Gene Expression Signature for Spontaneous Cancer Regression in Melanoma Pigs\textsuperscript{1,2}

Florian Rambow\textsuperscript{*,,†}, Guillaume Piton\textsuperscript{*,,†}, Stephan Bouet\textsuperscript{*,,†}, Jean-Jaques Leplat\textsuperscript{*,,†}, Sylvain Baulande\textsuperscript{‡}, Angelique Marrau\textsuperscript{‡}, Mark Stam\textsuperscript{*,,†}, Vratislav Horak\textsuperscript{§} and Silvia Vincent-Naulleau\textsuperscript{*,,†}

\textsuperscript{*}INRA, UMR 314, Laboratoire de Radiobiologie et d’Etude du Génome, F-78350 Jouy-en-Josas, France; \textsuperscript{†}CEA, DSV, iRCM, SREIT, Laboratoire de Radiobiologie et d’Etude du Génome, F-78350 Jouy-en-Josas, France; \textsuperscript{‡}PartnerChip, Genopole, F-91000 Evry, France; \textsuperscript{§}Institute of Animal Physiology and Genetics, 27721 Libechov, Czech Republic

Abstract
Incomplete spontaneous regression of melanoma is common. However, complete melanoma regression is still a very rare phenomenon. Because melanoma is the most immunogenic human malignancy, the mechanisms leading to regression, based on accumulative evidence, are the host’s immune responses. Unfortunately, therapies aiming to enhance the patient’s natural immunity against melanoma have yet to meet their expectations. Reasons for failure include various immune escape mechanisms, induced by the tumor, that subsequently lead to tolerance. Here, we performed time-dependent gene expression profiling to unravel molecular changes involved in the transition of progressive melanoma to complete tumor regression using a porcine model. The melanoblastoma-bearing Libechov minipigs are highly suitable for this study because these animals exhibit naturally occurring and regressing melanomas. We were able to identify a molecular signature of the melanoma regression process. Genes regulated in this signature were associated with 1) cell cycle, 2) immune response, and 3) melanocyte differentiation. These genes may shed light on molecular mechanisms involved in complete melanoma regression and indicate what improvements are needed for successful antimelanoma therapy.

Neoplasia (2008) 10, 714–726

Introduction
Complete regression of cancer is the ideal outcome of any antitumor therapy. At present, no such treatment exists for advanced melanoma because melanoma cells exhibit an extraordinary resistance to chemotherapy, radiotherapy, and even immunotherapy \cite{1}. Hence, its resistance to treatment and aggressiveness make it the most fatal of all skin cancers, with mortality of patients with metastasis reaching >95\% within 5 years \cite{2}. Interestingly, total regression of advanced melanoma occurs spontaneously, where spontaneous regression refers to the disappearance of the malignant tumor mass without treatment or as a consequence of an indirect action (i.e., treatment against another disease or symptoms) \cite{3}. Complete regression of metastatic melanoma is an extremely rare occurrence with only 38 well-documented cases \cite{4}. However, the regression could be more common than reported because it is prone to escape detection \cite{5}. Nevertheless, partial regression is observed more frequently with 7\% to 61\% in thin melanoma \cite{6}. Clinically, partial regression is mainly characterized by a heterogeneous pigmentation of the tumor site. Whereas on a histopathologic level, the process starts with a dense infiltrate of lymphocytes and ends with fibrosis and/or melanosis within a thickened papillary dermis \cite{7}. Different mechanisms such as immune recognition, virus infection of tumor cells, cytokine-
induced apoptosis, high levels of stress-induced steroids, hypoxic conditions, telomeric breaks, and gene mutations have been discussed as mediators of regression but clear evidence is missing [8].

The melanoblastoma-bearing Libechov minipigs (MeLiM) have been described as a suitable animal model to study melanoma and its regression because the tumors occur and vanish naturally and melanocytes are localized at the basal layer of the epidermis. In addition, large litters allow studies of homogenous genetic background. Spontaneous complete tumor regression occurs in 96% of MeLiM and is characterized by tumor flattening, tumor drying, depigmentation, and infiltration of firstly melanophages and then lymphocytes [9]. The biggest difference between humans and pigs is the early onset of regression in MeLiM, which occurs during childhood, and its extreme efficiency. The elucidation of regression mechanisms is of valuable interest to find a more specific therapy to treat the disease. Therefore, we aimed to study the molecular changes leading to melanoma regression in MeLiM using Porcine Genome Arrays (GeneChip, Affymetrix, High Wycombe, UK). We have conducted time-dependent gene expression profiling to characterize transcriptomic changes leading from melanoma progression to spontaneous regression. We were able to identify characteristic gene signatures and significant molecular pathways associated with spontaneous and complete melanoma regression.

Materials and Methods

Biologic Samples

Time-dependent gene expression profiling of spontaneously regressing melanomas was performed at five different time points, namely, \(t_0 = \text{day } 8\) after birth \((d + 8)\), \(t_1 = d + 28\), \(t_2 = d + 49\), \(t_3 = d + 70\), and \(t_4 = d + 91\). Six MeLiM of the same litter were chosen, which were homozygous for predisposition quantitative trait loci located on Sus scrofa chromosome (SSC) 1 and SSC6 to ensure the presence of multiple lesions with high aggressiveness [10]. Tumors were excised surgically from MeLiM swines under complete anesthesia.

