mechanisms [reviewed in [46]].

| Probe Set ID | Gene Title | Gene Symbol | Fold | Function |
|--------------|------------|-------------|------|----------|
| 206498_at    | oculocutaneous albinism II | OCA2 | −11.4 | eye pigment biosynthesis |
| 241600_at    | transcribed locus | — | −11.0 | — |
| 205297_s_at  | CD79b molecule, immunoglobulin-associated | CD79B | −10.9 | immune response |
| 228256_s_at  | erythrocyte membrane protein band 4.1 likeEPB41L4A | — | −10.9 | — |
| 223693_s_at  | hypothetical protein FLJ10324 | FLJ10324 | −10.3 | signal transduction |
| 243727_at    | copine VIII | CPNE8 | −10.1 | — |
| 232687_at    | CDNA FLJ33091 fs, clone TRACH2000660 | — | −9.9 | — |
| 232443_at    | hypothetical gene supported by AF131741 | LOC441052 | −9.9 | — |
| 236377_at    | transmembrane protein 132D | TMEM132D | −9.1 | — |
| 211748_x_at  | prostaglandin D2 synthase 21kDa (brain) | PTGDS | −9.0 | lipid biosynthesis |
| 204112_s_at  | histamine N-methyltransferase | HNMT | −8.7 | respiratory gaseous exchange |
| 228057_at    | DNA-damage-inducible transcript 4-like | DDI4L | −8.6 | — |
| 51158_at     | hypothetical gene | LOC400451 | −8.3 | — |
| 209550_at    | neoddin homolog (mouse) | NDN | −8.3 | regulation of cell cycle |
| 214255_at    | ATPase, Class V, type 10A | ATP10A | −7.9 | cation transport |
| 1553485_at   | hypothetical protein LOC151278 | FLJ32447 | −7.8 | — |
| 204273_at    | endothelin receptor type B | EDNRB | −7.6 | G-protein signaling |
| 235758_at    | paraneoplastic antigen like 6A | PNMA6A | −7.6 | — |
| 213816_s_at  | met proto-oncogene | MET | −7.2 | cell proliferation |
| 227704_at    | full-length cDNA clone C50CAP008Y07 | — | −7.1 | — |
| 224566_at    | trophoblast-derived noncoding RNA | TncRNA | −6.9 | — |
| 207610_s_at  | EGF-like module containing, mucin-like | EMR2 | −6.8 | signal transduction |
| 232504_at    | hypothetical protein LOC285628 | LOC285628 | −6.8 | — |
| 241966_at    | myosin VA (heavy polypeptide 12, myoxin) | MYOSA | −6.7 | actin-based movement |
| 229925_at    | solute carrier family 6, member 17 | SLC6A17 | −6.6 | neurotransmitter transport |
| 229251_s_at  | two pore segment channel 2 | TPCN2 | −6.2 | cation transport |
| 216059_at    | paired box gene 3 (Waardenburg syndrome PXA3) | — | −6.2 | development |
| 227949_at    | phosphatase and actin regulator 3 | PHACTR3 | −5.7 | — |
| 209685_s_at  | protein kinase C, beta 1 | PRKCB1 | −5.6 | intracellular signaling cascade |
| 204995_at    | cyclin-dependent kinase 5, regulatory subunit 1 | CDK5R1 | −5.3 | neuron differentiation |
| 206020_at    | suppressor of cytokine signaling 6 | SOCS6 | −5.0 | regulation of cell growth |
| 221036_s_at  | anterior pharynx defective 1 homolog B | APH1B | −5.0 | Notch signaling pathway |
| 205458_at    | melanocortin 1 receptor | MC1R | −5.0 | G-protein signaling |

*Genes with greater than five-fold differential expression are shown.
^Fold represents average expression ratio of aggressive metastatic melanoma samples (group2) over normal primary human melanocyte samples.
\*Gene with multiple probesets are shown with a representative probeset.

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critical NF-κB effectors including CXCL1, FMN2, MMP1, IL-8, IGFBP3 which have been implicated in the regulation of tumor cell proliferation, motility, migration, and/or invasion (Figure 3D).