At \(t_0\) and \(t_1\), \(n = 6\) tumors, at \(t_2\) and \(t_3\), \(n = 5\) tumors, and at \(t_4\), \(n = 3\) tumors were processed for chip hybridization. Number of excised tumors \((n)\) is equal to the number of used microarrays. In total, \(n = 25\) microarrays were used. Tumor samples were obtained from \(N\) different animals (Table 1). Due to reduced RNA integrity of tumor samples at \(t_4\), only three tumors passed the microarray quality controls. Ulcerated or necrotic regions of tumor sections were maximally macrodissected before RNA extraction. Collected tumor samples were stored in liquid nitrogen for RNA extraction and in 10% buffered formalin for histologic diagnosis. Hematoxylin-stained paraffin-embedded sections were evaluated histologically according to human classification and for different criteria namely ulceration, vascularity, fibrosis, hyperkeratosis, and infiltration by inflammatory cells. Clinically, signs of regression included drying surface, flattening, and depigmentation of the tumor. Histologically, regression was characterized by an extensive infiltration of melanophages, lymphocytes, dermal fibrosis, and telangiectasia at the tumor site. All experiments were performed in accordance with the French law for animal experimentation (Décret: 2001-464 29/05/01). The presented study was approved by the local ethic committee for animal experimentation (Comité régional d’éthique Ile de France-Sud, project no. 05-030).

RNA Isolation

Total RNA was extracted from several 16-μm tumor tissue cryosections using the PicoPure RNA extraction kit (Arcturus Engineering, Mountain View, CA). The RNA quality was carefully assessed by capillary electrophoresos using the 2100 BioAnalyzer (Agilent, Massy, France). The RNA quantity and the 260/230 nm ratio, 260/280 nm and 405/415 nm (detection of melanin contamination) were determined by spectrometry using Nanodrop (Thermo Fisher Scientific, Palaiseau, France).

Target Production and Hybridization

Microarray experiments and part of data analysis were performed by PartnerChip (Evry, France) following the Affymetrix-recommended procedure. Target was prepared and hybridized according to the Affymetrix 1-cycle technical protocol as described before [11]. Fluorescent images were detected in a GeneChip Scanner 3000 (Affymetrix). Expression data and raw expression data (CEL files) were generated using GCOS software (Affymetrix). Quality control was assessed based on 3′/5′ ratios of glyceraldehyde 3-phosphate dehydrogenase and β-actin control probe sets.

Normalization and Statistical Analysis

Normalization and statistical analysis of microarray data have been performed using R resources, Bioconductor statistical packages (http://www.bioconductor.org/), and the ArrayAssist software (Stratagene Europe, Amsterdam, The Netherlands) for the analysis of variance (ANOVA) and \(k\)-means analysis. Raw intensity values were subjected to a preprocessing step using the GC Robust Multiarray Average algorithm that summarizes and normalizes data into gene expression values. The log₂ scale transformation is integrated into this process, so output values are then log₂-transformed and ready to be used for test and one-way ANOVA. The time after birth was considered as a central parameter for one-way ANOVA. Multiple hypotheses testing was controlled by applying Benjamini–Hochberg false discovery rate (FDR) correction. \(P\) values of the ANOVA were adjusted using the Benjamini–Hochberg algorithm (FDR or adjusted \(P\) value < .01).

### Table 1. Tumor Characteristics of Biologic Samples Used for Microarray Analysis

<table>
<thead>
<tr>
<th>Time Point</th>
<th>(n)</th>
<th>(N)</th>
<th>Clinical Aspect</th>
<th>Histologic Aspect</th>
</tr>
</thead>
<tbody>
<tr>
<td>(t_0) = day 8</td>
<td>6</td>
<td>6</td>
<td>7.6–9.2 NM/IV–V</td>
<td>Ulcerated nodule – plateau-shaped tumor</td>
</tr>
<tr>
<td>(t_1) = day 28</td>
<td>6</td>
<td>6</td>
<td>7.6–8.9 NM/IV–V</td>
<td>Bleeding ulcerated exophytic tumor</td>
</tr>
<tr>
<td>(t_2) = day 49</td>
<td>5</td>
<td>5</td>
<td>7.4–8.6 NM/IV–V</td>
<td>Dry plateau-shaped – exophytic tumor</td>
</tr>
<tr>
<td>(t_3) = day 70</td>
<td>4</td>
<td>5</td>
<td>7.3–8.5 UC</td>
<td>Gray plateau-shaped, dry exophytic, ulcerated plateau-shaped tumor</td>
</tr>
<tr>
<td>(t_4) = day 91</td>
<td>3</td>
<td>3</td>
<td>7.1–7.8 UC</td>
<td>Gray plateau-shaped tumor</td>
</tr>
</tbody>
</table>

\(N\) indicates the number of different animals from which tumors were excised; \(n\), number of excised tumors equal to the number of used microarrays; NM, nodular melanoma; RIN, RNA integration number; TILs, tumor-infiltrating lymphocyte; UC, unclassified.
For the $t$ tests, $P$ value adjustments were performed individually for each comparison. Probe sets were defined as differentially expressed for $t_n$ versus $t_0$ time points if the fold change (FC) was bigger than 2 and $P$ value was lower than .05 after unpair $t$ test. Furthermore, probe sets also found significant after ANOVA were used for $k$-means clustering ($k = 6$). We used $k = 6$ clusters because most of the time, the number of clusters is close to the number of time points. In addition, we grouped our data by $k = 9$ clusters. Subsequent functional analysis, however, showed an “overclustering” of the data, as many genes of the same biologic function were arranged in different $k$-means clusters. So, using $k = 6$ clusters was an experience-based choice but justified by following functional analysis. Microarray data were submitted to ArrayExpress (http://www.ebi.ac.uk/microarray-as/aer-entry): Experiment (E-MEXP-1152).

**Reannotation of Porcine Affymetrix Probe Sets**

The porcine Affymetrix microarray was poorly annotated with less than 10% of the immobilized probe sets describing a gene and was therefore reannotated using the method by Tsai et al. [12]. Using this strategy, 19,675 (82%) of 24,123 transcripts on the Affymetrix porcine microarray could be identified, representing 11,256 unique human genes. The limit of significant homology with human sequence was set at $e$-value $< e^{-10}$.

**Functional Pathway Analysis by Ingenuity**

A total of 1411 $k$-means clustered genes ($e$-value $< e^{-10}$) were analyzed by Ingenuity Pathways Analysis (Ingenuity Systems; http://www.ingenuity.com). Swiss Prot identifiers were imported into the Ingenuity Pathway Analysis (IPA) Software. Of these genes, 1396 were mapped to the Ingenuity database. Cluster 1 with 406 IPA-mapped genes (IMGs) contained 235 network-eligible genes (NEGs), cluster 2 (280 IMGs) contained 184 NEGs, cluster 3 (with 243 IMGs) contained 178 NEGs, cluster 4 (with 242 IMGs) contained 172 NEGs, cluster 5 (with 128 IMGs) contained 105 NEGs, and cluster 6 (with 97 IMGs) contained 62 NEGs. The identified genes were mapped to genetic networks available in the Ingenuity database and were then ranked by score. The score is the probability that a group of genes equal to or greater than the number in a network (maximum 35 genes) could be achieved by chance alone. Furthermore, comparison analysis of the six gene clusters and the different time points ($t_1$–$t_4$) was performed. Ingenuity pathway analysis mapped the clustered genes for each time point relative to $t_0$ as follows: $t_1 = 53$ genes (42 NEGs), $t_2 = 234$ genes (183 NEGs), $t_3 = 1049$ genes (719 NEGs), and $t_4 = 1256$ genes (833 NEGs). After single IPA core analysis for each time point, IPA comparison was performed to analyze changes in biologic states over time. Functional pathway analysis over time identified highly significant biologic classes that changed during the progression and regression of melanoma. The readout of the comparison analysis were histograms representing the significance ($-\log P$ value) of the functional association that is dependent on the number of genes in a class and in biologic relevance. The threshold line that appears in the bar chart represents a $P$ value of .05. In addition, network analysis was performed for each time point to identify gene interaction.

**Quantitative Real-Time Polymerase Chain Reaction Validation**

To validate differentially expressed genes identified by microarray analysis, we performed quantitative real-time polymerase chain reaction (qRT-PCR) as previously described [13]. We tested 18 genes in total (CCNB1, CDC2, BUB1B, BIRC5, KIF11, CKAP2, SCIN, KLRK1, CCL5, IL15, TVB1, SLC37A2, ATP6V0D2, IGHG1, TRGV9, MITF-M, PRF1, and MLANA). Eighteen genes were chosen because we wanted to validate genes of the major gene signatures of interest (cell cycle and immune response). We chose six genes representative for the cell cycle signature (CCNB1, CDC2, BUB1B, BIRC5, KIF11, and CKAP2) and nine genes for the different facets of the immune response (SCIN, KLRK1, CCL5, IL15, TVB1, SLC37A2, ATP6V0D2, IGHG1, and TRGV9). Three additional genes were selected that were of interest but not responsive or present on the microarray, PRF1 was represented on the porcine GeneChip with two probe sets, but both were unresponsive. MITF-M and MLANA were not present on the porcine microarray (Table W2).

**Gene List Comparison**

To confront our data with human melanoma expression profiles, we compared our MeLiM data against three human melanoma expression studies conducted with melanoma tissue and primary melanoma cell lines [14–16]. The aim was to identify overlapping genes as an indicator of biologic relationship. To evaluate an overall gene overlap with a certain significance, we applied the hypergeometric distribution test (HDT) [14]. Briefly, the test calculates the probability of obtaining by chance a number $k$ of annotated genes for a given term among a data set of size $n$, knowing that the reference data set contains $m$ such annotated genes out of $N$ genes. In our case, $m$ was the number of genes in the target gene list, $k$ was the number of genes in our gene list, $n$ was the number of overlapping genes between $m$ and $k$, and $N$ was the number of all possible genes. Because our porcine microarray did not represent an approximate 33K human pan genomic GeneChip, we corrected $m$ (number of genes in the reference data set) by subtracting genes that were not immobilized. Because the HDT calculates the probability of having exactly $n$ overlaps, we repeated the calculation up to four times by increasing each time $n + 1$. Adding up these probabilities and using the cumulative results permitted to state the probability of finding at least $n$ overlaps. The cumulative results were rounded up to the next order of magnitude to be more conservative.

**Tumor-Infiltrating Lymphocyte Isolation and Flow Cytometry**

Single-cell suspension of melanoma tissue was obtained by collagenase B (Roche, Meylan, France) treatment. Double staining was performed with mouse anti–swine-CD4 at 1:500 (IgG2a, clone PT90A; VMRD, Inc, Lisseu, France) and mouse anti–swine-CD8α antibody at 1:500 (IgG2b, clone PT81B; VMRD, Inc). Primary antibody staining was revealed by R-phycocerythrin–labeled antimouse IgG2a at 1:500 (Southern Biotech, Montrouge, France) and fluorescein isothiocyanate (FITC)–labeled antimouse IgG2b at 1:300 (Southern Bio- tech). Flow cytometry analysis was performed using a FACSCalibur (BD Biosciences, Le Pont de Clai, France).