In addition, the putative NF-κB target gene GAGE7B which we identified in our melanoma invasion-specific signature, has been associated with apoptotic resistance and worse prognosis in other tumors [56]. In addition, several of our invasion-specific signature genes are chemokines including CXCL1, CXCL2, and IL-8 which have been implicated in the promotion of tumor-associated angiogenesis, a critical feature of invasive tumors [57].

In summary, our gene expression profiling studies of melanoma cell lines from varying stages of malignant progression and primary human melanocytes have identified several important melanoma signatures including: 1) Aggressive melanomas are characterized by upregulation of genes associated with cell cycle progression, DNA replication and repair and apoptotic resistance as well as loss of genes associated with apoptotic susceptibility, 2) Melanomas notably differ from their cell of origin, primary human melanocytes, due to a loss of cellular adhesion and differentiation-associated genes, and 3) Invasive melanomas are characterized by a signature indicative of global activation of NF-κB and downstream effector genes associated with tumor cell migration, invasion, chemotaxis, and proliferation. Since pathways associated with tumor progression may have clinical utility as prognostic tumor markers and therapeutic targets, we expect novel melanoma signature genes identified in this study will be further developed for such translational endpoints. Moreover, the important information regarding melanoma biology gleaned from these studies on renewable cell resources cannot be understated. A major roadblock to advances in melanoma therapy has been the relative paucity of informative tissue specimens available for analysis in profiling studies as well as the notoriously heterogeneous nature of this malignancy. The use of surrogate tissue resources including tumor cell lines for the early discovery phases in melanoma, as used in this study, will undoubtedly allow for the conservation of precious tissue specimens for use in more advanced validation studies. It is expected that the novel melanoma progression-associated genes identified in this study will provide new insights into the molecular defects associated with this malignancy and ultimately pave the way for the development of new melanoma biomarkers and novel targeted therapies.

MATERIALS AND METHODS

Cells

Ten melanoma cell lines (WM35, SBC12, and WM1552C, WM902B, WM278, WM983A, and WM793, WM852, WM983B, 1205Lu) were obtained from M. Herlyn (The Wistar Institute, Philadelphia, PA). These cell lines were maintained in modified complete melanocyte growth medium (Cell Application Inc., San Diego, CA) which lacked 12-0-tetradecanoyl phorbol-13-acetate and was supplemented with 2 % fetal bovine serum.

Figure 3. Identification of an invasion-specific gene signature for melanoma. A) The three-step data reduction algorithm used for identification of a melanoma invasion-specific signature. (see detailed description in Data Extraction and Statistical Analysis section of Methods). B) Relative expression levels of melanoma invasion-specific signature genes in all cells analyzed including human primary melanocytes (HPM1, HPM2). C) Validation of differential expression for selected genes by semi-quantitative duplex RT-PCR. Four genes (IL-8, IGFBP3, CXCL1, CXCL2) that are upregulated in invasive melanomas were selected and their differential expression was verified. D) Promoter analysis of selected genes from the melanoma invasion-specific signature identifies putative NF-κB binding cis elements. E) Immunofluorescence staining of NF-κB in invasive (WM902B) vs. non-invasive (WM1552C) melanoma cells demonstrates constitutive activation and nuclear trafficking of NF-κB in invasive melanomas.

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Table 3. Melanoma invasion-specific signature genes that are upregulated in VGP compared to RGP melanoma cells.a