**Immunohistochemistry**

Cryosections were fixed in 95% ethanol for 10 min. Nonspecific binding sites were blocked by incubation with goat serum. Melanoma sections were incubated overnight with mouse anti–swine-CD3 (IgG1 at 1:50, clone PPT3; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti–swine-CD8α (IgG2b at 1:400, clone PT81B; VMRD, Inc), anti–swine SWC3a (IgG2b at 1:200, clone 74-22-15a; BD Pharmingen, Le Pont de Clai, France), CD3, CD8, and SWC3a
protein stainings were revealed by Alexa Fluor 555–labeled, goat anti-mouse IgG1 at 1:500 (Invitrogen, Cergy Pontoise, France), and FITC–labeled goat antimouse IgG2b at 1:500 (Southern Biotech), respectively. Negative controls were assessed by replacing the primary antibodies with the nonimmune goat serum at the same concentration. Staining patterns were assessed independently by two different investigators.

Results

Sample Characteristics

Tumors at t₀ and t₁ were exclusively growing melanomas of nodular type with a Clark level of IV–V, with a tumor area consisting mainly of homogenously distributed melanoma cells (up to 98%; Table 1). The first signs of regression appeared at t₂ (d + 28) with the occurrence of tumor-infiltrating lymphocytes (TILs; three of five tumors), fibrosis, and melanophages. This observation pursued, whereas tumors at t₃ and t₄ showed clear signs of regression namely extensive infiltration of melanophages, TILs, dermal fibrosis, and telangiectasia. The change of the tumor microenvironment during regression was also reflected in the steady decrease of RNA integrity over time. Whereas the highest RNA integration number (RIN) at t₀ was 9.2, the maximal RIN at t₄ was 7.8 (Table 1) due to regression-related phenomenon such as necrosis.

Microarray Analysis and Clustering

After normalization and statistical analysis, 1761 probe sets representing 1411 unique human genes (e-value < e⁻¹⁰) were regulated over time and grouped into six clusters according to similar expression behavior. Cluster 1 (Figure 1A) contained the most genes (409), whereas clusters 2, 3, and 4 were almost equal in numbers (248, 245, and 244, respectively). Clusters 5 and 6 harbored fewer genes of 131 and 98, respectively (Table W1). The expression profiles of clusters 2, 3, and 5 indicated a general up-regulation of genes over time, with cluster 5 showing the strongest up-regulation at t₅. Clusters 1, 4, and 6’s profiles showed a down-regulation over time, with cluster 6 marking a dramatic decrease in gene expression from t₃ onward. Only minor differences were detected between t₀ and t₁ among the six clusters. However, at t₂, more prominent changes in gene expression profiles were observed (129 genes significantly up-regulated, and 107 down-regulated) reflecting observed histologic changes. Functional classes were assigned to gene clusters by IPA. Genes of cluster 6 (Figure 1B) were mainly associated with Hair and Skin Development and Function (P = 1.36 × 10⁻¹¹), whereas cluster 5 contained mainly Immune Response– (P = 3.28 × 10⁻¹⁰) and Cell Death– (P = 1.7 × 10⁻⁹) related genes. Cluster 4 genes were highly associated with Cell Cycle (P = 9.39 × 10⁻¹⁶) and DNA Replication, Recombination, and Repair (P = 1.44 × 10⁻¹¹) functions. Genes of cluster 3 were mainly linked to Cellular Movement (P = 1.61 × 10⁻¹⁰) and Immune Response (P = 1.62 × 10⁻⁹). Clusters 1 and 2, containing most genes, showed only minor regulatory changes over time, and functional comparison to the other clusters did not render highly specific gene classes.

Time-Dependent Analysis by IPA

To focus on expression changes at each time point, functional interpretation and biologic interaction of the clustered genes were achieved by IPA. As demonstrated in Figure 2A, highly significant classes namely Cellular Movement, Cell Cycle, Cell Death, Immune Response, DNA Replication, Recombination, and Repair, and Hair and Skin Development and Function changed remarkably over time. To discern the change in regulation, we included counts for up- and down-regulated genes for each time point, as well as their range of FCs as a measure for their “weight” of regulation. Considering these criteria, t₁ (d + 28) showed only minor transcriptomic changes relative to t₀ (d + 8). However, 18 genes were associated with Cell Death, of which 12 were up-regulated. In addition, at t₁, 8 of 9 genes linked to Immune Response were up-regulated (~10-fold) and 4 of 10 genes linked to Cell Cycle functions were down-regulated (~2.5-fold). Interestingly, at t₂ (d + 49), a remarkable change occurred in the Cell Cycle class that contained a total number of 52 genes, of which 38 (~73%) were down-regulated with up to approximately seven-fold compared to t₀. DNA Replication, Recombination, and Repair, a related class, showed the same trend with 86% of the genes down-regulated at t₂. The cell cycle genes affected were mainly cyclin-dependent kinases (CDC2 and CDC6) and cyclins (CCNA2, CCNB1, and CCNB3), as shown in Figure 2B, and spindle-associated proteins such as members of the kinesin family (KIF11). Canonical pathway analysis confirmed this finding, showing genes for G₂/M phase, Checkpoint Regulation being down-regulated at t₂ (Figure W1). Furthermore, 48 of 72 genes involved in cell death were up-regulated at t₂. Evidence for an immune response onset was suggested by the fact that 29 of 30 immune response genes were increased up to ~40-fold at t₂. This immune response seemed to be established at t₃, with ~82% of the immune genes being strongly up-regulated (up to ~100-fold). There were no less than six canonical pathway classes demonstrating immune response–associated signaling (Figure W1). These canonical classes indicated a complex immune invasion because signaling pathways of chemokines, leukocyte extravasation, and different immune cells such as T cells, natural killer (NK) cells, and B cells were augmented. The invasion of immune cells was mirrored by the up-regulation of ~70% of the cellular movement genes up to 28-fold at t₃. The phenomenon of depigmentation was clearly reflected on the expression level because 50% of pigmentation genes classified under Hair and Skin Development and Function were strongly down-regulated.

Comparison to Human Melanoma Expression Studies

We compared our gene lists to those of different human melanoma gene expression profiling studies to identify overlapping genes [14–16]. All groups studied the progression of the disease using either melanoma tissue [16] or primary melanoma cell lines [14,17]. The probability of finding common genes between two independent gene lists by random was calculated using the HDT test [14].

Compared to the 174 known genes associated with metastatic dissemination of cutaneous melanoma [16], we found 4 overlapping genes at t₃, 27 at t₂ (P < 2.86 × 10⁻²⁹), 36 at t₃ (P < 9.69 × 10⁻²¹), and 33 at t₄ (P < 1.50 × 10⁻¹⁵; Table W3). Interestingly, common genes were all down-regulated in our melanoma regression model, whereas in humans, they were up-regulated suggesting a shorter survival. In addition, comparison with signatures of aggressive melanoma cell lines [15] again showed common genes that are associated with cell cycle progression, DNA replication and repair, and apoptotic resistance, namely, HELLs, BIRC5, GINS1, NUSAP1, MELK, and NCLAP2 (Table W3). Again, we found inverse regulation of those genes because they were down-regulated from t₃ onward during regression. Ryu et al. [17] also identified a signature for down-regulated genes in aggressive melanoma compared to primary human melanocytes harboring genes of cell adhesion and differentiation-associated genes. We found a common decrease of mainly differentiation-associated
Figure 1. k-Means clustered genes regulated during regression and their biologic interpretation. (A) Gene expression profiles of k-means clustered genes over time. Left upper panel represents the data as a heat map, whereas upper right panel represents the data in a line chart. Y-axis represents the log2 FC, whereas the X-axis represents the five consecutive time points when microarray analysis was performed. (B) Functional comparison of k-means gene clusters. Y-axis indicates the significance (−log P value) of the functional association that is dependent of the number of genes in a class as well as biologic relevance.
genes namely KIT, OCA2, TYR, and CITED1 from t₀ onward. Hoek et al. [14] identified 223 cohort-specific genes overexpressed in melanoma cell cultures, whereas one cohort (neural crest signature) seemed to be linked with a high proliferative and weak metastatic potential of melanoma cells, and the other cohort [transforming growth factor beta (TGFβ) signature] seemed to be associated with a weak proliferative and high metastatic potential. At t₀, we identified 19 common genes (P < 9.91 × 10⁻⁶). Of these 19 genes, 15 were associated with the neural crest signature, and of these 15, 10 were being down-regulated and 5 were up-regulated (Table W3). The last four common genes associated with the TGFβ signature were up-regulated in our model. At t₀, 20 overlapping genes (P < 3.34 × 10⁻³) were detected, of which 16 were neural crest-related and 4 were TGFβ signature-related. Eleven of the neural crest genes were down-regulated, mostly melanocytic markers, namely, TYR, CITED1, SILV, and GPR143. Of four TGFβ signature-related genes, three were up-regulated but with low FCs.

**Immune Response**

Focusing on immune response-related genes revealed distinct signatures that were regulated throughout spontaneous melanoma regression (Figure 3). A monocyte/macrophage-like signature (Figure 3A) was detected, which reached maximal up-regulation on day 70 (t₅). This signature contained macrophage-specific genes, such as the recently described sugar transporter SLC37A2 [18], B7 family-related phagocytic receptor VSIG4, and a T-cell regulator (19), and genes highly expressed in osteoclast-like cells such as tartrate-resistant phosphatase (ACP5), transcription factor PU.1 (SPP1), calcitonin receptor (CALCR) [20], the lysosomal ATPase V0 subunit D₂ (ATP6V0D2) [21], and integrin beta 3 (ITGB3). Moreover, genes involved in differentiation and multinucleation of osteoclasts (CALCR and ATP6V0D2) were the most up-regulated at t₃. Markers for the monocyte lineage included sialic acid binding Ig-like lectin 5 (SIGLEC5) [22] and C-type lectin domain family 5 member (CLECS5A) [23] that were approximately 25-fold up-regulated at t₃ (Figure 3A). Classic activation markers such as CD83 and CD86 remained stably expressed between t₀ and t₅ with minor FCs. VSIG4 and CD74, as well as reactive oxygen species (ROS) production-related genes such as CYBB and CYP2C9, reached maximal expression at t₅. By immunohistochemical analysis, we confirmed an increase of tumor-infiltrating monocytes during regression, characterized by SWC3 (SIRPA) expression (Figure 4B).