| Probe Set ID | Gene Title | Gene Symbol | Fold b | Biological Process                      |
|--------------|------------|-------------|--------|-----------------------------------------|
| 204470_at    | chemokine (C-X-C motif) ligand 1 | CXCL1 | 62.3 | chemotaxis/inflammatory                  |
| 1554771_a_at | formin 2   | FMN2        | 38.8  | development                              |
| 204475_at    | matrix metalloproteinase 1 | MMP1  | 34.2 | proteolysis/ECM organization             |
| 202196_s_at  | dickkopf homolog 3 (Xenopus laevis) | DKK3c | 24.5 | Wnt receptor signaling                   |
| 208494_at    | major histocompatibility complex, class II, Drx | HLA-DRD | 21.6 | immune response                         |
| 211506_s_at  | interleukin 8 | ILB       | 19.9  | chemotaxis/inflammatory                  |
| 212730_at    | desmuslin  | DMN         | 17.6  | —                                        |
| 210095_s_at  | insulin-like growth factor binding protein 3 | IGFBP3c | 17.5 | regulation of cell growth                |
| 206640_x_at  | G antigens | GAGEs       | 15.5  | cellular defense response                |
| 227566_at    | neurotrigin | HNT        | 15.2  | cell adhesion                           |
| 212327_at    | hypothetical protein DKFZP686A01247c | —     | 14.8 | actomyosin structure                    |
| 211776_s_at  | erythrocyte membrane protein band 4.1-like 3 | EPB41L3 | 13.3 | cytoskeleton organization               |
| 221729_at    | collagen, type V, alpha 2 | COL5A2 | 12.7 | ECM organization                        |
| 226189_at    | integrin, beta 8 | ITGB8 | 12.4 | cell-matrix adhesion                    |
| 226847_at    | follistatin | FSTc       | 11.4  | development                              |
| 222450_at    | transmembrane, prostate androgen induced RNA | TMEPAIc | 11.3 | androgen receptor signaling             |
| 212942_s_at  | KIAA1199   | KIAA1199    | 11.2  | sensory perception of sound              |
| 205207_at    | interleukin 6 (interferon, beta 2) | IL6    | 11.0 | immune response/inflammatory            |
| 209619_at    | CD74 molecule, major histocompatibility complex | CD74 | 10.7 | immune response                         |
| 211571_s_at  | chondroitin sulfate proteoglycan 2 (versican) | CSPG2c | 10.3 | cell adhesion/development                |
| 223614_at    | chromosome 8 open reading frame 57 | C8orf57 | 10.3 | —                                        |
| 207030_s_at  | cysteine and glycine-rich protein 2 | CSRP2c | 10.0 | muscle development                      |
| 229800_at    | Doublecortin and CaM kinase-like 1 | DCAMKL1 | 10.0 | nervous system development              |
| 211538_s_at  | heat shock 70kDa protein 2 | HSPA2 | 9.9  | protein folding                         |
| 223638_at    | neuroblastoma breakpoint family, member 3 | NBP3 | 9.9  | —                                        |
| 209312_x_at  | major histocompatibility complex, class II, DR, j1 | HLA-DRB1c | 9.6 | immune response                         |
| 1561691_at   | hypothetical protein LOC285735 | LOC285735 | 9.0  | —                                        |
| 209774_s_at  | chemokine (C-X-C motif) ligand 2 | CXCL2 | 8.6  | chemotaxis/inflammatory                  |
| 209392_at    | pyrophosphatase/phosphodiesterase 2 | ENPP2c | 8.3  | cell motility/chemotaxis                 |
| 231756_at    | zona pellucida glycoprotein 4 | ZP4 | 7.8  | fertilization/cell adhesion              |
| 209309_at    | alpha-2-glycoprotein 1, zinc | AZGP1 | 7.7  | cell adhesion                           |
| 241803_s_at  | —          | —           | 7.4   | —                                        |
| 204469_at    | protein tyrosine phosphatase, receptor-type, Z | PTPRZ1 | 7.2  | nervous system development              |
| 205885_s_at  | integrin, alpha 4 | ITGA4 | 7.2  | cell adhesions                          |
| 228293_at    | DEP domain containing 7 | DEPDC7 | 7.0  | regulation of transcription              |
| 213075_at    | olfactomedin-like 2A | OLFM2A | 7.0  | —                                        |
| 219230_at    | transmembrane protein 100 | TMEM100 | 7.0  | —                                        |
| 204681_s_at  | Rap guanine nucleotide exchange factor 5 | RAPGEF5 | 6.6  | signal transduction                     |
| 211980_at    | collagen, type IV, alpha 1 | COL4A1 | 6.4  | ECM organization                        |
| 211991_s_at  | major histocompatibility complex, class II, DP, 1 | HLA-DPA1 | 6.3 | immune response                         |
| 216959_x_at  | neuronal cell adhesion molecule | NRCAM | 6.3  | neuron cell adhesion                    |
| 209967_s_at  | cAMP responsive element modulator | CREM | 6.2  | signal transduction                     |
| 222162_s_at  | ADAM metallopeptidase | ADAMTS1 | 6.1  | proteolysis                             |
| 233903_s_at  | Src domain-containing guanine exchange factor | SGEFc | 6.1  | Rho protein signaling                   |
| 207034_s_at  | GLK-Kruppel family member GLI2 | GLI2 | 6.1  | morphogenesis                           |
| 222853_at    | fibronectin leucine rich transmembrane protein 3 | FLRT3 | 6.1  | cell adhesion                           |
| 226436_at    | Ras association (RalGDS/AF-6) domain family 4 | RASSF4 | 6.0  | signal transduction                     |
| 219872_at    | chromosome 4 open reading frame 18 | C4orf18 | 5.9  | —                                        |
| 211966_at    | collagen, type IV, alpha 2 | COL4A2 | 5.8  | ECM organization                        |
| 209859_at    | tripartite motif-containing 9 | TRIM9 | 5.8  | —                                        |
Normal human primary melanocytes were isolated from neonatal foreskins and grown in complete melanocyte growth medium (Cell Applications, Cat. No. 135–500). The complete melanocyte growth medium is consisted of the melanocyte basal medium (Cell Applications, Cat. No. 134-500) and growth supplement cocktails containing hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), 12-O-tetradecanoylphorbol-13-acetate (10 ng/ml), bovine pituitary extract (21 μg/ml), bFGF (1 ng/ml), heparin (1 μg/ml), FBS (0.5 %), gentamycin sulfate (50 μg/ml), amphotericin B (5 ng/ml), and NaCl (45 mM).

### Gene Expression Profiling

Total RNA was isolated from exponentially growing melanoma cell lines using RNeasy column purification per manufacturer’s protocol (Qiagen). Two sets of short-term cultured (2 to 3 passage numbers) normal human melanocytes were prepared from neonatal foreskins. In order to minimize genetic variability melanocytes from 4–5 individuals were pooled for each culture. Total RNA from normal melanocytes was extracted and purified by a combination of phase extraction and chromatography using TRIzol reagent (Invitrogen Life Technologies Inc.) and RNeasy columns (Qiagen) in order to remove melanin. In brief, exponentially growing melanocytes were lysed with TRIzol reagent and lysate was incubated at 65°C for 2 minutes to inactivate melanin. Lysate was then subjected to phase extraction and RNeasy column purification. RNA quality checks, double stranded complementary DNA synthesis, hybridization with Human Genome U133 Plus 2.0 Array Chips (Affymetrix Inc. Santa Clara, CA), and initial data extraction were performed at The Gene Array Core Facility in the Malaria Research Institute (JHMRI) at The Johns Hopkins Bloomberg School of Public Health (http://malaria.jhsph.edu/jhmri/resources_education/gene_array_core/).

### Data Extraction and Statistical Analysis

SAM [19], Gene Cluster 3.0 [58] and TreeView (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/index.html), Access, and Excel (Microsoft, Seattle, WA) programs were used. For all of the statistical analysis beyond the initial description of datasets, microarray data were normalized [Dataset S1] and a subset of the 12 microarray data (10 from melanoma cell lines and 2 from normal human melanocytes) was obtained by filtering to include each gene probe to have at least one observation in the expression intensity resulting in a ‘present’ call from all 12 samples. This produced a subset of data containing a total of 32,632 affymetrix gene probes [Dataset S2]. This filtered subset of data was used for all of the additional analysis. Cluster Analysis. Unsupervised hierarchical clustering analysis was performed on the subset of data (without log transformation) with Gene Cluster 3.0 (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/index.html) by using the correlation (uncentered) similarity metric and centroid linkage clustering method. The resulting tree-images were visualized using Java TreeView. Statistical Analysis of Microarray (SAM). SAM was performed on the subset of array data without log transformation using SAM software package. Groups are defined based on the hierarchical clustering; for example, group 1 = less-aggressive primary melanomas (RGP melanomas: WM852, 80E2, WM1552C and VGP melanomas: WM902B and WM270), and group 2 = aggressive metastatic melanomas (Metastatic melanomas: WM832i, WM903B, and 1205Lu and VGP melanomas: WM903A and WM793) as seen Figure 1. Delta was chosen to limit the output gene list so that minimum predicted false-positives would be included. Three-step Data Reduction Algorithm. In order to identify melanoma invasion-specific gene signature, uniquely designed three-step data reduction algorithm was applied to the subset of expression data. First step is that the probeset should be called as ‘present’ in three samples out of four VGP melanoma cells and two samples out of three RGP melanoma cells. Second step is that the candidate probeset should be expressed five folds or more in VGP melanoma cell lines than RGP melanoma cell lines. The last step is that the gene probesets were retained only when the expression level is greater than three folds in VGP melanoma cell lines compared to that of primary human melanocytes. The last step is implemented because the candidate gene expression level should be higher if the gene products have certain degree of functional roles in the invasion processes of malignant melanoma. The probesets, those that pass through three-step filtration criteria are subjected to probeset to gene mapping using NetAffx, a web interface program from Affymetrix Inc. Gene annotation for the gene name, gene symbol, and GO Biological Procession Analysis also performed by the NetAffx.