Furthermore, we detected genes related to a T/NK response and different T-cell receptor chains such as alpha, beta, and gamma (TC4, TVB1, and TRGV9). Genes of the T/NK signature were slightly up-regulated at t₀ and were highly up-regulated at t₅ (d + 91; Figure 3B).

By flow cytometry and immunohistochemical analysis, we confirmed an extensive infiltration of T cells at t₅ (Figures 4B and 5), where ~43% and ~22% were CD8⁺ and CD4⁺-positive, respectively.

Interestingly, the humoral response signature, containing different immunoglobulins, was most highly up-regulated in the regression process. Immunoglobulin heavy chain (IGHM), kappa variable (IGKV1–5), and the Ig lambda constant (IGHL1) were increased more than 70-fold at t₅ (Figure 3C). The cytokine gene signature showed, as expected, late up-regulation of T/NK cell-related cytokines and receptors such as CCL5, IL2RG, IL15, and CCR5 (Figure 3D).

**Microarray Validation**

To validate the microarray results, nine immune response–related genes that were up-regulated over time and six cell cycle–related genes that showed a decrease over time were selected for qRT-PCR analysis (Table W2). In addition, MITF and MLANA were also quantified by qRT-PCR.

The steady augmentation of the immune genes was confirmed by qRT-PCR. The up-regulation of molecules (TVB1, TRGV9, KLRR1, and PRF1) characteristic for immune effector cells such as cytotoxic T cells (CTLs) and NK cells, their signaling molecules (IL15 and CCL5), and SLC37A2, ATP6V0D2 (an ATPase mainly active in vacuoles of macrophages/osteoclasts), and IGHG1 represented a complex immune response including innate and adaptive components. Interestingly, SCIN, an actin-related gene that was shown to play a role in tumor cell resistance against CTL pressure [24], was also up-regulated over time. MLANA was mostly down-regulated (~27-fold) at t₅ whereas T-cell response genes were expressed at the highest. MITF-M already showed a decrease in expression (~4-fold) at t₅ whereas at t₆ it was down-regulated up to 80-fold. Furthermore, we validated six genes associated with an early retardation of the cell cycle, namely, CCNB1, CDC2, BUB1B, BIRC5, KIF11, and CKAP2. All genes were at least twofold down-regulated from t₀ onward, except BIRC5.

**Discussion**

We used the MeliM model to conduct the first genome-wide time-dependent gene expression profiling to analyze molecular mechanisms involved in spontaneous melanoma regression. A total of 1411 genes were significantly regulated during melanoma regression. Functional analysis by IPA revealed highly significant functional gene classes namely Cell Death, Cell Cycle, Cellular Movement, Immune Response,
Hair and Skin Development and Function, and DNA Replication, Recombination, and Repair that changed over time.

The reference of our gene expression profiling study consisted of growing tumors excised at \( t_0 \) (\( d + 8 \)) that showed extensive dermal invasion. The expression profiles of the reference \( t_0 \) could therefore not be exploited. However, we showed previously by subtractive suppression hybridization (SSH) that genes up-regulated at \( t_0 \) were connected to a highly proliferative gene signature [13]. Expression

Figure 3. Regulation of immune response–related genes during melanoma progression and regression. Fold changes relative to \( t_0 \): (A) monocyte/macrophage–related gene signature; (B) T-cell– and NK-cell–related gene signature; (C) immunoglobulin signature; (D) cytokine/chemokine and their receptors.

Figure 2. Functional analysis of clustered genes regulated over time (IPA). (A) Functional gene classes changing over time. \( Y \)-axis indicates the significance (\(-\log P \) value) of the functional association that is dependent of the number of genes in a class as well as biologic relevance. Furthermore, the number of genes up- and down-regulated as well as their FC of regulation is noted. (B) Most significant gene network detected at \( t_2 \) by IPA. A total of 25 focus genes were identified and mapped to top IPA functional classes such as cancer, cell cycle, and cellular compromise (significance: score, 44). A score of three indicates that there is a 1/1000 (score = \(-\log_{10}(P \) value)) chance that the focus genes are assigned to a network randomly. The maximal number of focus genes per network is set to be 35 by IPA network algorithm. Whereas green gene symbols refer to down-regulation, red gene symbols refer to up-regulation. The color intensity is correlated with FC. Uncolored gene symbols were not identified in this study but suggested to be in functional proximity by IPA network algorithm. Straight lines suggest direct gene to gene interactions whereas dashed lines indirect ones.
profiles at \( t_1 \) were not very different from \( t_0 \), indicating an ongoing growth phase. Nevertheless, immunoglobulin-related genes (IGHM and IGLC1) and fibronectin (FN1), which is involved in tumor invasion, were up-regulated at \( t_1 \).

Interestingly, we observed an early down-regulation of genes involved in Cell Cycle and DNA Replication, Recombination, and Repair starting at \( t_1 \) and most remarkably at \( t_2 \) and \( t_3 \), suggesting a slowdown of the cell cycle and therefore reduced proliferative capacity of melanoma cells. This could be the first indicator of spontaneous regression. At least 11 of the top 15 down-regulated genes at \( t_2 \) were connected with cell cycle and DNA repair mechanisms. The most significant gene network, detected by IPA analysis, was mainly composed of genes (86%) involved in cell cycle mechanisms. Cyclins such as CCNB1 and CCNA2, cyclin-dependent kinases such as CDC2 and CDC6, and spindle and chromosome structure-related proteins such as kinesins (KIF11 and KIF2C), NEK2, MELK, TOP2A, BUB1, and BIRC5, playing roles in chromosome segregation and cell cycle checkpoint control, showed the most drastic down-regulation during spontaneous regression suggesting cell cycle retardation. CCNB1 and CDC2 form the maturation-promoting factor (MPF) complex and thereby regulate mitosis; CKAP2 and KIF11 are spindle-associated molecules critical for mitosis, suggesting a mitotic arrest of melanoma cells at early regression.