### Quantitative Real-time PCR

cDNA was generated by using the SuperScript™ First-Strand Synthesis System for RT-PCR according to manufacturers instructions (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed with an Applied Biosystems Prism 7900 HT Sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The thermal cycling conditions for quantitative real-time RT-PCR analysis to validate gene expression changes were as follows: hold for 10 minutes at 95°C, followed by three-step PCR for 40 cycles of 95°C for 15 seconds, 55°C to 60°C for 25 seconds, and 72°C for 30 seconds. Optimal annealing temperatures were predetermined
to ensure single amplified product. All samples were performed in triplicate. Amplification data were analyzed with an Applied Biosystems Prism Sequence Detection Software Version 2.3 (Applied Biosystems, Forster City, CA). Human GAPDH gene was used as endogenous control. To normalize the relative expression of the genes of interest to the GAPDH control, standard curves were prepared for each gene and GAPDH in each experiment.

**Semi-quantitative Duplex PCR**

Semi-quantitative duplex RT-PCR was performed by an MJ Research Programmable Thermal Controller (PTC-100, Inc., Watertown, MA) and the amplified products were separated on an agarose gel. Our duplex PCR utilized 20 bp oligonucleotides to amplify regions of 300–400 bp from the genes of interest. Initial optimization experiments were conducted to establish the most favorable primer concentrations between the genes of interest and internal control GAPDH, yielding 0.8 µM and 0.04 µM, respectively. The PCR was carried out in a total volume of 25 µL, containing 2.5 µL of 10X PCR Buffer (containing 15 mM MgCl2), 0.2 mM dNTPs, and 0.3 ul AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). Thirty to thirty-five amplification cycles were performed by the MJ Research Programmable Thermal Controller (PTC-100, Inc., Watertown, MA), using a denaturing temperature of 95°C for 25 seconds, an annealing temperature varying between 55°C–60°C (depending on gene) for 30 seconds, and primer extension at 72°C for 30 seconds. Each amplification experiment also included two negative PCR controls, a no-RNA control from reverse transcriptase to ensure single amplified product. All samples were performed in triplicate. Amplification data were analyzed with an Applied Biosystems Prism Sequence Detection Software Version 2.3 (Applied Biosystems, Forster City, CA). Human GAPDH gene was used as endogenous control. To normalize the relative expression of the genes of interest to the GAPDH control, standard curves were prepared for each gene and GAPDH in each experiment.

**Immunofluorescence Labeling**

RGP and VGP Cells were plated on glass slides and cultured in melanocyte growth media overnight without any stimulation. Cells were fixed at room temperature for 15 minutes using 3.5% paraformaldehyde solution. Cells were washed briefly with PBS and then permeabilized with either 0.5% Triton X-100 for 10 minutes or −20°C cooled methanol for 15 minutes. Slides were blocked with 16% normal goat serum (Santa Cruz Biotech., Santa Cruz, CA) for 1 hour and then incubated with rabbit polyclonal IgG p65 antibody (Santa Cruz Biotech., Santa Cruz, CA) at 1:100 dilution. Subsequent to overnight incubation at 4°C, the slides were washed with PBS and incubated with goat anti-rabbit IgG-Alexa 594 (Molecular Probes Eugene, OR) at 1:200 dilution at room temperature for 1 hour. Stained slides were washed with PBS and viewed under a fluorescence microscope (Eclipse TS100, Nikon, Tokyo, Japan).