**Figure 4.** Clinical, histologic, immunohistologic, and gene expression analysis of spontaneous melanoma development and regression. (A) Kinetics of clinical and histologic progression and regression of MeLiM melanomas. (B) Immunohistologic evaluation of tumors used for microarray analysis, characterizing monocytes by SWC3, T cells by CD3, and CTLs by CD3 and CD8. (C) Gene expression analysis. \( t_0 \) was used as transcriptomic reference. Top 15 up-regulated and top 15 down-regulated genes per time point compared to \( t_0 \) are demonstrated.
We identified 10 genes down-regulated (PLP1, EDNRB, CITED1, TFAP2A, RAB38, TYR, MITF, GPR143, GPM6B, and GPRC5B), while in humans, the up-regulation of these 10 genes was associated with a highly proliferative and weakly metastatic potential in primary melanoma cell lines [14].

The use of melanoma tissue in expression studies is often stated as a drawback because normal or reactive tissue constituents (e.g., vessels, inflammatory cells, normal epithelium) are interpreted as “contaminants” [27]. In our case, the interest was to obtain a global understanding of regression mechanisms related to melanoma cells and their surrounding, because the tumor microenvironment has recently been found to influence the progression of cancer by inducing phenotypic changes in cancer cells [28] and is therefore also very likely to be involved in regression [29].

To address questions of expression specificity, we laser-microdissected tumor regions of homogenous melanoma cells at early time points such as t₀, t₁, and t₂ and confirmed the early down-regulation of CCNB1 and CDC2 by qRT-PCR, indicating that the cell cycle retardation phenomenon is mainly linked to melanoma cells. Furthermore, immunohistochemical analysis identified melanoma cells positive for KIF11, CCNB1, and BIRC5 protein expression (data not shown).

The question, why early cell cycle retardation occurs, needs further investigation.

Possible mechanisms include senescence, which is defined as the irreversible loss of division capacity, but melanoma senescence–associated genes such as HDAC1, p16Ink4a, and p21 were not regulated, suggesting that an induction of senescence is not very likely to be involved in the regression process. Terminal differentiation of melanoma cells into pigment-laden macrophages was also suggested as an early event in spontaneously regressing melanoma using the Sinclair swine, another spontaneously regressing melanoma model [30]. Indeed, differentiation occurs in human malignant melanoma through diverse pathways as reviewed by Banerjee and Eyden [31]. However, genes frequently involved in human melanoma differentiation, such as NCAM and SYPI, were not regulated. Induction of melanoma regression could either be initiated by the tumor cells itself or is a consequence of extrinsic mechanisms such as an antitumor immune response or a beneficial interaction of both.

Recent studies demonstrated that spontaneous regression is mediated by infiltration of leukocytes mostly of innate immunity [32,33]. Spontaneous melanoma regression in MeLiM is highly correlated with an increase of immune response–related genes. Apparently, the tumor does not succeed in escaping the immune attack, as it is mostly the case in human melanoma [34]. In our model, we detected a strong up-regulation of monocyte/macrophage–related genes from t₂ to t₅, which corresponded with the appearance of histiocyte-like cell infiltrates on a histologic level. Tumor-infiltrating macrophages were shown to play a dual role in carcinogenesis because they can enhance and inhibit tumor growth depending on their activation and secretion of effector molecules [35]. Molecules associated with an inhibitory effect, such as IL-10, IL-13, IL-4, and TGFB1, were present on our microarray but did not show significant regulation. Characteristics of
these cells include hyperpigmentation, aneuploidy, increased size, and irregular borders. Phenotypically similar cells in melanoma have been associated with osteoclast-like giant cells [36], melanophages [37], and macrophage–melanoma fusion hybrids [38]. In fact, overexpression of many genes in our case were characteristic but not exclusive for osteoclasts such as PU.1, SCIN, ITGB3, ATP6V0D2, and ACP5 [39], whereas high expression of ACP5 in mouse macrophages was shown to enhance cytotoxicity by increased ROS and superoxide production [40]. In the spontaneous regression/complete resistance (SR/CR) mouse model [32], macrophages were demonstrated to induce tumor apoptosis by cell contact–dependent secretion of ROS and serine proteases. Although antibodies against tumors were produced in SR/CR mice, they were not required for killing, suggesting that antibody-dependent cellular cytotoxicity is not an effector mechanism in this model. Regression in MeLiM is accompanied by an early up-regulation of immunoglobulins (kappa and lambda light chain and heavy chain loci) proposing also a humoral response. We detected a major increase in IGHM levels between t3 and t6, although at t1, IGHM was already 10-fold up-regulated compared to t0. One might suggest that a humoral response could be the initiator of melanoma regression, but qRT-PCR analysis revealed equal levels of IGHM expression in melanoma tissue at t1 and healthy skin (data not shown). Furthermore, a stronger up-regulation of IgM levels would be expected if it is the initiating mechanism, because normal pigs already show stable IgM serum levels throughout their first 3 weeks of life and are therefore fully armed to fight the tumor at t1 [41].

Spontaneous regression of melanoma in humans is rare and hard to study; however, larger numbers of CD4+ T cells were found in tumor infiltrates besides high percentages of Langerhans cells, macrophages, and other major histocompatibility complex II–expressing cells [42]. We detected a significant up-regulation of different T-cell receptor chains at a late stage of regression (t6), as well as T-cell–associated cytokines (CCL5 and IL13), effector molecules such as granzyme B, perforin (verified by qRT-PCR), and the NKG2D receptor. Flow cytometry and immunohistochemical analysis of MeLiM–TILs around t4 showed higher percentages of CD8+ T cells, suggesting a predominant CTL response in our case. γδ T cells as well as CD4+CD8+ T cells and CD16+CD8+ NK cells did not seem to play a role in regression because they were almost not detectable in tumor infiltrates compared to peripheral blood mononuclear cells (data not shown).

MeLiM show signs of autoimmune reactions such as localized or systemic vitiligo-like depigmentation. This phenomenon could be explained by cross-reactivity that occurs between melanoma and normal melanocytes. The presence of vitiligo in melanoma patients seems to improve the prognosis of melanoma by means of effective immunity against the tumor [43]. Autoantibodies isolated from vitiligo patients were shown to trigger apoptosis in melanocyte cultures [44]. Furthermore, antimelanoma antibodies were isolated from Sinclair swine, recognizing antigens expressed on the surface of normal melanocytes [45].

CD8+ T cells isolated from peripheral blood of vitiligo patients were also shown to frequently recognize the melanosomal protein MLANA and kill human leukocyte antigen–matched melanoma cells [46], playing therefore a role in the depigmentation process. We confirmed the down-regulation of MLANA expression during spontaneous regression by qRT-PCR analysis. The expression of additional genes involved in melanogenesis, such as S117, SLC24A5, OCA2, CITED1, and TYR, was dramatically down-regulated at t4, which confirmed a major loss of melanoma cells at the end of regression. Tumors at t0 showed severe hyperpigmentation, probably a consequence of genetically induced abnormal melanogenesis. High levels of melanin have been reported to be protective against ROS, including OH, O2·−, and H2O2 [47], whereas accumulation of melanin intermediates, produced in the absence of tyrosinase, TYRP1, and DCT, is cytotoxic [48]. Hence, one may hypothesize that high levels of melanin in our model could also mediate protective effects against macrophage produced ROS. Scavenging free radicals could be a reason for a change in melanin confirmation and subsequent change in color. At the same time, overexpression of genes involved in the melanosomal pathway might result in increased immunogenicity because more melanoma antigens would be presented on the surface that could be recognized by immune cells [49]. A main immune escape mechanism such as the down-regulation of tumor associated antigens would be in this case impaired.

The early detected monocyte/macrophage–like signature might represent the first attempt of the immune response to fight highly immunogenic melanoma cells, whereas mediated cytotoxicity might be buffered to some extent by the elevated melanin content. Proper antigen presentation, as suggested by the presence of major histocompatibility complex II molecule CD74 and costimulatory molecule CD86, seems to lead to the activation of CD8+ cytotoxic effector T cells. The major down-regulation of melanoma antigens at t3 is coherently accompanied by the up-regulation of T-cell response genes. One might expect the detection of a distinct apoptotic gene signature characteristic for T cell–mediated cytotoxicity including genes such as FAS/FASL, caspase 10, and caspase 3. In our case, apoptosis-related genes were associated with both intrinsic (TP53/11 and BIM) and extrinsic (TNFSF12 and GZMB) death signals lacking a distinct classification. A possible explanation could be that our chosen time points for microarray hybridization missed the molecular detection of the ongoing apoptotic process because programmed cell death is a rather rapid process. We oriented our choice by considering major histologic changes such as first signs of regression. Other apoptotic genes such as CLU, ELMO1, PLEKHF1, and THY1 were detected along with a strong increase of ubiquitin D expression at t6. Clusterin was maximally up-regulated at t3 (~12-fold) suggesting a role in early regression, as we have shown and confirmed earlier by SSH and qRT-PCR [13]. CLU codes for a glycoprotein whose nuclear isoform seems proapoptotic, and its secretory form antiapoptotic [50]. CLU was shown not to be expressed by normal melanocytes, whereas 30% of primary desmoplastic melanoma and only 13.5% of metastatic tumors were CLU-positive [51]. Future investigations include in vitro experiments to answer questions of cytotoxic key mechanisms and adoptive transfer of melanoma cells in mice (beige mice) with functionally defective monocytes, especially phagocytes and NK cells [52,53].

In summary, our time-dependent gene expression profiling study of spontaneously developing and regressing swine melanoma has identified several significant gene signatures. For the first time, expression profiles for complete melanoma regression were identified, harboring potential targets for either chemotherapy (down-regulation of a mitotic cell cycle gene signature) or immunotherapy (up-regulation of a complex immune response signature).

Acknowledgments
The authors thank G. Frelat for constant and active support. The authors also thank F. Andreoletti and P. Bacon for animal care. The
authors are grateful to C. Bevilacqua and S. Makhzmi (Institut National de la Recherche Agronomique, Plateau d’instrumentation et de compétences en transcriptomique) for technical assistance regarding laser capture microdissection.

References


Figure W1. IPA canonical pathway analysis.