**Gene Transcription Promoter Analysis**

Transcription factor binding cis element sequence profiling in the selected gene promoter was performed by using a web tool known as TESS (Transcription Element Search System, http://www.cbil.upenn.edu/ tess). Each genes promoter sequences from the expression data start site up to 2.0 kb of upstream of the gene were subjected to the TESS and screened by TRANSFAC database to identify matched consensus sequences of known DNA binding transcription factors.

**SUPPORTING INFORMATION**

**Dataset S1** A combined and normalized raw dataset from the 12 sets of microarray data (two sets of primary human melanocytes and ten melanoma cell lines). Found at: doi:10.1371/journal.pone.0000594.s001 (18.02 MB DOC)

**Dataset S2** A subset of the 12 microarray data (10 from melanoma cell lines and 2 from human primary melanocytes) was obtained by filtering to require each Affymetrix probe to have at least one observation in the expression intensity resulting in a total of 12 samples. Found at: doi:10.1371/journal.pone.0000594.s002 (4.50 MB DOC)

**Table S1** Results of SAM analysis between Group 1 and Group 2 melanoma cells. The results include the SAM Output showing the list of Affymetrix probesets and associated gene symbols that are differentially expressed in the two groups of melanoma cell lines. Found at: doi:10.1371/journal.pone.0000594.s003 (3.20 MB DOC)

**Table S2** Results of SAM analysis between HPMs and Group 2 melanoma cells. The results include the SAM Output showing the list of Affymetrix probesets and associated gene symbols that are downregulated in Group 2 melanoma cell lines. Found at: doi:10.1371/journal.pone.0000594.s004 (3.05 MB DOC)

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**MELK**

Forward: 5' - TGGCCTCTCCCGACTGACAT-3'

Reverse: 5' - TAGCAGCTGTTGCTCACCAG-3'

**GINS4**

Forward: 5' - CAGAGAGTCATTGCGACACA-3'

Reverse: 5' - TCTCCAAGTGCTGGGATAA-3'

**NCAPG**

Forward: 5' - TATGGTTGTCCTTTGTGA-3'

Reverse: 5' - CAGGGATATTTGGGATTTG-3'

**CDH3**

Forward: 5' - ACGACACCCTTTGTTGTC-3'

Reverse: 5' - GTCAACTGCCCAACATTCTT-3'

**KIT**

Forward: 5' - TAGCTTACGGACGGTCCTTG-3'

Reverse: 5' - AAGGAGTGAACAGCGCACA-3'

**DPP4**

Forward: 5' - CAGGGTTTGAGAGCTGAG-3'

Reverse: 5' - CAGGGCTTTGGAGATCTGAG-3'

**SYR**

Forward: 5' - GAGGACATATGCAAGGGGATGA-3'

Reverse: 5' - TGCAAGTTTGGGCGATGT-3'

**CXCL1**

Forward: 5' - TGTGCAGGATCTGCTAGGA-3'

Reverse: 5' - GATCTCATTGGGCTTTGCT-3'

**CXCL2**

Forward: 5' - TGGCGCTAATTTTGTTG-3'

Reverse: 5' - ATACATTTCCCTGGCGTTC-3'

**IL8**

Forward: 5' - AGGGTTTGCGCAGATGCAAA-3'

Reverse: 5' - AGCAGACTAGTGGGTGCCAG-3'

**IGFBP3**

Forward: 5' - GCTACAGGATGCGAGCGAAG-3'

Reverse: 5' - AACAGTGGGTGAGATCCAA-3'

**GAPDH**

Forward: 5' - GATCATCAGCAATGCCTCCT-3'

Reverse: 5' - TCCAGCTCAAGGGGATGACCT-3'
Author Contributions
Conceived and designed the experiments: BR, RA. Performed the experiments: DK, BR. Analyzed the data: RA, DK, BR. Contributed reagents/materials/analysis tools: BR. Wrote the paper: RA, BR.

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